Chapter 15
Sperm Cryopreservation of Aquatic Species

Ákos Horváth and Béla Urbányi

Abstract Cryopreservation of fish sperm is feasible, and methods have been developed for several hundred species. The procedure includes the collection of sperm and assessment of its quality, dilution in suitable extenders, addition of cryoprotectants, loading into freezing devices, cooling, storage, thawing, and finally post-thaw use of sperm. An alternative to traditional freezing methods is the vitrification of sperm which is promising primarily in smaller model fish species. A wide variety of protocols are available in the literature; however, in spite of the significant progress made by the scientific community, commercial application of fish sperm cryopreservation is still very limited.

Keywords Sperm · Quality · Freezing · Cryopreservation · Vitrification

15.1 Introduction

Cryobiology is a branch of biology that focuses on the survival of living matter at ultra-low temperatures. Sperm was among the first cell types to be cryopreserved with the earliest reports dating back to the late 1940s. Thus, the survival of sperm from various mammalian and avian species exposed to dry ice (−79 °C) and the beneficial effect of glycerol on post-thaw survival was reported in 1949 (Polge et al. 1949). This was soon followed by the birth of the first calf born of insemination with cryopreserved sperm (Stewart 1951). The first study in fish has reported the use of cryopreservation for the hybridization of fall- and spring-spawning varieties of the herring (*Clupea harengus*) which would have obviously been impossible in natural conditions (Blaxter 1953). Since then, cryopreservation has become a subject of extensive scientific studies as well as a routine procedure in assisted reproduction.

Á. Horváth (✉) · B. Urbányi
Department of Aquaculture, Szent István University, Gödöllő, Hungary
e-mail: Horvath.Akos@mkk.szie.hu

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In aquatic species—primarily finfish—sperm cryopreservation faces specific challenges. These include the typically short motility period of spermatozoa, activation of spermatozoa upon release into the aquatic environment, as well as increased volumes of sperm and eggs related to the body weight in comparison to terrestrial mammals. These need to be taken into account when developing cryopreservation protocols for fish.

15.2 Principles of Cryopreservation

The main goal of cryopreservation is the arrest of biological time which is possible at extreme low temperatures. Below −130 °C water exists only in a crystalline or amorphous solid form where the movement of molecules is limited; thus, its effects can only be measured in geological time. Consequently, at the boiling point of liquid nitrogen (the most commonly used coolant and storage environment), −196 °C, no physical or chemical processes requiring heat energy exist (Mazur 1984).

The principles of cryopreservation of live matter are closely related to the principles of freezing of water or aqueous solutions. The freezing point of water is 0 °C; however, water rarely freezes at that temperature. Water and its solutions have the tendency to supercool beyond the freezing point and remain in the liquid state until ice formation begins along so called ice nuclei. Ice nucleation can start spontaneously or more commonly as a result of ice nucleating agents (Mazur 1990). Energy (called latent heat) is released during transition from the liquid state to the solid, which causes the temperature of solution to increase to the freezing point. During freezing, ice crystals are primarily built of water molecules (and not those of the solutes); thus, the concentration of the solution increases with the process of freezing. This is continued to the eutectic point when the entire solution solidifies (Denniston et al. 2011).

During cryopreservation of live cell and tissues, the cell membrane plays a key role in osmotic regulation that allow cells to survive the process. As there are no efficient ice nucleating agents within the cells, ice formation typically starts in the extracellular space (Mazur 1970) which leads to an increased concentration of solutes outside the cells. Cells try to compensate for the difference in osmotic pressure by releasing water. According to the two-factor hypothesis of freezing injury, cell death can occur either if cells are cooled too slow or too fast (Mazur et al. 1972). Too slow cooling results in prolonged exposure of cells to an increasing concentration of solutes (to the point of cytotoxicity) while too fast cooling does not allow the cells to release sufficient volumes of water which freezes inside the cells in the form of lethal ice crystals. Thus, an optimal cooling rate must be found to maximize the cryosurvival of cells.

Extreme fast cooling results in the formation of an amorphous glassy solid in the process of vitrification. In this case, the solid state is attained by a sudden increase

of viscosity instead of crystallization. The absence of ice crystals—and the damages associated with them—would be ideal for cells; however, vitrification has several shortcomings. Successful vitrification requires high concentrations of toxic cryoprotectants (Fahy et al. 1984). Also, vitrified matter is metastable, it can only be sustained at very low temperatures (below −100 to −130 °C) as higher temperatures lead to recrystallization of ice. Nevertheless, even classical cell freezing protocols lead to partial vitrification, primarily in the intracellular space (Leibo et al. 1978; Hirsh et al. 1985).

The rate of thawing of cryopreserved cells or tissues is at least as important as that of their cooling. During slow thawing, small ice crystals present in cells can start growing and vitrified matter can recrystallize causing mechanical damage to the cells. When cells are thawed fast (at a rate of 1000 °C/min or faster), they quickly rehydrate and their survival improves (Denniston et al. 2011). For vitrified cells and tissues, it is commonly accepted that from the point of view of survival, the thawing rate is actually more important than the cooling rate (Mazur and Seki 2011; Seki et al. 2014).

15.3 Cryopreservation of Fish Sperm

Cryopreservation of fish sperm follows a set of important steps such as collection of sperm, determination of sperm quality, dilution with extenders and cryoprotectants, packaging (loading into straws or other devices), freezing or vitrification of sperm, storage, thawing, determination of post-thaw sperm quality, and finally fertilization with thawed sperm (Fig. 15.1). These steps are crucial for the development and application of a successful cryopreservation protocol.

15.3.1 Collection, Storage, and Determination of Sperm Quality

During the spawning season, the majority of fish species produces a relatively large volume of sperm that can be collected by hand-stripping. There are exceptions, such as many catfish species (members of the order Siluriformes) that are either oligospermic or stripping is impossible due to anatomical reasons. In these species, the testis or a part of it is surgically removed and sperm is squeezed through a sterile gauze or other mesh fabric (Legendre et al. 1996). A general rule during sperm collection is that contamination of sperm with water, urine, or feces should be avoided as these can either activate sperm or carry bacteria and enzymes that can damage spermatozoa. Collected sperm should be kept at a low temperature (0–4 °C) that slows the rate of chemical processes in it.

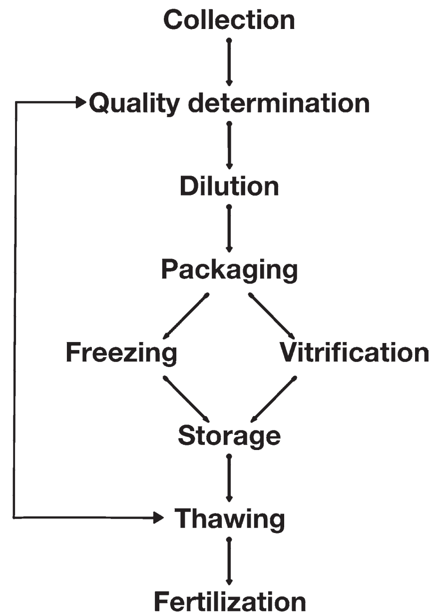

Fig. 15.1 Steps in the process of fish sperm cryopreservation

Following collection, the quality of sperm should be determined. In fish, sperm quality prior to cryopreservation is characterized almost exclusively by its motility determined either by visual estimation or by computer-assisted sperm analysis—CASA (Gallego and Asturiano 2018a,b). Visual estimation is often criticized for its subjectivity, especially in scientific circles. Sperm is typically activated on a microscope slide with an activating solution and observed under a light microscope at 10–25× magnification; finally the percentage of motile cells is estimated (Fauvel et al. 2010). The accuracy of visual estimation can be improved by pre-dilution of sperm in isotonic solutions prior to the investigations (Billard and Cosson 1992). CASA systems consisting of a phase contrast microscope, a camera, and a software analyzing the video sequences offer more objective results and more detailed information on the movement of cells such as motility or various velocity parameters (WHO 2010). On the other hand, they are more expensive, and their accuracy also depends on the analysis settings and dilution ratio of sperm; thus, their use also requires a training period. During the last decade, CASA became the standard procedure to determine sperm quality in fish sperm cryopreservation research (Beirão et al. 2011; Bernáth et al. 2016; Judycka et al. 2016; Riesco et al. 2017). This was further facilitated by the availability of free sperm analysis software packages such as the CASA plugin of the open-source image analysis software ImageJ (Wilson-Leedy and Ingermann 2007).

Sperm quality can be determined by other parameters, as well. These include sperm concentration expressed as the number of spermatozoa per milliliter of sperm that can be determined by the use of various hemocytometers (Bürker-Türk, Thoma, Neubauer, Neubauer Improved, etc.). Other devices whose primary function is not

the determination of sperm concentration can also be successfully used for this task such as spectrophotometers (Ciereszko and Dabrowski 1993; Fauvel et al. 1999) or cell counters such as flow cytometers or Coulter counters (Fauvel et al. 2010). Sperm quality can also be determined by the intact morphology of cells; however, in fish these are restricted to species with a distinct sperm head shape such as sturgeons or eels (Asturiano et al. 2007). Membrane integrity of sperm cells (often referred to as viability) is also used to characterize sperm quality. Differential staining methods are used to distinguish cells with intact and damaged membranes. The eosin/nigrosin staining developed originally for mammalian sperm (Blom 1950) is rarely used in fish, although nigrosin alone was used to evaluate membrane integrity of sea bass (*Dicentrarchus labrax*) sperm (Zilli et al. 2004). Currently, differential fluorescent staining procedures combined with flow cytometry are commonly practiced. One example is the use of SYBR-14 to stain membrane-intact cells in fluorescent green and propidium iodide (PI) to counterstain membrane-damaged cells in fluorescent red (Segovia et al. 2000; Flajšhans et al. 2004; Cabrita et al. 2011). Other fluorescent dyes have also been tested on fish sperm such as Hoechst 33258 (Asturiano et al. 2007) to investigate membrane integrity or Rh123 (Ogier De Baulny et al. 1997) to test mitochondrial function. Genotoxic damage to cells can be tested using the Comet assay (Zilli et al. 2003; Cabrita et al. 2005), whereas the energy content of spermatozoa can be described by the concentration of ATP in the cells (Perchec et al. 1995; Boryshpolets et al. 2009).

15.3.2 Dilution of Sperm

Prior to freezing, sperm needs to be diluted in a cryomedium. The cryomedium consists of two principle components: the extender and the cryoprotectants (note that the expressions cryomedium and extender are used interchangeably in the literature). Extenders are solutions of salts, sugars (or the combination of the two), and other chemicals, and their main function is the reversible immobilization of sperm (Glenn III et al. 2011). They exert their function by providing isotonic conditions for the cells or by maintaining an adequate K^+ concentration in species requiring that (salmonids, sturgeons). Their buffer systems prevent fluctuations of pH during freezing. Extenders allow dilution of sperm to the required ratio.

Cryoprotectants—as shown by their name—protect cells from the damaging effects of freezing and thawing. They decrease the freezing point of the solution, they bind to water and thus prevent its incorporation into ice crystals and probably stabilize cell membranes. Cryoprotectants are classified as external or non-penetrating and internal or penetrating depending on whether they exert their action outside or inside cells (Denniston et al. 2011). External cryoprotectants can be sugars or polymers that can be part of the extender (contributing to the problems of the nomenclature); thus, they also have multiple roles: they maintain the osmolality of the solution around the cells, protect cells from osmotic shock (osmoprotectants), decrease the freezing point of the solution and provide nutrients for spermatozoa

(Lahnsteiner et al. 1993). They are, however, almost exclusively used in combination with internal cryoprotectants.

Internal cryoprotectants are low molecular weight chemicals that penetrate the cell membrane and exert their action inside the cells. Glycerol was the first internal cryoprotectant to be discovered and used both in livestock and in fish (Polge et al. 1949; Blaxter 1953); however, since then a multitude of chemicals were found to have cryoprotective function. Nevertheless, in fish only a handful of cryoprotectants are used regularly. The most common ones include dimethyl-sulfoxide (DMSO or Me_2SO) (Mounib 1978; Legendre and Billard 1980; Kurokura et al. 1984), methanol (MeOH) (Harvey et al. 1982; Lahnsteiner et al. 1996; Horváth et al. 2003; Kása et al. 2017), *N*,*N*-dimethyl-acetamide (DMA) (McNiven et al. 1993; Horváth and Urbányi 2000; Morris et al. 2003; Warnecke and Pluta 2003), ethylene glycol (EG) (Jähnichen et al. 1999), propylene glycol (PG) (Conget et al. 1996), and methyl glycol (also known as 2-methoxyethanol) (Viveiros et al. 2012, 2014, Gallego and Asturiano 2018b).

External cryoprotectants are typically characterized by low toxicity while internal cryoprotectants have a varying degree of toxicity. Thus, their concentration also has to be chosen according to different principles. While in case of external cryoprotectants, the most important goal is the maintenance of osmotic balance and prevention of hypo- or hyperosmotic shock, in the case of internal cryoprotectants toxicity also needs to be taken into account.

Sperm dilution is followed by equilibration of osmotic pressure between the extracellular and intracellular spaces. The importance of equilibration time is controversial. The cryoprotective effect of glycerol was evident after equilibration for 30–60 min (Suquet et al. 2000), and a positive effect was observed following a 10-min equilibration of rainbow trout sperm exposed to DMA (Babiak et al. 2001). In case of DMSO, equilibration seemed to be more harmful rather than beneficial (Stoss and Holtz 1983; Gwo 1994), sometimes causing premature activation of spermatozoa in marine species (Peñaranda et al. 2009). Equilibration time does not play a major effect on post-thaw sperm quality when methanol is used as a cryoprotectant (Lahnsteiner et al. 1997).

15.3.3 Freezing of Sperm

Liquid nitrogen and its vapors are currently used almost exclusively for the cryopreservation of fish sperm. Earlier, dry ice was also used as a coolant (Mounib et al. 1968; Chao et al. 1987; Billard et al. 1993); however, it is seldom employed for fish sperm now. Dry ice cannot be used for the storage of cells as cells gradually die at its sublimation temperature due to the possible presence of unfrozen solution to which cells are exposed (Mazur 1984). Liquid nitrogen, on the other hand, ensures adequate cooling rates and storage conditions at its boiling temperature (−196 °C).

Sperm can be packaged into various containers before freezing. These can be cryotubes, ampoules, or French straws developed for the freezing of dairy bull

sperm. Straws were patented by Robert Cassou in France in 1949 and have seen worldwide use for dairy bull sperm. Straws are plastic tubes produced in various sizes (0.25, 0.5, and 1.2 mL) that contain a tripartite plug at one end consisting of a powder between two fibrous pads that gelifies upon contact with aqueous solutions and acts as a stopper. Macrotubes produced in volumes of 4–5 mL are of different construction, they are offered with metal or plastic balls to be inserted into one or both ends to act as stoppers.

Sperm filled into straws can be frozen in the vapor of liquid nitrogen in uncontrolled or controlled conditions. Uncontrolled cooling is typically done in polystyrene (Styrofoam) boxes filled with liquid nitrogen. Straws are laid at a certain distance from the surface of liquid nitrogen (e.g., on a polystyrene raft) and allowed to cool for several minutes before being plunged into the liquid. A standardized variant of these boxes was developed for the cryopreservation of salmonid sperm (Lahnsteiner 2011). Cooling rate can be regulated by the height and the duration of holding straws above the level of liquid nitrogen, and this can have a significant effect on post-thaw motility (Boryshpolets et al. 2017). An alternative to the cooling box is freezing in a dry shipper (a container used for the shipment of cryopreserved samples) that offers even less control over the cooling process; yet, it is used successfully for the freezing of fish sperm. The method was first used by Brazilian scientists (Viveiros and Godinho 2009) and was found to be more reliable than the cooling box when freezing varying numbers of straws (Horokhovatskyi et al. 2017).

A controlled-rate freezer allows a precise and replicable cooling of samples which is difficult to attain with the methods described above. These freezers consist of a freezing chamber and a computer. The software installed in the computer regulates the cooling rate according to a program set up by the user. In most system, individual probes measure the temperature of the chamber and the sample which gives the software feedback on the progress of cooling. Most controlled-rate freezers also use the vapor of liquid nitrogen; however, coolant-free devices such as an electric ultrafreezer (Diogo et al. 2018) also exist.

Following the required period of storage, samples need to be thawed before further use. Sperm samples need to be thawed quickly in order to avoid devitrification and recrystallization of partially vitrified solids that result in similar damages as the formation of large ice crystals during freezing. Typically, samples are thawed in a water bath at 25–40 °C for a varying period of time (Sarvi et al. 2006; Boryshpolets et al. 2017). For straws, thawing time can reach up to 30 s, while for macrotubes and cryovials up to 150 s (Cabrita et al. 2001; Riesco et al. 2017).

15.3.4 Post-thaw Use of Sperm

Following thawing, sperm quality is determined as described above (Sect. 15.3.1) and then sperm is used for fertilization. Post-thaw sperm motility is typically lower than that of fresh sperm; however, contrary results have also been reported (Viveiros et al. 2010). The maximum attainable post-thaw motility was earlier considered to

be taxon-specific; however, due to methodical refinement, the threshold values keep improving.

The principles of fertilization with cryopreserved sperm are identical to those using fresh sperm. Sperm–egg ratio is regularly taken into account when maximizing fertilization success. As fertilizing capacity of frozen-thawed sperm is typically lower than that of fresh sperm, in many cases higher sperm–egg ratios are recommended and are crucial to optimize the use of cryopreserved sperm (Ciereszko et al. 2014; Nynca et al. 2017; Judycka et al. 2018). Fertilization success is usually measured as the hatch rate of larvae although sometimes larval deformities are also assessed (Horváth and Urbányi 2000; Young et al. 2009).

Unlike the use of cryopreserved sperm in dairy bull farming, fish sperm cryopreservation is not anticipated to be employed commercially for direct aquaculture production in the near future. This is mainly due to the fact that domestication and genetic improvement in aquatic species is still in its infancy, and higher yields can be achieved by other means (e.g., nutrition) (Asturiano et al. 2017). Also, the technologies developed for dairy bull sperm (intended to fertilize a single egg) cannot be adapted directly to fish species that produce much larger volumes of both sperm and eggs; thus, alternative methods are sought such as the use of 4–5 mL straws or macrotubes (Horváth et al. 2007, 2010). In spite of the recent efforts for the commercial use of cryopreserved sperm in the genetic improvement in the Atlantic salmon (*Salmo salar*, www.cryogenetics.com), the general consensus is that cryopreservation of sperm in fish species is expected to become part of aquaculture practice when the industry shows a legitimate demand for it. Until then, it will be applied on individual basis and mostly using public funding (Asturiano et al. 2017). Efforts to standardize fish sperm cryopreservation technologies using high-throughput methods contribute to the improvement of fertilization with cryopreserved sperm which is a further step towards commercial use (Tiersch et al. 2011; Hu et al. 2013; Matthews et al. 2018).

15.4 Vitrification of Fish Sperm

Vitrification of fish sperm has recently gained attention as an alternative to conventional freezing (Cuevas-Uribe et al. 2011a, b, 2015; Merino et al. 2012; Figueroa et al. 2013). What makes it interesting is applicability to the sperm of model fish species characterized by small individual body size such as the zebrafish (*Danio rerio*) or the Mexican swordtail (*Xiphophorus hellerii*). In these species, the volume of sperm that can be collected is very low, e.g., in zebrafish, it is in the range of 1–1.5 μL which makes most available freezing methods impractical, although recent systematic optimization of protocols resulted in stable and high fertilization rates (Matthews et al. 2018). Vitrification is efficient for the cooling of low liquid volumes such as 20–30 μL (Schuster et al. 2003) or according to our own experiences 2.5–5 μL (Kása et al. 2017). Successful vitrification requires the use of high concentrations (up to 40% v/v) of cryoprotectants which can be highly toxic to the cells;

thus, the combination of two or three cryoprotectants is recommended. For vitrification the use of devices in which the liquid forms a thin layer or film is recommended. These include devices specifically developed for vitrification such as Cryotops or even the inoculating loops used in cell cultures. Unfortunately, post-thaw motility and fertilization rates using vitrified fish sperm are typically very low; thus, the currently available vitrification methods are not suitable alternatives to conventional freezing in this field.

15.5 Conclusion

Fish sperm cryopreservation is an efficient tool in the preservation of genetic diversity of fish species. Protocols have been described in many species that result in viable spermatozoa following thawing; however, commercial application of sperm cryopreservation in aquatic species is still very limited. Future research is anticipated to concentrate on the standardization and development of high-throughput methods in order to improve fertilization results with cryopreserved sperm.

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