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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

General overview

Sturgeon is an ancient fish of the order *Acipenseriformes*, which comprises 27 species in two families, *Acipenseridae* (sturgeon, 25 species) and *Polydontidae* (paddlefish, two species) (Zhang et al., 2013). Most sturgeon species are considered threatened or endangered due to severe decline of natural stocks as a consequence of overfishing and environmental degradation (Raymakers, 2007; Bronzi et al., 2011). Sterlet *Acipenser ruthenus*, listed as a vulnerable species (Gesner et al., 2010), has small size and a short reproductive cycle relative to other sturgeon species. It is widely used as a model sturgeon species for study of artificial reproduction and larviculture. Due to the shortage of broodstock, storage of good quality fish gametes is critical to an effective conservation program and synchronization of stages in artificial reproduction (Lahnsteiner et al., 2002; Sarvi et al., 2006).

Cryopreservation is a process in which cells are preserved by cooling to very low temperatures, usually -196 °C, but remain viable after warming to temperatures above 0 °C. It makes possible almost indefinite storage of desirable genetic material and ensures the availability of cryopreserved semen for artificial insemination, transportation of semen among hatcheries, conservation of populations, and preserving genetic diversity of endangered species (Martínez-Páramo et al., 2017). Effective cryopreservation of sperm was first demonstrated in 1949 with the discovery that glycerol acts as a potent cryoprotectant (Polge et al., 1949). Soon after, Blaxter (1953) reported fish sperm cryopreservation, and studies demonstrating the feasibility of cryopreserving the semen of a large number of marine and freshwater species followed. To date, sperm of over 200 species of freshwater and marine fish has been successfully cryopreserved (Tsai and Lin, 2012). A variety of protocols have been developed for fish and optimized to obtain high post-thaw sperm motility and fertilization rates (Urbányi et al., 2004; Boryshpolets et al., 2011). These methods are primarily based on conventional sperm cryopreservation, in which extracellular water is partially crystallized, or on sperm vitrification in which both intra- and extra-cellular liquids are vitrified. With both techniques, the quality of frozen/thawed sperm and its fertilization rate are inferior to that of fresh sperm. Traditional cryopreservation and vitrification present advantages and disadvantages for sperm storage. An optimal protocol for cryopreservation of sterlet sperm, based on its unique characteristics, needs to be developed.

Proteins represent a major molecular component of sperm and play important roles in sperm structure and function, including in acrosome reactions, metabolic activity, and maintenance of motility (Li et al., 2009). It has been demonstrated that large quantities of proteins related to the spermatozoon nucleus, midpiece, flagellum, plasma membrane, and cytosol are released during cryopreservation, consequently impairing sperm quality and fertilizing capacity (Dietrich et al., 2015; Nynca et al., 2015). In post-thawed common carp *Cyprinus carpio* sperm, 183 proteins were reported to be released from spermatozoa during the cryopreservation process. These proteins were associated with metabolism and energy production, response to stress, apoptosis, small GTPase-mediated signal transduction, transcription, translation, protein folding and turnover, and DNA repair (Dietrich et al., 2015). In sturgeon species, the protein profile and its associations to sperm function with respect to cryopreservation is unknown.

The stability of the spermatozoon plasma membrane is critical to sperm quality and must be optimized to reduce damage incurred in the freezing process, since cytosol proteins may be released from spermatozoa through a compromised plasma membrane during cryopreservation procedures. Antifreeze proteins (AFP) are present in arctic organisms that confer the ability to resist freezing temperatures by modifying ice-crystal formation, preventing recrystallization, and interacting with the plasma membrane at low temperatures (Robles et al., 2006; Kim

et al., 2017). They have long been studied as cryoprotectants and are reported to preserve sperm quality relative to motility parameters and, especially, plasma membrane integrity during cryopreservation (Hezavehei et al., 2018).

Vitrification, an alternative procedure of sperm cryopreservation

Vitrification is a method by which a substance is transformed to a non-crystalline solid "glass." Vitrification has become increasingly widespread for preserving cells, due to its simplicity, speed, and utility for field applications with no requirement for specialized equipment (Morris, 2006). The process uses cryoprotectant concentrations that prevent ice crystallization, allowing the material to solidify as an amorphous glass (Cuevas-Uribe et al., 2017). The concentrations of cryoprotectant required are extremely high and, therefore, potentially harmful to cells. Optimization of temperature and the rate of introduction and removal of the cryoprotectant is critical. The required cryoprotectant concentration can be reduced if rapid cooling and even more rapid warming are used (Tavukcuoglu et al., 2012). Specialized containers and procedures have been designed to keep the volume of vitrification solution to a minimum, hence increasing cooling/warming rates, including open-pulled straws (Vajta et al., 1997), copper loops (Isachenko et al., 2004), droplets (Isachenko et al., 2008; Figueroa et al., 2013), glassy cell layers (Andreev et al., 2009), nylon cryoloops (Desai et al., 2004), cryovials (Merino et al., 2011), and aluminum boats partially immersed in liquid nitrogen for solid-surface vitrification (Satirapod et al., 2012). Sperm vitrification has been applied in fish species, including green swordtail *Xiphophorus hellerii* (Cuevas-Uribe et al., 2011a), Russian sturgeon *Acipenser gueldenstaedtii* (Andreev et al., 2009), Persian sturgeon *Acipenser persicus* (Abed-Elmdoust et al., 2015), channel catfish *Ictalurus punctatus* (Cuevas-Uribe et al. 2011b), rainbow trout *Oncorhynchus mykiss* (Merino et al., 2011, 2012; Figueroa et al., 2013), Atlantic salmon *Salmo salar* (Figueroa et al., 2015), spotted seatrout *Cynoscion nebulosus*, red snapper *Lutjanus campechanus*, red drum *Sciaenops ocellatus* (Cuevas-Uribe et al., 2015) and southern flounder *Paralichthys lethostigma* (Cuevas-Uribe et al., 2017) with limited success.

In our studies, sterlet was selected as an easily-manipulated model sturgeon species for conservation and maintaining biodiversity. A comprehensive literature search revealed that vitrification of small sperm quantities is not yet feasible for commercial applications and is unlikely to be suitable for sterlet sperm storage, due to the large volume and low concentration of sperm produced.

Sperm protein alteration during cryopreservation

Freeze-thaw procedures can irreversibly damage sperm, impairing spermatozoon acrosome structure (Psenicka et al., 2008), motility (Linhart et al., 2000), and viability (Li et al., 2010b), thus directly affecting success of fertilization (Linhart et al., 2000) and embryo development (Kopeika et al., 2003). The damage is likely in part the consequence of protein alteration during the freeze/thaw process (Lahnsteiner et al., 1996; Zilli et al., 2003; He and Woods, 2004). The functional state of numerous proteins in the sperm nucleus, midpiece, flagellum, plasma membrane, and cytosol has been shown to be affected by cryopreservation in many fish species (Gwo and Arnold, 1992; Zilli et al., 2014, Nynca et al., 2015, Figueroa et al., 2016a). Specific membrane proteins such as N-ethylmaleimide-sensitive fusion attachment protein alpha, cofilin 2, and annexin A4 that are involved in membrane trafficking, organization, and cell movement, are significantly altered by cryopreservation (Li et al., 2010a). Transferrin, a multi-task protein, is affected by freeze-thaw stress, which is suggested to be associated with the spermatozoon membrane and protection against oxidative damage (Li et al., 2010a).

Sterlet sperm cryopreservation has been established, but sperm quality and fertilization capacity are reduced compared with the fresh sperm (Boryshpolets et al., 2011). Knowledge of the sturgeon sperm protein profile and its function with respect to cryopreservation is limited. A proteomic study of sterlet sperm was undertaken to expand knowledge of the impact of cryopreservation-altered proteins on sperm function and quality, and to provide insight into improved sperm cryopreservation techniques.

Antifreeze proteins as sperm cryoprotectant

Since the extreme temperature change during cryopreservation can lead to lethal and sublethal injuries to spermatozoa, efforts have been made to improve techniques and obtain higher quality thawed sperm. Addition of proteins such as antioxidants and antifreeze proteins to cryopreservation media has been recommended (Beirão et al., 2012; Figueroa et al., 2017; Magnotti et al., 2018). The ability of AFPs to protect sperm during the freeze-thaw procedure has been tested in mammals with inconsistent results (Payne et al., 1994; Prathalingam et al., 2006). Limited studies have been carried out to determine the effectiveness of antifreeze proteins in fish sperm cryopreservation (Abed-Elmdoust et al., 2015). Antifreeze proteins, especially AFPIII, have shown a beneficial effect on post-thawed sperm quality and were reported to contribute to the stabilization of sperm plasma membrane organization in gilthead seabream *Sparus aurata* (Beirão et al., 2012; Zilli et al., 2014). Information with respect to the effects of AFPs on the quality and fertilization capacity of cryopreserved sterlet sperm is not currently available. Research into cryopreservation of sterlet sperm using a medium containing antifreeze proteins may not only improve cryopreservation techniques to maintain sperm quality, but also reveal the mechanisms by which antifreeze proteins preserve the cell at freezing temperatures.

Research Goals

The primary goal of this research was to determine the optimal method of sterlet sperm cryopreservation, assess sterlet spermatozoon cryoinjury, and improve cryopreservation protocols to preserve sperm quality and comprised the following objectives:

1. To summarize the advantages and disadvantages of vitrification of fish sperm and discuss recent progress in vitrification of fish spermatozoa.
2. To assess the effects of cryopreservation on motility variables of sterlet spermatozoa and compare the proteomes of fresh and frozen/thawed spermatozoa and seminal plasma.
3. To characterize the protective role of antifreeze protein type I and antifreeze protein type III on sterlet sperm motility, velocity, membrane integrity, and fertilization rate following cryopreservation.

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MOLECULAR AND SUBCELLULAR CRYOINJURY OF FISH SPERTMATOZOA AND APPROACHES TO IMPROVE CRYOPRESERVATION

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Molecular and subcellular cryoinjury of fish spermatozoa and approaches to improve cryopreservation

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Abstract

The quality of frozen/thawed fish sperm is generally lower than that of fresh sperm. Extremely low temperatures are associated with damage to fish spermatozoon subcellular compartments and associated molecules such as DNA and proteins. Cryodamage can negatively affect DNA integrity, spermatozoon metabolism and motility, consequently impairing fertilization and embryo development. To preserve sperm efficiently, the addition of proteins including seminal plasma proteins, antioxidants, antifreeze proteins and antifreeze glycoproteins, and bovine serum albumin before and/or after freezing is suggested to minimize cryoinjury. An appropriate quantity of seminal plasma components in cryopreservation medium can prevent spermatozoon membrane damage by maintaining antioxidant enzymes and their proper distribution on the spermatozoon surface. Under storage conditions, the semen antioxidant system is ineffective in protecting spermatozoa from reactive oxygen species. The addition of antioxidants can provide protection for spermatozoa and reduce cryoinjuries during storage, but the effect of a given antioxidant is species-specific. Antifreeze proteins and antifreeze glycoproteins can stabilize the cell membrane via interaction with phospholipid components and inhibit ice-crystal growth to reduce damage related to osmotic stress and ice crystallization. However, they may exhibit a cytotoxic effect. This review summarizes the sources and characteristics of subcellular spermatozoon cryoinjury and discusses approaches to improve outcome of fish sperm cryopreservation.

Key words: antifreeze glycoproteins, antifreeze proteins, antioxidants, cryodamage, seminal plasma, spermatozoa.

Introduction

Sperm cryopreservation provides a stable supply of sperm for hatchery production, simplifies sperm transfer among hatcheries, helps in conservation of endangered species, is valuable in selective breeding programmes and provides material for research, including gene transfer (Lahnsteiner *et al.* 2002; Sarvi *et al.* 2006; Tiersch 2008; Martínez-Páramo *et al.* 2017). Despite gains in cryopreserving sperm of more than 200 freshwater and marine fish species (Tsai & Lin 2012), the sperm quality of most fish species declines significantly during the freeze–thaw process, affecting fertilization and hatching rates (Suquet *et al.* 1998; Li *et al.* 2006). In order to improve the protocol to reduce the sperm damage suffered in the freezing process, the freeze–thaw steps which are the most critical aspects of cryopreservation, should be optimized (Yoon *et al.* 2015).

Fish spermatozoon subcellular compartments, including the acrosome (sturgeons), nucleus (head), midpiece, flagellum, cytosol and plasma membrane (Golpour *et al.* 2016, 2017), are vulnerable to cryodamage leading to impairment of cell function (Nynca *et al.* 2015). Herein, we summarize potential negative effects on spermatozoon function of cryopreservation process to subcellular compartments and their associated molecules (DNA, proteins, phospholipids).

In order to improve the frozen/thawed sperm quality and minimize the cryoinjury during the freeze–thaw process, a variety of approaches have been employed, including the addition of various proteins to protect sperm during the pre-freeze phase and after thawing (Figueroa *et al.* 2017). Seminal plasma plays critical roles in creating an optimal environment for spermatozoa stored in the reproductive system. It is demonstrated that supplementing epididymal sperm of red deer with its seminal plasma was highly positive for cryopreservation (Martínez-Pastor *et al.* 2006). Moreover, incubation with seminal plasma proteins has been reported to restore spermatozoon of ram membrane structural damage induced by cooling (Barrios *et al.* 2000). Mogielnicka-Brzozowska and Kordan (2011) discussed the potential of adding selected plasma proteins to sperm for the retention of qualities critical to effective fertilization after storage in mammals. The addition of nonenzymatic and enzymatic antioxidants to freezing medium has also been shown effective in defence of fish spermatozoa against oxidative attack during the freeze–thaw process (Cabrita *et al.* 2011; Shaliutina-Kolešová *et al.* 2014).

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) inhibit the growth of ice crystals and depress the freezing point, as well as stabilize cell membrane via interaction with phospholipids (Goddard & Fletcher 2002), making them of interest in cryopreservation of both fish and mammalian sperm (Wang 2000; Kim *et al.* 2017). Type III AFP has been demonstrated to function in stabilizing plasma membrane organization to improve post-thaw spermatozoon motility in gilthead seabream (*Sparus aurata*) and chimpanzee (*Pan troglodytes*) (Younis *et al.* 1998; Beirão *et al.* 2012). Prathalingam *et al.* (2006) reported that Type I AFP resulted in increased osmotic resistance in cryopreserved bovine spermatozoa. Although these findings show a benefit in cryopreservation, the function and mechanisms of proteins with respect to the preservation of fish sperm during storage is not well understood.

The objectives of this review are to collect and summarize data addressing (1) injury to fish spermatozoa incurred during cryopreservation with respect to subcellular compartments and their related molecules; (2) the protective effects of seminal plasma proteins on cryopreserved spermatozoa; (3) the improvement of sperm quality by addition of antioxidants during cryopreservation; and (4) the protective effects of AF(G)Ps and selected proteins on cryopreserved spermatozoa.

Molecular and subcellular cryoinjury and consequences on cell function

Cryoinjury can be a major challenge for practical application of spermatozoa cryopreservation in fish aquaculture and conservation programmes. Sperm cryopreservation involves extreme freezing and thawing that can result in lethal and sublethal alterations to the spermatozoon even when using the best available techniques. Sublethal injury can occur in cell compartments including the acrosome, nucleus, midpiece, flagellum, plasma membrane and cytosol, leading to impairment of cell structure and function including alterations in DNA integrity (Gwo & Arnold 1992; Zilli *et al.* 2003), mitochondrial damage and metabolic deficiency (He & Woods 2004), reduction in spermatozoon motility and velocity (Linhart *et al.* 2000), disintegration of plasma membrane (Lahnsteiner *et al.* 1996) and protein leakage (Dietrich *et al.* 2015; Nynca *et al.* 2015).

Nuclear damage and DNA fragmentation

The adverse effects of sperm cryopreservation on DNA integrity are of special concern when cryopreservation is used for the purpose of gene banking. Spermatozoon DNA fragmentation has been shown to be induced by low temperatures, possibly related to DNA oxidation (Ciereszko *et al.* 2005; Dietrich *et al.* 2005; Thomson *et al.* 2009). However, an increase in DNA fragmentation in post-thaw sex-reversed rainbow trout (*Oncorhynchus mykiss*) spermatozoa suggested that freezing *per se* could be involved in DNA fragmentation, as opposed to oxidative stress (Pérez-Cerezales *et al.* 2009). Significant DNA fragmentation was seen in frozen/thawed European sea bass (*Dicentrarchus labrax*) spermatozoa with and without the use of a cryoprotectant (Zilli *et al.* 2003). In contrast, cryoprotectant toxicity was demonstrated to be a primary factor in DNA damage of cryopreserved sperm of striped catfish (*Pangasianodon hypophthalmus*) and bocachico (*Prochilodus magdalenae*) (Martínez *et al.* 2012; Rani & Munuswamy 2014). A study of human embryos suggested that vitrification of gametes (high concentration of cryoprotectant) affects DNA integrity to a much lesser extent than does slow freezing (Kopeika *et al.* 2014). Some evidence suggested an increase in DNA single-strand breaks and DNA condensation or fragmentation in spermatozoa after cryopreservation. Dietrich *et al.* (2015) reported release of nucleoplasmic and DNA-binding proteins such as adenosine kinase isoform 1 from common carp (*Cyprinus carpio*) spermatozoa during cryopreservation. These proteins are

associated with the regulation of DNA replication and transcription, and DNA repair processes (Dietrich *et al.* 2015); hence, their loss is detrimental to DNA stability. Nynca *et al.* (2015) reported in rainbow trout spermatozoa that cryopreservation can negatively affect nuclear signal transduction proteins such as members of the Ras family, but not the nuclear structure proteins, protamines and histones.

DNA fragmentation is associated with a decrease in fertilization capacity and abnormal embryo cleavage (Gwo *et al.* 2003). Lower hatching rates were found in rainbow trout, sea bass and common carp eggs fertilized by cryopreserved sperm with high levels of DNA fragmentation (Cabrita *et al.* 2001b; Zhou *et al.* 2006; Morrow *et al.* 2017). A study of Atlantic croaker (*Micropogonias undulatus*) cryopreserved sperm showed greater damage to spermatozoon mitochondria, plasma membrane and the 9 + 2 axoneme than to the nucleus (Gwo & Arnold 1992), while other research has provided evidence that DNA damage does not affect motility or fertilization capacity of cryopreserved spermatozoa (Labbe *et al.* 2001; Zilli *et al.* 2003). Cabrita *et al.* (2005) found no significant difference in fragmentation of DNA of fresh and cryopreserved gilthead sea bream *Sparus aurata* spermatozoa with 1:6 sperm:extender (1% NaCl, 300 mOsm/kg, 5% Me_2SO, and 10mg/mL bovine serum albumin (BSA)) dilution. Human spermatozoon chromatin is considered less susceptible to damage compared to that in somatic cells, due to its high level of chromatin packaging (Singh *et al.* 2003). Sperm DNA fragmentation has been shown to be reversible in oocytes and early embryos during fertilization and embryonic development (González-Marín *et al.* 2012; Fernández-Díez *et al.* 2016; Herráez *et al.* 2017). While it has been demonstrated that rainbow trout sperm can maintain fertilization capacity in spite of DNA damage, embryo survival was affected and damage was only partially repaired in the egg during the first cleavage (Pérez-Cerezales *et al.* 2010). Pérez-Cerezales *et al.* (2011) stated that fertilization with cryopreserved DNA-damaged spermatozoa of rainbow trout significantly affects offspring, detectable as an increase in telomere length as well as alterations in gene transcription in surviving embryos and larvae. Therefore, DNA fragmentation in cryopreserved spermatozoa may contribute to the decrease in sperm quality and hatching rate.

Damage to the midpiece, mitochondria and metabolism

Spermatozoa require a plenty of ATP to support multiple cell functions and biochemical events, such as sustaining motility and activating phosphorylation for successful fertilization (Miki 2007; Madeddu *et al.* 2010). Cryopreservation has been demonstrated to result in a decrease in sperm metabolic activity (Figueroa *et al.* 2017). The major reason for the decrease in metabolic activity and low post-thaw sperm quality is alteration of mitochondrial structures and membrane in the midpiece during cryopreservation (Madeddu *et al.* 2010; Figueroa *et al.* 2015). These changes exert extreme impact on the function of mitochondria, including the biochemical processes involved in ATP production (Figueroa *et al.* 2015, 2017), consequently reducing the energy available for motility. In addition, it is demonstrated that freeze–thaw process can spontaneously activate common carp spermatozoa, accompanying the significant decrease in the ATP level with post-thaw time (46 and 10 nmol ATP/10^9 spermatozoa after 25 s and 10 min thawing respectively) (Boryshpolets *et al.* 2009). Thus, we suppose that spontaneous activation after freeze–thaw process may be also one of the reasons for the decrease in spermatozoa metabolic activity in some species, such as common carp and Eurasian perch (*Perca fluviatilis*) (Dzyuba *et al.* 2010).

Membrane damage can result in leakage of the internal components of spermatozoa leading to deficiency in, or reduced activity of enzymes (Dietrich *et al.* 2015). Proteomic investigations have revealed that proteins most commonly found to decrease in spermatozoa during cryopreservation are those with ATP-binding and catalytic activity (O'Connell *et al.* 2002; Chen *et al.* 2014; Dietrich *et al.* 2015; Nynca *et al.* 2015). These proteins are related to the breakdown of carbon compounds and the delivery of energy for spermatozoon movement. For instance, diminished expression of mitochondrial ATP synthase subunit β and enolase B, members of a superfamily of glycolytic enzymes, may be associated with impaired spermatozoon motility and viability following the freeze–thaw process, as these enzymes play a primary role in metabolic pathways including gluconeogenesis and glycolysis (Xin *et al.* 2018a). Identification of proteins, such as malate dehydrogenase, isocitrate dehydrogenase, succinyl-CoA ligase, aspartate aminotransferase, ornithine carbamoyltransferase and aconitate hydratase, in the extracellular fluid of cryopreserved rainbow trout semen implied severe damage to the mitochondria during cryopreservation (Nynca *et al.* 2015; Ciereszko *et al.* 2017). In addition, reduction in adenosine triphosphatase, creatine kinase, succinate dehydrogenase, lactate dehydrogenase, malate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase activity has been reported to lower the generation of ATP by the oxidative phosphorylation system and by glycolytic pathways (Miki *et al.* 2004; Huang *et al.* 2014; Zilli *et al.* 2014). However, it is not clear that the altered proteins associated with ATP after cryopreservation were from the dead spermatozoa, in which the proteins are poured out from the damaged membrane, or from live spermatozoa, in which some proteins leaked out of spermatozoa because of the organization, fluidity, permeability and lipid composition alteration of membrane during cryopreservation. Comparison of sterlet

(*Acipenser ruthenus*) sperm proteomes obtained from fresh and cryopreserved sperm under subpopulations selection showed that cryopreservation leads to minimal proteomic changes in spermatozoa population with high motility and viability parameters. Furthermore, the results also indicated that global differences in protein profiles between unselected fresh and cryopreserved samples are mainly due to protein loss or changes in the damaged and non-viable spermatozoa population (Horokhovatskyi *et al.* 2018).

Therefore, it can be suggested that protein leaking from the spermatozoon during cryopreservation may contribute to reduced mitochondrial function and metabolic activity (He & Woods 2004) and consequently disrupt the ATP production and regeneration (Ogier de Baulny *et al.* 1997) crucial to the initiation and maintenance of spermatozoon motility.

Damage to the cytoskeleton, flagellum and spermatozoon motility

Motility parameters represent the common standard by which to assess the quality of both fresh and cryopreserved spermatozoa. Spermatozoon motility generally presents a positive correlation with fertilizing capacity, as it affects the spermatozoon ability to reach the oocyte for successful fertilization (Rurangwa *et al.* 2004; Boryshpolets *et al.* 2013a, b). A low percentage of motile spermatozoa and low velocity, as well as shorter duration of spermatozoon movement, have been observed in cryopreserved sperm of mammals including human (Nallella *et al.* 2004; Moore *et al.* 2005), and in fish, significant impairment of post-thaw spermatozoon motility parameters has been recorded. In common carp, motility rate after cryopreservation was 33% compared to 95% in fresh spermatozoa (Boryshpolets *et al.* 2017); in rainbow trout, 49% compared to 90% (Ciereszko *et al.* 2014) and in burbot (*Lota lota*), 46% compared to 87% (Lahnsteiner *et al.* 2002). Spermatozoon motility rates up to 50% were obtained in post-thaw spermatozoa of European eel (*Anguilla anguilla*) (Asturiano *et al.* 2007) and sterlet, albeit with low fertilization rates (~20%) (Boryshpolets *et al.* 2011).

Inadequate post-thaw sperm motility may be due to cryodamage to cytoskeletal proteins, such as dynein and tubulin that act in cell motility (Nynca *et al.* 2015). Tubulins, for example, are the major constituents of microtubules in the spermatozoon flagellum. Significant changes in the abundance and distribution of cytoskeleton-related proteins have been demonstrated in human (Desrosiers *et al.* 2006), boar (Chen *et al.* 2014), common carp (Dietrich *et al.* 2015) and rainbow trout (Nynca *et al.* 2015) spermatozoa following cryopreservation. Cytoskeleton proteins are sensitive to thermal shock during cryopreservation. The decreased quantity of tubulin during cryopreservation may affect the spermatozoon flagellum and its movement symmetry, resulting in impaired motility and, consequently, fertilizing ability (Correa *et al.* 2007; Nynca *et al.* 2015). Cofilin is a protein associated with the spermatozoon flagellum and plays a role in its motility in mammals and fish (Dietrich *et al.* 2014; Megnagi *et al.* 2015; Chen *et al.* 2016). Li *et al.* (2010a) described low motility rate of cryopreserved common carp spermatozoa as a result of loss of cofilin from the cell. A recent proteomic study suggested that, in common carp sperm, a high level of cofilin in the seminal plasma can be considered an indirect sign of sublethal flagellum damage and indicates sperm unsuitable for cryopreservation (Dietrich *et al.* 2017). Impairment of the ubiquitin–proteasome system may negatively affect motility in the cryopreserved semen of rainbow trout (Nynca *et al.* 2015).

Damage to the plasma membrane

The spermatozoon plasma membrane is rich in polyunsaturated fatty acids and a variety of proteins, and its function is associated with ion regulation, signal pathways, acrosome reaction and sperm–egg fusion (Li *et al.* 2009). An intact plasma membrane is critical to the fish spermatozoa fertilization, since it is capable to fuse with the oocyte by fluidity, fusogenicity and permeability. Structure and function integrity of the spermatozoon plasma membrane is a commonly used indicator of fish sperm quality, especially in cryopreserved spermatozoa in which plasma membranes may be damaged (Ogier de Baulny *et al.* 1997). Common carp sperm cryopreserved with dimethyl sulfoxide (DMSO) and ethylene glycol (EG) showed significantly lower membrane integrity compared to fresh sperm (Li *et al.* 2010b). The plasma membrane integrity of red drum (*Sciaenops ocellatus*) spermatozoa was decreased significantly after vitrification with various cryoprotectant combinations of DMSO, EG, glycerol and trehalose (Cuevas-Uribe *et al.* 2015). Impaired membrane integrity could be related to the inferior motility parameters of cryopreserved spermatozoa.

Membrane-permeable cryoprotective agents, such as DMSO and ethylene glycol, were demonstrated to stabilize membranes and modulate the rate of cellular dehydration and incidence of intracellular ice formation, whereas di- and polysaccharides increase the glass transition temperature of freezing extender and facilitate preservation in a more stable glassy matrix for storage (Oldenhof *et al.* 2013). Damage to the sperm membrane upon cryopreservation may be due to osmotically induced cryoprotectant toxicity or to insufficient concentration of cryoprotectant in the cell interior allowing the formation of intracellular ice crystals (Mazur 1984; Gao *et al.* 1992; Martínez *et al.* 2012). He *et al.* (2016) demonstrated that intercellular ice crystallization can damage the spermatozoon membrane by

changing the organization, fluidity, permeability and lipid composition. In striped bass (*Morone saxatilis*) and *P. magdalenae* spermatozoa, both glucose and DMSO as cryoprotectants were found to alter plasma membranes through changes in osmotic pressure (Martínez *et al.* 2012). This may be because of the high concentration of membrane-permeable cryoprotectants used, giving great osmotic force to the spermatozoa by a rapid displacement of the intracellular water and a quick spread throughout the membrane towards the interior of spermatozoa. It is probable that membrane could not endure it, generating damages in two possible ways: 1) rupture of the membrane during freezing or thawing through the processes of dehydration or rehydration, when the friction between water and bilayers exceeds the membrane's diffusion capacity (Muldrew & McGann 1990), 2) formation of pores in bilayers of palmitoylphosphatidylcholine, provoked by DMSO molecules (Notman *et al.* 2006). Similarly, an increase in external osmolality by membrane-impermeable cryoprotective agents such as glucose could also have driven a larger contraction of the membrane or a greater dehydration, which could have resulted in lesions of the membrane (Kopeika & Kopeika 2008).

Increase in reactive oxygen species (ROS) during cryopreservation was reported to induce oxidative attack on sperm phospholipid-bound PUFAs, resulting in lipid peroxidation (LPO) (Chen *et al.* 2010; Li *et al.* 2010b). Lipid peroxidation is particularly important for aquatic animals, since they normally contain greater amounts of highly unsaturated fatty acids than do other species (Shaliutina-Kolešová *et al.* 2013). It can trigger loss of plasma membrane fluidity and integrity required for fusion events associated with fertilization (Ohyashiki *et al.* 1988; Shiva *et al.* 2011) and has usually been indicated by thiobarbituric acid reactive substances (TBARS) level in fish (Oakes & Van der Kraak 2003). Compared to fresh spermatozoa and seminal plasma, a significantly higher LPO level was observed in cryopreserved Pacific cod (*Gadus microcephalus*) spermatozoa and seminal plasma without extender/cryoprotectant, while no significant increase in LPO level was detected after cryopreservation with 12% propylene glycol or 12% propylene glycol and 0.1 mol/L trehalose (Wang *et al.* 2016).

Proteins play important roles in plasma membrane integrity, and the degradation of plasma membrane proteins during cryopreservation has been well documented (Dietrich *et al.* 2015; Nynca *et al.* 2015). Certain forms of apolipoprotein A-I are suggested to contribute to superior semen freezability by maintaining the spermatozoon membrane structure (Dietrich *et al.* 2017). The plasma membrane proteins *N*-ethylmaleimide-sensitive fusion attachment protein alpha and annexin A4, involved in membrane trafficking and organization, were found to be reduced in common carp sperm after freeze–thaw process, impacting the stability of the spermatozoon membrane structure (Warren *et al.* 2003; Li *et al.* 2010a). Flotillin, a marker of lipid rafts, was reported to be reduced in cryopreserved common carp sperm compared to fresh, indicating alteration of the plasma membrane structure (Dietrich *et al.* 2015). Damage to the spermatozoon plasma membrane may result in leakage of intracellular proteins involved in metabolism, signalling, biological regulation, and cytoskeleton organization, conferring a deleterious effect on various aspects of sperm structure and function (Zilli *et al.* 2005; Dietrich *et al.* 2015; Nynca *et al.* 2015; Westfalewicz *et al.* 2015). An increase in spermatozoon intracellular enzymes in the extracellular environment after thawing is primarily the result of damage to the plasma membrane (Ciereszko & Dabrowski 1994; Nynca *et al.* 2015).

The alteration of plasma membrane proteins during sperm cryopreservation indicates the necessity of further research to characterize the mechanism of cryoinjury to the spermatozoon membrane structure and, consequently, to cell function and novel cryopreservation techniques preventing such damage. Thus, appropriate cryoprotectant and its concentration should be selected to balance the osmolality and minimize the cryoinjuries on spermatozoa plasma membrane in different species (Kopeika *et al.* 2007).

Damage to the cytosol

Cytosol as the environment surrounding organelles and subcellular compartments contains numerous proteins and other molecules that carry out a variety of sperm functions. In cryopreserved human spermatozoa, Cao *et al.* (2003) observed degradation, rather than leakage, of heat-shock protein 90, a cytosolic protein that may affect stress tolerance, signal transduction and protein folding. However, Nynca *et al.* (2015) showed that 32% of the proteins leaked from cryopreserved rainbow trout spermatozoa were components of cytosol. In cryopreserved common carp spermatozoa, 65% of leaked proteins were from the cytosol (Dietrich *et al.* 2015). The leakage of cytosolic proteins may compromise sperm physiological function, impacting motility and fertilization ability. For instance, creatine kinase, the most abundant leaked cytosolic protein can affect energetics of the gamete (Ciereszko *et al.* 2017). However, most studies focused on the negative effects of cryopreservation on spermatozoa membrane, metabolic and cytoskeleton proteins. More studies are needed to clarify effects of cryopreservation on the abundance and function of cytosolic proteins in fish spermatozoa.

Cryoinjury of subcellular compartments, their molecular biomarkers and the consequences on spermatozoon function are given in Figure 1.

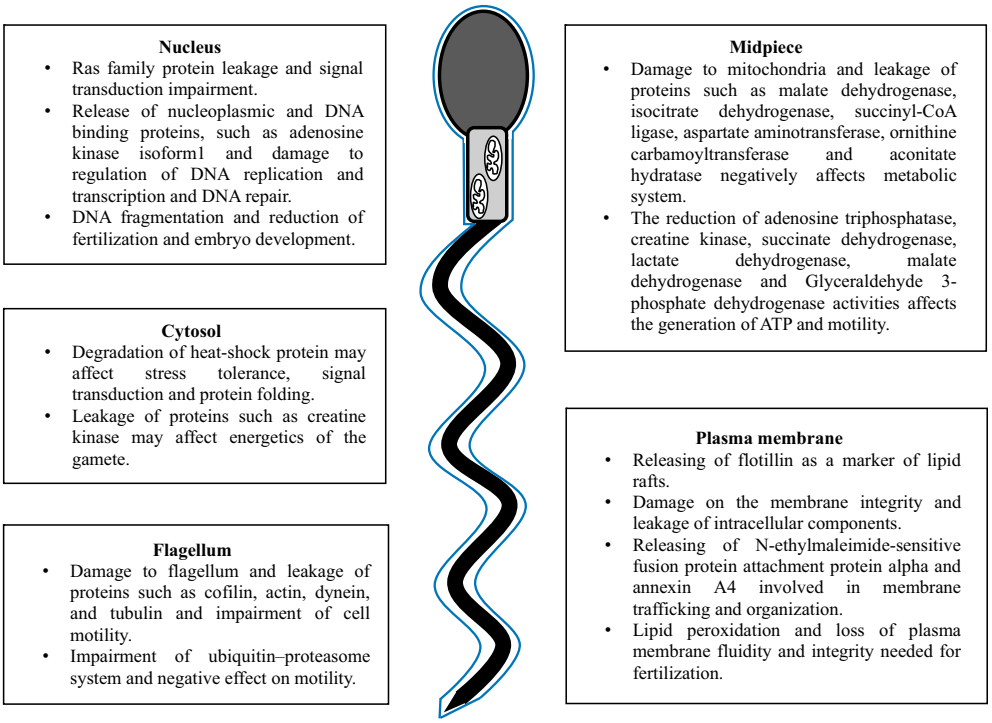

Figure 1 Cryoinjury of subcellular compartments, their molecular biomarkers and the consequences to spermatozoon function (Cao *et al*. 2003; Li *et al*. 2010a; Dietrich *et al*. 2015; Nynca *et al*. 2015; Ciereszko *et al*. 2017).

Proteins as additives for cryopreservation

Seminal plasma proteins, among many other substances present in the seminal plasma, are very promising biomarkers for predictive of sperm freezability and a number of proteins have been found to be more or less abundant in high and low freezability groups in buffalo and ram (Asadpour *et al*. 2007; Rickard *et al*. 2016). In some fish species, seminal plasma proteins have been shown to preserve the viability of mature sperm during cryopreservation, including maintaining spermatozoa in the quiescent state via sperm motility-immobilizing proteins, supporting adequate nutrient levels for sperm metabolism, controlling and regulating final maturation processes, and protecting sperm against damage resulting from proteolytic or oxidative attack (Ciereszko *et al*. 1999; Ciereszko 2008). Antifreeze proteins are specific proteins found in arctic fish species that exhibit the capacity to resist freezing temperatures. They offer protection to cells by depressing the freezing point, modifying ice-crystal formation, preventing recrystallization and interacting with the plasma membrane at low temperatures (Kim *et al*. 2017). The addition of seminal plasma proteins, antioxidants, antifreeze proteins and BSA to reduce the deterioration of sperm quality caused by freeze–thaw procedures has been investigated, with mixed results (Cabrita *et al*. 2011; Beirão *et al*. 2012; Figueroa *et al*. 2015).

Seminal plasma and seminal plasma proteins

Fish seminal plasma is a complex fluid responsible for many biological processes. Disturbances in the composition of the seminal plasma will impair its protective functions and, consequently, lead to a decrease in sperm quality (Ciereszko 2008). Furthermore, the protein composition of seminal plasma is important for preservation of spermatozoon viability during storage in the reproductive system (Billard 1983; Li *et al*. 2009). Researchers have concluded that male fertilization potential is highly dependent, not only on sperm ultrastructure and spermatozoon motility,

but also on organic components, including protein composition of the seminal plasma (Loir *et al.* 1990; Ciereszko 2008; Li *et al.* 2009, 2010a).

Adding seminal plasma to the cryo-medium before cold-shock prevented membrane damage and reduction in the life span of spermatozoa by inhibiting protein tyrosine phosphorylation (Pérez-Pé *et al.* 2002; Barbas & Mascarenhas 2009) and maintaining antioxidant enzyme activity and distribution on the spermatozoon surface (Marti *et al.* 2008), leading to a viable sperm population (Pérez-Pé *et al.* 2001). The supplementation of seminal plasma with BSA protected Atlantic salmon (*Salmo salar*) spermatozoa during vitrification (Figueroa *et al.* 2015). Similar studies on mammals showed that the addition of seminal plasma consistently improved membrane integrity, mitochondrial function, and motility of boar spermatozoa during *in vitro* storage and cryopreservation (Caballero *et al.* 2004; Okazaki *et al.* 2012). High viability of post-thaw goat semen was shown associated with the presence of seminal plasma and a 10% concentration of egg yolk in extender (Ferreira *et al.* 2014). In contrast, some researchers have reported that adding seminal plasma to extender before freezing did not increase the viability of post-thaw sperm (Herold *et al.* 2004; Moore *et al.* 2005).

The addition of seminal plasma proteins with molecular weight smaller than 60 kDa and larger than 10 kDa to mammalian sperm was found to protect sperm against cold-shock and oxidative stress (Calvete *et al.* 1996; Barrios *et al.* 2000; Rueda *et al.* 2013). Similar results have been observed in fish such as rainbow trout, in which protein fractions of 54, 47 and 16 kDa were found to be the most effective in promoting spermatozoon longevity (Lahnsteiner 2007). Significantly higher motility rates and swimming velocities were observed in rainbow trout spermatozoa incubated in artificial spermatozoon motility inhibiting saline solution (SMIS) containing a total seminal plasma protein fraction <50 kDa, or pure seminal plasma, than in SMIS without seminal plasma proteins or containing >50 kDa protein fraction (Lahnsteiner *et al.* 2004). These results indicated that seminal plasma proteins exhibit physiological functions in prolongation and stabilization of spermatozoon motility parameters (Lahnsteiner *et al.* 2004). Although many studies have reported success using supplementary seminal plasma proteins to improve cryopreserved fish sperm quality, the mechanisms and functions of seminal plasma for prevention of spermatozoon injury are not fully understood.

In addition to protein content, the concentration of seminal plasma proteins is crucial to prevention of cryoinjury. The use of a vitrification medium containing 50% seminal plasma in Atlantic salmon *Salmo salar* sperm provided spermatozoa with more acceptable levels of DNA fragmentation, plasma membrane integrity, mitochondrial membrane integrity, motility and fertility compared to 30% and 40% seminal plasma (Figueroa *et al.* 2015). Similarly, Fernández-Gago *et al.* (2016) reported that frozen/thawed boar semen with the presence of 50% seminal plasma proved more effective in preserving of sperm motility and chromatin content compared to 0% and 10%.

The quantity of proteins in seminal plasma also plays an important role in protecting spermatozoa during cryopreservation and may be related to interindividual variation in semen post-thaw quality. Dietrich *et al.* (2017) categorized the freezing capacity of common carp semen as *good* or *poor* based on levels of selected seminal plasma proteins. They concluded that, while high content of proteins related to immune response could be a biomarker for poor freezability, the presence of proteins involved in the maintenance of membrane integrity and antioxidant protection could signify semen of good potential for cryopreservation. The abundance of seminal plasma proteins involved in the immune response, such as apolipoprotein E that can be attributed to infection and inflammation in the reproductive tract, may have a minor effect on the quality of fresh semen but could significantly reduce its freezability (Dietrich *et al.* 2017). Fetuin showed higher abundance in the seminal plasma of good quality cryopreserved semen than in poor (Dietrich *et al.* 2017). Research on cryopreservation of rabbit semen demonstrated that fetuin plays an important role in cell motility, protection against oxidative stress and maintenance of plasma membrane integrity (Sarıözkan *et al.* 2015).

Determination of seminal plasma proteins content and their optimal concentrations as additives for cryopreservation medium may be a topic for further research.

Antioxidants

The antioxidant system comprising glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) has been described as a defence mechanism against lipid peroxidation (LPO) in semen of numerous animals and fish species (Shaliutina-Kolešová *et al.* 2014; Ahmadi *et al.* 2016). However, the sperm antioxidant systems in fish are not effective in protecting spermatozoa from ROS *in vitro* storage condition (Lahnsteiner *et al.* 2010). It has been showed that the activity of major seminal plasma enzyme antioxidants such as SOD and CAT significantly decreased throughout cryopreservation process in red seabream (*Pagrus major*) sperm (Chen *et al.* 2010). Other work reported the differences in total SOD activity between the sterlet control group and sperm cryopreserved with 10% EG (Shaliutina-Kolešová *et al.* 2015). Therefore, antioxidant supplementation has been developed to improve techniques for sperm cryopreservation (Cabrita *et al.* 2011). Kopeika *et al.* (2014) suggested

that the addition of antioxidants to cryopreservation medium and the employment of well-controlled cooling regimes could potentially decrease DNA fragmentation. Supplementation with antioxidants naturally present in seminal plasma, especially taurine and hypotaurine, protected DNA against strand breaks, significantly reducing fragmentation in cryopreserved gilthead seabream sperm (Cabrita *et al.* 2011) (Table 1). The primary reason for this effect is that the amine group of taurine exhibits an affinity to nucleic acids and can neutralize generation of ROS to limit DNA damage (Zhang *et al.* 2004; Sokól *et al.* 2009). Enzymatic antioxidants including CAT, SOD and glutathione peroxidase (GPx) have been shown to provide effective protection against cold-shock and oxidative damage in common carp spermatozoa (Li *et al.* 2010b; Shaliutina-Kolešová *et al.* 2014). The capacity of GPx was found to counteract ROS stress in spermatozoa and minimize cryoinjury (Li *et al.* 2010b).

Lahnsteiner and Mansour (2010) reported that non-enzymatic antioxidants including uric acid, carnitine, glutathione and methionine in freezing medium of brown trout (*Salmo trutta*), burbot (*Lota lota*) and European perch (*Perca fluviatilis*) sperm were associated with preservation of motility and membrane integrity during short-term storage. In contrast, supplementation with the antioxidants cysteine and ascorbic acid has not been shown to be of value for brook trout, rainbow trout or Arctic char (*Salvelinus alpinus*) sperm during routine cryopreservation (Lahnsteiner *et al.* 2011; Ekici *et al.* 2012; Sarosiek *et al.* 2013). Although the effect of a given antioxidant is species-specific, the use of suitable antioxidants for cryopreservation of fish sperm can prevent injury caused by oxidative stress (Martins-Bessa *et al.* 2007; Cabrita *et al.* 2011).

Antifreeze proteins and antifreeze glycoproteins

A diverse group of antifreeze proteins and antifreeze glycoproteins in teleost fish offer protection from freezing in subzero seawater and have been studied for use in cryopreservation of mammalian and fish sperm (Younis *et al.* 1998; Abed-Elmdoust *et al.* 2015). These proteins can stabilize the cell membrane via interaction with the phospholipid components at low temperatures (Beirão *et al.* 2012). Antifreeze proteins, especially AFPIII, were shown to improve post-thawed sperm quality and contribute to stabilization of plasma membrane organization by maintaining membrane phospholipid composition and the degree of saturation/unsaturation of their fatty acid components during cryopreservation of sea bream sperm (Beirão *et al.* 2012). The addition of AFPIII can reduce the changes in sperm protein profiles during the freeze–thaw procedure (Zilli *et al.* 2014). Additionally, involving AFPI and AFPIII to cryopreservation medium has shown to improve the membrane integrity of sterlet spermatozoa significantly, but not motility, velocity and fertility (Xin *et al.* 2018b,c). The effect of AF(G)Ps on sperm during cryopreservation depends on the type of protein, as well as its concentration (Table 2). Different concentrations of AFPIII showed different effects on post-thaw motility in Persian sturgeon *Acipenser persicus* spermatozoa, suggesting that AFPIII effectiveness is highly dose dependent (Abed-Elmdoust *et al.* 2015). Low concentrations of AFPI (5–150 μM) in winter flounder (*Pseudopleuronectes americanus*) enhanced survival rate of red blood cells, while a higher concentration (1.54 mM) was associated reduced survival (Carpenter & Hansen 1992). Qadeer *et al.* (2016) applied beetle (*Dendroides canadensis*) recombinant antifreeze proteins (DAFP) at 0.1, 1.0 and 10 μM to cryopreserved Nili-Ravi buffalo (*Bubalus bubalis*) sperm. A quantity of 10 μM concentration of DAFP in the extender improved motility and plasma membrane integrity after freeze/thawing, and yielded numerically higher, although statistically nonsignificant, *in vitro* cleavage and *in vivo* fertility rate compared to the cryopreserved sperm without DAFP. Koshimoto and Mazur (2002) reported decreased survival of mice spermatozoa cryopreserved with AFPI and AFPIII, as well as AFGP, at concentrations of 1–100 μM. Antifreeze protein I or AFGP at 0.1 μM and >10 μM significantly decreased ram spermatozoon motility after chilling to 5 °C (Payne *et al.* 1994).

In addition to stabilizing the cell membrane, a well-recognized capacity of AF(G)Ps is to bind to, and inhibit the growth of ice crystals. Antifreeze proteins and antifreeze glycoproteins are called ice structuring proteins, since they are capable of binding to non-basal surface planes on the crystals (Goddard & Fletcher 2002) and inhibiting further crystal growth in specific directions, altering ice morphology (Clarke *et al.* 2002) and reducing damage to spermatozoa caused by ice crystals (Table 2). The mechanism of antifreeze proteins on ice crystallization is to inhibit the ice nucleation process by adsorbing onto both the surfaces of ice nuclei and dust particles (Du *et al.* 2003). Antifreeze protein type I was demonstrated to alter ice-crystal structure following freezing and increase osmotic resistance in spermatozoa after thawing, reducing mechanical stress to the cell membrane and consequently increasing fertilization capacity of bovine sperm (Prathalingam *et al.* 2006). Antifreeze proteins and antifreeze glycoproteins of polar fish and insects were found to adsorb to ice surfaces and inhibit their growth; hence, addition of insect AF(G)Ps can increase antifreeze effects (Raymond & DeVries 1977; Drori *et al.* 2015). Low concentrations of AF(G)Ps were reported to be remarkably effective in preventing ice recrystallization (Knight *et al.* 1984).

The knowledge of AF(G)Ps effects on fish sperm during cryopreservation is still rudimentary. They appear to display both protective and cytotoxic actions in spermatozoa

Table 1 The protective effect of antioxidants on cryopreserved sperm

Species	Antioxidant	Concentration	Success						References
			Motility	Velocity	Membrane integrity	post-thaw fertility	DNA fragmentation	Duration of motility	
Brook trout (*Salvelinus fontinalis*)	Oxidized and reduced glutathione	1.5 mM	B	A	B	B	A		Lahnsteiner *et al*. (2011)
	Reduced methionine	1.5 mM	B	A	B	B	A		
	Catalase	100 U L^{-1}	B	C	B	B	B		
Gilthead seabream (*Sparus aurata*)	Taurine	1 mM	B	B	B		A		Cabrita *et al*. (2011)
		10 mM	B	B	C		A		
	Hypotaurine	1 and 10 mM	B	B	B		A		
European seabass (*Dicentrarchus labrax*)	α-tocopherol	0.1 and 0.5 mM			B		C		
	Ascorbic acid	1 and 10 mM			B		C		
	α-tocopherol	0.1 mM	A	A	B				Martínez-Páramo *et al*. (2012)
	Ascorbic acid	1 mM	A	A	B				
	taurine	1 mM	B	A	B		A		Martínez-Páramo *et al*. (2013)
	hypotaurine	1 mM	A	B	B		A		
Rainbow trout (*Oncorhynchus mykiss*)	Taurine	50–70 mM	B					B	Ekici *et al*. (2012)
	Uric acid	0.25 mM	A	C		B		A	Kutluyer *et al*. (2014)
	L-methionine	1.5 mM	A	B		B		A	
	Superoxide dismutase	250 U L^{-1}	A	A		B		A	
	Carnitine	0.5 mM	A	B		B		B	
	α-tocopherol	2.0 mM	A	B		B		A	
	Reduced glutathione	1.5 mM	A	B		B		A	
Curimba (*Prochilodus lineatus*)	Ascorbic acid	0.001 mM	B					A	Navarro *et al*. (2014)
scaly carp (*Cyprinus carpio*)	Taurine	75 mM	A			A		A	Yavas *et al*. (2014)
Goldfish (*Cyprinus auratus*)	L-methionine	1.5 mM	A				A	A	Kutluyer *et al*. (2015)
Red seabream (*Pagrus major*)	Taurine	50 mM	A	B	A			A	Liu *et al*. (2015)
Common carp (*Cyprinus carpio*)	Cysteine	20 mM	A			B	A	A	Öğretmen *et al*. (2015)
Beluga (*Huso huso*)	Glutamine	2.5–20 mM	A			A		A	Aramli and Nazari (2016)
Persian sturgeon (*Acipenser persicus*)	Ascorbic acid	2.5, 5 and 10 μM	B					B	Shaluei *et al*. (2017)
Atlantic salmon (*Salmo salar*)	α-tocopherol and ascorbic acid	0.1 and 1 mM	A			A			Figueroa *et al*. (2018)
		1 and 10 mM	A			A			

A: significantly positive effect on sperm cryopreserved with antioxidants compared to sperm cryopreserved without antioxidants; B: no significant effect on sperm cryopreserved with antioxidants compared to sperm cryopreserved without antioxidants; C: significantly negative effect on sperm cryopreserved with antioxidants compared to sperm cryopreserved without antioxidants; BSA, bovine serum albumin.

Table 2 Protective effect of antifreeze proteins on sperm during short-term storage and cryopreservation

Species	AF(G)Ps	Concentration	The effect of AF(G)Ps on sperm	References
Middle-Russian Carp (*Cyprinus* sp.)	AFGP	20 mM	Increase spermatozoon motility	Karanova *et al*. (1997)
Sea bream (*Sparus aurata*)	AFPIII	1 μM	Increase spermatozoon motility and stabilize the plasma membrane organization	Beirão *et al*. (2012)
		0.1, 1 and 10 μM	Increase spermatozoon motility, viability, and reduce protein changes in spermatozoon plasma membrane	Zilli *et al*. (2014)
Persian sturgeon (*Acipenser persicus*)	AFPIII	10 μM	Increase spermatozoon motility	Abed-Elmdoust *et al*. (2015)

AFPIII, Type III antifreeze protein; AFGP, antifreeze glycoprotein.

at low storage temperatures. The effect depends on the protein used, dose, storage protocol, composition and concentration of cryoprotectant, and features of spermatozoa of the species (Wang 2000). Application of AF(G)Ps in fish sperm cryopreservation is an important field of study in artificial fish reproduction.

Other proteins and their effects

Impact of other proteins on quality of frozen sperm has been investigated. Cabrita *et al*. (2001a) evaluated combinations of BSA, egg yolk and soy protein complex (DanPro S760) mixed with freezing extender on cryopreserved rainbow trout sperm quality and fertility. Freezing extender with BSA and egg yolk provided the best resistance to hypo-osmotic shock, whereas higher fertility rates were obtained with a soy protein complex (DanPro S760), alone and in combination with BSA (Cabrita *et al*. 2001a). A similar study in Persian sturgeon found supplementation with 10 mM BSA in freezing medium to be associated with increased motility in cryopreserved sperm (Shaluei *et al*. 2017). Kumaresan *et al*. (2006) argued that incorporation of oviductal proteins into the extender reduced the lipid peroxidation levels in Murrah buffalo spermatozoa during cryopreservation and improved post-thaw sperm quality. A commercial cell antioxidant supplement (Sigma, A1345) applied in cryopreservation of gilthead seabream and European seabass (Cabrita *et al*. 2011) was associated with lower DNA fragmentation in gilthead seabream but showed no positive effects on DNA fragmentation in the seabass or in spermatozoon motility, velocity or viability in either species (Cabrita *et al*. 2011). Information on protective effects of other proteins on cryopreserved sperm is lacking and is a candidate for future research.

Conclusion and future perspectives

This review will help to better understand the progresses and the obstacles of fish artificial reproduction, and provide bases for carrying out studies to gain an understanding of the effects of cryopreservation on the morphology, physiology and function of fish spermatozoa. The alteration of sperm molecules including DNA, phospholipids and proteins and, consequently, their related subcellular compartments during cryopreservation is at least partially responsible for reduced spermatozoon function and quality. Currently, studies exploring protein alterations of fish spermatozoa during cryopreservation are not comprehensive, especially with respect to the mechanisms by which cryoinjury causes degradation of proteins and resulting effects on cell function. Further research using cutting-edge cellular and molecular techniques such as omics is needed to clarify precise roles of the affected molecules in their related subcellular compartments and in functions such as activation, egg–sperm fusion, fertilization and embryo development. Recent studies have primarily focused on acquiring better quality of post-thaw spermatozoa and optimizing sperm storage techniques by supplementation of the storage medium with seminal plasma proteins, antioxidants, or AF(G)Ps. The molecular basis of the protective mechanisms of these proteins during cryopreservation is still unclear, and future studies of their function will contribute to knowledge and improvement of fish semen cryopreservation.

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CHAPTER 2

PROGRESS AND CHALLENGES OF FISH SPERM VITRIFICATION

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Progress and challenges of fish sperm vitrification: A mini review

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ABSTRACT

To survive low temperature is required for a long-term storage (cryopreservation), cells should be vitrified to a state in which intracellular water is solidified without ice crystal formation. Two different approaches are described for fish sperm cryopreservation: 1) sperm conventional cryopreservation, in which extracellular water is partially crystallized and 2) sperm vitrification, in which both intra- and extra-cellular liquids are vitrified. Sperm vitrification has been applied to some fish species with limited success. Traditional vitrification requires rapid cooling/warming rates, small sample carriers, and using high permeable cryoprotectant concentrations. The latter cause cytotoxic effects which must be well managed and will require continuous effort to match an appropriate cryoprotectant with suitable apparatus and warming methods. Novel cryoprotectant-free sperm vitrification approach has been applied to several fishes. This review summarizes development of basic procedures and discusses advantages and disadvantages of vitrification when applied it to fish sperm.

1. Introduction

Vitrification has become an increasingly popular method of preserving the cells and tissues by using permeable cryoprotectant concentrations which will develop an amorphous glassy state, while preventing intracellular and extracellular ice crystallization [1,2]. First reported in 1937, there has been recent renewal of interest after a long latent period [3]. During the last decade, scientists keep their attention on human sperm vitrification without any permeable cryoprotectants [4]. Researchers feel that vitrification might offer the capability to cryopreserve cells using simple and fast procedures needing no specialized equipment [4,5].

Successful vitrification of fish gametes requires high concentrations of permeable cryoprotectants, and a rapid temperature change. Vitrification has been applied for fish primordial germ cells [6], oocytes [7], eggs [8], testicular tissues [9] and embryos [10]. Spermatozoa were the first mammalian cell to be cryopreserved by slow-cooling using glycerol as a cryoprotectant [11]. The use of vitrification on fish spermatozoa is relatively a new application. Several scientists have tested vitrification on fish sperm; they have mainly focused on permeable cryoprotectant toxicity at various concentrations, exposure times, and temperatures [12,13]. Traditional vitrification on spermatozoa has been tested with limited success on: Russian sturgeon *Acipenser gueldenstaedtii* [14], Persian sturgeon *A. persicus* [15], rainbow trout *Oncorhynchus mykiss* [16], channel catfish *Ictalurus punctatus* [17], green swordtail *Xiphophorus hellerii* [18], spotted seatrout *Cynoscion nebulosus*, red snapper *Lutjanus campechanus*, red drum *Sciaenops ocellatus* [19], Atlantic salmon *Salmo salar* [20], Tambaqui *Colossoma macropomum* [21], Eruasian perch *Perca fluviatilis*, and European eel *Anguilla anguilla* [22], while only few studies carried out on fish sperm by cryoprotectant-free vitrification [23,24].

The aim of this review is to (1) summarize the basic procedures of vitrification of fish sperm (2) discuss the current progresses in vitrification application for fish spermatozoa (3) compare the advantages and disadvantages of vitrification, and (4) to provide

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recommendations for the future research.

2. Vitrification approaches

The basic sperm vitrification procedures are to suspend the spermatozoa in a vitrification solution and then plunge the sample into liquid nitrogen to obtain a vitreous transparent state [25]. Success for fish spermatozoa by traditional vitrification depends on several factors, including high quality sperm, suitable cryoprotectants, equilibration time, and cooling and warming rates [26]. To prevent intracellular ice crystal formation, high concentrations of permeable cryoprotectants and rapid cooling rates are important [12]. Also the temperature and equilibration time need to be carefully controlled to reduce chemical toxicity, this is particularly critical because high concentrations of cryoprotectants are used [27]. For cryoprotectant-free vitrification, osmotic stress and chemical toxicity should be avoided and extremely high cooling rate is necessary to reduce ice crystal formation.

2.1. Development of traditional vitrification solutions

Most gametes will not survive cryopreservation without cryoprotectants, which reduce cryo-damage and protect them from ice crystallization [28]. Two forms of cryoprotectants are classified according to their capacity to penetrate the plasma membrane. Dimethyl sulfoxide (DMSO), glycerol (Gly), ethylene glycol (EG), methanol (MeOH), and propylene glycol (PG) are permeating cryoprotectants. They increase viscosity within the cell, thereby preventing water molecules to form ice crystals [29]. Non-permeating cryoprotectants include sucrose, albumins, dextran, egg yolk, hydroxyethyl, and polyethylene glycols. These cryoprotectants prevent cellular damage caused by freeze-thaw events, like crystallization and recrystallization [30]. Vitrification solutions should be considered relative to toxicity of the permeable cryoprotectants, the role or effect of temperature, the interactions between various intracellular biomolecules and membranes, inclusion of serum proteins, osmolytes, and buffers as supplements to the cryoprotectant solution that promote vitrification and protect the cells against cryoinjury [30]. Salinity of an extender can play an important role in sperm protection [26]. Multi-component salt media are effective for cryopreservation of salmonid spermatozoa [31]. Additionally, the extender composition with certain osmolality and pH can affect the sperm activation and motility in several fish species [32].

High molecular weight additives, disaccharides, can significantly reduce the required concentration of permeable cryoprotectant without passing through the cell membrane, as well as the reduction of toxicity of permeable cryoprotectants [33,34]. Sucrose benefiting plasma membrane of cells during cryopreservation [35], while trehalose can enhance glass formation [34]. Good post-thaw fertility was obtained by adding 0.6 M sucrose to semen of Asp *Aspius aspius* [36]. Similarity, high fertilization rates (82%) were acquired with thawed sperm of orange-spotted grouper *Epinephelus coioides* by adding trehalose into cryopreservation solution [37]. However, in contrast, sucrose was ineffective when added to the vitrification solution for rainbow trout spermatozoa [23]. Isachenko et al. [38] showed that putting sucrose and 1% human serum albumin into the vitrification medium of human sperm had a visible cryoprotective effect on mitochondrial membrane integrity.

Permeating cryoprotectants with low molecular weight, are widely used in the cryopreservation of fish spermatozoa [13,39]. Dimethyl sulfoxide is the most common cryoprotectant used for sperm cryopreservation of marine fishes because of the fast cell permeability which is little affected by temperature [40,41]. The basic requirement of vitrification is to determine the effective concentration of permeable cryoprotectants that is needed to form glass (Table 1). Glass formation can be distinguished by the appearance after plunging samples into liquid nitrogen as the sample remains transparent while the sample becomes milky white if crystallization occurs [30]. A high concentrations of permeable cryoprotectants is required to reach vitrification, consequently, toxicity is an obvious consideration in the selection of permeable cryoprotectants. Most permeable cryoprotectants are toxic and have hypertonic effects at concentrations needed for vitrification [42]. Also the reaction to a permeable cryoprotectant differs from one species to another (Table 2). An experiment performed in a marine fish demonstrated that ethylene glycol has the lowest toxicity followed by dimethyl sulfoxide [19], but Wayman et al. [43] found that dimethyl sulfoxide was the least toxic cryoprotectant and glycerol was among the most toxic.

Mixtures of several permeable cryoprotectants and stepwise addition have been used to reduce toxicity but attain the necessary concentration [44,45]. In general, reduced toxicity of permeable cryoprotectant mixtures has been explained by vitrification that occurs at lower concentrations and an aggregate effect of the combined properties of each permeable cryoprotectant [30]. One of the most commonly used cryoprotectant mixture for vitrification in mammalian gametes is dimethyl sulfoxide and ethylene glycol [46]. Dimethyl sulfoxide is a better glass former, while ethylene glycol is less toxic and permeates faster than dimethyl sulfoxide [46,47]. Average post-thaw motilities of 44% for spotted seatrout, 43% for red snapper and 20% for red drum were reported with a combination of dimethyl sulfoxide and ethylene glycol, along with trehalose [19]. Similar results were also achieved on Atlantic salmon spermatozoa by using a combination of a standard buffer (Cortland medium), 10% dimethyl sulfoxide, 2% bovine serum albumin (BSA), 0.13-M sucrose and 30%, 40%, and 50% of seminal plasma [20]. Employing acetamide in the vitrification of fish embryos and sperm achieved little success [19,48]. In addition, after evaluating three different addition methods, Cuevas-Uribe et al. [17] suggested that there is no advantage in adding the

Table 1
Appearance of cryoloop and droplets containing different concentrations of cryoprotectants after direct plunging into liquid nitrogen.

Cryoprotectant		Cryoloops (mm)			Droplet
		2	3	4	
methanol (MeOH)	10% MeOH	M	M	M	M
	20% MeOH	M	M	M	M
	30% MeOH	M	M	M	M
	40% MeOH	T	T	T	M
ethylene glycol (EG)	10% EG	M	M	M	M
	20% EG	M	M	M	M
	30% EG	M	M	M	M
	40% EG	T	T	T	I
dimethyl sulfoxide (DMSO)	10% DMSO	M	M	M	M
	20% DMSO	M	M	M	M
	30% DMSO	M	M	M	M
	40% DMSO	T	T	T	T
propylene glycol (PG)	10% PG	M	M	M	M
	20% PG	M	M	M	M
	30% PG	M	M	M	M
	40% PG	T	T	T	T
20% (MeOH)+ 20% (EG)		T	T	F	T
20% (MeOH)+10% (EG)+ 10% (PD)		T	T	F	T
5% (MeOH)+10% (EG)+ 20% (PD)		T	T	T	T

Note: M, milky; T, transparent; I, intermediate; F, fail to handle. 1, 2, 3 represent different size of cryoloops with diameter of 2 mm, 3 mm, 4 mm, individually; while droplets performed with 7 μL.

Table 2
Summary of studies on sperm vitrification of different fish species.

Fish species	Cryoprotectant	Volume	Warming rate	Vitrification success			Reference
				Motility	Membrane integrity	Fertilization	
Atlantic salmon *Salmo salar*	Cortland®+10%DMSO +2%BSA+ 0.13M Suc+50% seminal plasma	30 μL	37 °C for 45 s	44%	47%	46%	[18]
Eurasian perch *Perca fluviatilis*	15%MeOH+15%PG	2 μL Cryotop	Room temperature	14%	–	2%	[20]
European eel *Anguilla Anguilla*	20%MeOH+20%PG+10% Foetal Bovine Serum	2 μL Cryotop	Room temperature	5%	–	–	[20]
Green swordtail *Xiphophorus hellerii*	20%Gly+20%EG	10–15 μL	24 and 37 °C	8%	6%	5 of 10	[16]
	15%DMSO+15%EG+10%Gly + XZ	10–15 μL	24 and 37 °C	58%	19%	–	
Persian sturgeon *Acipenser persicus*	10 μM fish antifreeze protein type III	Drop 5 mm diameter	37 °C for 5–10 min	16%	–	–	[13]
Rainbow trout *Oncorhynchus mykiss*	Cortland®+10%DMSO+2%BSA+ 0.13M Suc+40% seminal plasma	30 μL	37 °C for 60 s	97% Viability	98%	31%	[14]
	None (Cortland® medium+ 1%BSA+40% seminal plasma)	20 μL	37 °C for 30 s	81%	84%	–	[21]
	None (Cortland® medium)	20 μL	37 °C for 30 s	86%	30%	–	[22]
Red drum *Sciaenops ocellatus*	10%DMSO+30%EG+0.25M Tre	10 μL	24 °C for 10 s	17%	19%	–	[17]
	15%DMSO+15%EG+10%Gly + XZ	10 μL	24 °C for 10 s	27%	20%	–	
Red snapper *Lutjanus campechanus*	10%DMSO+30%EG+0.25M Tre	10 μL	24 °C for 10 s	43%	22%	–	[17]
	40%EG+0.25M Tre	10 μL	24 °C for 10 s	40%	12%	–	
Russian sturgeon *Acipenser gueldenstaedtii*	10% DMSO	10 μL	34-36 °C for 20–40 s	12%	–	90%	[12]
Southern flounder *Paralichthys lethostigma*	20%Gly+20%EG	10 μL	21 and 37 °C	13%	11%	12%	[86]
Spotted seatrout *Cynoscion nebulosus*	10%DMSO+30%EG+0.25M Tre	10 μL	24 °C for 10 s	44%	22%	–	[17]
	15%DMSO+15%EG+10%Gly + XZ	10 μL	24 °C for 10 s	58%	19%	–	
Tambaqui *Colossoma macropomum*	5, 10, 15 and 20% DMSO	0.25 mL straws	45 °C for 5 s	0%	10%	7%	[19]

The cryoprotectants used were: bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol (Gly), methanol (MeOH), propylene glycol (PG), sucrose (Suc), Trehalose (Tre), X-1000™ (X), and Z-1000™ (Z).

cryoprotectants in successive steps.

2.2. Non-equilibration time

Generally, in order for permeable cryoprotectants to penetrate into the cells, some equilibration time (10–20 min) is needed before freezing. However, this usually results in a decrease of the motility after thawing [49]. Equilibration time plays a key role in the toxicity of permeable cryoprotectants. One method to reduce the required concentration of single permeable cryoprotectants is to limit exposure time at high concentrations to a minimum [17]. After 10 min in a high permeable cryoprotectant concentration, most of spermatozoa were immotile. Likewise, it has been shown that long equilibration time is toxic for mammalian oocytes and embryos [50]. Spermatozoa have low tolerance for high concentrations of permeable cryoprotectants, because of lethal osmotic effects and chemical alterations [33]. Thus, a method of non-equilibration during vitrification is vital for viability of fish spermatozoa after freezing and thawing.

2.3. Development of cryoprotectant-free vitrification

Considering all information from past trials of traditional vitrification, nowadays researchers prefer to vitrify fish sperm without permeable cryoprotectants because of the resultant hyperosmoticity and toxicity [20,39]. The entire process of cryoprotectant-free vitrification takes only a few seconds by direct plunging of a sperm suspension into liquid nitrogen. After storage, the vitrified samples are warmed at higher rate [51,52]. However, a disadvantage of cryoprotectant-free vitrification is that the small volume of spermatozoa can be processed to increase the cooling/warming rate and surface-to-volume ratio of the sample [53]. Cryoprotectant-free vitrification of rabbit sperm were showed very low survival of spermatozoa, but could be improved by enriching bovine serum albumin (BSA) together with sucrose or trehalose [54]. Furthermore, studies on human sperm showed that vitrification with a non-permeable cryoprotectant (protein or in combination with sucrose) provides a high recovery rate of motile cells and effectively protects mitochondrial membrane and DNA integrity after warming [38,51,53]. In addition, cryoprotectant-free vitrification is still in infancy for fish sperm. Only a few studies were successfully conducted on rainbow trout sperm using 1% of BSA and 40% of seminal plasma by droplets [23,24] (Table 2). Based on the progress achieved in human and rainbow trout sperm, cryoprotectant-free vitrification of sperm is supposed to develop in the near future for other fishes.

2.4. Types of apparatus and technical aspects of vitrification

Decisive factors in successful vitrification is the avoidance of intracellular crystallization and toxicity. To achieve this goal, the volume of vitrification solution should be kept to a minimum so as to increase cooling/warming rates and to use specially designed carriers such as open-pulled straws [55], copper loop [51,56], droplets [16], pellets [38], glassy cell layers [14], nylon cryoloops [57], Cryovials [23], and aluminum boats partially immersed in liquid nitrogen, known as solid-surface vitrification [58].

Open-pulled straws (OPS) are made from a 0.25 mL French mini-straws which are heat-softened over a hot plate and drawn out manually, then cut at the narrowest point after cooling in air [55]. The major benefit of these narrow tubes is that they are safe and easy to manipulate. The development of aseptic vitrification techniques allows freezing 5–10 μL of sperm suspension in OPS placed inside of insemination straws (straw-in-straw method) [59].

Nichrome, polystyrene or copper cryoloops are other options. During vitrification, a thin solution film is applied to the hole of the loop and the semen is loaded onto this film. The film remains intact during immersion in liquid nitrogen. Although the system seems to

be fragile and sensitive, it has been widely used in fish sperm cryopreservation [19,60]; it is easy to apply but the small volume is limiting.

Compared to cryoloops or droplets, a relatively large sample volume can be placed onto copper foil which was previously cooled in vapor of liquid nitrogen to 160 °C or direct into liquid nitrogen. The size of the drop on the solid surface should be minimized so as to obtain the optimal cooling rate. After 5 min of cooling, the solidified droplets of sperm samples were placed into cryovials which had been pre-cooled in liquid nitrogen; the sample can be stored in liquid nitrogen until the time of use [24,61].

If the permeable cryoprotectant concentration is lowered, cooling and warming rates must be increased, this can be achieved by decreasing the volume of the suspension and increasing the surface-to-volume ratio of the sample [51]. Different carriers have been tested to cryopreserve fish sperm during vitrification; guiding principles are that carriers should be simple, easy to use and have small volumes to obtain vitreous state. Choosing suitable apparatuses is vital for successful sperm vitrification with low toxicity and high survival.

2.5. Cooling and warming

To achieve 'glassy solid state', both crystallization and recrystallization ice formation is eliminated during the whole vitrification process. The "critical cooling rate" is the rate at which appreciable ice formation is not observed [62]. On the other hand, the "critical warming rate" is the warming rate that completely or sufficiently suppresses ice formation during warming [62]. Using a high cooling rate to make possible lower permeable cryoprotectant concentration and thereby decrease the damage caused by osmotic stress and chemical toxicity [29]; it is equally essential to use a quick warming rate during fish sperm vitrification. Although it is reported that embryos and oocytes are not harmed by recrystallization significantly [63], but recrystallization will takes place and damage spermatozoa cells with fast freezing and slow warming [14]. Also it is known that recrystallization can result in the conversion of relatively innocuous small ice crystals into larger and more damaging ice crystals [64]. Thus, cooling and warming rate should be treated seriously for fish sperm vitrification.

3. Vitrification of sperm

Cryopreservation of fish sperm by vitrification is still in a developmental period. The first report of sperm vitrification was done by Luyet and Hodapp [65], who used sucrose to dehydrate frog sperm prior to immersion in liquid air (−192 °C). After the discovery of glycerol as cryoprotectant [11], studies on vitrification were sporadic and results varied, mainly with low viability [66]. The high concentrations of permeable cryoprotectants needed to achieve vitrification were believed to be extremely toxic to the sperm [67]. However, a breakthrough came in 2002, when human spermatozoa were vitrified with no conventional cryoprotectants; samples were frozen in copper cryoloops [56]. Successful vitrification of human spermatozoa also has been achieved with no permeable cryoprotectants [38,51,59,68]. Nevertheless, few studies have frozen fish sperm by vitrification with no cryoprotectant. The first report of successful cryoprotectant-free vitrification of fish sperm was done by plunging samples directly into liquid nitrogen [23]. A similar technique was done on channel catfish; sperm without cryoprotectant was frozen in cut standard straws, yielding fertilization of <2% in two of sixteen trials [17]. A encouraging result that 31% fertilization of spermatozoa vitrification without significant loss of important physiological characteristics was achieved on sex-reversed rainbow trout in a medium containing 10% DMSO + 2% BSA + 0.13 sucrose + 50% seminal plasma by droplet [16].

It is generally recognized that sperm from marine fishes have a higher quality following vitrification compared with freshwater species [19]. It is largely because exposure of fish sperm to high cryoprotectants concentrations can damage spermatozoa by chemical toxicity and osmotic effects such as changes in plasma membrane integrity, inner or outer mitochondrial membrane, and nuclear DNA [13,69]. The mitochondrial damage, altering the biochemical process involved in ATP production, can cause a reduction in sperm motility and fertilization ability [13]. Sperm from freshwater fishes are not generally adapted to deal with high osmotic pressures, as they typically become immotile in response to increased osmotic pressure. Sperm of marine fishes respond in the opposite manner, with motility being activated by increased osmotic pressure [70].

4. Advantages and disadvantages of vitrification

All cryopreservation methods, including vitrification, expose tissues and cells to an environment that they would not normally experience and have no intrinsic genetically coded capacity to survive [71]. Therefore, the challenge is to establish a process where the injuries are minimal and defensive-regenerative capacities are supported [71]. Vitrification usually has been achieved with a drastic elevation of permeable cryoprotectant concentration, along with an increased cooling/warming rate. Complete vitrification eliminates concerns for the known damaging effects of intra and extracellular crystallization, and vitrification can decrease chilling injury to fish spermatozoa, also fish sperm can be vitrified with the need for equilibration time. Further, vitrification requires no specialized equipment which provides more user-friendly techniques, especially useful for on-farm procedures and field work at remote sites [72]. Conversely, this technology faces a number of significant challenges for commercial deployment.

4.1. Elimination of ice crystal formation

As we know, successful vitrification can reduce ice crystallization, with a high solute concentration and cooling rate, it is possible to progress to the glass transition temperature without significant ice formation [45]. Vitrification is typically achieved by partial replacement of intracellular water with permeable cryoprotectants, which readily form (glass), and by drawing out the intracellular water via non-permeating cryoprotectants. During cryoprotectant-free vitrification, practically it is very hard to avoid ice formation because of being unable to reach sufficient high cooling rate to vitrify and devitrify of cell. The cooling rate of vitrification could be hampered by Leidenfrost effect, since the vapor layer formed around the sperm sample insulates it from the cold liquid nitrogen [73]. Fortunately, high cooling/warming rate vitrification can be achieved by the application of slush nitrogen [74] and minimal carrier device (minimum sperm volume and maximal the surface area). Ice crystals reduce survival of spermatozoa not only due to their growth to critical size in the process of crystallization and recrystallization, but also because of various modification of ice crystal [14]. The good results on sperm by cryoprotectant-free vitrification could be due to non-lethal ice (i.e. cubical vs. hexagonal "killer ice") which is formed during cooling/warming [4]. According to our present knowledge, the practical approach for achieving better post-thaw sperm motility by vitrification is to increase the cooling/warming rates and decrease the volume of the solution [33,38,51,59,68], as well as keep the cryoprotectant concentration at a level which could be tolerated by spermatozoa [17].

4.2. Reduction of chilling injury

Another benefit of vitrification is the possibility to decrease dramatically chilling injury. Chilling injury is observed both in nature, at temperatures above 0 °C [75,76], and in the laboratory conditions, at temperatures well below zero [77,78]. Chilling injury is observed during slow cooling; rapid cooling can counter this problem if the system is not subject to injury from thermal shock [64]. Chilling injury may result from protein denaturation based on protection by prior heat shock [79], both of the production of heat shock proteins and cold shock proteins in response to chilling [79,80]. During vitrification, the rate at which samples pass through the dangerous temperature zones (15 to −5 °C) determines chilling injury; a rapid transition decreases the chilling injury [71]. So cells may be rapidly cooled from a temperature at which chilling injury and cold shock play no role [29].

4.3. Challenges relating to permeable cryoprotectant

The high concentration of permeable cryoprotectant necessary to facilitate traditional vitrification are potentially toxic, and can kill cells by direct chemical toxicity, or indirectly by osmotically induced stress during addition or removal [81]. Therefore, use of permeable cryoprotectant is the most problematic procedure in vitrification; relatively sophisticated methods of adding and removing these agents and careful selection of the most suitable cryoprotectant is required [64]. Chemical toxicity of permeable cryoprotectant is temperature dependent, but in even subzero exposure does not assure safe use [81]. Osmotically induced stress depends on the properties of permeable cryoprotectant [24]. Therefore, a better understanding of permeable cryoprotectant molecular properties that cause cell injury is needed. Several approaches exist to toxicity of permeable cryoprotectants can be addressed through avoiding osmotic injury, employing cryoprotectant mixtures, maintaining temperature as low as possible, selecting an appropriate apparatuses, and minimizing exposure time [45]. The demonstration of the tolerance of sex-reversed rainbow trout to vitrifiable concentration of permeable cryoprotectant, indicated that the constraints of permeable cryoprotectant toxicity and fluxes can be overcome by selection and optimization of appropriate conditions [16]. Therefore, additional inquiry should be made on lowering permeable cryoprotectant and conducting vitrification without permeable cryoprotectant.

4.4. The potential danger of disease transmission

Another problem associated with vitrification is the potential for disease transmission. A wide range of infectious diseases can be transmitted via artificial insemination, including bacterial, viral and other diseases [82]. It has been demonstrated that there is much less danger of transmitting viruses or bacteria with embryo transfer than by artificial insemination [83]. The small size samples used in vitrification are fragile [71]. The main sources of infection may through straws and cryovials. Special caution should be paid to hygiene with rigorous disinfection of the collecting equipment used during collection of sperm [84]. One approach to eliminate all possible sources of infections is to separate the cooling and warming phase from storage, and to use a relatively low amount of clean liquid nitrogen for cooling, then to wrap the sample in a precooled sterile container and seal it hermetically before placing it into the common storage tanks [71].

5. Conclusion and recommendation for future research

Vitrification is a promising option for cryopreservation. Recently some technical improvements have been facilitated success in sperm processing of some fishes. Moreover, it is simple, inexpensive and can be easily performed. However, vitrification has had little practical impact on fishes. Perhaps because of the wide variety of carriers and different solution used for vitrification, it is difficult to standardize a single approach. In addition, low survival rates of fish sperm during vitrification has discouraged for practical application. Although, the recent application of sperm vitrification in other aquatic organisms, such as the white shrimp *Litopenaeus vannamei* have been demonstrated promising results [85]. We should understand that vitrification is a technology that must be deployed with care and diligence, if procedures are to become a more reliable strategy with simple and high commercial efficiency and better outcome.

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CHAPTER 3

IMPACT OF CRYOPRESERVATION ON STERLET, *ACIPENSER RUTHENUS* SPERM MOTILITY AND PROTEOME

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Impact of cryopreservation on sterlet, *Acipenser ruthenus* sperm motility and proteome

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ABSTRACT

Fish sperm cryopreservation is a well-established technique allowing for artificial insemination on a commercial scale. The extent of proteome alterations in seminal plasma and sperm due to cryopreservation, however, is not known. This study was conducted to evaluate the effect of cryopreservation on motility variables of sterlet *Acipenser ruthenus* sperm and to detect the differences in protein profiles of fresh and cryopreserved sterlet sperm and seminal plasma. Fresh sperm had 89 ± 3% motility and 160 ± 14 μm/s curvilinear velocity at 15 s post-activation. The motility rate of cryopreserved sperm (37 ± 5%) was less at 15 s post-activation. No difference (ANOVA; $P > 0.05$) in mean curvilinear velocity of fresh and cryopreserved sperm was detected. The protein profiles of seminal plasma and sperm were characterized using comparative proteomics to determine the influence of cryopreservation. Six altered protein spots in seminal plasma and thirteen altered spots in sperm were detected in fresh and thawed sperm. Subsequent protein characterization suggested that the proteins identified were involved in sperm metabolism, cytoskeleton, and stress response. The results broaden the understanding of the effects of cryopreservation and identify the proteins associated with cryo-injury. These data may help to determine the function of altered proteins and provide new insights into improving sperm cryopreservation.

1. Introduction

Sturgeon populations have drastically decreased due to intensive-fishing, habitat destruction, and water pollution, and sturgeon are classified as an endangered species by many international organizations (Billard and Lecointre, 2000). Sterlet *Acipenser ruthenus* is a common Eurasian sturgeon that survives in most parts of its historical distribution range, including rivers draining into the Black, Azov, and Caspian Seas; Siberia from Ob eastward to the Yenisei drainage areas; and in Danube estuaries. The sterlet has been listed

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as a vulnerable species (Gesner et al., 2010). Careful broodstock management and reliable information on its biology and biogeography are important for the development of sterlet aquaculture and conservation programs (Lenhardt et al., 2010; Shaliutina et al., 2012).

Cryopreservation is an important technique that allows sperm to be stored indefinitely especially for endangered animals (Sarvi et al., 2006; Martínez-Páramo et al., 2017). Sperm cryopreservation offers protection from the elimination of males by disease, natural disaster, or accidents such as oil spills. Cryopreservation also provides a stable supply of sperm for hatchery production, simplifies sperm transfer among hatcheries, is valuable in selective breeding programs, and provides material for research, including gene transfer. Cryopreservation methods for sturgeon sperm are established (Ciereszko et al., 1996), and modifications of the procedures for species-specific optimization have been proposed (Drokin et al., 1993). Even though there have been improvements in cryopreservation technology, the quality of frozen/thawed sturgeon sperm remains less than that of fresh sperm.

The success of cryopreservation depends not only on preserving the motility of the spermatozoon but on maintaining its metabolic function (Ciereszko et al., 1996). Freeze-thaw procedures can induce irreversible and lethal damage to sperm, impairing spermatozoon motility (Linhart et al., 2000), viability (Li et al., 2010b) and acrosome structure (Psenicka et al., 2008) and directly impact successful fertilization and embryo development (Suquet et al., 1998; Kopeika et al., 2003). A few studies on the effect of cryopreservation at the molecular level have reported sperm protein content to be affected in mammals (Mostek et al., 2017), including humans (Wang et al., 2014; Bogle et al., 2017). In fish, negative effects of cryopreservation on sperm proteins have been described in sea bass *Dicentrarchus labrax*, common carp *Cyprinus carpio*, and rainbow trout *Oncorhynchus mykiss* (Zilli et al., 2005; Li et al., 2010a; Nynca et al., 2015). The proteins are involved in a variety of molecular functions in sperm, which could result in the impairment of spermatozoon function and a decrease in sperm quality (Li et al., 2010a; Zilli et al., 2014; Dietrich et al., 2015). Current knowledge of protein variation in sturgeon sperm with respect to cryopreservation, however, is limited. The objectives of this study were to assess the effect of cryopreservation on spermatozoon motility variables in sterlet and compare the proteome of sperm and seminal plasma of fresh and frozen/thawed sperm.

2. Materials and methods

All experiments were specifically approved by the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany, based on the EU-harmonized Animal Welfare Act of the Czech Republic.

2.1. Reagents

All medium components were purchased from Sigma-Aldrich/Merck Life Science (Darmstadt, Germany) or Bio-Rad (Hercules, CA, USA) unless otherwise stated.

2.2. Fish, spermiation induction, and sperm collection

The research was conducted at the experimental station of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Six mature male sterlet (7 year s, 2.5–3.0 kg) were used. Prior to experimentation, fish were maintained in 4 m^3 hatchery tanks with water flow rate of 0.2 L/s, O_2 of 7.0 mg/L and water temperature 15 °C. Spermiation was induced by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at 4 mg/kg body weight. After 24 h, sperm were collected from the urogenital tract using a 4 mm catheter connected to a 20 mL plastic syringe.

2.3. Spermatozoon motility and velocity assessment

Tap water with 0.1% Pluronic F-127 (3 mOsm/kg) was used as the activating medium. Motility of sterlet sperm was recorded microscopically (UB 200i, PROISER, Spain) at optical phase-contrast condenser and an ISAS digital camera (PROISER, Germany) set at 25 frames/s. Recordings were stored on a hard disk in AVI format. Analyses of the sperm recordings were performed by the Integrated System for Semen Analysis software (PROISER, Valencia, Spain) at 15, 30, and 45 s post-activation. Computer-assisted sperm analysis (CASA) included spermatozoon curvilinear velocity (VCL, μm/s) and percent of motile cells (motility rate, %). Analyses were conducted in triplicate. All observations were performed at room temperature (21.1 °C).

2.4. Seminal plasma preparation

Sperm samples from six male were separately centrifuged at 300 × *g* 4 °C for 30 min followed by 10 min at 13,000 × *g* at 4 °C according to methods described by Li et al. (2010a). The supernatant (seminal plasma) was carefully collected and divided into two parts, for cryopreservation and for use as fresh seminal plasma. The pellets (spermatozoa) was collected to be used as fresh sperm.

2.5. Sperm cryopreservation

Sperm and seminal plasma from six sterlet males were frozen individually. Samples were diluted (1:1) in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris–HCl, pH 8.0, and 10% methanol (Glogowski et al., 2002, modified). The diluted seminal

plasma and sperm were loaded into 0.5 mL plastic straws (CRYO-VET, France) and immersed in liquid nitrogen vapor after 10 min equilibration. Samples were frozen 3 cm above liquid nitrogen in a Styrofoam box for 10 min and then plunged into liquid nitrogen. Thawing was performed in a water bath at 40 °C for 5 s. The thawed seminal plasma was stored at −80 °C as cryopreserved seminal plasma samples for later protein analysis, and the thawed sperm was centrifuged using the method of fresh sperm described above and the pellets was collected as cryopreserved sperm.

2.6. Preparation of protein samples

The pellets (sperm) from fresh and cryopreserved sperm were washed twice in sperm immobilization solution (IM) (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) by centrifugation at 7000 × *g* at 4 °C for 30 min. The pellets were re-suspended in protein extraction buffer (IM with 1% Triton X-100). The prepared samples were held for 1 h on ice, shaken every 10 min and subsequently centrifuged for 10 min at 13,000 × *g* at 4 °C. The supernatant was collected and diluted using columns (Thermo Fisher Scientific, USA) for desalting and exchanging buffer of protein samples, and stored at −80 °C as fresh sperm and cryopreserved sperm proteins samples, respectively. A bicinchoninic acid assay was used to determine the protein concentration in the samples, using the Infinite M200 photometer (Tecan, Switzerland).

2.7. 2D-PAGE separation

Spermatozoon and seminal plasma proteins were analyzed by 2D electrophoresis. Isoelectric focusing was performed on ReadyStrip IPG strips (pH 4–7 for seminal plasma, pH 3–10 for sperm, 7 cm length) using PROTEAN IEF (Bio-Rad). A total of 150 μg of protein in 130 μL rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 50 mM dithiothreitol, 0.4% Bio-Lyte 3–10 buffer) was applied to the IPG strip. The conditions for separation were active rehydration at 50 V for 14 h, and, for isoelectric focusing (IEF), 500 V for 1 h, 1000 V for 1 h, 3000 V 1 h (gradient), and 8000 V for 2 h (gradient). After IEF, IPG strips were equilibrated with a buffer containing 6 M urea, 29.3% glycerol, 2% SDS, 75 mM Tris–HCl pH 8.8, and 2% dithiothreitol for 15 min followed by a second step with a similar solution containing 2.5% iodoacetamide instead of dithiothreitol for a further 15 min. The strips were laid onto 12% SDS-PAGE gel by sealing with 0.5% agarose in the SDS-PAGE running buffer. After separation, protein spots were visualized by staining with Coomassie Brilliant Blue R-250. The gels were assessed and analyzed using Melanie 8.0 (Geneva Bioinformatics SA, Geneva, Switzerland).

2.8. In-gel digestion and mass spectrometry

The altered protein spots in fresh seminal plasma and sperm were excised from gels and de-stained by incubation in 200 μL of 200 mM $(NH_4)HCO_3$ and 40% (v/v) acetonitrile (ACN) for 30 min at 37 °C twice, according to a modified protocol of Hellman et al. (1995). Subsequently, the gel pieces were dried in a speed-vac (Labconco, Kansas City, MO, USA), rehydrated with 5 μL of 40 mM $(NH_4)HCO_3$ containing 100 ng modified sequencing grade trypsin, and incubated 16 h at 37 °C. After digestion, the resulting peptides were extracted twice with 20 μL of 40 mM $(NH_4)HCO_3$ and 50% (v/v) ACN. The protein digests were subsequently dried in a speed-vac and re-suspended in a mixture of 6 μL of 0.1% trifluoroacetic acid (TFA) and 30% (v/v) ACN prior to mass spectrometry (MS) analysis. For the spot preparation, aliquots of 1.3 μL were applied onto a ground steel plate, dried, overlaid with 0.8 μL of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 40% ACN, 0.1% TFA, dried, and subsequently recrystallized with 0.8 μL of 80% ACN, 0.1% TFA. The MS spectra of protein digests and tandem MS spectra of the 30 most abundant peptides were obtained using an Autoflex Speed matrix-associated laser desorption-ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) instrument (Bruker Daltonics, Bremen, Germany). Prior to sample spectra acquisition, optimal laser power and number of shots per MS and MS/MS were determined.

Further data processing used Flex Analyses software (Bruker Daltonics), applying the same peak detection and annotation parameters to all sample spots. Protein identification was performed by searching the extracted peak lists against the NCBInr database using the Mascot (Matrix Science, London, UK) search engine (MS/MS ion search, 1 mis-cleavage, precursor tolerance ± 0.1 Da, fragment mass tolerance ± 0.5 Da).

2.9. Data analysis

Statistical comparison was made by analysis of variance (ANOVA) followed by Tukey's HSD test for each variable analyzed. All analyses were performed at a significance level of 0.05 using SPSS 15.0 for Windows.

3. Results

3.1. Spermatozoon motility variables

Direct observation of undiluted sperm indicated most sperm were immotile, with straight flagella and slightly quivering (Fig. 1). After dilution in activation medium, the spermatozoon in fresh samples had 89 ± 3% motility and VCL of 160 ± 14 μm/s at 15 s post-activation. Cryopreservation resulted in significantly lesser values for sperm motility variables. The motility rate recorded for cryopreserved sperm was 37 ± 5% at 15 s post-activation. No significant difference (ANOVA; $P > 0.05$) in VCL of fresh and cryopreserved samples, however, was detected at 15 s post-activation. There was a significantly lesser motility rate of cryopreserved

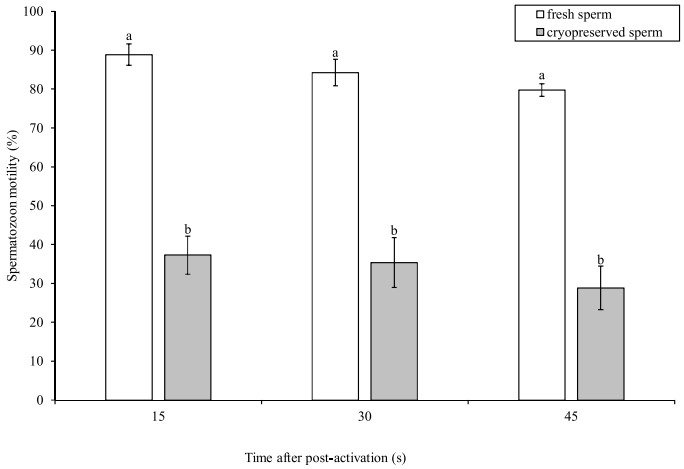

Fig. 1. Spermatozoon motility in sterlet *Acipenser ruthenus* before and after cryopreservation. Data are expressed as mean ± SD. Values with the same superscript are not different ($P > 0.05$).

sperm following the 30 and 45 s post-activation compared with fresh sperm. No significant decrease of VCL was observed in fresh and cryopreserved sperm following 30 and 45 s post-activation, except the cryopreserved samples at 30 s (92 ± 14 μm/s) and 45 s (77 ± 8 μm/s) post-activation compared to fresh samples at 15 s post-activation (Fig. 2).

3.2. Protein separation by 2-DE and gel imaging

Two-dimensional IEF/SDS-PAGE gels were processed on fresh and cryopreserved seminal plasma and spermatozoon samples. The intensity of several seminal plasma and spermatozoon proteins changed in fresh and cryopreserved samples (Figs. 3A, B and 4A, B). Six protein spots differed in cryopreserved seminal plasma compared to fresh: the staining intensity of Spots 1, 4, 5, and 6 was greater in fresh seminal plasma (Fig. 3A, B), while Spots 2 and 3 from the fresh seminal plasma were not detected in the cryopreserved seminal plasma (Fig. 3A, B). Isoelectric points (pI) of these spots were in the range 5.6 to 7 and molecular weights in the range of 25–45 kDa.

Proteins from thirteen altered spots were submitted to protein identification in the fresh sterlet sperm. Representative reference

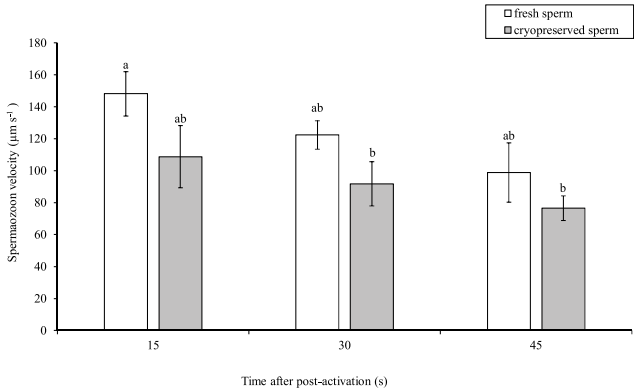

Fig. 2. Spermatozoon velocity in sterlet *Acipenser ruthenus* before and after cryopreservation. Data are expressed as mean ± SD. Values with the same superscript are not different ($P > 0.05$).

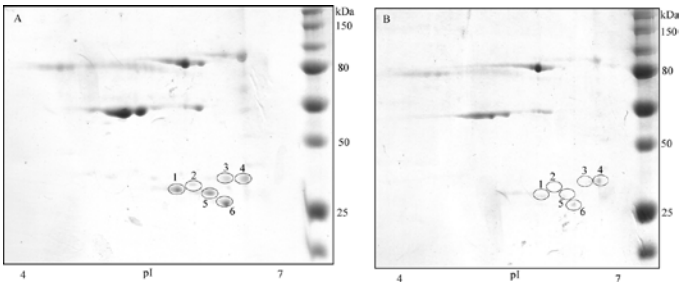

Fig. 3. Representative seminal plasma protein profiles before (A) and after freezing (B) of sterlet sperm. Each specimen was analyzed five times by two-dimensional gel electrophoresis. Circles show differential spots. Molecular weight marker is on the right. pI = Isoelectric points.

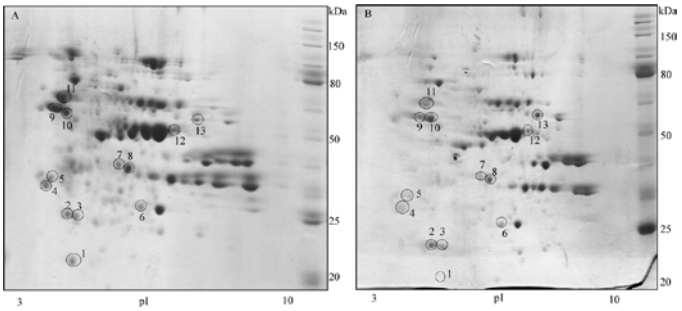

Fig. 4. Representative spermatozoon protein profiles before (A) and after freezing (B) of sterlet sperm. Each specimen was analyzed five times by two-dimensional gel electrophoresis. Circles show differential spots. Molecular weight marker is on the right. pI = Isoelectric points.

maps of spermatozoon proteins indicated the intensity of staining of protein Spots 2 through 12 was greater in the fresh sperm in comparison with cryopreserved samples (Fig. 4A, B). In contrast, the intensity of staining of protein in Spot 13 was greater in the cryopreserved sperm. Protein spot No. 1 was present in the fresh samples, but absent in cryopreserved samples (Fig. 4A, B). Spots 1–13 had a pI range of 3.5–8 and a molecular weight range of 20–78 kDa.

3.3. Identification of changed proteins

The six changed spots in seminal plasma and thirteen in sperm gels were excised and subjected to MALDI-TOF/TOF MS. In the six protein spots analyzed in seminal plasma, two proteins were identified (Table 1). Proteins identified in seminal plasma were the mitochondrial ATP synthase subunit α (Spot 1) and heat shock protein 70 (HSP 70) (Spot 6). Details of the proteins identified in fresh sterlet seminal plasma, together with the source organism, accession number, Mascot score, molecular mass, and sequence coverage are given in Table 1.

The amino acid sequence of six of the thirteen protein spots in gels of fresh sperm was positively determined (Table 2). These proteins were triosephosphate isomerase, glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic, ATP synthase subunit beta, mitochondrial, ATP synthase subunit beta, mitochondrial, tubulin beta chain, tubulin alpha chain, testis-specific, and enolase B. Proteins in Spots 1–5, 7 and 13 were not identified. A possible hit in Spot 7 was the Hoxa5 α transcription factor, however, with a very low MASCOT score. Results from these unidentified protein spots were compared with available protein sequences deposited in public databases; however, no positive sequence coverage for these protein spots was detected.

4. Discussion

In the present study, there were no significant differences in sperm curvilinear velocity associated with cryopreservation, while motility rate varied significantly. The use of MALDI-TOF/TOF MS allowed for identification of eight proteins that changed following cryopreservation in seminal plasma and sperm. Several types of proteins were not identified by MALDI-TOF/TOF MS, although the obtained spectra were of high quality, probably due to the incomplete genomic/proteomic information for sterlet and related fish in the databases. The sturgeon genome is also not completely annotated. The sturgeon species diverged from teleost hundreds of

Table 1
Proteins in sterlet *Acipenser ruthenus* seminal plasma identified by Mascot MS/MS Ion Search.

Spot no.	Protein name [organism]	UniProt accession No.	MASCOT Score	MW (kDa)/pI	Precursor ion m/z	Sequence
SP1↓	ATP synthase subunit alpha, mitochondrial [*Bos taurus*]	ATPA_BOVIN	116.25	59.72/ 9.21	892.488	LELAQYR
					1000.579	VLSIGDGIAR
					1026.595	AVDSLVPIGR
					1553.733	EAYPGDVFYLHSR
					1624.882	TGAIVDVPVGEELLGR
					1667.782	NVQAEEMVEFSSGLK 7: Oxidation (M)
					1683.772	
SP6↓	Heat shock protein 70 [*Huso dauricus*]	A0A0C5BIF3	74.51	71.13/ 5.28	989.54	LSKEDIER
					1228.64	VEIIANDQGNR
					1691.746	STAGDTHLGGEDFDNR
					2774.341	QTQTFTTYSDNQPGVLIQVYEGER

SP = fresh seminal plasma; Precursor ions selected for MS/MS are listed; Probability Based score is −10*Log (*P*), where *P* is the probability that the observed match is a random event; Scores greater than recorded significant hit were significant ($P < 0.05$); The MS/MS peak lists were searched against a NCBInr protein database using MASCOT™ software with the following settings: enzyme chemistry - trypsin, max missed cleavages 1, variable modifications: Oxidation (M), peptide mass tolerance ± 0.1 Da, and fragment mass tolerance ± 0.5 Da. ↓ indicates proteins with low intensity after cryopreservation.

millions of years ago. Sturgeons have different reproductive features compared to teleost such as several micropyles in eggs (Psenicka et al., 2010), acrosome in sperm (Golpour et al., 2016), and different amounts of ploidy (Havelka et al., 2017) that make it difficult to annotate the sturgeon genome using genomic data of well-known teleost such as zebrafish (Trifonov et al., 2016; Niksirat et al., 2017).

Motility is an important characteristic reflecting the quality of fresh as well as cryopreserved fish sperm (Billard, 1983) and generally, there is a positive correlation with fertilizing capacity (Rurangwa et al., 2004). In the present study, the fresh sperm had 89 ± 3% motile cells at 15 s post-activation. Cryopreservation was associated with significantly lesser motility. The greatest spermatozoon motility rate recorded for cryopreserved sperm was 37 ± 5% at 15 s post-activation. These data are consistent with those of Psenicka et al. (2008) and Boryshpolets et al. (2011). There was no significant difference in curvilinear velocity between fresh and cryopreserved sperm at any time post-activation. Similar results have been reported in lake sturgeon, *A. fulvescens,* after cryopreservation with dimethyl sulfoxide and sucrose (Ciereszko et al., 1996), with a velocity slightly greater in fresh, as compared with cryopreserved sperm. It is presumed that the decrease in sperm motility rate may be associated with membrane damage and alteration of some sperm proteins during cryopreservation.

At present, only a few proteins of sturgeon sperm, such as arylsulphatase, and a few phosphorylases for specific proteins involved in phosphorylation of molecules related to sperm motility have been identified (Sarosiek et al., 2004; Gazo et al., 2017). The protein variations induced by cryopreservation processing have not been reported. Research on cryopreservation effects on proteins in fish sperm has been based on the assumption that the freeze/thaw process induces alteration of proteins of spermatozoon (Dietrich et al., 2015). Zilli et al. (2014) reported that cryopreservation can lead to the loss of membrane proteins in fish spermatozoa. In the present study, seminal plasma was cryopreserved separately so as to detect the effects of cryopreservation on seminal plasma proteins and avoid the interference of proteins from damaged sperm. The results indicate there is a significant effect of cryopreservation on the seminal plasma proteome. The investigation revealed, for example, that the mitochondrial ATP synthase subunit alpha (Spot 1) in seminal plasma decreased after cryopreservation which could be associated with inadequate bioenergetics for providing ATP for spermatozoon movement (Zilli et al., 2014). This protein, associated with metabolism, has also been detected in the seminal plasma of infertile humans, and is associated with the amount of reactive oxygen species (Agarwal et al., 2015). Van Tilburg et al. (2013) reported that ATP synthase subunit was a sperm membrane-enriched protein in Morada Nova rams. ATPase and some other metabolic enzymes were present in seminal plasma of rainbow trout, but the presumption was that these proteins had leaked out of sperm (Lahnsteiner et al., 1998). The less visible spot of ATP synthase subunit in cryopreserved seminal plasma suggests that cryopreservation might damage this protein causing inactivation. In addition, if this protein originated from sperm, this effect could be important for sperm physiology because of the potential loss of this protein impacting cell motility.

Heat shock protein 70 (Spot 6) has been detected in a variety of species and cell types, such as boar sperm, oviduct, carp sperm and rainbow trout seminal plasma (Huang et al., 2000; Lloyd et al., 2012; Dietrich et al., 2014; Nynca et al., 2014). Its primary function is to protect cells from thermal and oxidative stress (Roberts et al., 2010). In addition, heat shock protein 70 is associated with cell immune, apoptotic, and inflammatory processes (Roberts et al., 2010). Relatively lesser amounts of heat shock protein 70 in sperm can negatively influence sperm quality (Huang et al., 2000; Zhang et al., 2015; Varghese et al., 2016). The heat shock protein 70 in seminal plasma could be involved in proteolytic degradation of proteins (Nynca et al., 2014).

Alterations in mitochondrial, cytoskeletal, nuclear, and cytosolic proteins of sperm after cryopreservation could damage sperm structure and alter function (Nynca et al., 2015). A large number of spermatozoon proteins are released as a result of cryopreservation (Nynca et al., 2015; Dietrich et al., 2015). In the present study, the staining intensity of several proteins was altered in fresh/frozen

Table 2
Proteins in sterlet *Acipenser ruthenus* spermatozoa identified by Mascot MS/MS Ion Search.

Spot no.	Protein name [organisms]	UniProt accession No.	MASCOTscore	MW (kDa)/pI	Precursor ion m/z	Sequence
S6↓	Triosephosphate isomerase [*Acipenser brevirostrum*]	Q90XG1	395.72	26.85/6.32	850.486	VVFEQTK
					954.492	FFVGGNWK
					1082.583	KFFVGGNWK
					1141.513	MGVAAQNCYK 8: Carbamidomethyl (C)
					1240.629	THVSEEVGGAVR
					1307.663	GAFTGEVSPAMIK
					1323.659	GAFTGEVSPAMIK 11: Oxidation (M)
					1340.681	IIYGGSVTAATCK 12: Carbamidomethyl (C)
					1368.745	SLGELIHTLNSGK
					1452.703	TASPQQAQEVHEK
					1458.721	HVFGESDELIGQK
					1602.884	VVLAYEPVWAIGTGK
					1614.78	DCGVSWVILGHSER 2: Carbamidomethyl (C)
					1864.995	VAHALQEGLGVIACIGEK 14: Carbamidomethyl (C)
					2239.114	INADTDVVCGAPTIYLDFVR 9: Carbamidomethyl (C)
S8↓	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic [*Homo sapiens*]	GPDA_HUMAN	82.97	37.57/5.81	1135.549	ELMQTPNFR
					1151.544	ELMQTPNFR 3: Oxidation(M)
					1646.855	KVCIVGSGNWGSAIAK 3: Carbamidomethyl (C)
					1997.93	NVVAVGAGFCDGLGFGDNTK 10: Carbamidomethyl (C)
S9↓	ATP synthase subunit beta, mitochondrial [*Cyprinus carpio*]	ATPB_CYPCA	212.27	55.24/5.05	941.54	VLDTGAPIR
					975.561	IGLFGGAGVGK
					1038.591	IPVGPETLGR
					1088.632	VVDLLAPYAK
					1262.635	TIAMDGTEGLVR
					1385.704	IMNVIGEPIDER
					1406.68	AHGGYSVFAGVGER
S10↓	ATP synthase subunit beta, mitochondrial [*Bos Taurus*]	ATPB_BOVIN	251.15	56.28/5.15	1038.587	IPVGPETLGR
					1262.634	TIAMDGTEGLVR
					1385.704	IMNVIGEPIDER
					1406.678	AHGGYSVFAGVGER
					1435.752	FTQAGSEVSALLGR
					1439.786	VALTGLTVAEYFR
					1601.81	VALVYGQMNEPPGAR
					1650.915	LVLEVAQHLGESTVR
					1921.962	DQEGQDVLLFIDNIFR
S10↓	Tubulin beta chain [*Paracentrotus lividus*]	TBB_PARLI	103.85	50.05/4.73	1028.514	TAVCDIPPR 4: Carbamidomethyl (C)
					1053.604	YLTVAAIFR
					1130.59	FPGQLNADLR
					1143.629	LAVNMVPFPR
					1159.62	LAVNMVPFPR 5: Oxidation (M)
					1229.594	ISEQFTAMFR
					1601.81	AVLVDLEPGTMDSVR
					1617.798	AVLVDLEPGTMDSVR 11: Oxidation (M)
S11↓	Tubulin alpha chain, testis-specific [*Oncorhynchus mykiss*]	TBAT_ONCMY	114.66	49.99/4.98	1023.449	EDAANNYAR
					1071.606	EIVDLVLDR
					1267.513	YMACCMLYR 4: Carbamidomethyl (C) 5: Carbamidomethyl (C)
					1687.897	AVFVDLEPTVVDEVR
					2330.023	AFVHWYVGEGMEEGEFSEAR
S12↓	Enolase B [*Acipenser baerii]*	A4ZDY5_ACIBE	107.00	47.20/7.60	704.531	GVPLYR
					732.518	EILDSR
					775.545	NFRNPK

(*continued on next page*)

Table 2 (*continued*)

Spot no.	Protein name [organisms]	UniProt accession No.	MASCOTscore	MW (kDa)/pI	Precursor ion m/z	Sequence
					806.582	YNQLLR
					814.53	SPDDPKR
					877.178	ACNCLLLK
					971.703	RIQQAVEK
					976.694	TGAPCRSER
					1005.735	KACNCLLLK
					1072.668	MSITKIHAR 1: Oxidation (M)
					1143.766	IGAEVYHNLK
					1295.811	FMLELDGTENK
					1359.85	HITGDQLGDLYK
					1390.882	GNPTVEVDLFTAK
					1541.937	LAQSNGWGVMVSHR
					1542.928	LAQSNGWGVMVSHR
					1544.962	IVIGMDVAASEFHR
					1558.925	LAQSNGWGVMVSHR 10: Oxidation (M)
					1560.962	IVIGMDVAASEFHR 5: Oxidation (M)
					1663.997	VNQIGSVMESIQACK 14: Carbamidomethyl (C)
					1805.138	AAVPSGASTGIYEALELR
					1833.113	IVIGMDVAASEFHRSGK 5: Oxidation (M)
					1868.125	LAMQEFMILPVGASSFK
					2047.26	FGANAILGVSLAVCKAGAAEK 14: Carbamidomethyl (C)
					2176.308	AGYPDKIVIGMDVAASEFHR
					2220.332	AAVPSGASTGIYEALELRDGDK
					2353.374	SGETEDTFIADLVVGLCTGQIK 17: Carbamidomethyl (C)
					2481.408	ACNCLLLKVNQIGSVMESIQACK 16: Oxidation (M)
					2525.341	NYPVVSIEDPFDQDDWGNWTK
					2743.56	DATNVGDKGGFAPNILENNEALELLK
					3036.832	HIADLAGNPELILPVPAFNVINGGSHAGNK

S = fresh sperm; Precursor ions selected for MS/MS are listed; Probability Based score is -10*Log (P), where P is the probability that the observed match is a random event; Scores greater than recorded significant hit were significant ($P < 0.05$); The MS/MS peak lists were searched against a NCBInr protein database using MASCOT™ software with the following settings: enzyme chemistry - trypsin, max missed cleavages 1, variable modifications: Carbamidomethyl (C), Oxidation (M), peptide mass tolerance ± 0.1 Da, and fragment mass tolerance ± 0.5 Da. ↓ indicates proteins with low intensity after cryopreservation.

sperm. Based on cellular component, the proteins were categorized into several groups. Triosephosphate isomerase (Spot 6) and mitochondrial ATP synthase subunit β (Spots 9, 10) were mitochondrial enzyme and mitochondrial membrane proteins, respectively. Glycerol-3-phosphate dehydrogenase [NAD(+)] (Spot 8) is located in the cytoplasm. Enolase B (Spot 12) is a cytosolic protein. These proteins are members of a superfamily of glycolytic enzymes, having a primary role in metabolic pathways such as gluconeogenesis and glycolysis, which are essential to efficient energy production for spermatozoon movement. The lesser quantities of these four proteins in cryopreserved sperm could be associated with an ATP deficiency and reduced spermatozoon motility.

Tubulin α chain (Spot 10) and Tubulin β chain, testis-specific (Spot 11) are the major constituents of sperm microtubules. These proteins have important roles in the organization of the microtubule cytoskeleton. There is a decrease of cytoskeleton related proteins after cryopreservation that occurs in human, boar, carp, and rainbow trout sperm (Desrosiers et al., 2006; Chen et al., 2014; Dietrich et al., 2015; Nynca et al., 2015). The decrease of tubulin after cryopreservation in present study may affect the sperm flagellum structure and sperm movement symmetry, resulting in the decrease in values for sperm motility variables of sterlet sperm.

Some researchers hold the view that the decrease of protein amount in sperm following cryopreservation is mainly the consequence of leakage of proteins from sperm (Dietrich et al., 2015; Nynca et al., 2015). Reduction of various proteins in cryopreserved seminal plasma and sperm in comparison to fresh seminal plasma and sperm in the present study indicates that protein degradation resulted in a decrease in protein amount of sperm during cryopreservation.

Results of the present study indicate that cryopreservation affected motility of sterlet sperm. Proteomic profiling also indicated there are differences in fresh and frozen/thawed sperm and seminal plasma. Most sperm proteins affected by cryopreservation were associated with metabolism, response to stress, and sperm cytoskeleton. This study of the proteome differences between fresh and cryopreserved sperm may expand understanding of effects of cryopreservation and identify proteins that are possibly associated with spermatozoon motility, viability and other functions. Further research is needed to understand the mechanisms behind the differences in protein profiles and to characterize the relationship between the protein profile and sperm quality.

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CHAPTER 4

PROTECTION OF ANTIFREEZE PROTEINS ON STERLET, *ACIPENSER RUTHENUS* SPERM QUALITY AND FERTILITY DURING CRYOPRESERVATION

4.1. **Xin, M.M.,** Sterba, J., Shaliutina-Kolesova, A., Dzyuba, B., Lieskovska, J., Boryshpolets, S., Siddique, M. A. M., Kholodnyy, V., Lebeda, I., Linhart, O., 2018. Protective role of antifreeze proteins on sterlet (*Acipenser ruthenus*) sperm during cryopreservation. Fish Physiology and Biochemistry 44, 1527–1533.

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4.2. **Xin, M.M.,** Tučková, V., Rodina, M., Kholodnyy, V., Dadras, H., Boryshpolets, S., Shaliutina-Kolešová, A., Linhart, O., 2018. Effects of antifreeze proteins on cryopreserved sterlet (*Acipenser ruthenus*) sperm motility variables and fertilization capacity. Animal Reproduction Science 196, 143–149.

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Protective role of antifreeze proteins on sterlet (*Acipenser ruthenus*) sperm during cryopreservation

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Abstract The loss of sperm quality in sterlet (*Acipenser ruthenus*) due to freeze-thaw process in cryopreservation was investigated in the present study. Two antifreeze proteins (AFPI or AFPIII) were used at different concentrations of 0.1, 1, 10, and 100 μg/mL. We compared motility, curvilinear velocity, and plasma membrane integrity of fresh, cryopreserved sperm, and sperm cryopreserved in the presence of antifreeze proteins. Fresh sperm (control) had 85 ± 4% motility and 160 ± 2 μm/s curvilinear velocity, respectively. After cryopreservation, the motility of frozen-thawed sperm without addition of antifreeze proteins significantly decreased (44 ± 9%), compared to the control. The highest motility of frozen-thawed sperm was obtained in cryopreserved sperm with addition of 1 μg/mL of AFPIII (58 ± 14%). No significant differences were observed in curvilinear velocity between fresh sperm and cryopreserved sperm with/without addition of AFPI or AFPIII. The flow cytometry analysis revealed that fresh sperm contained 94.5 ± 6% live cells, while the cryopreserved sperm only contained 26.6 ± 14% live cells. Supplementation of antifreeze proteins has significantly improved the percentage of live cells in frozen-thawed sperm, except 0.1 μg/ml of AFPI group. No significant difference in percentage of live cells was detected in the sperm cryopreserved with 10 μg/mL of AFPI or AFPIII, compared to fresh sperm. Thus, addition of antifreeze proteins to cryopreservation medium could be considered to improve the post-thawed sperm quality of sterlet.

Keywords Antifreeze proteins · Cryopreservation · Membrane integrity · Motility rate · Sperm quality

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Introduction

Sturgeon (Chondrostei, Acipenseriformes) are considered as threatened or endangered fish species based on significant decline of their natural stocks; causative factors are overfishing and environmental degradation, including changes affecting migration and reproduction (Billard and Lecointre 2000; Bronzi et al. 2011). The sterlet *Acipenser ruthenus* is in the family of Acipenseridae living in both Europe and Asia. It has a relatively small body size, and the reproductive cycle is one of the shortest among the group. It is widely used as a model fish among sturgeon for study of artificial reproduction and larva-culture. Due to the shortage of broodstock, sometimes the gametes from both male and female sterlet cannot be collected sufficiently at the same time, thus improving sperm storage methods resulting in good quality of gametes is very critical for efficient conservation program.

Cryopreservation has been successfully used for more than 60 years as a tool for fish hybridization and selective breeding, as well as for maintaining biodiversity in the conservation of endangered species (Lahnsteiner et al. 2002; Kopeika et al. 2007). Cryopreservation is considered as a suitable and effective method for the long-term storage of fish sperm (Sarvi et al. 2006; Tsai and Lin 2012). However, the cryopreservation process influences the morphological and functional integrity of spermatozoa (Li et al. 2006; Xin et al. 2017). Plasma membrane structure is one of the most important factors affecting viability of the sperm (Ogier de Baulny et al. 1997; Li et al. 2006). The sperm plasma membrane is rich with polyunsaturated fatty acids and a variety of proteins. These lipids and proteins with fluidity, fusogenicity, and permeability are associated with sperm acrosome reaction, regulations of sperm motility initiation, and sperm-egg fusion (Li et al. 2009). Thus, damage to sperm plasma membranes may affect sperm motility, fertilization capacity, and the early events following fertilization (Beirão et al. 2012; Zilli et al. 2014).

Non-permeating cryoprotectants (CPAs), such as some carbohydrates, lipids, and proteins are added to the freezing solution to stabilize the sperm membrane (Wang 2000; Marco-Jimenez et al. 2006). Antifreeze proteins (AFPs) offer protection to cells by inhibiting ice crystal growth and stabilizing cell membrane (Kim et al. 2017). The ability of AFPs to protect sperm during the freeze-thaw procedure has been tested in mammals (Prathalingam et al. 2006). Limited studies have been carried out to understand the role of AFPs for fish sperm cryopreservation (Abed-Elmdoust et al. 2015). It has been shown that AFPs, especially AFPIII, improved post-thawed sperm quality and has contributed to the stabilization of the sperm plasma membrane organization in gilthead seabream (*Sparus aurata*) (Beirão et al. 2012; Zilli et al. 2014). However, AFPs have had a mildly cytotoxic effect at certain concentrations on ram sperm motility (Payne et al. 1994). The studies of AFP action on the quality of cryopreserved sterlet sperm have not been previously performed. Thus, the present study assessed the protective role of antifreeze protein type I (AFPI) and antifreeze protein type III (AFPIII) on sterlet sperm motility, velocity, and membrane integrity following cryopreservation.

Materials and methods

Fish, spermiation, and sperm collection

Seven mature sterlet males were used in this study. Prior to experimentation, fish were transferred from fish farming ponds to 4 m^3 plastic tanks with a closed water recirculation system, and the temperature of the water was gradually increased from 3 to 15 °C by a 1 °C increment per day. Spermiation was induced by a intramuscular injection of carp pituitary powder dissolved in 0.9% (*w*/*v*) NaCl solution at a dose of 4 mg/kg body weight. Sperm was collected after 24 h using a syringe with an attached 4-mm plastic catheter inserted into the urogenital ducts, then placed on ice until experimentation within 4 h.

Sperm cryopreservation

The seven samples with motility higher than 80% were separately diluted (1:1) in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris–HCl, pH 8.0, 10% methanol (Glogowski et al. 2002, modified), and with/without 0.1, 1, 10, and 100 μg/mL of AFPI or AFPIII. The diluted sperm samples were immediately loaded into 0.5 mL plastic straws (CRYOVET, France) and equilibrated for 10 min on ice. Then, the samples were frozen 3 cm above liquid nitrogen level in a Styrofoam box for 10 min and then directly plunged into liquid nitrogen. Thawing was performed in a water bath at 40 °C for 6 s.

Sperm motility and velocity assessment

Sperm were activated in tap water with 0.1% Pluronic F-127 (catalog number P2443, Sigma-Aldrich). Sperm motility rate was determined under a microscope (UB 200i, PROISER, Spain) using 10× lens, negative phase-contrast condenser and recorded with an Integrated System for Semen Analysis (ISAS®) digital camera (PROISER, Spain) set at 25 frames/s. Recordings were stored on a hard disk in AVI format. Analyses of the sperm recordings were performed by the ISAS software (PROISER, Spain) at 15 s post-activation for curvilinear velocity (VCL, μm/s) and at 30 s post-activation for motility rate (%). Each sample was analyzed in triplicate at room temperature (21 °C).

Membrane integrity assessment

The plasma membrane integrity of fresh and cryopreserved sperm with/without different concentrations of AFPI or AFPIII was assessed by flow cytometry analysis using the LIVE/DEAD Sperm Viability Kit (Invitrogen/Thermo Fisher Scientific Inc., Waltham, USA). Sperm samples were pre-diluted, 2 μL of fresh or cryopreserved sperm samples with/without AFPI or AFPIII was suspended in 2 mL of phosphate-buffered saline (PBS) (PH 7.4). Then, 5 μL of 1:500 diluted SYBR Green I 10,000× (Sigma-Aldrich, USA) was added into the diluted sperm samples. After 5 min, 5 μL propidium iodide (PI) (Sigma-Aldrich, USA) was added to the sperm solutions. After incubating sperm samples with both fluorescent dyes for 30 min at room temperature. Flow cytometry was performed on FACS Canto II flow cytometer, and data were analyzed using FACS Diva software, v. 5.0 (BD Biosciences). Dead sperm were determined by number of positive events in FITC channel and in PE-Texas Red channel (region Q2). Live sperm (with intact plasma membrane) were determined as positive in FITC channel and negative in PE-Texasa Red channel (region Q4). Each sample was analyzed in triplicate.

Statistical analysis

The data (mean ± SD) on sperm motility rate, velocity, and plasma membrane integrity was made by analysis of variance (ANOVA) followed by Tukey's HSD test for each variable analyzed. All analyses were performed using SPSS 15.0 for Windows and p less than 0.05 was considered statistically significant.

Results

Sperm motility rate

The motility rate of fresh or cryopreserved sperm with/without AFPI or AFPIII varied (Fig. 1). The fresh sperm in the seminal plasma were immotile, and the flagella were straight and quivered slightly. After dilution with activation medium, the fresh sperm showed 85 ± 4% motility and a curvilinear velocity of 160 ± 2 μm/s. After cryopreservation, sperm motility parameters were significantly lower in the frozen-thawed sperm without addition of antifreeze proteins (44 ± 9%). However, cryopreserved samples with addition of 10 μg/mL of AFPI (56 ± 15%) and 1 μg/mL of AFPIII (58 ± 14%) had improved motility but had no significant difference compared to both fresh sperm and other frozen-thawed sperm. No significant differences were detected in curvilinear velocity between fresh and cryopreserved sperm with/without addition of antifreeze proteins (Fig. 2).

Sperm plasma membrane integrity

PI/SYBR Green I analysis of plasma membrane integrity in fresh sperm indicated that 94.5 ± 6% cells were alive (had intact plasma membrane). Significant decrease in viability with 26.6 ± 14% of live cells was observed in cryopreserved sperm (Fig. 3). Supplementation of different concentrations of AFPI or AFPIII in extender increased the percentage of live sperm with intact plasma membranes. The sperm cryopreserved with 10 μg/mL of AFPI or AFPIII obtained 65.4 ± 12 and 62.9 ± 12% live cells with intact plasma membranes respectively, which showed no significant difference with the fresh sperm samples (Fig. 3).

Discussion

Cryopreserved sturgeon sperm has been shown to have a significant decrease in motility rate compared to the fresh sperm (Boryshpolets et al. 2011). As a possible way to improve the quality of frozen-thawed sterlet sperm, antifreeze proteins (AFPI or AFPIII) were employed as cryoprotectants in the present study. The

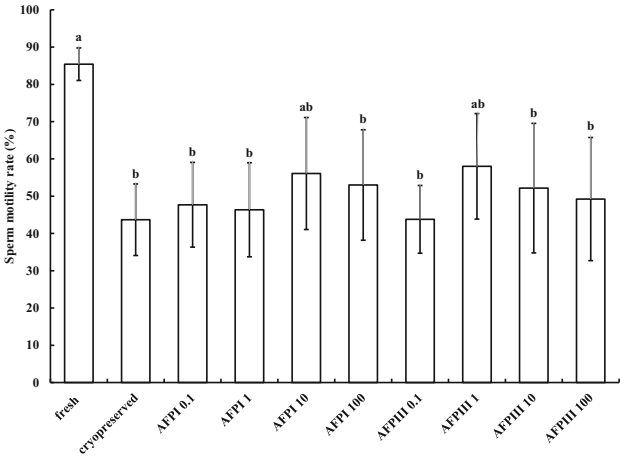

Fig. 1 Sperm motility rate (%) in sterlet *Acipenser ruthenus* before (fresh) and after cryopreservation with/without addition of antifreeze proteins (AFPI or AFPIII) at different concentrations of 0.1, 1, 10, 100 μg/mL at 30 s post-activation. Data is expressed as mean ± SD. Values with the same superscript are not significantly different ($p > 0.05$)

results showed that supplementation of different concentrations of AFPI or AFPIII to the cryopreserving extender improved the motility rate and plasma membrane integrity of frozen-thawed sterlet sperm. The results from the current study demonstrated that improvements from antifreeze proteins are possible for sturgeon sperm cryopreservation.

Antifreeze proteins (AFPs) are well-recognized specific proteins which can offer protection to cells by depressing the freezing point, modifying the ice-crystal formation process, preventing recrystallization, and interacting with plasma membrane at low temperatures (Robles et al. 2006; Kim et al. 2017). They have been studied as cryoprotectants for many years and are reported to improve the sperm quality relative to motility parameters and plasma membrane integrity during cryopreservation.

Motility is an important characteristic reflecting the quality of fresh or cryopreserved fish sperm and is positively correlated with fertilizing capacity as it enables spermatozoa to reach to the oocyte for successful fertilization (Rurangwa et al. 2004). The motility of

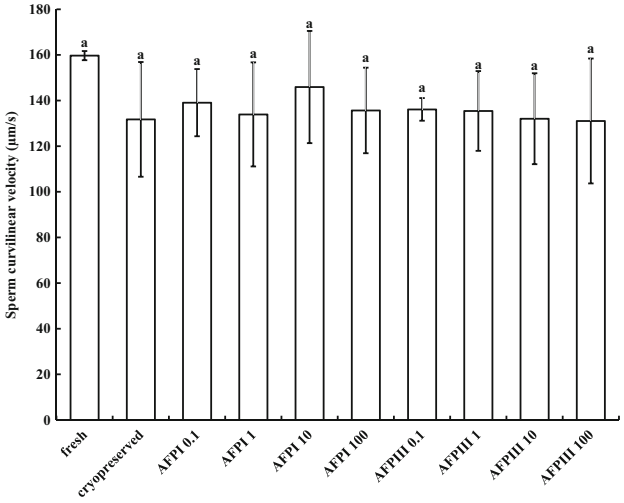

Fig. 2 Curvilinear velocity (VCL) (μm/s) in sterlet *Acipenser ruthenus* sperm before (fresh) and after cryopreservation with/without addition of antifreeze proteins (AFPI or AFPIII) at different concentrations of 0.1, 1, 10, and 100 μg/mL at 15 s post-activation. Data is expressed as mean ± SD. There are no differences between different samples ($p > 0.05$)

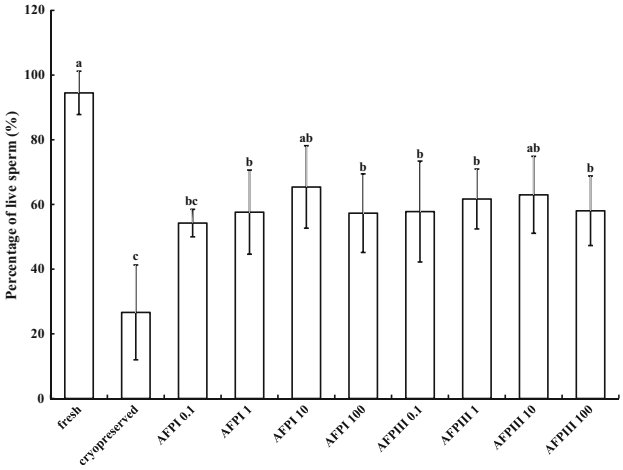

Fig. 3 Plasma membrane integrity in sterlet *Acipenser ruthenus* sperm before (fresh) and after cryopreservation with/without addition of antifreeze proteins (AFPI or AFPIII) at different concentrations: 0.1, 1, 10, and 100 μg/mL. Data is expressed as mean ± SD. Values with the same superscript are not significantly different ($p > 0.05$)

frozen-thawed sperm in the present study reached 58 ± 14 and 56 ± 15% in sperm cryopreserved with 1 μg/mL of AFPIII and 10 μg/mL of AFPI, respectively; neither was significantly different from fresh sperm (85 ± 4%) nor statistically significantly different from cryopreserved sperm without addition of AFPI or AFPIII (44 ± 2%). It seems that the effects of antifreeze protein on sterlet sperm during cryopreservation are not highly concentration dependent. AFPI from Winter flounder, *Pseudopleuronectes americanus* and antifreeze glycoprotein (AFGP) from Antarctic cod, *Dissostichus mawsoni* at concentration of 0.1 to 10 μg/mL were demonstrated to significantly reduce the loss in sperm motility of ram that occurs due to the freeze-thaw process (Payne et al. 1994). Similarly, its effect was not concentration dependent nor did it depend on which type of antifreeze protein was added (Payne et al. 1994). However, Abed-Elmdoust et al. (2015) reported that antifreeze proteins were dose dependent in Persian sturgeon *A. persicus* as AFPIII with a concentration of 10 μM improved the motility rate of post-thawed Persian sturgeon sperm, while other studied concentrations of 5 and 15 μM of AFPIII did not improve the motility rate (Abed-Elmdoust et al. 2015).

In the present study, no significant difference in curvilinear velocity was found between fresh and cryopreserved sterlet sperm with/without supplementation of AFP(s) after activation. Similar results were reported for sea bass sperm; no significant differences in velocity were obtained between fresh and cryopreserved sperm with/without addition of dimethyl sulfoxide and AFPI/AFPIII (Zilli et al. 2014). On the contrary, significant decrease of velocity was detected in sperm of Chimpanzee, *Pan troglodytes*, after cryopreservation with 1, 10, and 100 μg/mL of AFPIII compared to fresh sperm (Younis et al. 1998). There is no clear consensus, but the antifreeze proteins in cryopreservation of different cell types and different species are in controversy and dose dependent (Rubinsky et al. 1992; Wang 2000; Beirão et al. 2012; Abed-Elmdoust et al. 2015). However, our findings were not in accordance with the opinion of concentrate-dependent action of AFPI and AFPIII.

Spermatozoa, capability of reaching the oocyte and fuse with it, is characterized by fluidity, fusogenicity, and permeability of plasma membrane (Wassall and Stillwell 2009). The structural and functional integrity of sperm plasma membrane have been chosen as indicators of evaluating fish sperm quality, especially for the cryopreserved sperm whose plasma membranes are sensitive due to cryopreservation procedure (Ogier de Baulny et al. 1997; Aitken and Baker 2006; Muller et al. 2008). In our study, the plasma membrane of sterlet sperm was significantly damaged after cryopreservation and antifreeze proteins significantly improved the plasma membrane integrity of frozen-thawed sterlet sperm, except the sperm cryopreserved with 0.1 μg/mL of AFPI. Furthermore, no significant difference of plasma membrane integrity was observed between fresh sperm and sperm cryopreserved with 10 μg/mL of AFPI or

AFPIII. While the beneficial effects of AFPI or AFPIII on the plasma membrane integrity of sterlet sperm do not significantly increase the motility parameters of frozen-thawed sterlet sperm. This may be because except the plasma membrane damage, some other damages were also caused during cryopreservation, such as damage on sperm nucleus, mitochondria, and cytoskeleton (Cabrita et al. 2005; Li et al. 2006; Figueroa et al. 2017; Xin et al. 2018). On the other hand, the protective effects of antifreeze proteins could only partially, not completely, protect the plasma membrane of sperm from the freeze-thaw process. Qadeer et al. (2016) applied three different concentrations (0.1, 1, and 10 μM) of beetle *Dendroides canadensis* recombinant antifreeze proteins (DAFPs) to improve the quality and fertility of cryopreserved Nili-Ravi buffalo (*Bubalus bubalis*) sperm. This elucidated that only supplementation of 10 μM of DAFP with an extender improved the motility and plasma membrane integrity of Nili-Ravi buffalo sperm after freezing/thawing and yielded numerically higher, although statistically non-significant, in vitro cleavage and in vivo fertility rate (Qadeer et al. 2016). Further, it is reported that AFPIII interacted with unsaturated fatty acids and reduced the alteration of protein profile, stabilizing the plasma membrane organization during cryopreservation and contributing to improve post-thawed sperm quality in gilthead seabream *Sparus aurata* (Beirão et al. 2012; Zilli et al. 2014).

The cryopreserved sperm without addition of AFPI or AFPIII showed 44 ± 9% motility, while the flow cytometry analysis indicated that only 26.6 ± 15% live cells were observed in the cryopreserved sperm without addition of AFPI or AFPIII. This difference comes from differences in the experiment setup. The motility of frozen-thawed sperm was assessed immediately after thawing. While the frozen-thawed sperm for estimating plasma membrane integrity was incubated with fluorescence dye (SYBR Green I and PI) and measured after thawing for half an hour or more. Therefore, the data from motility and membrane integrity did not show the reality in exactly the same time and should be considered separately. On the other hand, sperm motility percentage is probably the most complicated parameter to evaluate in a CASA system. Since it is likely to be affected by CASA itself (Boryshpolets et al. 2013b) and some other trivial factors, such as immotile cells not being measured as a result of being out of focus and track collisions of overloaded sperm (Boryshpolets et al. 2013a). Thus, the deviation of motility rate which cannot be avoided should also be considered.

Results from our study provide an insight into the protective effects of antifreeze proteins on fish sperm motility parameters and plasma membrane integrity. Both of AFPI and AFPIII might be helpful in standardization of cryopreservation protocol for sturgeon and other fish species. Nevertheless, future studies are required to verify the effect of AFPs on sperm fertilization ability and compare the difference of protein and lipid components of sperm membranes with/without addition of AFPs into the freezing extender.

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Effects of antifreeze proteins on cryopreserved sterlet (*Acipenser ruthenus*) sperm motility variables and fertilization capacity

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ABSTRACT

The effect of antifreeze proteins on sterlet, *Acipenser ruthenus* sperm motility variables and fertilization rate were investigated after cryopreservation. Two types of antifreeze proteins (AFPI or AFPIII) were used at concentrations of 0.1, 1, 10 and 100 μg/mL. The motility variables of fresh and cryopreserved sperm with and without addition of antifreeze proteins were evaluated by the Computer Assisted Semen Analyzer (CASA). The fertilization rate using about 200,000 spermatozoa per egg was evaluated after 54 h incubation at 17 °C during the early stage of organogenesis. The motility, curvilinear velocity and straight-line velocity of fresh sperm was 93 ± 5%, 128 ± 13 μm/s and 89 ± 9 μm/s, respectively. There was a significant decrease of sperm motility rate between fresh sperm and cryopreserved sperm with/without addition of antifreeze proteins. The greatest motility among thawed samples was in the sperm cryopreserved with 10 μg/mL of AFPI (56 ± 20%), however, these data were not different compared to the sperm without antifreeze proteins (49 ± 14%). No statistical variations were detected in curvilinear velocity nor straight-line velocity. The fertilization rate with fresh sperm was 67 ± 7%. No significant differences were detected in fertilization rate between fresh and cryopreserved spermatozoa with/without addition of antifreeze proteins, except the sperm cryopreserved with 100 μg/mL of AFPIII (39 ± 14%). Thus, it is concluded that addition of antifreeze proteins to cryopreservation medium do not improve nor have toxicity effects on the quality and fertilization capacity of sterlet sperm after thawing.

1. Introduction

Sturgeons (Chondrostei, Acipenseriformes) are considered as threatened or endangered species due to intensive-fishing, habitat destruction, and water pollution (Billard and Lecointre, 2001; Bronzi et al., 2011). Sperm cryopreservation has become important for gene banking of endangered populations and for synchronization of the times of artificial reproduction in fish (Lahnsteiner et al., 2002; Sarvi et al., 2006).

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The first attempts at cryopreservation of Acipenseridae sperm dates back to the late 1960s. Researchers succeeded in freezing and thawing sterlet (*Acipenser ruthenus*) sperm, although the fertilization rate was less than 1% (Burtsev and Serebryakova, 1969). A variety of cryopreservation protocols for sturgeon species have been developed and optimized to achieve high frozen-thawed sperm motility and fertilization rates (Ciereszko et al., 1996; Urbányi et al., 2004; Boryshpolets et al., 2011). Although, improvements of cryopreservation technology have been made, the quality of frozen-thawed sturgeon sperm and fertilization rate with the frozen-thawed sperm remains less than that with use of fresh sperm. It, therefore, is necessary to improve cryopreservation methods of sperm in sturgeon species.

Cryopreservation processing of sperm involves extreme temperature change which provokes irreversible cryoinjuries to cell morphology, structure and physiology. These cryoinjuries alter the functional state of many proteins in the sperm nucleus, midpiece, flagellum, plasma membrane and cytosol, leading to the impairment of motility and result in the loss of fertilization capacity (Gwo and Arnold, 1992; Lahnsteiner et al., 1996; Linhart et al., 2000; Zilli et al., 2003; He and Woods, 2004; Figueroa et al., 2016a). To improve the sturgeon sperm cryopreservation technique and achieve greater frozen-thawed sperm quality, addition of various proteins, such as antioxidants and antifreeze proteins to cryopreservation medium has been recommended (Beirão et al., 2012; Figueroa et al., 2017,2018; Magnotti et al., 2018).

Antifreeze proteins (AFPs) are well-recognized specific proteins which can provide protection to cells by decreasing the freezing point, modifying the ice-crystal formation process, preventing recrystallization and interacting with plasma membranes at low temperatures (Robles et al., 2006; Kim et al., 2017). The capacity of AFPs to protect sperm during the freeze-thaw procedure has been tested in mammals with both positive and negative results (Payne et al., 1994; Prathalingam et al., 2006). A few studies have been conducted evaluating the possible effects of use of AFPs for cryopreservation of fish sperm. Results from these previous studies indicate AFPs contribute to the protection of sperm metabolism and the stabilization of the sperm plasma membrane lipids and proteins during cryopreservation (Beirão et al., 2012; Zilli et al., 2014; Abed-Elmdoust et al., 2017). Similarly, the action of AFPs on the cryopreserved sterlet sperm has indicated that antifreeze proteins can significantly improve the percentage of live cells with intact membranes in frozen-thawed sterlet sperm (Xin et al., 2018a). Information concerning the effect of AFPs on fertilizing capacity of sterlet sperm during cryopreservation, however, has not yet been studied. Consequently, the current experiment explored the potential benefits of applying the AFPI or AFPIII at different concentrations to improve sperm motility rate, velocity and fertilization rate of sterlet sperm following cryopreservation.

2. Materials and methods

All experiments were specifically approved by the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology, Vodnany, based on the EU-harmonized Animal Welfare Act of the Czech Republic.

2.1. Gamete collection

The research was conducted at the experimental station of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Three mature sterlet males and three mature sterlet females were used in this study. Prior to experimentation, sterlet males and females (6–7 years, 2–3 kg) were transferred from fish farming ponds to 4 m^3 plastic tanks with a closed water recirculation system, where the water temperature was gradually increased from 2 to 15 °C by a 1 °C increment per day. Before stripping (24 h), spermiation was induced by a intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at a dose of 4 mg/kg body weight (BW), and females were injected with the same carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at 24 h (0.5 mg/kg BW) and 12 h (4.5 mg/kg BW). Sperm was collected from the urogenital tract using a 4-mm catheter connected to a 20-mL plastic syringe with gentle abdominal massage, then the sperm with motility greater than 80% were selected and placed on ice with aerobic conditions no longer than 4 h before experimentation. Ova were stripped into a plastic bowl, then stored in aerobic conditions at 17 °C and used within 1 h post-collection.

2.2. Sperm motility and velocity assessment

The fresh (control of pooled sperm from 3 males), cryopreserved sperm and sperm cryopreserved with AFPs of individual samples were diluted in activation medium [tap water with 0.1% Pluronic F-127 (3 mOsm/kg)] with 1:50 ratio. All observations were performed at room temperature (21 °C). Motility was recorded microscopically (UB 200i, PROISER, Spain) using an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) set at 25 frames/s. Recordings were stored on a hard disk in AVI format. Analyses of the sperm recordings were performed by the Integrated System for Semen Analysis software (PROISER, Spain) at 15 s post-activation. Computer-assisted sperm analysis (CASA) included sperm curvilinear velocity over the actual path (VCL, μm/s), straight-line velocity (VSL, μm/s) and percentage of motile sperm cells (motility rate, %). Records were conducted in triplicate per sample.

2.3. Spermatozoa concentration

The spermatozoa concentration was determined for the fertilization experiment. Sperm samples were diluted in 0.9% (w/v) NaCl solution at 1:20 ratio and estimated using a spermtrack-10 ISAS (sperm counting chamber; PROISER, Spain) with 100 squares and at

an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain). The number of spermatozoa in each square varied from 5 to 25, with counting of spermatozoa occurring without difficulty. The number of spermatozoa in 10 diagonal squares were counted, respectively, and this finding was used to calculate the average number of spermatozoa in one square. The spermatozoa concentration was calculated as average number of spermatozoa in 10 diagonal squares × 100 (the total number of squares) × 20 (dilution ratio) × 100,000 (the volume of total square in 1 mL).

2.4. Sperm cryopreservation

The three freshly collected sperm samples were separately diluted (1:1) in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris – HCl, pH 8.0, 10% methanol (Glogowski et al., 2002, modified), and with/without addition of 0.1, 1, 10, 100 μg/mL of AFPI or AFPIII. The diluted sperm from individual males were immediately loaded into 0.5 mL plastic straws (CRYO-VET, France) and placed on a 3-cm thickness polystyrene raft for 10 min equilibration on ice. Subsequently, the polystyrene raft with straws were transferred and frozen in liquid nitrogen vapor in a styrofoam box. After 10 min, the straws were plunged directly into liquid nitrogen and stored in plastic goblets. Thawing was performed in a water bath at 40 °C for 6 s, followed by immediate use for further estimations and sample processing.

2.5. Fertilization assay

Eggs from three female were collected, pooled in an equal ratio and used to reduce variations in fertilization rates related to egg quality for this study. The 3.2 g of eggs (220–240 eggs) were placed in small beakers and fertilized. Pooled sperm from three males with sperm motility greater than 80% was used as a control. The frozen-thawed sperm with/without addition of different concentrations of AFPI or AFPIII from three males were fertilized separately. Calibrated volume of fresh (control) and frozen sperm samples with ratio 200,000 spermatozoa per egg was added. Subsequently, the eggs were activated with 20 mL of dechlorinated water at 17 °C and immediately added on a shaker table at 250 rpm. Each treatment group was kept in motion for 1 min, and then the fertilized eggs were allocated in four petri dishes and allowed to adhere. The eggs were incubated at 17 °C. Dechlorinated water in the petri dishes was replaced each 12 h. Eggs in each petri dish and the number fertilized were counted at neurulation stage at approximately 54 h post-fertilization under binocular microscope. The percentage of fertilization was calculated for each treatment from the ratio of fertilized eggs and total number of eggs placed in the petri dish (fertilized eggs/total number of eggs*100).

2.6. Statistical analysis

Proportional data were normalized using arcsine square root transformation. All the data (mean ± SD) on sperm motility, curvilinear velocity, straight velocity and fertilization rate were evaluated by analysis of variance (ANOVA) followed by LSD test for each variable analyzed. All analyses were performed using SPSS 15.0 for Windows and $P < 0.05$ were considered statistically significant.

3. Results

3.1. Sperm motility

After dilution with activation medium, the fresh spermatozoa had 93 ± 5% motility, 128 ± 13 μm/s curvilinear velocity and 89 ± 9 μm/s straight-line velocity, respectively. After cryopreservation, the motility rate of frozen-thawed sperm with/without addition of antifreeze proteins was less than that in control group (Fig. 1). The greatest motility was in samples cryopreserved with 10 μg/mL of AFPI (56 ± 20%), but there were no differences between the sperm cryopreserved without addition of antifreeze

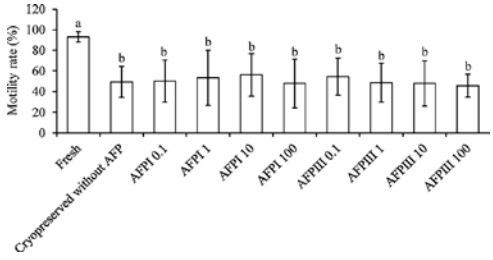

Fig. 1. Sperm motility (%) of sterlet *Acipenser ruthenus* before (fresh) and after cryopreservation with/without addition of antifreeze proteins (AFPI or AFPIII) at different concentrations of 0.1, 1, 10, 100 μg/mL at 15 s post activation; Data are expressed as mean ± SD; Values with the same superscript are not different ($P > 0.05$).

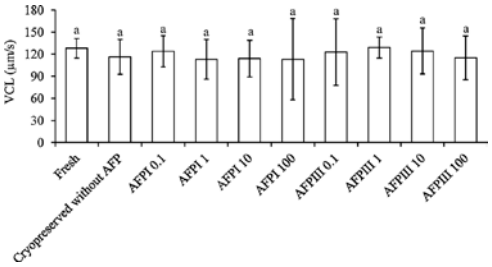

Fig. 2. Curvilinear velocity (VCL) (μm/s) of sterlet *Acipenser ruthenus* sperm before (fresh) and after cryopreservation with/without addition of antifreeze proteins (AFPI or AFPIII) at different concentrations of 0.1, 1, 10, 100 μg/mL at 15 s post activation; Data are expressed as mean ± SD; Values with the same superscript are not different ($P > 0.05$).

proteins (49 ± 14%). The least motility was with the sperm that were processed in 100 μg/mL of AFPIII (45 ± 11%). There were no differences (ANOVA; $P > 0.05$) in curvilinear or straight-line velocity between fresh and frozen-thawed sperm with/without addition of antifreeze proteins (Figs. 2 and 3).

3.2. Fertilization rate

The fertilization rate of sterlet in fresh sperm group was 67 ± 7%. There were no differences (ANOVA; $P > 0.05$) in fertilization rate between fresh and cryopreserved sperm without addition of antifreeze proteins (Fig. 4). The fertilization rate with sperm cryopreserved with 100 μg/mL of AFPIII (39 ± 21%) was less than that with all other samples (Fig. 4).

4. Discussion

Antifreeze proteins, a group of proteins from arctic creatures, offer protection from freezing in sub-zero seawater and have been studied as cryoprotectants for use in cryopreservation of mammalian and fish sperm (Karanova and Tsvetkova, 1994; Karanova et al., 1997a,b; Younis et al., 1998; Abed-Elmdoust et al., 2015, 2017). These proteins are capable of stabilizing the cell membrane, inhibiting the ice crystal formation and recrystallization during cryopreservation. In the current study, two types of antifreeze proteins (AFPI or AFPIII) were applied to investigate the motility variables and fertilization capacity of sterlet sperm after cryopreservation.

Sperm motility is the basic standard for evaluation of cryopreserved sperm quality (Fu et al., 2017). There is less sperm motility after cryopreservation (Boryshpolets et al., 2017; Xin et al., 2018b). In the present study, addition of antifreeze proteins did not result in an enhancement of motility rate of frozen-thawed sperm. There was a reduction of sperm motility when comparing fresh and cryopreserved sperm with/without addition of antifreeze proteins. Similar results were reported in previous studies where motility of sterlet sperm was less after cryopreservation (Boryshpolets et al., 2011; Xin et al., 2018a,b). Furthermore, in contrast to results of the present study, with previous studies there have been reports that cryopreservation of sea bream sperm with addition of 1 μg/mL of AFPIII and DMSO resulted in greater motility compared to cryopreservation with DMSO alone *Sparus aurata* (Zilli et al., 2014). The effects of antifreeze proteins on fish sperm cryopreservation are species-specific, which could be explained by the different

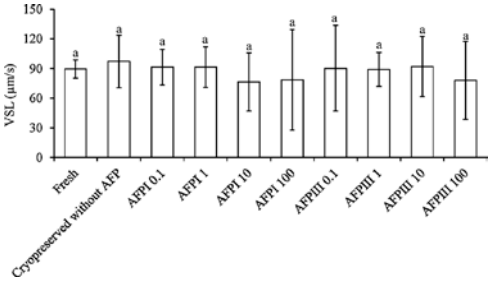

Fig. 3. Straight-line velocity (VSL; μm/s) of sterlet *Acipenser ruthenus* sperm before (fresh) and after cryopreservation with/without addition of antifreeze proteins (AFPI or AFPIII) at different concentrations of 0.1, 1, 10, 100 μg/mL at 15 s post activation; Data are expressed as mean ± SD; Values with the same superscript are not different ($P > 0.05$).

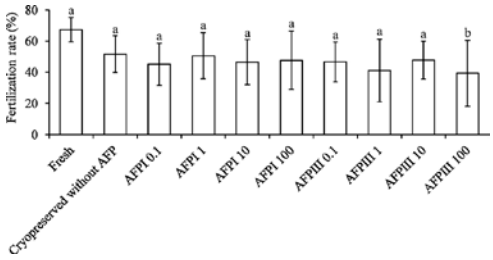

Fig. 4. Fertilization rate of sterlet *Acipenser ruthenus* with sperm before (fresh) and after cryopreservation with/without addition of antifreeze proteins (AFPI or AFPIII) at different concentrations: 0.1, 1, 10, 100 μg/mL; Data are expressed as mean ± SD; Values with the same superscript are not different ($P > 0.05$).

characteristics of sperm structure and morphology, and the different processes of sperm activation among species. Sterlet sperm appear to be more cryo-resistant than sperm of some other freshwater fish species, such as common carp, *Cyprinus carpio*. This may be because sterlet sperm have more resiliant plasma membranes that facilitate adaptation to osmolality changes as a result of being normally diluted by the hypotonic urine during its final maturation (Dzyuba et al., 2014). In the present study, the antifreeze proteins had neither beneficial effects, nor harmful effects on motility of frozen-thawed sterlet sperm. This could be because the plasma membrane of sterlet sperm has a cryo-resistance that is similar to that of antifreeze proteins that protects the plasma membrane and prevents ice crystal formation during cryopreservation.

Sperm velocity is one of the main determinants of the outcome of sperm competition that is related to fertilizing success (Moore and Akhondi, 1996; Gage et al., 2004; Malo et al., 2005), when there are many spermatozoa that surround the same egg. In the present study, there was no difference in either curvilinear or straight-line velocity between fresh and cryopreserved sperm with/without supplementation of AFPI or AFPIII. In previous studies by Zilli et al., 2014 and Xin et al., 2018a, there were no significant differences in curvilinear velocity of spermatozoa between fresh and cryopreserved sperm with/without addition of AFPI or AFPIII which is consistent with the findings in the current data. The addition of 1 μg/mL AFPIII increased the straight-line velocity of frozen-thawed sperm in sea bream (Beirão et al., 2012; Zilli et al., 2014); however, in contrast there was a significant decrease in straight-line velocity of sperm from the chimpanzee, *Pan troglodytes,* after cryopreservation with 1, 10 and 100 μg/mL of AFPIII, and sea bream after cryopreservation with 1 μg/mL of AFPI (Younis et al., 1998; Beirão et al., 2012). In addition, inconsistent with the results of the present study, there was a significant decrease of straight-line velocity in frozen-thawed sperm with addition of 1 μg/mL of AFPI or AFPIII to chimpanzee and sea bream sperm, compared with the fresh sperm from these species (Younis et al., 1998; Beirão et al., 2012; Zilli et al., 2014).

Freeze-thaw cycles during cryopreservation are known to be detrimental to sperm function and ultimately to fertility. Seeking capacity for greater fertilization with frozen-thawed sperm is the final goal of improving a cryopreservation technique. Results of the current study indicate there were differences of fertilization rate between sperm cryopreserved with 100 μg/mL of AFPIII (39 ± 21%) and sperm samples processed with other diluents. Similarly, Qadeer et al. (2016) applied three different concentrations (0.1, 1 and 10 μM) of beetle *Dendroides canadensis* recombinant antifreeze proteins (DAFPs) to improve the quality and fertility of cryopreserved Nili-Ravi buffalo (*Bubalus bubalis*) sperm. The results indicated that supplementation of DAFP with an extender did not improve the fertility rate of Nili-Ravi buffalo sperm after freezing/thawing (Qadeer et al., 2016). In contrast to results of the present study, there was a significant decrease in the fertilization rate with cryopreserved sterlet sperm, compared to the fresh sperm (Boryshpolets et al., 2011). One of the reasons that there was no significant variation in fertilization rate among sperm processed with different diluents, except for the diluent with 100 μg/mL of AFPIII can be explained by the high spermatozoa/egg ratio (200,000:1) in the current study, compared to the spermatozoa/egg ratio (100,000:1) in previous studies assessing sterlet egg fertilization (Boryshpolets et al., 2011; Dzyuba et al., 2014). The relatively greater spermatozoa/egg ratio, however, could explain why there was an increase in the success of fertilization, especially with the cryopreserved sperm, because there were differences in sperm motility between fresh and frozen-thawed sperm. Jähnichen et al. (1999) reported a marked loss of motility but not of fertilizing capacity in frozen-thawed sterlet sperm. Furthermore, it was suspected that this finding resulted from the use of excess sperm as compared to that in some other studies, which can result in a compensation in rate of fertility when there is a loss of sperm motility (Billard et al., 2004). Interestingly, in Atlantic salmon (*Salmo salar*) and carp, spermatozoa number is not the primary determinant of which sperm compete to the extent that a specific cell is involved in the fertilization process (Gage et al., 2004; Linhart et al., 2005), which contradicts the theory of competition (Parker, 1982) and comparative (Stockley et al., 1997) experimental findings in other species (Martin et al., 1974; Gage et al., 2004). It was demonstrated that fertilization rate is positively correlated with curvilinear and straight-line velocity in frozen-thawed sperm of salmonids (Lahnsteiner et al., 1996; Lahnsteiner, 2000; Figueroa et al., 2016b). In present study, there was no significant variation of curvilinear and straight-line velocity, as well as fertilization rate between fresh and frozen-thawed sterlet sperm, which is consistent with the findings with salmonids.

In conclusion, supplementation with different concentrations of AFPI/AFPIII in the extender had neither beneficial nor harmful effects on motility, velocity and fertilization capacity of sterlet sperm after freeze-thawing. Future studies could focus on the effects of

antifreeze proteins on the fish species which have more fragile sperm plasma membranes.

Declaration of conflict of interest

None.

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CHAPTER 5

GENERAL DISCUSSION

Sterlet *Acipenser ruthenus*, a species with relatively small size and short reproductive cycle, is widely used as a model fish for sturgeon research. Cryopreservation of its sperm has been established, albeit with decrease of motility, fertilization and hatching rates (Boryshpolets et al., 2011). The process of cryopreservation has been demonstrated to influence the morphological and functional integrity of spermatozoa (Li et al., 2006), but this has not been thoroughly researched in sturgeon. In our research, cryoinjury related to motility, velocity, protein profile, and plasma membrane integrity of sterlet sperm was assessed. Antifreeze proteins have been shown to inhibit ice crystallization, consequently exerting a protective effect on the spermatozoon membrane during cryopreservation (Zilli et al., 2014). Thus, the effect of antifreeze proteins on sperm quality, especially on the plasma membrane, was investigated to reveal the function of antifreeze proteins and to optimize cryopreservation protocols for fish breeding programs.

Progress and challenges in fish sperm vitrification

There are currently two primary methods used for long term storage of fish sperm: conventional cryopreservation, in which extracellular fluid is partially crystallized, and vitrification, when both intra- and extra-cellular liquids are frozen. Vitrification has economic advantages over conventional cryopreservation, being a simple and rapid procedure without requirements for specialized equipment (Tavukcuoglu et al., 2012). To prevent intracellular and extracellular ice crystallization, traditional vitrification uses a permeable cryoprotectant at concentrations of 30–50 %, compared to 5–10 % with slow freezing, which would have a lethal osmotic effect (Warnecke and Pluta, 2003; Cuevas-Uribe et al., 2017).

The quantity of permeable cryoprotectant can be reduced or eliminated by use of apparatus or techniques that dramatically increase freezing and warming rates by treating a much smaller quantity of sperm (Pegg, 2007). Usually with such techniques, the volume of sperm for vitrification is <30 µL, which is most suitable for fish species producing a small volume and highly concentrated sperm (Cuevas-Uribe et al., 2017). This novel cryoprotectant-free sperm vitrification has been applied to several fish species. Merino et al. (2012) first vitrified rainbow trout sperm with 1% BSA + 40% seminal plasma and no cryoprotectant by dropping a 20 µl suspension directly into liquid nitrogen. The process was successful without significant loss of critical parameters such as cytoplasmic membrane integrity and motility.

Vitrification of fish spermatozoa is a relatively new application compared to conventional cryopreservation, which is well developed in many fish species (Tsai and Lin, 2012). Although vitrification has made significant progress for sperm storage, standardization of the approach for individual species is technologically challenging (Xin et al., 2017). Sturgeon is an ancient fish group, with sizes to 2–3 m in length, producing high quantities of sperm but with low concentration compared to bony fish. At present, vitrification using a low quantity of sperm may be more suitable for fish producing small quantities of highly concentrated sperm. Meanwhile, vitrification is a promising technology that must be deployed with care and diligence to be developed into a reliable strategy with commercial applications.

Impact of cryopreservation on sterlet sperm proteome and motility parameters

In the present study (Chapter 3), sterlet sperm was stored using conventional cryopreservation methods that have been successfully applied in many fish species. In the fish-rearing industry, parameters including motility rate, velocity, membrane integrity, and metabolic activity are used to evaluate sperm quality and predict reproductive success. Motility rate is the most frequently used characteristic for evaluation of fresh or cryopreserved sperm quality, as it is simple, visually demonstrable, and positively correlated with fertilization capacity (Rurangwa et al., 2004; Fu et al., 2017). Cryopreservation has been shown to decrease spermatozoon motility in many fish species, including in sturgeon (Psenicka et al., 2008; Boryshpolets et al., 2017). Our findings were consistent with previous studies showing sterlet sperm motility rate to decrease significantly after freeze-thaw procedures, compared with fresh sperm. It is presumed that the decrease in motility may be associated with membrane damage and alteration of some sperm proteins during cryopreservation.

Spermatozoon velocity is one of the chief determinants of the outcome of competition that is related to fertilizing success when multiple spermatozoa surround an egg (Gage et al., 2004; Malo et al., 2005). No significant difference in curvilinear or straight-line velocity was found between fresh and cryopreserved sperm in the reported studies.

Sperm cryopreservation involves extreme temperature change, provoking irreversible cryoinjury to cell morphology, structure, and physiology and consequently leading to impaired motility and fertilization capacity (Lahnsteiner et al., 1996; Linhart et al., 2000). These injuries probably result from alteration of the functional state of proteins in the sperm nucleus, midpiece, flagellum, plasma membrane, and cytosol (Zilli et al., 2003; He and Woods, 2004; Figueroa et al., 2016a). Zilli et al. (2014) reported that cryopreservation can lead to the loss of membrane proteins in fish spermatozoa. The use of MALDI-TOF/TOF MS in the present study identified eight proteins in seminal plasma and spermatozoa that changed in intensity following cryopreservation (Xin et al., 2018a).

We (Xin et al., 2018a) froze sterlet seminal plasma separately to assess the effects of cryopreservation on seminal plasma proteins without influence of proteins from damaged spermatozoa. The results showed that mitochondrial ATP synthase subunit alpha and heat shock protein 70 were significantly impacted by cryopreservation. Mitochondrial ATP synthase subunit alpha, correlated with metabolism to provide ATP for spermatozoon movement, has been detected in the seminal plasma of infertile humans (Zilli et al., 2014; Agarwal et al., 2015). Van Tilburg et al. (2013) reported ATP synthase as a spermatozoon membrane-associated protein in Morada Nova rams. Our findings of lower levels of ATP synthase in cryopreserved sterlet seminal plasma suggest that cryopreservation might have an impact on this protein, causing inactivation. Heat shock protein 70 has been found in a range of species and cell types, including boar sperm, carp sperm, and rainbow trout seminal plasma (Huang et al., 2000; Dietrich et al., 2014; Nynca et al., 2014). Its primary function lies in protecting cells from thermal and oxidative stress as well as acting in cell immune, apoptotic, and inflammatory processes (Roberts et al., 2010). The heat shock protein 70 in seminal plasma may be involved in proteolysis (Nynca et al., 2014). Reduced quantities of heat shock protein 70 in seminal plasma after cryopreservation can negatively influence sperm quality (Zhang et al., 2015; Varghese et al., 2016).

Alterations in mitochondrial, cytoskeletal, nuclear, and cytosolic proteins during cryopreservation can damage sperm structure and function (Nynca et al., 2015). A large number of proteins are released from spermatozoa as a result of cryopreservation (Nynca et al., 2015; Dietrich et al., 2015). In the present study (Xin et al., 2018a), six of thirteen altered proteins were identified in spermatozoa following cryopreservation and categorized

into groups based on cellular components. Triosephosphate isomerase and mitochondrial ATP synthase subunit β were classified as mitochondrial enzyme and mitochondrial membrane proteins, respectively. Glycerol-3-phosphate dehydrogenase [NAD(+)] is located in the cytoplasm. Enolase B is a cytosolic protein. These proteins are members of a superfamily of glycolytic enzymes, playing primary roles in metabolic pathways such as gluconeogenesis and glycolysis. They are essential to production of energy for spermatozoon movement. The observed reduced quantities of these four proteins in cryopreserved sperm could be associated with ATP deficiency and reduced spermatozoon motility. The remaining two altered proteins, testis-specific tubulin α chain and tubulin β chain, are the major constituents of sperm microtubules, acting in the organization of the microtubule cytoskeleton. A decrease of cytoskeleton-related proteins after cryopreservation has been reported in human, boar, carp, and rainbow trout sperm (Desrosiers et al., 2006; Chen et al., 2014; Dietrich et al., 2015; Nynca et al., 2015). The decrease of tubulin with cryopreservation may affect spermatozoon flagellum structure and movement symmetry, resulting in impairment of sterlet sperm motility.

Some researchers suggest that the decrease of protein quantities in sperm following cryopreservation is primarily the consequence of leakage of proteins from spermatozoa (Dietrich et al., 2015; Nynca et al., 2015). In the present study (Xin et al., 2018a), we found that protein degradation was probably most responsible for the decrease of protein quantities of sperm during cryopreservation, since reduction of several proteins was detected in cryopreserved seminal plasma. To confirm this speculation, the proteomic profile should be investigated in both fresh and cryopreserved spermatozoa, as well as in fresh seminal plasma and frozen extracellular media. The latter analysis was not conducted in the present research.

Antifreeze proteins protect sterlet spermatozoon plasma membrane during cryopreservation

Spermatozoon ability to reach and fuse with the oocyte is dependent on fluidity, fusogenicity, and permeability of the plasma membrane (Wassall and Stillwell, 2009). The structural and functional integrity of the sperm plasma membrane is a commonly-used indicator of fish sperm quality, especially for cryopreserved sperm, in which plasma membranes are vulnerable (Ogier de Baulny et al., 1997; Muller et al., 2008). Cryopreserved sturgeon sperm has been demonstrated to show a significant decrease in plasma membrane integrity compared to the fresh sperm (Horokhovatskyi et al., 2018). We employed antifreeze proteins as a potential means of preserving the quality, especially plasma membrane integrity, of frozen-thawed sterlet sperm (Xin et al., 2018b). The results indicated that plasma membrane of sterlet sperm was significantly damaged upon cryopreservation. The addition of antifreeze proteins significantly improved the integrity of frozen-thawed sterlet spermatozoon plasma membrane, with the exception of that cryopreserved with 0.1 μg/mL of AFPI. No significant difference in plasma membrane integrity was observed between fresh sperm and sperm cryopreserved with 10 μg/mL of AFPI or AFPIII. However, the beneficial effect of AFPI and AFPIII on plasma membrane integrity of sterlet sperm was not associated with a significant increase in motility parameters of the post-thawed sterlet sperm. This may be reflect other cryopreservation-related injury in addition to the plasma membrane damage (Cabrita et al., 2005; Li et al., 2006; Figueroa et al., 2017).

A seemingly contradictory phenomenon occurred in the present study in that the cryopreserved sperm without addition of AFPI or AFPIII showed 44 ± 9% motility, while flow cytometry indicated only 26.6 ± 15% live cells. This discrepancy may be attributed to differences in the experiment setup. The motility of cryopreserved sperm was assessed immediately after thawing, while the sperm used to estimate plasma membrane integrity was incubated with fluorescent dye and measured 30 min or more after thawing. Therefore, data of motility and

membrane integrity are not comparable and should be considered separately. Spermatozoon motility rate is probably the most complex parameter to evaluate in the Computer Assisted Semen Analyzer (CASA) system, since it is likely to be affected by the CASA system used (Boryshpolets et al., 2013b) and factors such as immotile cells uncounted as a result of being out of focus or tracking collisions of overcrowded spermatozoa (Boryshpolets et al., 2013a). Thus, the motility rate obtained by CASA should be interpreted with caution.

Results of Xin et al. (2018b) provide insight into the protective effects of antifreeze proteins on sterlet sperm motility parameters and plasma membrane integrity. Antifreeze proteins can maintain the quality of sterlet sperm and standardize the cryopreservation protocol for sturgeon and other fish species.

Effects of antifreeze proteins on cryopreserved sterlet sperm fertility

As discussed, antifreeze proteins provide benefits by stabilizing the sterlet sperm plasma membrane during cryopreservation. Successful fertilization using frozen-thawed sperm is the ultimate goal of a cryopreservation technique. Xin et al. (2018c) evaluated the effects of antifreeze proteins on fertilization ability of cryopreserved sterlet sperm. The results indicated no differences in fertilization rate of sperm frozen with/without supplementation by antifreeze proteins, except in the case of sperm cryopreserved with 100 μg/mL of AFPIII (39 ± 21%).

Our similar fertilization results with and without antifreeze proteins may have been due to the high spermatozoon: egg ratio (200,000:1) used, since, in previous studies, a significant decrease in fertilization rate was detected for cryopreserved sterlet sperm compared to fresh sperm when using 100,000:1 spermatozoon: egg (Boryshpolets et al., 2011; Dzyuba et al., 2014). Further, we recently observed that the fertilization rate can reach 89% and 98% with 1000:1 and 5000:1 spermatozoon: egg ratio, respectively, when using fresh sperm (unpublished data). The relatively greater spermatozoon: egg ratio in the present study could explain the lack of difference in fertilization rate between fresh and cryopreserved sperm with/without antifreeze proteins despite their differences in motility rate. Our results were consistent with Jähnichen et al. (1999) who reported a loss of motility but not of fertilizing capacity in frozen-thawed sterlet sperm. It was suggested that this finding resulted from the use of excess sperm, which can result in compensation for the loss of sperm motility in functional fertility rate (Billard et al., 2004). Spermatozoon numbers and motility rate are not the primary determinants in fertilization (Gage et al., 2004; Linhart et al., 2005). Fertilization rate has been demonstrated to be positively correlated with curvilinear and straight-line velocity in frozen-thawed sperm of salmonids (Lahnsteiner et al., 1996; Lahnsteiner, 2000; Figueroa et al., 2016b). Xin et al. (2018c) found no significant variation in curvilinear and straight-line velocity or in fertilization rate between fresh and frozen-thawed sterlet sperm with/without antifreeze proteins, consistent with the findings in salmonids. Future studies investigating the relationships among sperm motility, velocity, and fertilization rate could be of practical value in commercial aquaculture and conservation.

Conclusions

This research reviews information of sperm vitrification, a promising option for cryopreservation. Vitrification currently has limited practical value in fish culture, because standardized protocols have not yet been developed. Future research into vitrification should be deployed with care and diligence to standardize protocols and achieve a more reliable strategy with simple and high commercial efficiency. Traditional cryopreservation has been applied for sterlet sperm preservation with effects on sperm motility, protein profile, and

membrane integrity. The proteome differences between fresh and cryopreserved sperm may expand understanding of effects of cryopreservation. The identified proteins are possibly associated with spermatozoon motility, viability, and other functions. Addition of antifreeze proteins to cryopreservation extender showed protective effects on sterlet spermatozoa quality, especially on membrane integrity. Thus, antifreeze proteins might be helpful in standardization of cryopreservation protocol for sturgeon and other fish species.

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ENGLISH SUMMARY

The role of some proteins in freezing fish sperm

Miaomiao Xin

Sperm damage during cryopreservation is considered a major obstacle to the expansion of sperm storage technology in fish. In-depth knowledge of cryoinjury and cryoprotectants with respect to the quality of fish sperm can enhance future use of cryopreservation. We used antifreeze proteins as cryoprotective agents to improve the quality of frozen/thawed spermatozoa, along with optimization of cryopreservation protocols.

Chapter 2 reviews vitrification, a promising cryopreservation technique for fish sperm storage. Vitrification requires rapid cooling/warming, small volume containers, and use of permeable cryoprotectants at high concentrations to solidify both intra- and extra-cellular materials. High concentrations of cryoprotectant are toxic to cells. The quantity of permeable cryoprotectant can be reduced or eliminated by use of apparatus or techniques that dramatically increase freezing and warming rates by treating a much smaller quantity of sperm. Thus, vitrification may be more suitable for fish producing small quantities of highly concentrated sperm, but not sturgeon producing high quantities of sperm with low concentration.

In Chapter 3, proteomic methods were applied to characterize the protein profiles of sterlet spermatozoa and seminal plasma and assess their effect on spermatozoon function in conventional cryopreservation. The motility variables of cryopreserved sterlet sperm were investigated. The motility rate of sterlet sperm significantly decreased after cryopreservation, while no difference in mean curvilinear velocity of fresh and cryopreserved sperm was detected. Six proteins were altered in seminal plasma and thirteen in spermatozoa following cryopreservation. Among them, eight proteins were positively identified: a) two (mitochondrial ATP synthase subunit alpha and heat shock protein 70) were from seminal plasma, associated with metabolism and response to stress; b) four (triosephosphate isomerase, mitochondrial ATP synthase subunit β, glycerol-3-phosphate dehydrogenase [NAD(+)], enolase B) in spermatozoa are involved in metabolic pathways such as gluconeogenesis and glycolysis to provide efficient energy for spermatozoon movement; c) the remaining two (testis-specific tubulin α chain and tubulin β chain) in spermatozoa are major constituents of sperm microtubules, playing important roles in the organization of the microtubule cytoskeleton. These results broaden the understanding of protein-related cryoinjury in sperm, which may help to determine the function of altered proteins and provide new insights into improving sperm cryopreservation.

In Chapter 4, since cryopreservation is known to cause lethal and sublethal damage to sperm, different concentrations of antifreeze proteins (AFPI or AFPIII) were employed as cryoprotectants. The flow cytometry analysis revealed that supplementation with antifreeze proteins was associated with significantly higher membrane integrity in cryopreserved sterlet sperm, except with the use of 0.1 μg/ml of AFPI. However, motility rate, curvilinear velocity, straight-line velocity, and fertilization rate of frozen-thawed sperm did not differ from no addition of antifreeze proteins. It was concluded that addition of antifreeze proteins to cryopreservation medium was the source of the protective effects on sperm plasma membrane integrity.

CZECH SUMMARY

Úloha některých proteinů při zmrazování spermatu ryb

Miaomiao Xin

Poškození spermií během kryokonzervace je považováno za hlavní překážku expanze technologie uchovávání spermatu ryb. Precizní znalost kryokonzervace spermatu a kryoprotektantů s ohledem na kvalitu rybích spermií může rozšířit budoucí využívání této technologie. Pro zlepšení kvality zmrazených a rozmrazených spermií byly použity antifreeze proteiny jako kryoprotektivní látky spolu s optimalizací protokolů kryokonzervace.

V kapitole 2 byla popsána vitrifikace jako slibná kryokonzervační technika pro uchovávání rybích spermií. Tradiční vitrifikace vyžaduje prudké zmrazení/rozmrazení, malé nosiče vzorků s použitím permeabilních kryoprotektantů o vysokých koncentracích, a to k vitrifikaci jak intra, tak extracelulárních kapalin. Nicméně vitrifikace rybích spermií je nepoužitelná v praktické akvakultuře, protože se používají permeabilní kryoprotektanty s cytotoxickými účinky na buňky. Množství permeabilního kryoprotektantu však může být sníženo nebo eliminováno použitím zařízení nebo technik, které dramaticky zvyšují míru zmrazení/rozmrazení ošetřením mnohem menšího množství spermií. Vitrifikace může být tudíž vhodnější pro ryby produkující malé množství koncentrovaného spermatu, ale není vhodná u jeseterů produkujících velké množství spermií s nízkou koncentrací ve srovnání s kostnatými rybami.

V kapitole 3 byly využity proteomické metody pro charakterizaci proteinového profilu u spermií a v semenné plazmě a studován jejich vliv na funkci spermií při konvenční kryokonzervaci. Dále byly zkoumány změny v motilitě spermií jesetera malého během kryokonzervace. Míra motility spermií jesetera malého významně poklesla po kryokonzervaci spermatu, kdežto u čerstvých a zmrazených spermií nebyl zjištěn rozdíl v průměrné nelineární rychlosti. Změna v proteinovém profilu po zmrazení byla zaznamenána celkem u šesti proteinů v semenné plazmě a u třinácti v případě spermatu. Mezi nimi bylo pozitivně identifikováno osm proteinů: a) dva (mitochondriální podjednotka ATP syntházy alfa a protein tepelného šoku 70) pocházející ze semenné plazmy, spojené s metabolismem a odpovědí na stres; b) čtyři (triosefosfát izomeráza, mitochondriální ATP syntházová podjednotka β, glycerol-3-fosfátdehydrogenáza [NAD (+)], enolasa B) ve spermiích se podílejí na metabolických drahách jako je glukoneogeneze a glykolýza a zajišťují účinnou energii pro pohyb spermie; c) další dva (řetězec α tubulinu a řetězec tubulinu β, specifický pro testes) ve spermiích jsou hlavními složkami mikrotubulů spermií, které hrají důležitou roli v organizaci cytoskeletu mikrotubulu. Dosažené výsledky rozšiřují znalosti v proteinovém složení spermatu během kryokonzervace, souvisejících s jejich poškozením během zmrazení, což může pomoci určit funkci těchto změněných proteinů a poskytnout nové představy o tom, jak zlepšit kryokonzervaci spermií.

Je známo, že kryokonzervace způsobuje letální a subletální poškození spermií, proto byly v kapitole 4 použity během kryokonzervace různé koncentrace antifreeze nemrznoucích proteinů (AFPI nebo AFPIII) jako kryoprotektantů. Analýza průtokovou cytometrií ukázala, že přidáním nemrznoucích proteinů došlo významně ke zlepšení membránové integrity spermatu jeseterů malých po kryokonzervaci s výjimkou koncentrace 0,1 μg/ml AFPI. Ve zmrazených spermiích s přídavkem nemrznoucích proteinů však nebylo pozorováno žádné statistické zlepšení rychlosti motility, nelineární rychlosti, lineární rychlosti a fertility ve srovnání se spermiemi bez přídavku nemrznoucích proteinů. Z toho vyplývá, že přidávání nemrznoucích proteinů do kryokonzervačního média poskytuje určité ochranné účinky integritě plazmatické membrány spermií.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Dadras, H., Dzyuba, V., Golpour, A., **Xin, M.M.,** Dzyuba, B., 2019. In vitro antioxidant enzyme activity and sperm motility at different temperatures in sterlet *Acipenser ruthenus* and rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem. Doi: https://doi.org/10.1007/s10695-019-00675-w (IF 2019 = 1.729)

Xin, M.M, Niksirat, H., Shaliutina-Kolešová, A., Siddique, M.A.M., Sterba, J., Boryshpolets, S., Linhart, O., 2019. Molecular and subcellular cryoinjury of fish spermatozoa and approaches to improve cryopreservation. Rev. Aquacult. doi: 10.1111/raq.12355 (IF 2018 = 7.190)

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Zhang, S.H., Wei, Q.W., **Xin, M.M.,** Wang, D.Q., Li, C.J., Yue, H.M., 2015. The genetic diversity in broodstocks of the endangered Chinese sturgeon, *Acipenser sinensis*, using novel microsatellite markers by EST sequencing. In: Book of abstracts "World Aquaculture for Healthy People, Planet and Profit", 26–30 May 2015, Korean.

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Xin, M.M., Shaliutina-Kolesova, A., Sterba, J., Konika, P., Boryshpoletsa, S., Rodina, M., Linhart, O., 2017. Impact of cryopreservation on sterlet (*Acipenser ruthenus*) sperm motility and proteome. In: Book of abstracts "8th international symposium on sturgeon", 10–17 September 2017, Vienna, Austria.	2017
Xin, M.M., Shaliutina-Kolesova, A., Sterba, J., Siddique, M., Dzyuba, B., Boryshpolets, S., Kholodnyy, V., Li, P., Linhart, O., 2017. Protective role of antifreeze proteins on sterlet (*Acipenser ruthenus*) spermatozoa during cryopreservation. In: Book of abstracts "6th International Workshop on the Biology of Fish Gametes", 4–7 September 2017, České Budějouvice, Czech Republic.	2017
Zhang, S.H., Wei, Q.W., Xin, M.M., Wang, D.Q., Li, C.J., Yue, H.M., 2015. The genetic diversity in broodstocks of the endangered Chinese sturgeon, *Acipenser sinensis*, using novel microsatellite markers by EST sequencing. In: Book of abstracts "World Aquaculture for Healthy People, Planet and Profit", 26–30 May 2015, Korean.	2015

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