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Cryopreservation of fish gametes: A remarkable tool for breeding conservation

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Abstract
To overcome the non-availability of sperm and asynchronous breeding, Cryopreservation is a method for long term preservation of gamete by using an ultra-cold temperature of -196 ºC. It allows to maintain the bigger and effective breeding population and helps in conservation of endangered species by preservation of genetic diversity by making gene bank.

Keywords: Cryopreservation, -196 ºC, conservation

Introduction
Cryopreservation is the technique by which of living things (cells, tissue, gametes) are preserved viable for an indefinite period by storing them at an ultra-cold temperature (-196 ºC). At this temperature, the metabolic activities of the cells are arrested, but they remain viable. Their normal functions can be reactivated after proper thawing. The cryopreserved semen of improved strains of fishes are also nowadays used for the purpose of selective breeding and hybridization. The objectives of cryopreservation of fish gametes are:

- For long-term preservation of desired gametes for future use.
- For conservation of endangered species and preservation of genetic diversity by making gene bank.
- For improvement in induced breeding, hybridization and selective breeding.
- To curb the problem of asynchronous maturity time in males & females during artificial breeding.
- For interbreeding of different stock/ species maturing at different time/ seasons.
- For easily transportation of gamete to different areas.
- In the case of seasonal breeders such as major carps, seed can be produced and supplied to farmers at any time of year as per the market demand.
- It helps in the development of selective breeding, hybridization, induced breeding and other techniques meant for genetic up gradation of cultivable fishes aimed at generating a superior strain that shell exhibits faster growth rate, better disease resistance better adaptability to extremes of climates and better feed conversion efficiency.
- It can help in preventing inbreeding depression.
- It can reduce the cost of male broodstock maintenance through the exploitation of limited stock.

Types of Preservation
Short term Preservation
It can be done for some hours to a few days by keeping the gamete at 0 ºC- 4 ºC with an addition of antibiotics and oxygenation to facilitate the prolonging spermatozoa survival. The ova stripped out after ovulation and stored in an undiluted state retain fertilizing ability for a few hours. The serializability of ova can also be preserved by keeping them in the ovarian fluid or isotonic electrolytic solution for few hours to few days at cold temperature 0 ºC- 4 ºC. e.g. In Tilapia, fertility of eggs remains better in the ovarian fluid at temperature of 10-15 ºC.

Cryopreservation Method
It involves three basic steps
(1) Pre-freezing
(2) Freezing

(3) Storage
(4) Thawing
(5) Post-thawing (6) Insemination.

Cryoprotectants
The cryo-injuries can be prevented or reduced by certain protective chemicals called cryoprotectants and diluting solutions called extenders. The cryoprotectant helps to avoid or minimize the process of formation of a crystal in the intracellular fluid. An ideal cryoptotectant should be permeable, soluble and least toxic to the cell.

Function of a normal cryprotectant
1. Reducing the rate of diffusion of water from cell to extracellular ice crystal.
2. Reducing the cell volume change.
3. Reducing the rate of ice crystal growth.
4. Lowering the homogeneous nucleation temperature.

Types of cryprotectant
There are two types of cryoprotectants: The ideal cryoprotectants should be least toxic and permeable to cell.
1. **Permeating cryoprotectant:** They can directly permeate into the cell. Ex-Glycol, Dimethyl Sulphoxide (DMSO), Ethylene glycol and Methanol.
2. **Non-permeating cryoprotectants:** They are not permeable to the cell membrane. They form an external coating around the cell which prevents the crystal formation in its vicinity. They act by depressing the freezing point and raising the ice formation temperature of the extracellular solution. Ex- Polyvinyl Pyrrolidone (PVP), Glucose, Sucrose, egg yolk serum, dextran, and skimmed milk.

a) **Partial vitrification:** When the cooling rate is moderate, osmotic equilibrium is not maintained. The only part of the freezable water leaves the cell. The remaining water vitrifies which are tolerable if thawing is fast enough to avoid recrystallization. The use of cryoprotectants will increase the rate of vitrification, thus optimizing post-thaw survival.
b) **Total vitrification:** When the cooling rate is very high, water vitrifies without forming ice. Such a cooling rate is unfeasible.

Extender
The extender is basically a solution of salts or some organic compounds which helps to maintain the viability of cell during refrigeration. It also inhibits the activation of spermatozoa and functions as a medium for cryoprotectant. An ideal extender provides osmotic stability by its buffering action and protects the membrane from injury along with preservation of the biological activity of the cell. The doses of extender are species specific.

Table 1: Dose of Extender

Sl.no	Extender	Quantity (gm.)
1	NaCl	6.5-8.0
2	KCl	0.2-3.0
3	$CaCl_2$	0.16-0.30
4	$NaHCO_3$	0.2-0.35
5	Sucrose	1.0

For long-term storage, the liquid nitrogen is the most suitable medium which has an ultra cold freezing point, i.e., 196 ^oC. The sperm and diluents (Extender + Cryoprotectants) are taken in cryovials in 1:9 ratios with the help of a syringe. After that, both the open ends of the straw are sealed under the flame and transferred into liquid nitrogen container. The cooling rate of about 20-30 ^oC/ minute is found to be optimum. When cryo-preserved sperm is needed for utilization, the cryo-vials are taken out from the liquid nitrogen container and thawed at 37 ^oC in water bath for 5-10 minutes. When suspension (sperm) is visible by cutting one end of the vial, the sperm is directly used for fertilization with ova of desired species. The diluent used for milt - a combination of extender and cryoprotectant. Kept in the fridge, as it is an exothermic reaction.

Table 2: Quantity of Extender used for diluent preparation

Components	Extender-A	Extender-B	Extender-C
NaCl	400 mg	600 mg	750 mg
$CaCl_2$	23 mg	23 mg	20 mg
KCl	38 mg	38 mg	20 mg
$NaHCO_3$	100 mg	100 mg	20 mg
NaH_2PO_4	41 mg	41 mg	-
$MgSO_4$	23 mg	23 mg	-
Distilled water	100 ml	100 ml	100 ml

Three categories of diluents are prepared by mixing cryoprotectants with extenders
- Extender A 90ml, DMSO- 5 ml, Glycerol- 10ml.
- Extender B 90ml, DMSO- 8 ml, Glycerol- 10ml.
- Extender C 65ml, DMSO- 15 ml.

Any one of the diluents – mixed with milt in a proportion-4:1(diluent: milt)

Function of different salts used in extenders
$NaHCO_3$, $KHCO_3$: As the buffer
Na Cl: For tonicity
KCl^-: To prevent the sperm activation
Mg^{++}, Ca^{++} ions: Normal components of seminal fluid
Sugar: For the source of energy
Glycine: For improved survival of sperm
Mannitol: To protect against the toxic effect of DMSO

Cryopreservation Protocol
It can be described in 5 phases

1) Pre-Freezing Phase
A. Collection of milt from the milter
During the breeding season, milt is collected from the milter for cryopreservation. The males are induced by carp pituitary extract 5 mg/kg or any synthetic compound sac as Ovaprim or Ovatide at a dose of 2 ml/kg. After a gap of 3-4 hours,
the milt is collected by hand stripping into a cold sterilized tube taking care to avoid contamination with urine, mucus, fecal matter, etc. The collected milt samples are kept in a refrigerator. Then the milt is diluted.

B. Equilibration of milt-diluent mixture
The milt-diluents mixture is kept at a low temperature for equilibration & time varies depending on the type of cryoprotectant used. In such case of major Indian carps equilibration period is around 45min. The low temperature

reduces the toxicity of the cryoprotectant on the cell as permeability is reduced at low temperature for DMSO & higher temperature for glycerol.

Freezing Phase

During freezing process, several physicochemical changes takes place within the cell and surrounding area. The plasma membrane that surrounds the cell gets affected by cold shock. Initially, ice crystal formation occurs in the extra-cellular medium due to freezing. As a result, the extra-cellular medium became hypertonic to the cell. These changes cause mechanical damage to the cell. It is observed that most of the cryo-injuries take place between 0 to -40° C. Therefore, to prevent the mechanical injuries, the freezing rate should be very high so that it does not let sufficient time for the water molecules to crystallize.

There are three ways of freezing

A. Pellet method
B. Straw method
C. Vial method

A. Pellet method

Size of the pellet may vary from 50 - 200 ml for which the freezing rate can be 20-35 ºC /minute. In this method, the sperms diluents mixture is formed into a pellet in a small hole drilled/melted into solid dry ice -79 ºC. The pellet so obtained is removed and kept in liquid nitrogen for storage. For pellet freezing, no equilibration time is required, and immediately after mixing sperm and diluent, it can be used for pellet making.

B. Straw method

It is available in different volumes (0.25 - 4 ml). This method is more popular during recent years as it has been found to yield better post-thaw quality of milt as chances for recrystallization during thawing is less in the straw method. After putting the equilibrated milt diluents mixture in straw, it is cooled on liquid nitrogen vapors/solid CO_2 ice. The optimum freezing rate for freshwater fish milt has been reported to be 20°-160 °C /minute and for saltwater fishes 5°-150 °C/minute respectively.

C. Ampoules /vial/visitubes method

Ampoules/vial/visitubes are used for storing the diluents milt mixture for freezing and cryopreservation.

Storage Phase

Frozen milt (pellet/vial/straw) is stored at -196 ºC using liquid nitrogen kept in cryo cans. At this temperature, spermatozoa can remain indefinitely without deterioration of cell quality.

Thawing Phase

For sperms frozen by pellet method, a thawing solution is required. This solution helps not only in absorbing heat but also in providing a suitable medium for post-thaw survival and induction of motility. 1% $NaHCO_3$ or 119 ml mol $NaHCO_3$ and 120 ml mol NaCl have been used as thawing solution for salmonid sperm. One pellet may be placed in 1-2 ml of thawing solution warmed to 25-30 ºC. The pellet begins to liquefy after 5-10 second, and this solution can be poured over the egg. The straws are usually thawed in a water bath at 30-40 °C. The fast thawing is preferred as slow thawing may cause crystallization in sperm cells. The French straws are made to slush, swirling them in a water bath at 38±2 °C for 7-9 seconds. Then drops of fresh water are added to the milt. This reactivates the sperm and restores their motility.

Post Thawing Phase & Insemination

During this phase, the sperms that have survived the cryopreservation are ready for artificial insemination. It is desirable to estimate the motility of sperm after thawing. Artificial insemination is to be done immediately after thawing as there is a reduction in the motility period of spermatozoa after thawing.

Conclusion

The cryopreservation of gametes is already routinely applied in animal husbandry. It has a significant role to play in maintaining the aquatic biodiversity and conservation of endangered, economically important and representative species from various aquatic habitats. The artificial insemination and embryo transfer in the livestock industry are facilitated using cryopreserved sperm and embryos. Regarding aquatic organisms, sperm cryopreservation has been successful in a number of finfish and shellfish species. Nowadays, the cryopreservation technique is used on a large scale for the conservation of monosex sperm and sperm from special brood-stock. The cryopreservation of female gametes (ova) of fish could not be done successfully so far because of its large size, complex structure and the existence of several membranes with variable permeability.

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