

Review on Cryopreservation in Fisheries Science: Applications and Perspectives

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Abstract: Cryopreservation is of interest not only for fish farming but also for the conservation and genetic improvement of resources. This technique has been well established in some freshwater fish species mainly, salmonid, sturgeons and carps, however, only in the last decade research was focused in marine fish species. The benefits of sperm cryopreservation include: (i) synchronization of gamete availability of both sexes, (ii) sperm economy; (iii) simplification of broodstock management, (iv) transport of gametes from different fish farms, and (v) germplasm storage for genetic selection programs or conservation of species. These issues would certainly benefit the aquaculture industry. The tremendous impact that biotechnology is having in aquaculture has been particularly obvious in recent years. Successful cryopreservation of fish sperm have been achieved for more than 200 fish species and many fish species have been adequately for the purpose of cryobanking. Cryopreservation of fish embryo is not viable, mainly because of the same limitations as in fish oocytes, i.e., high chilling sensitivity and low membrane permeability. However, cryopreservation of isolated embryonic cells is another option for preserving both maternal and paternal genome. In this paper, an overview of the current state of aquatic species is followed by a discussion on the sperm, embryos, oocytes and embryonic cells blastomeres.

Keywords: Aquaculture Cryopreservation, Fisheries Science, Gamete, Germplasm

1. Introduction

Cryopreservation is a long-term storage technique with very low temperatures to preserve the structurally intact living cells and tissues for extended period of time at a relatively low cost. Cryopreservation is to preserve and store the viable biological samples in a frozen state over extended periods of time. A very important part research in cryopreservation is to reveal the underlying physical and biological responses of the cell and cause of cryoinjury, especially those associated with the phase change of water in extracellular and intracellular environments [1]. From the original slow-cooling study, another cryopreservation approach has moved to easier and more efficient technique vitrification, Cryoprotective agents has to gain access to all the parts of the system. Cryopreservation considers the effects of freezing and thawing. Therefore, the diffusion and osmosis processes have important effects during the introduction of cryoprotective agents, the addition or removal of cryoprotectants, the cooling process, and during thawing. These phenomena are amenable to the experimental design and analysis. Thus, reliable methods can be developed for preserving a very wide range of cells and some tissues. These methods have found widespread applications in biology, biomedical technology and conservation. Germplasm cryopreservation includes storage of the sperm, eggs and embryos and contributes directly to animal breeding programmes. Germplasm cryopreservation also assist the *ex situ* conservation for preserving the genomes of threatened and endangered species. The establishment of germplasm banks using cryopreservation can contribute to conservation and extant populations in the future. Since the first successful cryopreservation of bull semen [2], cryopreserved bull semen has been used to propagate the rare and endangered species using assisted reproduction techniques. Every year, more than 25 million cows are

artificially inseminated with frozen-thawed bull semen [3] and many bovine calves have been produced using the transfer of cryopreserved embryos into cow. Tissues, cultured cell lines, DNA and serum samples could be frozen and store in cryogene bank. For example, mice and sheep have been generated from frozen-thawed pieces of ovary that have been replaced in a female and stimulated to ovulation [4], [5], [6].

2. Cryobiologic Principles

Nature dictates that biological material will decay and die. The structure and function of organisms are changed and lost with the time. An attempt to stop the biological clock, experiments with temperature and water contents of the cell is the basic theme of cryopreservation research. The use of much lower temperatures has proved a means of storing living organisms in a state of suspended animation for extended periods. The removal of water from biological material in the frozen state (freeze-drying) provides another means of arresting the biological clock by withholding water, and commencing again by its addition. This is all attempted after realization that water is the major component of all living cells and essential for the chemical processes of life to occur. Cellular metabolism stops when all water in the system is converted to ice during cooling and freezing. Further, reduced temperature lowers the motion of molecules. At absolute zero (-273°C) all molecular motions are inhibited and all biological and biochemical events cease. It is those events at ultra-low temperatures that provide basic mechanisms for long term preservation of biological material in genetically stable form. In practice, no significant change of biological importance occurs below -150°C and therefore material can be conveniently stored in liquid nitrogen vapour or liquid nitrogen at -196°C [7].

3. Sperm

In 1949, [2] successfully cryopreserved the avian spermatozoa using glycerol as a cryoprotectant. Thereafter, cryopreservation of male gamete became possible. [8] applied a similar approach for fish gametes and reported success with Atlantic herring spermatozoa, achieving approximately 80% cellular motility after thawing. Since then, cryopreservation of fish sperm has been studied and has been successful in more than 200 species [9], [10], [11] and techniques of sperm management have been established for freshwater and marine fish species, including carp, salmonids, catfish, cichlids, medakas, white-fish, pike, milkfish, grouper, cod, and zebrafish [12] [13], [14], [15].

Many studies on cryopreservation of fish sperm have been carried out on economically important freshwater species and attempts to cryopreserve sperm from the marine fish species tended to be more successful when compared with

those obtained from the freshwater fish. Although freshwater fish sperm are generally more difficult to cryopreserve, the fertilization rates obtained from the cryopreserved marine fish sperm are similar to those obtained with mammalian species [16]. Controlled-rate slow cooling in cryopreservation has been mainly used for fish sperm. Common carp has been studied using frozen-thawed sperm with 95% fertilization and hatching rate (Table 1).

Successful cryopreservation of the sperm of aquatic invertebrate has been carried out for sea urchin, oyster, starfish, abalone and coral [17], [18], [19]. Dimethyl sulfoxide has also been reported as a successful cryoprotectant for sperm cryopreservation; the concentration range used was 5 to 30% for these species. Various levels of motility, ranging from <5% to 95%, have been reported for the cryopreserved aquatic invertebrate sperm [20].

Table 1: Extender composition, cryoprotectant concentration and semen: extender dilution ratio used for cryopreserve sperm from several fish species

Species	Extender	Extender composition	[Cryoprotectant] extender	Dilution ratio	References
Rainbow trout	6 Erdahl and Graham	0.70 mM CaCl ₂ .2H ₂ O, 1.08 mM MgCl ₂ .6H ₂ O, 1.49 mM Na ₂ HPO ₄ , 34.30 mM KCl, 99.95 mM NaCl, 0.52 mM citric acid, 55.51 mM glucose, 2.26 mM KOH, 6.50 mM bicine, 1% BSA	7% DMSO	1 : 3	[21]
Brown trout	6 Erdahl and Graham	0.70 mM CaCl ₂ .2H ₂ O, 1.08 mM MgCl ₂ .6H ₂ O, 1.49 mM Na ₂ HPO ₄ , 34.30 mM KCl, 99.95 mM NaCl, 0.52 mM citric acid, 55.51 mM glucose, 2.26 mM KOH, 6.50 mM bicine, 10% egg yolk	7% DMSO	1 : 5	[22]
Northern pike	–	102.67 mM NaCl, 42.25 mM KCl, 1.02 mM CaCl ₂ .2H ₂ O, 0.81 mM MgSO ₄ .7H ₂ O, 19.72 mM Hepes, 14.61 mM sucrose, 1.5% BSA, 7% egg yolk	10% MeOH	1 : 3	[23]
Artic char	–	300 mM glucose	10% MeOH	1 : 3	[24]
European and Japanese eel	Tanaka modified	137 mM NaCl, 76.2 mM NaHCO ₃ , 20 mM TAPS, 0.14% phosphatidylcholine	10% DMSO	1 : 2	[25]
Turbot	Mounib modified	125 mM sucrose, 100 mM KHCO ₃ , 6.5 mM reduced glutathione, 1% BSA	10% DMSO	1 : 2	[26]
Winter flounder	Mounib	125 mM sucrose, 100 mM KHCO ₃ , 6.5 mM reduced glutathione	10% PG	1 : 3	[27]
Summer flounder	–	423 mM NaCl, 8.99 mM KCl, 9.25 mM CaCl ₂ .2H ₂ O, 22.92 mM MgCl ₂ .6H ₂ O, 25.52 mM MgSO ₄ .7H ₂ O, 14.05 mM NaHCO ₃	12% Glycerol	1 : 2	[28]
Halibut	–	70 mM NaCl, 1.5 mM KCl, 2.7 mM CaCl ₂ , 25 mM NaHCO ₃ , 6.1 mM MgCl, 200 mM glucose, 1% BSA	10% DMSO, DMA, MeOH	1 : 3	[29]
Gilthead seabream	–	170 mM NaCl, 1% BSA	5% DMSO	1 : 6	[30]
Sharpsnout seabream	–	100 mM Na citrate	12.5% DMSO	1 : 4	[30]
European seabass	–	59.89 mM NaCl, 1.47 mM KCl, 3.51 mM CaCl ₂ , 20 mM NaHCO ₃ , 12.92 mM MgCl ₂ , 0.08 mM glucose, 1% BSA	10% DMSO	1 : 3	[31]
Striped bass	–	239.56 mM NaCl, 5.36 mM KCl, 23.81 mM NaHCO ₃ , 5.55 mM glucose, 75 mM glycine	7.5% DMSO	1 : 3	[32]
Malaba grouper	–	300 mM glucose or 150 mM NaCl	20% DMSO	1 : 9	[33]
Dusky grouper	–	171.12 mM NaCl, 1% BSA	10% DMSO	1 : 9	[34]
Cod and haddock	Mounib	125 mM sucrose, 100 mM KHCO ₃ , 6.5 mM reduced glutathione	10% PG	1 : 3	[35]
African catfish	Ginsburg fish Ringer	123.2 mM NaCl, 3.75 mM KCl, 3 mM CaCl ₂ , 2.65 mM NaHCO ₃	11.1% MeOH	Testicular sperm 1 : 9	[36]
European catfish	Cryo-fish	CRYO-FISHTM, 10% egg yolk	10% DMA	Testicular sperm 1 : 3	[37]
Common carp	–	3500 mM glucose, 30 mM Tris	11.1% MeOH	1 : 9	[38]
Silver carp	–	68.38 mM NaCl, 27.2 mM sodium citrate, 11.01 mM glucose	10% DMSO	1 : 2	[39]

					Alvarez et al. (2008)
Paddlefish	Tsvetkovas modified	30 mM tris, 23.4 mM sucrose, 0.25 mM KCl 5–	10% MeOH	1 : 1	[40]
Sturgeon	–	23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris	5–10% MeOH	1 : 9	[40]
Brazilian characids	–	277.53 mM glucose, 10% egg yolk	10% DMSO	1 : 3; 1 : 4	[41]
Piracanjuba	–	150 mM NaCl, 0.5% egg yolk,	10% methylglycol	1 : 9	[42]
Zebrafish	BSMIS	75 mM NaCl, 70 mM KCl, 2 mM CaCl ₂ , 1 mM MgSO ₄ , 20 mM Tris	11.2% DMA	Testicular sperm 1 : 9	[43]
Medaka	–	600 mM sucrose	10% DMSO	1:9	[44]
Pacific bluefin tuna	–	171.12 mM NaCl	20% DMSO or 10–20% Glycerol or 10% MeOH	1 : 9	[45]
Red drum	HBSS	136.89 mM NaCl, 5.37 mM KCl, 0.44 mM KH ₂ PO ₄ , 5.55 mM glucose, 0.34 mM Na ₂ HPO ₄ , 0.81 mM MgSO ₄ , 1.26 mM CaCl ₂ , 4.17 mM NaHCO ₃	7.5–15% DMSO	Testicular sperm	[46]
Black drum	HBSS	136.89 mM NaCl, 5.37 mM KCl, 0.44 mM KH ₂ PO ₄ , 5.55 mM glucose, 0.34 mM Na ₂ HPO ₄ , 0.81 mM MgSO ₄ , 1.26 mM CaCl ₂ , 4.17 mM NaHCO ₃	5–10% DMSO	Testicular sperm	[47]
Walleye	Rathbun extender modified	1.05 mM CaCl ₂ , 0.07 mM MgCl ₂ .6H ₂ O, 1.66 mM Na ₂ HPO ₄ , 25.11 mM KCl, 112.56 mM NaCl, 55.51 mM glucose, 0.52 mM citric acid, 2.26 mM KOH, 24.51 mM bicine, 0.75% ProFam, 0.4% BSA	7% DMSO	1 : 15	[48]
Ocean Pout	–	1.45 mM CaCl ₂ , 0.84 mM MgSO ₄ , 10.25 mM KHCO ₃ , 183 mM NaCl, 0.15 mM glucose	20% DMSO	1 : 3	[49]
Sea perch	Riger solution modified	60.35 mM NaCl, 1.80 mM NaH ₂ PO ₄ , 3 mM NaHCO ₃ , 5.23 mM KCl, 1.13 mM CaCl ₂ .2H ₂ O, 1.13 mM MgCl ₂ .6H ₂ O, 55.55 mM glucose	10%DMSO	1 : 1	[50]
Burbot	–	100 mM NaCl, 0.2 mM KCl, 1 mM CaCl ₂ , 1 mM MgSO ₄ , 20 mM hepes, 7% egg yolk	10% MeOH	1 : 5	[23]

4. Embryos

Cryopreservation of embryos has become an integral part of assisted reproduction. Successful cryopreservation of embryos is important because the biodiversity of both the paternal and maternal genomes will be preserved. While cryopreservation techniques have been largely established for the mammalian embryos, successful cryopreservation of intact fish embryos has not yet been achieved. Factors limiting fish embryo cryopreservation include their multicompartmental biological systems, high chilling sensitivity, low membrane permeability and their large size, which gives a low surface area to volume ratio [51]. The effect of such low ratio is a reduction in the rate at which water and cryoprotectants can move into and out of the embryo during cryopreservation [1].

Fish embryos are osmoregulators; they are released into the external medium and activated. Then the vitelline envelope separates from the plasma membrane and forms chorion. Studies on the chorion permeability of zebra fish embryos clearly showed that it was permeable to electrolytes and a range of cryoprotectant, including propane-1,2-diol, methanol, DMSO, ethylene [52]. The chorion structure plays a crucial role as flexible filter for the transport of some materials [53] (Toshimori and Tsuzumi 1976) and protects against the microorganisms [49] Studies on zebra fish embryos have shown that the water permeability of the

plasma membrane at different developmental stages remained relatively stable. The permeability to methanol (cryoprotectant) appeared to decrease during embryo development [54]. This also indicated that there was a gradual reduction in the permeability following the fertilization in zebra fish embryos, as opposed to the generally held belief that the membrane permeability of fish embryos reduced rapidly to minimum shortly after the fertilization [55].

Cryopreservation studies on the embryos and larvae have been conducted on marine invertebrate such as oysters, sea urchins, polychaete worms, coral and penaeid shrimp species [56]. However, survivals of most of these species has been inadequate in maintaining the structure and activity of embryos and larvae after freezing to cryogenic temperatures. Embryonic and larval development of marine invertebrates after cryopreservation often showed abnormalities in structure and colour [57]. The problems with invertebrate embryo cryopreservation associated with those identified with the fish embryos are their low membrane permeability and high chilling sensitivity. Although cryopreservation of the embryos has not been fully achieved, considerable progress has been made in understanding the conditions required for fish embryo cryopreservation and this would undoubtedly assist the successful protocol design in the future.

5. Oocytes

Oocyte cryopreservation is potentially the best way to preserve the female fertility. Cryopreservation of fish oocyte has been studied [58] which offers several advantages such as the smaller sizes range, much lower water content in oocytes and absence of a fully developed chorion that the permeability to water and solutes in oocyte is higher than embryo. Fish embryos are too large to apply traditional cryopreservation protocol. Immature oocytes can be an alternative for the mature eggs because of their smaller size. However, there is no practical technique available to induce the small oocyte to mature *in vitro*. A technique to obtain the mature eggs from the late stage oocytes is available. Thus, the combination of this technique and their cryopreservation could be a breakthrough. However, at present, late stage oocytes cannot be successfully cryopreserved because their size is still not small enough to result in much lower surface area to volume ratio. These reduce the rate at which water and cryoprotectant move into and out of oocytes during the cryopreservation. Developing the methods for cryopreservation of oocytes requires the screening of potential cryoprotectant treatments, evaluation of tolerance to chilling, determination of the appropriate rate of freezing to cryogenic temperatures and rate of thawing [59].

The permeability of the zebra fish oocyte membrane to water and cryoprotectants has been studied [51] and membrane permeability was shown to decrease with the temperature and permeability was generally lower than those obtained from sea urchin eggs [60] but higher than the immature medaka oocyte [61]. Studies on zebra fish oocyte chilling sensitivity showed that those oocytes were very sensitive to chilling and their survival decreased with decreasing temperature [58]. Chilling sensitivity in zebra fish oocytes was thought to be due to lipid phase transition of the oocyte membrane. The phase transition in zebra fish oocytes showed that chilling injury could occur when oocytes were exposed to temperatures between 12 to 22°C above the water freezing temperatures [62].

Studies on the cryopreservation of invertebrate oocytes and eggs over the past several decades have been extraordinarily difficult to achieve. However, it was found that intracellular crystallization occurred in the starfish oocytes at relatively high temperature that was very close to the temperature of extracellular ice formation [63]. In order to avoid this problem, [64] successfully cryopreserved starfish oocytes using ultra-rapid freezing technique, called vitrification. High chilling sensitivity [65] and low membrane permeability [66] of zebra fish oocytes are major obstacles to the development of a successful protocol for their cryopreservation as chilling sensitivity or cold shock can hinder slow cooling processes. Vitrification may be another option to achieve successful cryopreservation for the oocytes.

6. Blastomeres

Blastomeres are the cells produced as the result of cell division and cleavage in the fertilized egg. They are totipotent and pluripotent (depending on the stage of embryonic development) having the ability to differentiate

into any of the three germ layers or entire organism. They are different from the muscle cells, blood cells or nerves cells. Although cultured somatic-cells from fish have been cryopreserved successfully, their value is limited because of loss of development potential. Cryopreservation of blastomeres can maintain the genetic diversity of both, nuclear genome and mitochondrial DNA [67].

Blastomeres from the early embryos of fish still retain pluripotency [68] and their cryopreservation may be a promising approach to preserve the genotypes of zygotes and reconstitution of the organism. Indeed, there are several reports of germ-line chimeras created using the transplantation of blastomeres into goldfish [69] and rainbow trout [70] embryos. [71] transplanted the frozen-thaw blastomeres into goldfish embryos and the blastomeres differentiated into primordial germ cells. This report demonstrated that germ-line cells from the cryopreserved blastomeres could develop into mature gametes of chimeric fish because the blastomeres were not damaged by cryopreservation. Therefore, the cryopreservation techniques are very important.

Vitrification of zebra fish blastomeres was studied more recently and the highest blastomere survival was 93.4% [72]. Cryopreservation of blastomeres was also carried out in rainbow trout, carp and medaka after post-thawing. Rainbow trout blastomeres have been cryopreserved using the controlled slow freezing procedures with a survival of 95%. It has been reported that the controlled slow freezing protocol adopted for rainbow trout was successfully applied to carp blastomeres with survivals of 94% and 96%. Lower survival rates of cryopreserved blastomeres using controlled slow freezing have also been reported for other fish species such as whiting (20%), medaka (34%), pejerrey (67%) and chum salmon (59%) [73].

7. Conclusion

A large number of studies have been performed during recent decades to design cryopreservation protocols. Some of these efforts have contributed to the application of this technology in fish farming, but some concerns are delaying the optimization of the use of freezing sperm for reproduction and selection management, and many of the advantages of using this tool are, at present, being missed. Future research must focus on particular aspects in order to definitively provide homogeneous and optimal results after freezing/ thawing. Some of the areas in which research will contribute with significant advances are (i) the identification of the key parameters defining sample susceptibility to cryopreservation, in order to define criteria for the selection of samples to be frozen, (ii) the study of different approaches for increasing pre and post freezing / thawing quality, including breeder nutrition, sperm incubation with particular compounds and other physicochemical treatments, and (iii) given the high potential of cryopreservation in gene banking and genetic selection, the analysis and evaluation of the preservation and transmission of the faithful genome. Progress in these areas would undoubtedly contribute to the standardization of methods and would provide a guaranty of the success and safety of the use of cryopreservation for fish aquaculture.

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