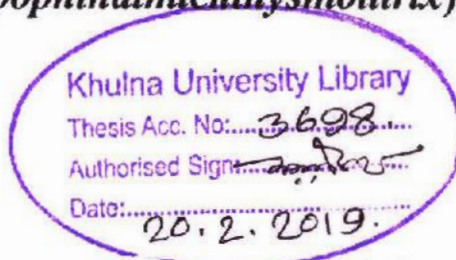


**Cryopreservation studies on Silver carp
(*Hypophthalmichthys molitrix*) embryos**



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**Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna
Bangladesh**

November, 2018

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A Thesis

By

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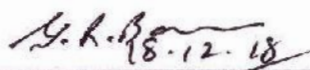
Khulna University, Khulna

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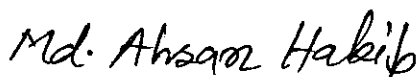
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Declaration

Cryopreservation studies on Silver carp (*Hypophthalmichthys molitrix*) embryos

The results submitted in this project thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it being concurrently submitted for any degree.



(Signature of the Candidate)

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Examination Roll No: MS-170625

Session: 2016-2017



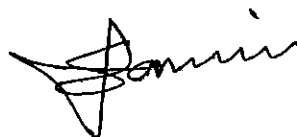
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**DEDICATED TO
MY
BELOVED PARENTS**

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ABSTRACT

The experiment was conducted to investigate the effect of different cryoprotectants and mixture solution on the hatching rate of different development stage in Silver carp (*H. molitrix*) embryos. This study also conducted to evaluate the relative efficiency of ultrasound to promote the incorporation of cryoprotectants (CPA) in three different embryonic developmental stages (Morula, 6-somites and tail elongation) of silver carp. Embryos at three developmental stages were immersed in five single cryoprotectants (DMSO, MeOH, PG, DFA, EG), in concentrations of 10, 15, 20 and 25% for 20 min and then hatching rates were determined. Twelve mixture solution, prepared based on the toxicity of single CPA were used at different concentration to test the toxicity in embryos. In case of ultrasound, embryos were exposed to ultrasound for 2, 3, 4, 5, 6, 7, 8, 9, and 10 min (1st trial), exposed to ultrasound for 1, 2, and 3 min in the presence of CPA mixture solutions (2nd trial), and pre-exposed to CPA mixture solution for 8 min (4 steps of 2 min) prior to ultrasound treatment in mixture solution for 2 min (3rd trial). Hatching rates decreased with increasing cryoprotectant concentrations and toxicity to embryos varied in the order of PG < DMSO < EG < MeOH < DFA. The tail elongation stage was more tolerant to cryoprotectant solutions than other stages. Hatching rates of embryos exposed to cryoprotectant mixture solutions in ultrasound varied with the type of mixture solution. Embryos up to 2 min resulted in rapid decline of survival rate (without any CPA). The mixture solution of CS3, CS6, CS12 at 2 min in ultrasound and 4 × 2 min + 2 min in ultrasound gave hatching rate of 8-11% and 6-8% respectively. For both 6-somite and tail elongation stage, Embryos that were subjected to ultrasound before cooling showed higher good embryos (%) than the embryos that were not subjected to ultrasound. Although cryopreservation trial after ultrasound-mediated impregnation of 6-somites and tail elongation embryos with a CPA solution did not yield live embryos after freeze-thawing but resulted in the increase of the proportion of morphologically good embryos (%) after freeze-thawing. These results suggest that ultrasound treatment might be useful for silver carp fish embryo cryopreservation.

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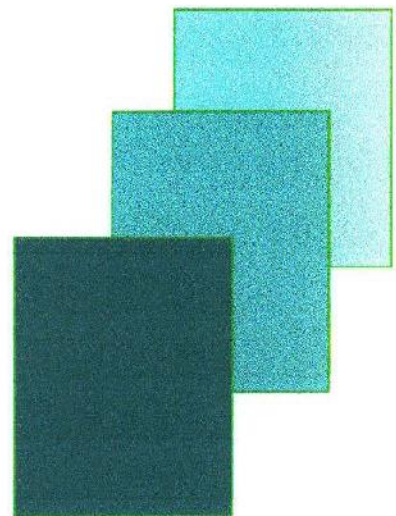


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Chapter 1

INTRODUCTION



CHAPTER-1

INTRODUCTION

1.1 Justification of the study:

Fish gamete and embryo cryopreservation are desirable techniques in aquaculture and fish resource management. Their major benefits for aquaculture include a reduction in the cost of seed production (because they obviate the need for broodstock fish rearing), the continuous supply of seeds for year-round production in farms, the easy exchange of germplasm between hatcheries, and their contribution to selective breeding (Jamieson and Leung, 1991). By helping preserve pure genetic materials, these techniques can also help mitigate the loss of valuable germplasm due to environmental changes, pollution, overfishing, and genetic introgression between populations as a result of anthropogenic introductions. Unfortunately, successful cryopreservation of fish germplasm has been achieved only for sperm cells and isolated blastomeres (early cells of the embryos) of some species, while techniques for the cryopreservation of eggs and whole embryos have proved elusive. Cryopreserving spermatozoa, of course, serves only the parental genome that is not necessarily sufficient to preserve genetic diversity. Molecular studies reveal that both the parental and maternal genomes play important roles in embryogenesis. Some genetic manipulations, such as androgenesis (all-paternal inheritance) and chimera production, are possible in teleosts and might protect genetic diversity, but these techniques are extremely labor-intensive and result in low survival rates (Thorgaard and Cloud, 1993). The ability to preserve both paternal lines through embryonic genome would greatly improve opportunities for conserving and protecting valuable fish species and argues strongly for cryopreservation of embryos. In principle, the problem could be solved by cryopreserving both sperm and mature eggs, however, mature eggs share many of the same cryopreservation problems as embryos.

Based on the report of the red data book of the "International Union for Conservation of Nature and Natural Resources (www.iucnredlist.org), 2,514 species of teleost are extinct in the wild or threatened to various degrees. Many subspecies, varieties, geographically isolated subpopulations or stocks, which are often of particular interest as fishing resources, are also in danger of extinction (Martinez-paramo et al., 2009; Ayyappan et al., 2014). On the other hand,

the quality of seeds has been deteriorated over the years due to inbreeding, hybridization and improper brood stock management (Alam and Khan, 2004). The recent dramatic worldwide declines in natural fish stocks (New, 1997) largely resulting from overexploitation and anthropogenic changes in the environment, have led to increasing interest in the creation of gene banks for wild aquatic organisms (Harvey, 1996). Furthermore, the global growth of intensive aquaculture has further necessitated the search for efficient and effective means of conserving fish gametes (Donaldson, 1997).

The ideal strategy for conservation of threatened and endangered species is through in-situ protection/restoration of the native habitat of the species. Unfortunately, this requires a great deal of time and money as habitat restoration is clearly a slow process. One alternative is to maintain ex-situ live or cryopreserved gene banks. Establishment of ex-situ gene banks in the immediate term would ensure the maintenance of genetically pure stocks of fish whereas “buying us the time” necessary to improve habitat conditions for restocking. Cryogenic preservation of embryos could facilitate dramatic increase in the effective breeding program in future restoration efforts, which would ultimately pave the way for conservation of valuable aquatic resources in the world.

Methods for the cryopreservation of semen from many species has been well documented and cryopreserved semen has been used for reproduction of many wild and farmed species. Attempts to cryopreserve fish embryos have been conducted over the past three decades, nevertheless successful cryopreservation protocol for long-term storage still remains elusive and cryopreservation of eggs or embryos has made little progress.

In 1989, Zhang et al. observed viable embryos of carp, *Cyprinus carpio* after slow-freezing while Chen and Tian (2005) claimed successful vitrification of Japanese flounder, *Paralichthys olivaceus* embryos. However, Chen and Tian's (2005) claim was subsequently refuted by Edashige et al. (2006). Fish embryos have a low surface area to volume ratio, a thick chorion, a complex, multi-compartmental structure, and a syncytial layer covering the yolk is considered as the major obstacle for the permeation of cryoprotectant (CPA) agent as well as their subsequent removal (Hahedorn et al., 1996 and 1997).

Insufficient permeation of cryoprotectant into the complex multi-structured embryos results in physical damage to the embryos during cryopreservation by the formation of intracellular ice crystals, particularly during slow cooling and thawing. The low permeability of fish embryos to CPAs also prevents the application of vitrification, an ice-free cryopreservation technique which combines high CPA concentrations and rapid freezing rates (Hagedorn et al., 1997 and Zhang and Rawson., 1996). Thus, the lack of a suitable CPA impregnation protocol is currently the great hurdle for successful cryopreservation of fish eggs and embryos (Bart and Kyaw, 2003).

CPA permeability and tolerance by the embryos varies with type and concentration of the CPA, the impregnation protocol, as well as the species and developmental stage of the embryos, and these are all aspects that need to be optimized prior to cryopreservation (Routray et al., 2002 and Suzuki et al., 1995).

The major constraint to the successful cryopreservation of fish eggs and embryos, as in other biological materials, appears to be the formation of ice crystals during cooling, which causes damages and results in the death of the cells.

Vitrification, an ice-free cryopreservation technique using high cryoprotectant concentrations and rapid freezing rates, has been suggested as a tool to overcome this problem. However, though tremendous efforts have been pursued in the last decades, cryopreservation is still not feasible either by slow cooling or vitrification. The insufficient impregnation of cryoprotectants into fish embryos is singled out as the major constraint for their cryopreservation (Robles et al., 2003; Cabrita et al., 2006 and Edashige et al., 2006). This problem appears to be related to the fact that fish embryos have a low surface area to volume ratio, a thick chorion, and a complex, multi-compartmental structure. Because of this, it is very difficult to achieve a swift and uniform permeation of the cryoprotectants into the various compartments of fish embryos, resulting in toxicity to some cells while in others the concentration of cryoprotectants is not sufficient to prevent cryoinjuries (Hagedorn et al., 2006 and Zhang and Rawson, 1995).

Permeability and low toxicity are the most important properties in cryopreservation protocols, particularly when high concentrations are required for vitrification. Regarding vitrification, the following suggestions have been made by a number of authors for an optimal protocol: (i) choose a cryoprotectant that is the least toxic; (ii) apply "toxicity neutralizers" when possible

(Baxter and Lathe, 1971; Fahy, 1983; Fahy et al., 1984 and Fahy et al., 1987); (iii) minimize embryo exposure time to the solution (Fahy et al., 1984 and Rall and Fahy, 1985); (iv) remove cryoprotectant immediately after thawing (Fahy et al., 1984; Rall and Fahy, 1985 and Rall, 1993); and (v) expose embryos to cryoprotectants at lower temperatures (Baxter et al., 1971 and Fahy et al., 1984).

The key to successful cryopreservation appears to involve the development of methods for faster, even impregnation and removal of CPAs into these biological materials. Several innovative techniques including impregnation with CPA after dechoriation, under negative or positive pressure, after microinjection of aquaporins, or by direct delivery of the CPA into embryos through microinjection and electroporation have been developed to accelerate the permeation of CPA into fish embryos. Some of these techniques showed promising results but none has led to successful cryopreservation. Besides, it is unclear whether sophisticated and time consuming techniques would be feasible in practical situations.

Cavitation level ultrasound is commonly used for drug delivery through mammalian skin, medical practices for diagnostic and therapeutic purposes (Mitragotri et al., 1996). Mitragotri et al. (1995) reported that during the process of cavitation, oscillation and the collapse of air bubbles effectively disorganize the lipid bilayers allowing larger molecules to diffuse through the skin. It has been reported recently that ultrasound enhanced the permeation of CPA into embryos of zebrafish (Wang et al., 2008 and Silakes and Bart, 2010) and marine whiting (Rahman et al., 2013b) while no such studies have been conducted for any freshwater commercial carp and catfish fish species in Bangladesh.

In this regards, this study was examined the suitability of ultrasound for the cryopreservation of freshwater fish embryos. This study was also determined the toxicity of cryoprotectant (CPA) for the different development stage of silver carp (*Hypophthalmichthys molitrix*) embryos.

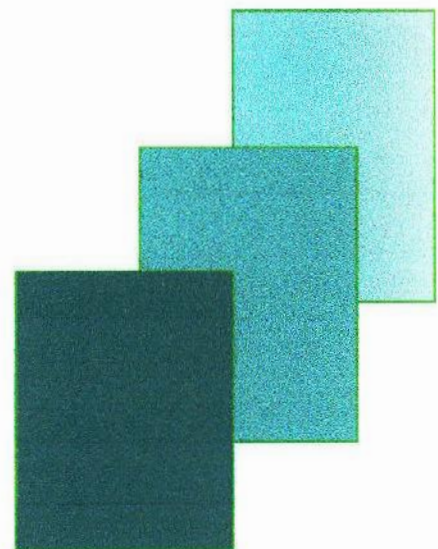
1.2 Objective:

In this study, the embryos of Silver carp, *H. molitrix* have been chosen due to the fact that these species can spawn in captive condition; produce numerous eggs and finally short embryonic development. The broad objectives of this proposal are:

- To examine the toxicity of CPA single and mixture solutionsto the fish embryos at different developmental stages.
- To observed the effect of ultrasound in the presence or absence of CPA mixture solution.
- To test the effectiveness of ultrasound in CPA mixture solution to prevent ice formation during rapid cooling.

Chapter 2

LITERATURE REVIEW



CHAPTER-2

REVIEW OF LITERATURE

Cryopreservation of embryos involves a series of complex and dynamic physiochemical processes of temperature and water transport between an embryo and the surrounding medium. Understanding these process and their interactions with the embryo's biology is essential prior to developing a successful cryopreservation methodology. Unfortunately, the technologies developed for mammalian embryo cryopreservation are unlikely to be successful for multi-compartment, highly differentiated and yolk-filled embryos, such as those of fish. Although, methods for cryopreservation of fish sperm have been developed for over 200 species of fresh water and salt water fish (Rana, 1995 and leung and Jaemison, 1991), cryopreservation of ova or embryos has not been successful and consequently, there are few publications on this topic.

The earliest reported cryopreservation of ova or embryos dates back to 1953 (Blaxter, 1953). Erdahl et al. (1984) reported viable eggs and embryos protected with 8 to 14% dimethylsulfoxide (DMSO); eggs were frozen at -20°C and embryos at -55°C . Neither of the reports specified the rate of cooling. It appears that these eggs and embryos were super cooled and not cryopreserved. A more recent set of studies has examined chilling injuries (Liu et al., 1998 and Zhang and Rawson, 1995) and cryopreservation toxicity at various stages of development (Liu et al., 1993; Robertson and Lawrence, 1988 and Suzuki et al., 1995).

Using magnetic resonance and electron spin resonance microscopy; Hagedorn et al. (1997) found that cryopreservation permeated into zebrafish embryos. However, the permeation was not uniform due to the yolk syncytial layer (YSL) and differentiating blastoderm cells.

Suzuki et al. (1995) studied on relation between toxicity of cryoprotectant dimethylsulfoxide (DMSO) and its concentration in several fish embryos. When the embryos of several fish species, medaka, pejerrey, rainbow trout and carp, were immersed in solutions of various DMSO concentrations, the amounts of DMSO penetrating into the embryos were measured by HPLC and simultaneously their mortalities were determined from observation under Microscope. It was concluded that the tolerance to DMSO becomes higher in the order of carp, rainbow trout, pejerrey, and medaka.

Wang (2006) studied on fish embryo responses to the treatment of cryoprotective chemicals using impedance spectroscopy. The permittivity and conductivity spectra were obtained during embryo exposure to different concentrations of methanol (1.0M, 2.0M and 3.0M) and DMSO (0.5M, 1.0M and 2.0M) solutions. The results showed significant permittivity and conductivity changes after embryo exposure to methanol and DMSO at the optimum embryo loading level (6 embryos). Embryos in different concentrations of methanol and DMSO also resulted in quantitative responses shown in the permittivity and conductivity spectra.

Antony et al. (2014) conducted a study on the milt quality of Common carp (*Cyprinus carpio* var. *communis*) and Koi carp (*Cyprinus carpio* var. *koi*). The milt samples were diluted with Cortland Medium (pH 7.56) and protected with Glycerol and DMSO (Dimethyl Sulphoxide) were used as cryoprotectants at 5%, 10% and 15% concentrations. Comparative studies were also made on the effectiveness of DMSO and Glycerol as cryoprotectants. It was observed that motility rates were higher (60%) for DMSO than Glycerol (40%) at 15% concentration levels. The samples were stored in LN₂ up to 60 days. The post thaw motility rates were observed based on motility rate. The equilibration periods were also studied and it was observed that motility rate was 60% for samples stored using DMSO as cryoprotectant. Among the cryoprotectants used it was also observed that DMSO proved better than Glycerol as cryoprotectant for both the samples.

Rahman et al. (2015) studied on evaluating the suitability of cryoprotectants and cryopreservation solutions for olive barb, *Puntius sarana* embryos. Embryos at three developmental stages (gastrula, somites, and tail elongation stages) were exposed to 10, 15, 20, 25, and 30 % solutions of five cryoprotectants such as propylene glycol (PG), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethylformamide (DFA), and ethylene glycol (EG). Hatching rates decreased with increasing cryoprotectant concentrations and toxicity to olive barb embryos varied in the order of PG < EG < MeOH < DMSO < DFA.

Xiao et al. (2008) studied on effect of cryoprotectants on hatching rate of red seabream (*Pagrus major*) embryos. Heart-beat embryos were immersed in: five permeable cryoprotectants, dimethyl sulfoxide (DMSO), glycerol (Gly), methanol (MeOH), 1,2-propylene glycol (PG), and ethylene glycol (EG), in concentrations of 5–30% for 10, 30, or 60 min; and two non-permeable

cryoprotectants: polyvinylpyrrolidone (PVP), and sucrose (in concentrations of 5–20% for 10 or 30 min). The hatching rate of the embryos treated with permeable cryoprotectants decreased with increased concentration and duration of exposure. In addition, PG was the least toxic permeable cryoprotectant, followed by DMSO and EG, whereas Gly and MeOH were the most toxic.

Cabrita et al. (2003) studied on effect of different cryoprotectants and vitrificant solutions on the hatching rate of turbot embryos (*Scophthalmus maximus*). They observed the toxicity of permeable cryoprotectants increased with concentration and exposure time. There were no significant differences between permeable cryoprotectants. However, embryos tolerated higher concentrations of dimethyl sulfoxide than other cryoprotectants.

Zhang et al. (2005) studied on toxicity and protective efficiency of cryoprotectants to flounder (*Paralichthys olivaceus*) embryos. They tested the toxicities, at culture temperature (16 degrees C), of five most commonly used cryoprotectants- dimethyl sulfoxide (Me₂SO), glycerol, methanol (MeOH), 1,2-propylene glycol (PG) and ethylene glycol (EG). The results showed that the toxicity to flounder embryos of the five cryoprotectants are in the following sequence: PG < MeOH < Me₂SO < glycerol < EG.

Kirichek (2016) studied on segregated water observed in a putative fish embryo cryopreservative. Water in cryoprotective solution, forms nano-clusters encapsulated by the surrounding matrix of cryoprotectant solute molecules. These small volumes inhibit ice formation, because water does not have space for the structural relaxation required to crystallize on the timescale of the cooling process.

Higaki (2009) reported on feasibility of cryopreservation of zebrafish (*Daniorerio*) primordial germ cells by whole embryo freezing. He examined the glass-forming properties and embryo toxicities of 5 cryoprotectants: methanol (MeOH), ethylene glycol (EG), dimethyl sulfoxide (DMSO), propylene glycol (PG), and 1, 3-butylene glycol (1,3-BG). Embryos at the blastula and gastrula stages had high sensitivities to cryoprotectant toxicities and were fragile against mechanical damage.

In 1989, Zhang et al. observed viable embryos of carp *Cyprinus carpio* after slow-freezing and, more recently, Chen & Tian (2005) claimed successful vitrification of Japanese flounder

Paralichthys olivaceus embryos. However, their success remains to be reproduced. The major constraint to the successful cryopreservation of fish eggs and embryos, as in other biological materials, appears to be the formation of ice crystals during cooling, which causes damages and results in the death of the cells. The insufficient impregnation of cryoprotectants into fish embryos is singled out as the major constraint for their cryopreservation (Robles et al., 2003; Cabrita et al., 2006 and Edashige et al., 2006, Rahman et al., 2011). This problem appears to be related to the fact that fish embryos have low surface area to volume ratio, a thick chorion, and a complex, multi-compartmental structure. Because of this, it is very difficult to achieve a swift and uniform permeation of the cryoprotectants into the various compartments of fish embryos, resulting in toxicity to some cells while in others the concentration of cryoprotectants is not sufficient to prevent cryoinjuries (Hagedorn et al., 1997 and Zhang and Rawson, 1995).

Dinnies et al. (1998) studied on chilling sensitivity of carp (*Cyprinus carpio*) embryos at different developmental stages in the presence or absence of cryoprotectants: work in progress. Toxicity increased in the order of methanol < Me₂SO < glycerol in morula and half-epiboly stages, and methanol < glycerol < Me₂SO in the heartbeat stage. The results show morulae are partially protected against chilling in Me₂SO and sucrose, half-epiboly in Me₂SO, sucrose and methanol, and heartbeat-stage in methanol and glycerol. The results further suggest that carp embryos are sensitive to chilling and that toxicity and protective effects against chilling of cryoprotectants are stage-dependent.

Chao and Liao (2001) studied on cryopreservation of finfish and shellfish gametes and embryos. Modest success has been achieved in the cryopreservation of shellfish embryos and early larvae. On the other hand, cryopreservation of finfish ova and embryos has not been successful so far. Unlike shellfish eggs and embryos, finfish ova and embryos are large, contain a large amount of yolk and are covered with a relatively thick chorion. Uniformity in the penetration of conventional cryoprotectants and in cooling during the freezing process has not been attained in these large and dense specimens.

Beirao et al. (2006) studied on cryoprotectant microinjection toxicity and chilling sensitivity in gilthead seabream (*Sparus aurata*) embryos. Permeabilized seabream embryos were microinjected with 2.35 nl dimethyl sulfoxide (Me₂SO), methanol (MeOH), ethylene glycol (EG) (5 M, 10 M

and pure) or sucrose (10% and 15%). In a second experiment, 29.5 nl and 154.0 nl of the highest concentration of each cryoprotectant were used in the same embryo stage. To test the effect of microinjected cryoprotectants on embryo chilling resistance, 29.5 nl of pure Me₂SO or 15% sucrose was microinjected into the yolk sac of tail bud stage embryos and then at a later stage, (tail-bud-free), were exposed to 3 M Me₂SO solution at -10 °C for 30 min. The results showed that microinjection technique did not affect the viability of tail bud stage embryos as is shown by the high hatching and survival rates. Hatching and larval survival rate at first feeding were not affected with any of the CPAs tested, showing percentages higher than 75% and 90%, respectively, when embryos were microinjected with a smaller quantity of cryoprotectant. Sucrose was the cryoprotectant better tolerated at higher concentration and volume. Cryoprotectant concentration inside the yolk higher than 1.18 M for Me₂SO, 1.5 M for EG and 2 M for methanol decreased the hatching rate.

Adam et al. (1995) conducted a study on effect of cryoprotectants on activity of selected enzymes in fish embryos. In this study, Rosy barb (*Puntiusconchonus*) and zebra fish (*Brachydaniorerio*) embryos at cleavage, epiboly, and closure of blastopore stages were exposed to Me₂SO and EG at 1.0, 2.0, 3.0, and 4.0 M for 0.25, 0.5, 1.0, 2.0, and 3.0 at room temperature. In both species, EG was more toxic than Me₂SO. Zebra fish embryos were more resistant to cryoprotectant enzymatic denaturation than rosy barb embryos.

Newton and Subramoniam (1996) studied on cryoprotectant toxicity in Penaeid prawn embryos. This study focuses on the toxicity of seven well known permeating, low molecular weight cryoprotectants: glycerol, formamide, acetamide, methanol, propylene glycol, dimethyl sulfoxide (Me₂SO), and ethylene glycol (EG). In the cryoprotectant toxicity experiments embryos were exposed to cryoprotectant solutions at 15°C for a period of 15 min, whereas the toxicity neutralization experiments were conducted at 28°C with an exposure time of 15 min. The widely used permeating cryoprotectant, glycerol, was toxic to morula stage embryos at concentrations >0.5M. This study shows that the toxicity tolerance of *Penaeusindicus* embryos varies with the developmental stage, the later stages being more resistant. Morulae did not tolerate any cryoprotectant above 5M, whereas the nauplii had survival rates of 45% in 12.5M methanol and 78% in 6.4M EG. The toxicity neutralization experiments do not seem to indicate any specific toxicity-blocking mechanism in Me₂SO toxicity reduction by EG. Based on the response to

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cryoprotectant toxicity, nauplius stage larvae of penaeid prawns can be considered a suitable stage for cryopreservation, both by the conventional slow cooling method and also the vitrification technique as it allows use of high levels of cryoprotectant.

Cabrita et al. (2006) studied on the cryopreservation of gilthead seabream (*Sparus aurata*) embryos. Permeabilized embryos were incubated in dimethyl sulfoxide (DMSO), methanol (MeOH), ethylene glycol (EG) and 1, 2-propanediol (PROH) in concentrations ranging from 0.5 to 6 M for 10 and 30 min and in 5%, 10% and 15% polyvinyl pyrrolidone (PVP), 10%, 15% and 20% sucrose for 2 min. The toxicity of permeable cryoprotectants increased with concentration and exposure time. EG was best tolerated by the embryos.

Ahammad et al. (1998) studied on effect of different concentrations of cryoprotectants and extender on the hatching of Indian major carp embryos (*Labeorohita*, *Catla catla*, and *Cirrhinus mrigala*) stored at low temperature. Rohu (*Labeorohita*), catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*) embryos were examined for hatching success after short-term (8-13 h) storage at low temperature (4°C) using three concentrations of three cryoprotectants, methanol (1, 2, and 3 M), dimethyl sulfoxide (1.282, 1.923, and 2.564 M, equal to 10, 15, and 20%), and glycerol (1.087, 1.63, and 2.174 M, equal to 10, 15, and 20%), separately as well as together with a single concentration of sucrose (0.5 M). Of the cryoprotectants used, methanol was found to be most suitable for low-temperature storage in the refrigerator. The hatching rate of embryos was significantly higher when sucrose was added to methanol when compared with methanol alone.

Ding et al. (2007) preliminary studied on the vitrification of red sea bream (*Pagrus major*) embryos. The toxicity of five single-agent cryoprotectants, dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), glycerol (GLY), and methyl alcohol (MeOH), as well as nine cryoprotectant mixtures, were investigated by comparing post-thaw hatching rates. Exposure to single-agent cryoprotectants (10% concentration for 15 min) was not toxic to embryos, whereas for higher concentrations (20 and 30%) and a longer duration of exposure (30 min), DMSO and PG were better tolerated than the other cryoprotectants.

El-Battawy and Linhart (2014) studied on cryopreservation of common carp (*Cyprinus carpio*) embryos using vitrification. The sensitivity to various methanol (MeOH) and glycerol (gly) concentrations at 4°C was tested to carp (*Cyprinus carpio*) embryos. Results indicated that no hatching embryos were recorded when 2-h stage embryos were incubated with different MeOH levels. No hatching embryos were also recorded when 6-h stage embryos were incubated with 30% and 35% MeOH. In contrast, low and very low hatching rates were recorded when they were incubated with 10% or 20% MeOH respectively. Moreover, no hatching was observed when 22-h stage embryos were incubated with 35% MeOH while it was high, low and very low when they were incubated with 10%, 20% and 30% MeOH respectively.

El-Battawy and Linhart (2009) studied on cryopreservation of common tench (*Tinca tinca*) embryos. Tench embryos at four developmental stages (11, 17, 23 and 29 h) treated with alcalase (2 ml/998 ml, 2 min at 22°C) were exposed to chilling with/without warming. Other embryos were exposed to methanol and glycerol at the concentration of 10% and 20% for periods of 20 min. the hatching rates of embryos decreased significantly ($p < 0.001$) after exposure to 0 degrees C at all developmental stages except the 29-h stage compared with the controls. The embryo stage most sensitive to chilling was 11-h stage. The 29-h stage exhibited the least sensitivity to low temperature while 17-h and 23-h stages were intermediate in their sensitivity to chilling. The toxicity of methanol increased significantly ($p < 0.001$) with developmental stage for 11, 17 and 23-h stages. The highest hatching rates of tench embryos were obtained with 29-h embryos using various concentrations of methanol. The hatching rates of tench embryos exposed to glycerol concentrations were approximately similar to those embryos exposed to methanol concentrations except for 11-h embryos that showed no hatching.

Rahman et al. (2008) reported on suitability of cryoprotectants and impregnation protocols for embryos of Japanese whiting (*Sillago japonica*). Exposure of gastrula, somites, tail elongation, and pre-hatching embryos to 10%, 15%, and 20% solutions of propylene glycol (PG), methanol (MeOH), dimethyl sulfoxide (Me₂SO), dimethylformamide (DFA), ethylene glycol (EG), and glycerol (Gly) in artificial sea water (ASW; 33 psu) for 20 min revealed that CPA toxicity for whiting embryos increased in the order of PG < Me₂SO < DFA < EG < MeOH < Gly. Relative CPA permeability, estimated by proton nuclear magnetic resonance spectroscopy, showed the same trend as toxicity except for Gly, which was highly toxic but showed only

moderate permeability. There were no marked differences in CPA tolerance between developmental stages except for a slight decrease in pre-hatching embryos.

Shaluei et al. (2013) studied on effect of different concentrations of permeable and non-permeable cryoprotectants on the hatching rate of goldfish (*Carassius auratus*) embryos. Heart-beat embryos were immersed in: five permeable CPAs, dimethyl sulfoxide (DMSO), methanol (MeOH), 1,2- propylene glycol (PG), glycerol (Gly), ethylene glycol (EG), and two non-permeable CPAs: polyvinylpyrrolidone (PVP) and sucrose in concentrations of 5%, 10%, 15% or 20% for 5, 15, or 30 min. The results showed that the toxicity to goldfish embryos of the five permeable CPAs were in the following sequence: PG < DMSO < MeOH < EG < Gly. Promising results have been obtained in some cases but it has not been possible so far to achieve successful optimized cryoprotectants solution for cryopreservation.

Robert Y. et al (2007) studied on ultrasound enhanced methanol penetration of zebra fish (*Danio rerio*) embryos measured by permittivity changes using impedance spectroscopy. Zebra fish (*Danio rerio*) embryos at 50% epiboly stage were exposed to 2 M methanol for 25 min before ultrasound treatment for 5 min at 22°C. Embryos were treated with ultrasound in different frequencies (24 and 48 kHz) and voltages (50, 100, 150 and 175 V) combinations. The results showed a clear increasing trend of permittivity from voltage 50 to 175 V over lower impedance frequency range of 10–103 Hz indicating increased methanol penetration into the embryos after ultrasound treatment. The embryo survival was not compromised after ultrasound treatment under conditions used in the present study.

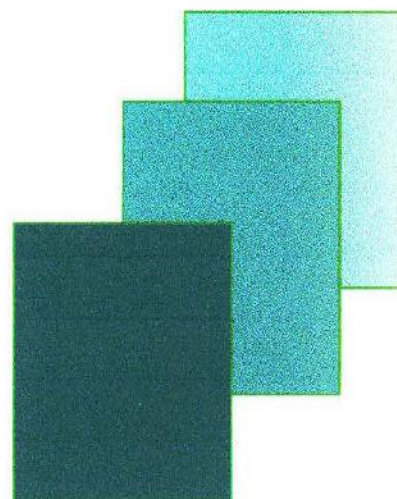
S. M. Rahman et al. (2017) studied on effects of ultrasound on permeation of cryoprotectants into Japanese whiting *Sillago japonica* embryos. The relative efficiency of ultrasound to promote the incorporation of CPAs in two different embryonic developmental stages (somites and tail elongation) of Japanese whiting *Sillago japonica* and performed a preliminary cryopreservation trial using the best conditions determined during the study. A preliminary cryopreservation trial after ultrasound-mediated impregnation of somites embryos with a CPA solution containing 20% PG and 10% MeOH did not yield live embryos after freeze-thawing but resulted in a significant decrease of nucleation temperature and increase of the proportion of morphologically intact embryos after freeze-thawing. These results suggest that sonication might be useful for fish

embryo cryopreservation although it may require combination with other techniques to enhance CPA permeation.

SawitriSilakes, and Amrit N. Bart (2010) studied on Ultrasound enhanced permeation of methanol into zebrafish, *Daniorerio*, embryos. The study determined the effect of ultrasound on methanol (MeOH) penetration in three types (dechorionated, soft chorion and intact embryos) and three stages (90% epiboly, bud and 4-somite stages) of zebrafish (*Daniorerio*) embryos. Ultrasound clearly helped to improve the permeation level of methanol into zebrafish embryos (pb0.05). A high level of methanol was found in soft chorion embryos. Dechorionated embryos had the highest relative difference in methanol levels between ultrasound and controls. Although, ultrasound significantly affected survival (pb0.05), survival was generally high (64±2% to 100%). Even the highest level of methanol (85.3±8.1 µmol) in the best treatment was far lower than that needed for vitrification.

Chapter 3

MATERIALS AND METHODS



CHAPTER- 3

MATERIALS AND METHODS

3.1 Species selection:

In this study, depending on availability a freshwater species was selected that had commercial as well as conservation interests like Silver carp (*Hypophthalmichthys molitrix*) (Figure 1).



Figure 1: Silver carp (*H. molitrix*)(Valenciennes, 1844)

Taxonomic classification:

Common Name: Silver carp

Local Name: Silver carp, Silver cup

Phylum: Chordata

Class: Actinopterygii

Order: Cypriniformes

Family: Cyprinidae

Genus: *Hypophthalmichthys*

Species: *Hypophthalmichthys molitrix* (Valenciennes, 1844)

3.2 Brood collection and maintenance:

Mature healthy broods of silver carp were collected from nature and local market and transferred to the fish hatchery named Shiromoni fish hatchery, Khulna. The broods (average weight: 2.9 kg, average age: 2.5 year) were kept in the closed aquaria until induced breeding. They were fed pellets (35% protein) at a maintenance level of 5 % of body weight daily.

3.3 Solution preparation:

Hatchery water was used (as extender) for the preparation of single CPA solution and for the incubation of embryos to monitor survival after exposure to CPA solutions or ultrasound in the presence or absence of CPA solutions. The composition of single CPA solution is mentioned in Table 1.

Table 1: Composition of Single CPA (cryoprotectant) solution. Propylene glycol (PG), Dimethyl sulfoxide (DMSO), Ethylene glycol (EG), Methanol (MeOH) Dimethyl formamide (DFA).

CPA Solutions	Chemicals (%)	Extender (%)
10% CPA	10 (PG / EG/ DMSO/ DFA/ MeOH)	90 hatchery water
15% CPA	15(PG / EG/ DMSO/ DFA/ MeOH)	85 hatchery water
20% CPA	20(PG / EG/ DMSO/ DFA/ MeOH)	80 hatchery water
25% CPA	25(PG / EG/ DMSO/ DFA/ MeOH)	75 hatchery water

3.4 Experimental Design:

A total of 21,447 (approximately) morphologically normal carp embryos at different developmental stages were used for the experiments of chilling sensitivity with/without cryoprotectants (CPA), effect of basic solution and cryopreservation trials in various solutions. Firstly embryos were collected and incubated until desired stage occurred such as Morula, 6-Somites, and Tail elongation stage. Embryos exposed to different treatment (Single CPA, Mixture solution, Ultrasound). Embryos also treated without chorion to see whether chorion

had any effect or not on CPAs uptake. Embryos did not receive CPA considered as control. Based on different experiment, selected CPAs solution were used to immersed the embryos in liquid nitrogen (-196°C) and after thawing morphologically good embryos were determined.

3.5 Embryos collection:

Spawning was performed by PG (Pituitary gland) and Ovaprim administration. Six hour after injection, eggs and sperm were collected and mixed thoroughly with a soft and clean feather. Inseminated eggs were then washed with freshwater for several times and incubated in jar until the desire stages to occur (Figure 2).

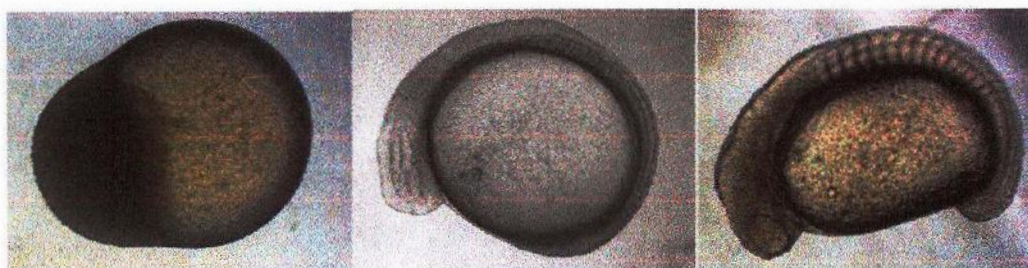


Egg collection

Egg & Sperm mixation Fertilized egg in Hapa

Figure 2: Embryos collection

In this experiment, three developmental stages including the morula (approximately 4 h after fertilization), 6-somite (approximately 10 h after fertilization), and tail elongation stage (approximately 13 h after fertilization) were used (Figure 3). The developmental stages were determined by using a light microscope (4x resolution).



Morulastage

6-somite stage

Tail elongation stage

Figure 3: Developmental stages of silver carp embryos used in the experiment.

3.6 Toxicity of single cryoprotectant (CPA) solutions to embryos:

Five cryoprotectants such as dimethylformamide (DFA), propyleneglycol (PG), methanol (MeOH), ethylene glycol (EG), and dimethyl sulfoxide (DMSO) at various concentrations were used to evaluate the toxicity of solution to different development stage of silver carp embryos. Each cryoprotectant was diluted with hatchery water to obtain 10, 15, 20, and 25% cryoprotectant solutions. Embryos at morula, 6-somites, and tail elongation stages exposed to single CPAs at concentrations of 10, 15, 20 and 25% for 20 min. Immediately after exposure, embryos were carefully washed with water for about 10 min and then transferred to a plastic bucket several cup-sized mesh net are attached with cork sheet (Figure 4).

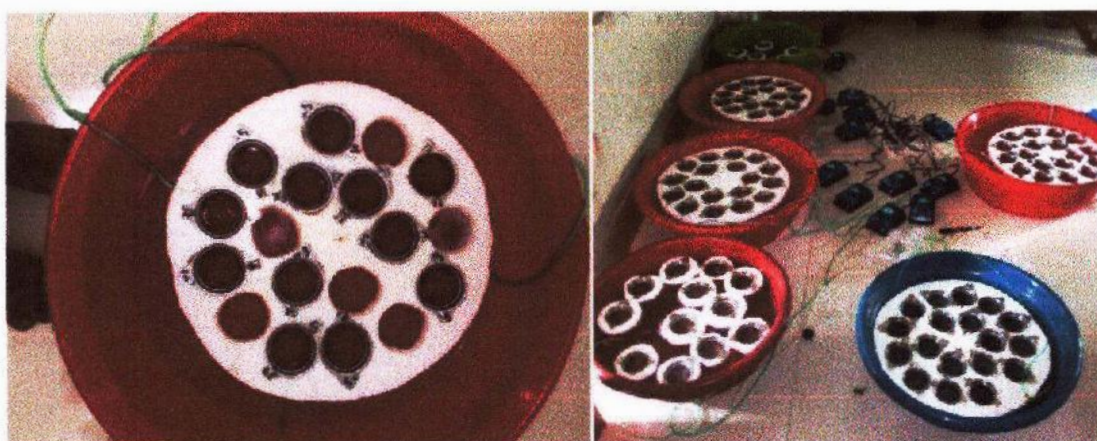


Figure 4: Embryos hatching system after treatment

Each treatment consisted of five replicates with approximately 50 embryos per replicate. Hatching rates were the indication of embryo viability. Embryos not receiving any cryoprotectant exposure were considered as controls.

3.7 Toxicity of mixture CPA solutions and impregnation protocols to embryos:

Five CPAs was used to prepare twelve mixture solutions of four/five CPAs. The composition of the mixture solutions is summarized in Table 2. Concentration and type of CPA were selected

based on the toxicity of single CPA to embryos. Toxicity of the CPA mixture solutions was tested during stepwise impregnation (5 steps of 2 min) of development stage embryos at room temperature. The five steps were represented increases in CPA concentration of 20% per step until 100%. After impregnation, embryos were treated as in the previous experiment for observation of survival. Five replicates, each consisting of approximately 50 embryos were used for each treatment. Untreated embryos were used as controls.

Table 2: Composition of the mixture solutions

Mixture solution	PG (%)	DMSO (%)	EG (%)	MeOH (%)	DFA (%)	Water (%)
CS 1	25	20	20	5	0	30
CS 2	25	15	15	5	0	40
CS 3	25	10	10	5	0	50
CS 4	20	20	20	5	0	35
CS 5	20	15	15	5	0	45
CS 6	20	10	10	5	0	55
CS 7	25	15	15	5	5	35
CS 8	20	15	10	5	5	45
CS 9	20	10	10	5	5	50
CS 10	15	15	15	5	5	45
CS 11	15	15	10	5	5	50
CS 12	15	10	10	5	5	55

Cryoprotectant Solution (CS), Propylene glycol (PG), Dimethyl sulfoxide (DMSO), Ethylene glycol (EG), Methanol (MeOH) Dimethyl formamide (DFA).

3.8 Effect of ultrasound in hatchery water and CPA mixture solutions:

In first trial, embryos were exposed to ultrasound (Figure 5) in hatchery water for 2, 3, 4, 5, 6, 7, 8, 9, and 10 min with continuous pulse. In second trial, embryos at selected development stages were exposed to ultrasound for 1, 2, and 3 min in the presence of CPA mixture solutions. In third

trail, embryos that were pre-exposed to CPA mixture solution for 8 min (4 steps of 2 min) prior to ultrasound treatment (Figure 6) in mixture solution for 2 min.



Figure 5: Ultrasonic machine



Figure 6: Embryo treatment system in ultrasound

After impregnation, embryos were treated as in the previous experiment for observation of survival. Four replicates, each consisting of approximately 50 embryos were used for each treatment. Untreated embryos were used as controls.

3.9Puncture treatment:

Embryos at tail elongation stages were punctured (Chorion removed) prior to treatment by dropper and then the punctured embryos were exposed to selected CPA mixture solution for 10 min (5 steps of 2 min). After impregnation, embryos were treated as in the previous experiment for observation of survival. Three replicates, each consisting of approximately 50 embryos were used for each treatment. Untreated embryos were used as controls.

3.10Analysis of ice formation during cooling and post thawing morphology:

Before subjecting the embryos to cryogenic temperatures, the occurrence of ice formation in selected CPA solutions during rapid cooling (Plunging into liquid nitrogen) and thawing (immersion in a water bath at 40°C) was examined. The reason that an ice-free condition during cooling and thawing would decrease the likelihood of embryo damage.

Embryos at selected development stages were impregnated stepwise (five steps of two min) at room temperature (24°C) with the CPA mixture solutions (Figure 7) and exposed to ultrasound.

Immediate after ultrasound, hatching tray were loaded with embryos, sealed, and then plunged into liquid nitrogen in a 2 L container (Figure 8).

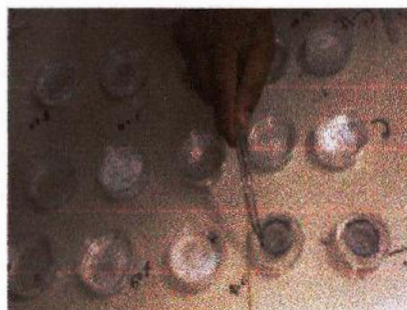


Figure 7: Embryos treatment before freezing

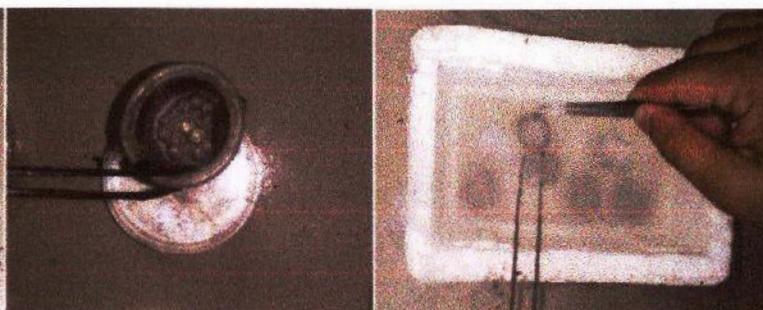


Figure 8: Embryos in liquid nitrogen

Three replicates were prepared for each solution and each contained approximately 10 embryos. Hatching tray containing frozen embryos were quickly removed from LN2 and immersed in a water bath at 40°C for rapid thawing.

After 2-3 hour storage in liquid nitrogen, each were quickly removed from flask and immersed for 5 second in a monitoring survival and observed within 10 min to determine the degree of morphological damage incurred during cooling and thawing. The morphological changes during the cooling-thawing process were recorded using a microscope (Figure 16, 17 & 19).

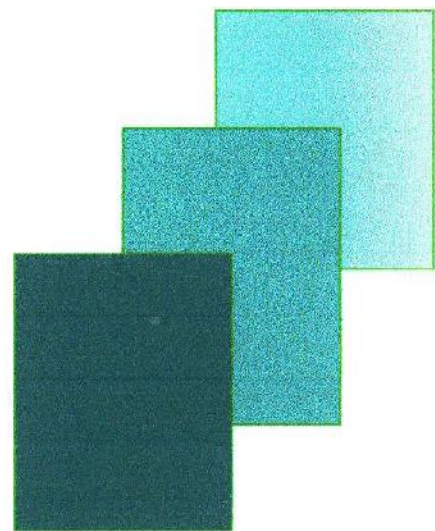
Accordingly, embryos will be classified as “good embryos” when they will undistinguishable from the pre-freezing controls or as “damage” when they have evident morphological abnormalities or damage.

3.11 Data Analysis:

All numerical results were represented as mean $\pm$ SD. Shapiro–Wilk Test were employed to check for the heterogeneity of variance of the data to make sure that the data were normally distributed. Data were then compared by one-way analysis (one-way ANOVA) followed by the Tukey’s multiple comparison test. Differences were considered as statistically significant at a probability value of $P < 0.05$.

Chapter 4

RESULTS



CHAPTER- 4

RESULT

4.1 Toxicity of single cryoprotectant (CPA) solutions to embryos:

Hatching rates of silver carp (*H.molitrix*) embryos exposed to one of the five cryoprotectants varied markedly with the type and concentration of cryoprotectant (Figure 9-11). Embryos at morula stages were less tolerant to the CPAs than other stages. In morula stage, embryos were highly toxic exposed to DFA and no embryos hatched even at lower concentration. Embryos exposed to PG were the least affected followed by those exposed to DFA, EG and MeOH. Hatching rates dropped significantly (Tukey's test, $p < 0.05$) when embryos were exposed to increasing concentration of most CPAs. For example, no surviving embryos were observed after treatment with 25% of DFA and MeOH.

Embryos at the tail elongation stage were more tolerant to CPAs than morula or 6-somite stages. The embryos at the tail elongation stage exposed to 10 % DFA solution had about 31.65 % hatching rates (Figure 11) in sharp contrast to morula and 6-somite stages showing 0% (Figure 9) and 12.24 % (Figure 10) hatching rates respectively.

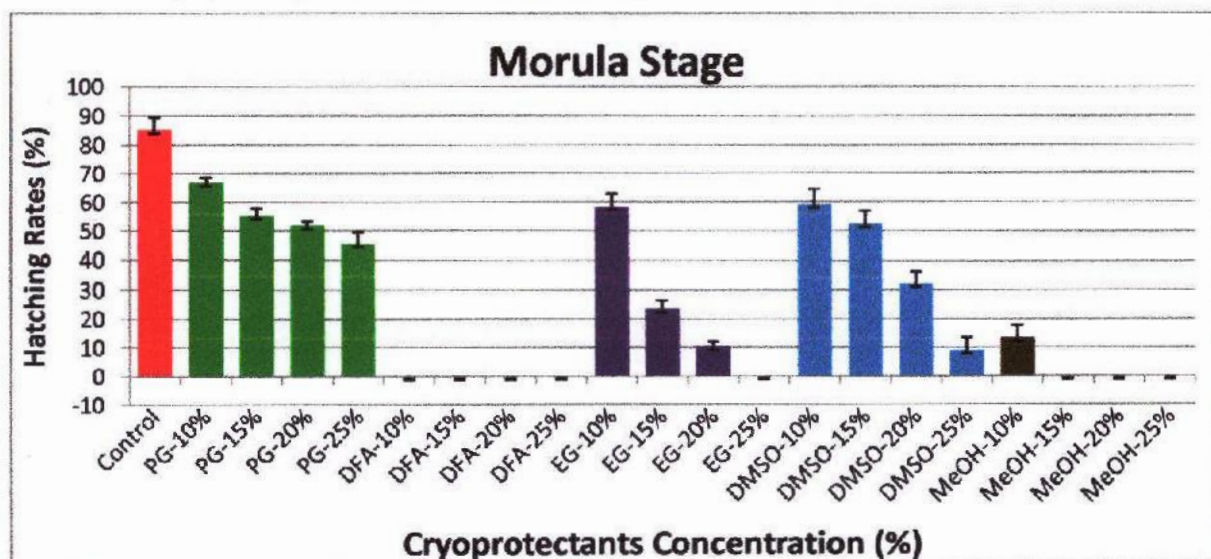


Figure 9: Hatching rates of silver carp embryos at morula stage exposed to different cryoprotectants and cryoprotectant concentrations for 20 min. Data shown as mean and SD of 5 replicates with approximately 50 embryos each. DFA= dimethylformamide, PG= propylene glycol, MeOH= methanol, EG=ethylene glycol, and DMSO= dimethyl sulfoxide.

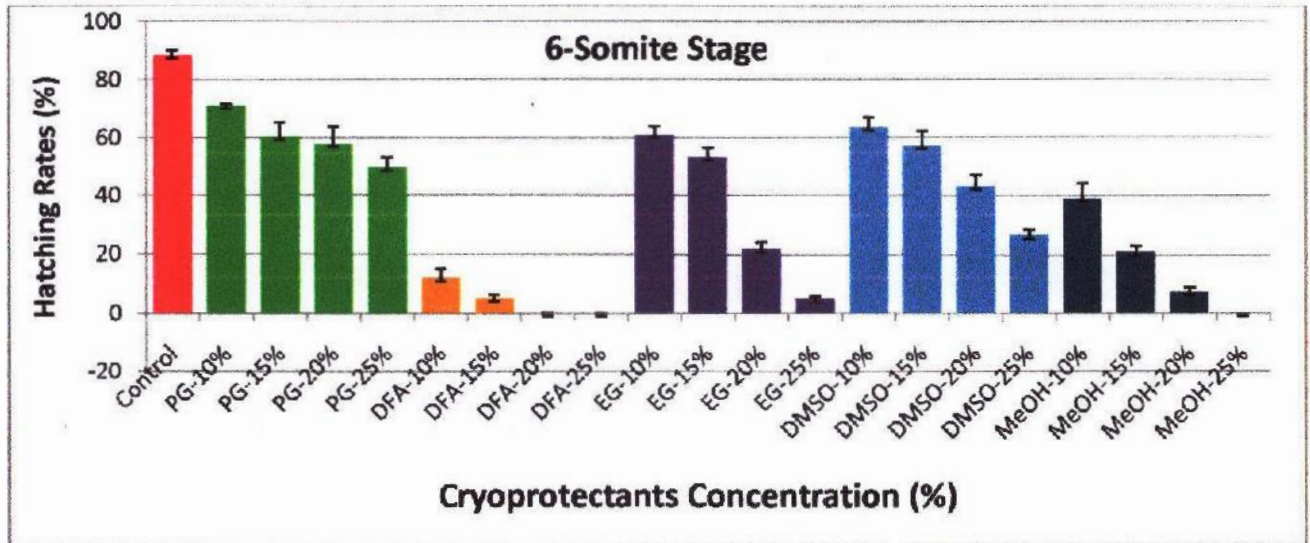


Figure 10: Hatching rates of silver carp embryos at 6-somite stage exposed to different cryoprotectants and cryoprotectant concentrations for 20 min. Data shown as mean and SD of 5 replicates with approximately 50 embryos each. DFA= dimethylformamide, PG= propylene glycol, MeOH= methanol, EG=ethylene glycol, and DMSO= dimethyl sulfoxide.

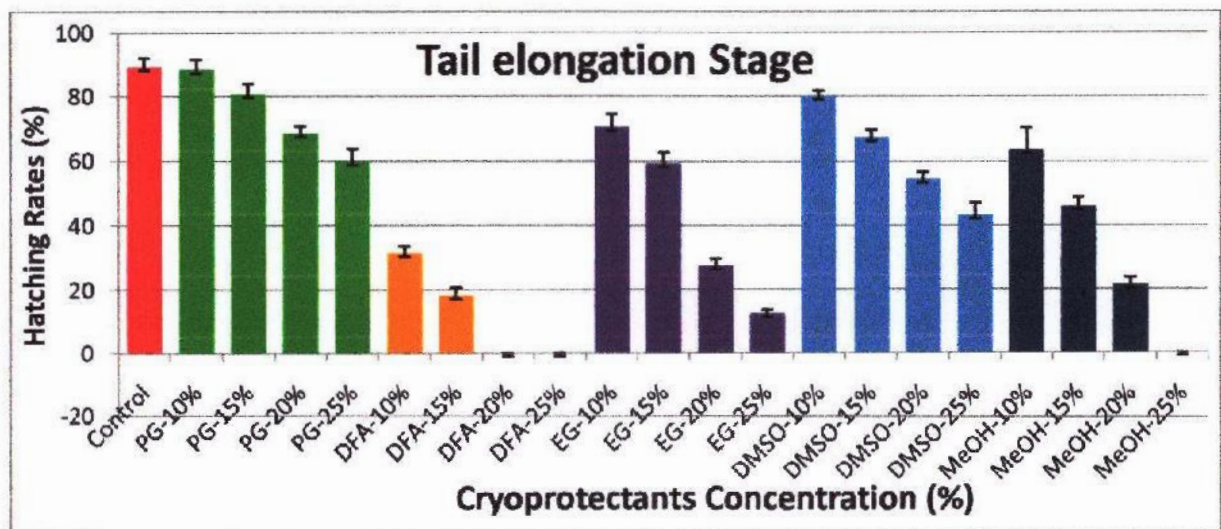


Figure 11: Hatching rates of silver carp embryos at tail elongation stage exposed to different cryoprotectants and cryoprotectant concentrations for 20 min. Data shown as mean and SD of 5 replicates with approximately 50 embryos each. DFA= dimethylformamide, PG= propylene glycol, MeOH= methanol, EG=ethylene glycol, and DMSO= dimethyl sulfoxide.

4.2 Toxicity of mixture CPA solutions and impregnation protocols to embryos

The survival rates of tail elongation stage embryos that were impregnated with CPA mixture solutions in five steps of 2 min were higher than those impregnated in five steps of 3 min regardless of CPA solution (Figure 12). Significant reduction (Tukey's test, $P < 0.05$) in hatching rate compared to the control was observed when embryos were treated with all CPA mixture solutions.

The survival rates of silver carp embryos (tail elongation stage) that were impregnated with CPA mixture solutions in five steps of 2 min were higher than those impregnated in five steps of 3 min regardless of the CPA solution (Figure 12). Significant reduction (Tukey's test, $P < 0.05$) in hatching rate compared to the control was observed when embryos were treated with all CPA mixture solutions. The survival rates of tail elongation embryos impregnated with CPA mixture solutions (5 steps of 2 min) were sharply reduced to half for CS3 (48%), CS6 (40%), and CS12 (48%) than that of control (84%) while CPA solutions CS2, CS5, CS8, CS9, CS10 and CS11 gave hatching between 7% and 26%. On the other hand, in case of five steps of 3 min, CPA solutions, CS3, CS5, CS6, CS11, and CS12 gave hatching rates about 7, 6, 10, 8, and 13, respectively while remaining solutions (CS1, CS2, CS4, CS7, CS8, CS9, and CS10) did not give any viable embryos. Silver carp embryos exposed to mixture solutions for five steps of 4 min did not show any survival except CS12 (4%) and thus their results were not shown in the figure.

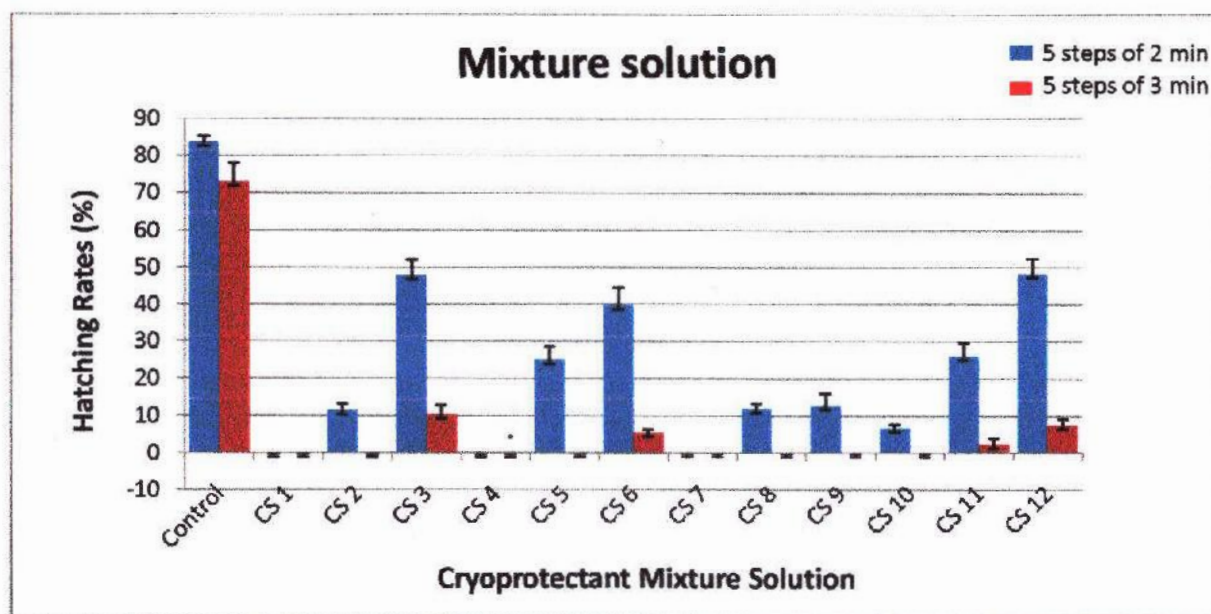


Figure 12: Hatching rates of silver carp (*H. molitrix*) embryos exposed to different cryoprotectant mixture solutions and impregnation protocols. Data shown as mean and SD of 5 replicates with approximately 50 embryos each.

4.3 Effect of ultrasound in hatchery water and CPA mixture solutions

Figure 13 shows the hatching rates of silver carp embryo (tail elongation stage) after ultrasound treatment at various exposure times without CPA solution. Hatching rates decreased gradually with increasing exposure time and varied significantly (Tukey's test, $P < 0.05$). Hatching rates of embryos exposed to ultrasound for 2, 3, 4, 5, 6, and 7 min were found as 78, 50, 37, 24, 19, and 2%, respectively. None of the embryos survived after 8 min of exposure. On the other hand, hatching rates reduced sharply when the embryos were exposed to ultrasound in the presence of CPA mixture solutions (Figure 14). Only CPA solutions CS3, CS6, and CS12 gave survival about 11, 17, and 6%, respectively. Other CPA mixture solutions were found toxic to the embryos (data not shown). Hatching rates further declined about 30% when the embryos were pre-exposed to CPA mixture solutions for 8 min (4 steps of 2 min) before exposed to ultrasound in the same solutions for 2 min (Figure 15). For example, CS3, CS6, and CS12 gave hatching rates about 6, 11, and 5%, respectively.

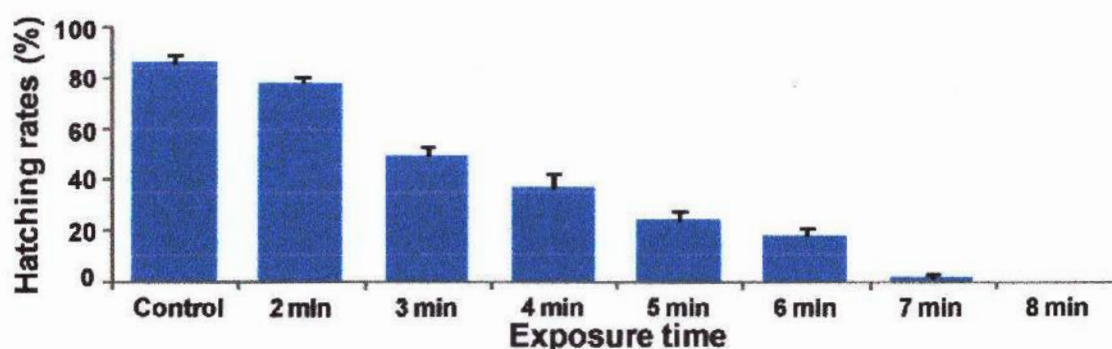


Figure 13: Hatching rates of silver carp embryos at tail elongation stage exposed to ultrasound without CPA mixture solution for 2 to 8 min. Control embryos did not receive ultrasound treatment. Each treatment consisted of five replicates with 50 embryos each. Data represents as mean and $\pm$ SD of each treatment.

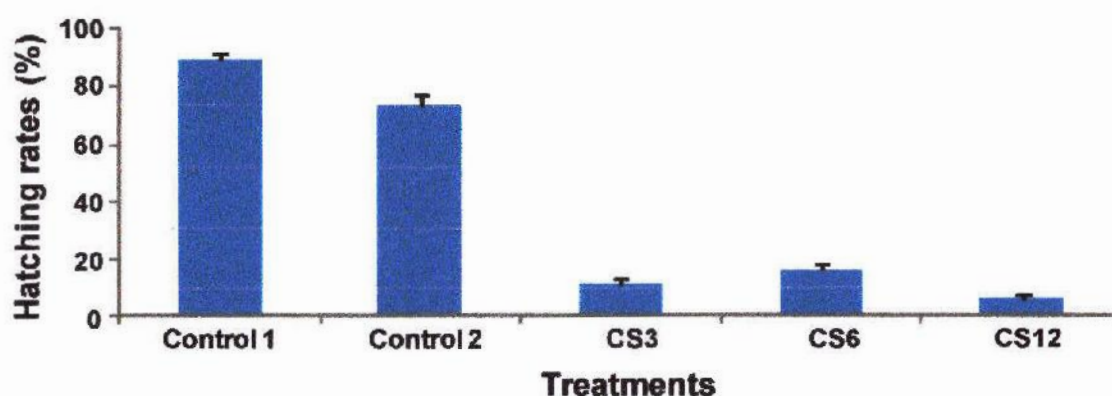


Figure 14: Hatching rates of silver carp embryos at tail elongation stage exposed to ultrasound in the presence of CPA mixture solution for 2 min. Control 1 embryos did not exposed ultrasound and CPA solution while control 2 embryos only exposed to ultrasound but not CPA solution. Each treatment consisted of five replicates with 50 embryos each. Data represents as mean and $\pm$ SD of each treatment.

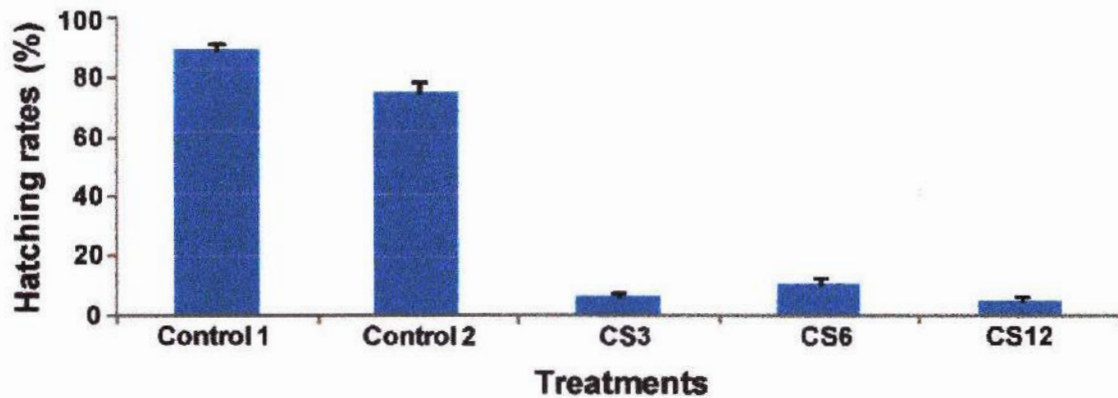


Figure 15: Hatching rates of silver carp embryos at tail elongation stage pre-exposed to CPA mixture solutions for 8 min (4 steps of 2 min) prior to ultrasound in the presence of CPA mixture solution for 2 min. Control 1 embryos did not exposed ultrasound and CPA solution while control 2 embryos only exposed to ultrasound but not CPA solution. Each treatment consisted of five replicates with 50 embryos each. Data represents as mean and $\pm$ SD of each treatment.

4.4 Puncture treatment:

Punctured embryos were highly sensitive to CPA mixture solution. None of embryos survived when embryos at tail elongation stage were exposed to CPA mixture solution (CS3, CS6, CS12) for 10 min (5 steps of 2 min) while punctured embryos without CPA drifted embryos gave hatching rates about 43% which varied significantly (Figure 16) to that of control (82%).

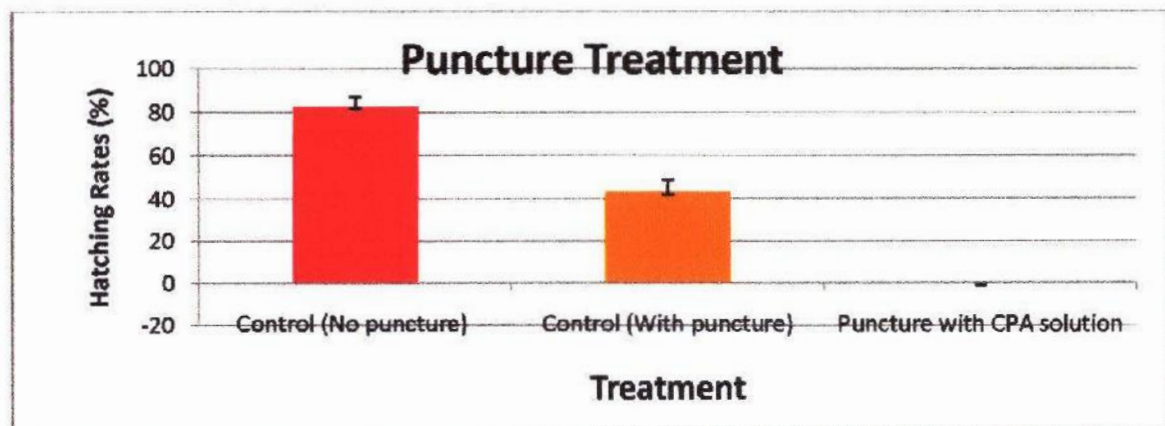


Figure 16: Hatching rates of silver carp (*H. molitrix*) punctured embryos (Chorion removed) exposed to mixture solution. Data shown as mean and SD of 3 replicates with approximately 30 embryos

4.5 Analysis of ice formation during cooling and post thawing morphology:

There were marked differences between groups impregnated with different CPA mixtures as regards embryo transparency during cooling and post-thawing embryo morphology. Embryos plunged into LN₂ after ultrasound-mediated CPA impregnation showed varied appearance after freeze-thawing ranging from apparently intact morphology (Figure 17C) to evident damage including breaking down of the chorion and leakage of contents (Figure 17D).

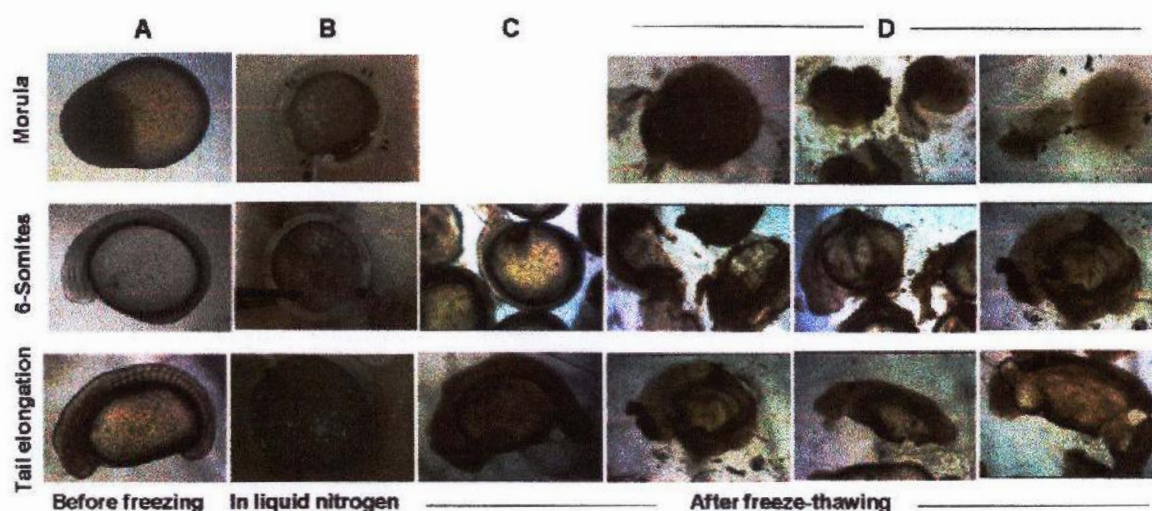


Figure 17: Embryo morphology of silver carp after freeze-thawing. A) Control embryos, B) Treated embryos plunged in liquid nitrogen, C) Intact embryos after freeze-thawing, and D) Damaged or ruptured embryos after freeze-thawing. Morula stage did not show any intact embryos after freeze-thawing

Embryo opaqueness, an indication of the presence of ice, was noticed in all CPA solutions during cooling in liquid nitrogen (Figure 17B). The highest percentages of embryos with normal appearance were observed in CS3 for tail elongation embryos (30 and 32% for without and with ultrasound exposure), CS3 for 6-somited embryos (12 and 11% for without and with ultrasound exposure) (Figure 18). In contrast, all embryos impregnated with CS3 and CS12 for morula stage showed clearly visible abnormalities. The majority, however, had clear damages such as mottling, loss or protuberances in the yolk mass, increased perivitelline space, and ruptured chorion (Figure 17D). Tail elongation stage is more tolerant to cooling than 6-somites and morula stages. However, even embryos with intact morphology did not show any developmental ability during incubation.

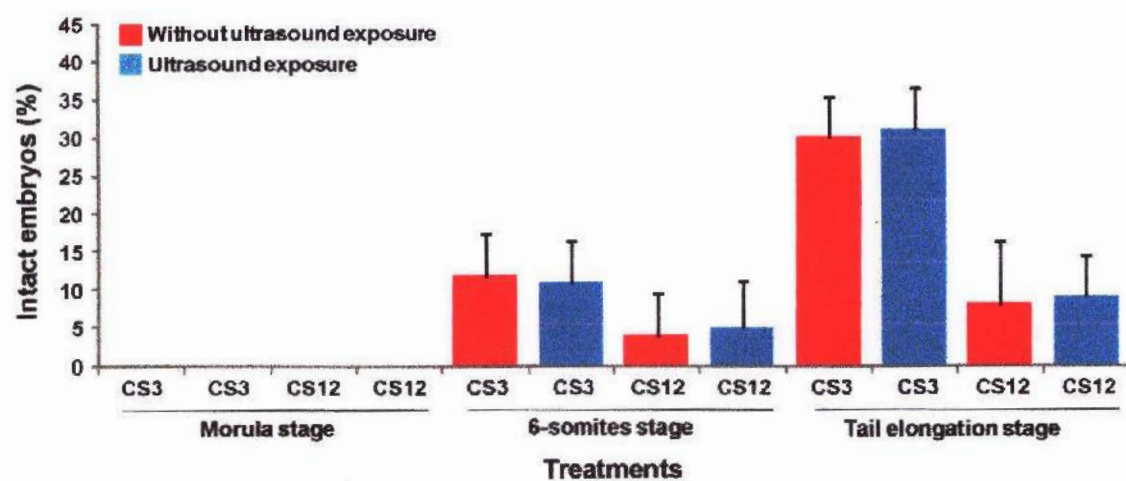
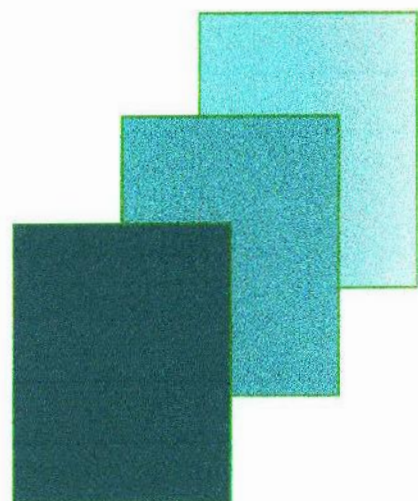


Figure 18: Percentage of intact embryos (morula, 6-somites, and tail elongation stage) of silver carp fish after freeze-thawing. Each treatment consisted of six replicates with approximately 10 embryos each. Data shown as mean and SD.

Chapter 5

DICUSSION



CHAPTER- 5

DISCUSSION

There are significant challenges for the cryopreservation of fish embryos due to their size and physical complexity which affect the permeation of cryoprotectants and water, sensitivity to chilling and toxicity of cryoprotectants. Vitrification offers considerable promise for simplifying and improving the cryopreservation of cells because potential injury associated with ice formation in the suspension is obviated. For vitrification, high cryoprotectant concentrations are required which are toxic to embryos for long term exposure. The use of ultrasound could be promising to improve the CPA permeability sharply and minimize the toxic effects.

Successful cryopreservation depends not only on the right choice of cryoprotectant and extender, but also on the protocols used for embryo immersion in cryoprotectants. The most commonly used cryoprotectants for farm animals are DMSO, glycerol and more recently ethylene glycol. Results relevant to cryoprotectant toxicity obtained from different species including silver carp used in the present study ascertained that some fish embryos are lethal to particular cryoprotectant due possibly to the biological structures of each fish embryos. Cryoprotectant toxicity also varies depending on the developmental stages within the same species. Due to controversy over the species-specificity of cryoprotectants toxicity with regard to different species and stages of embryo development, special attention should be given to determine the concentration and exposure time that minimize toxicological effects to the specific biological material to be cryopreserved.

The present study used 10, 15, 20, and 25% of five cryoprotectants, DFA, PG, MeOH, EG, and DMSO to evaluate the toxicity of three developmental stages of silver carp embryos. Toxicity studies revealed that PG was the least toxic followed by DMSO, EG, MeOH, and DFA. Embryos treated with PG and DMSO gave the similar pattern of hatching rates. No viable embryos were recorded when the embryos (tail elongation stage) were impregnated with 20 and 25% of DFA and 25% of MeOH. Working with olive barb (*P. sarana*), Rahman et al. (2015) reported that embryos exposed to PG were the least affected followed by those exposed to EG, MeOH, DMSO, and DFA, in this order. Chen and Tian (2005) and Edashige et al. (2006) reported that PG and MeOH were less toxic to flounder embryos (*P. olivaceus*) than DMSO and DFA while

no embryos were survived after treatment with EG and Gly at the concentration of 20% for 20 min. Cabrita et al. (2003) mentioned that DMSO impregnated turbot embryos tolerated well than MeOH and EG treated embryos, an observation also previously reported by Chereguini et al. (1995). On the other hand, studies on gilthead sea bream (*Sparus aurata*) embryos showed that EG was the least toxic to embryos than DMSO, MeOH, and PG (Cabrita et al., 2006). Although DMSO has been preferred choice for cryo-biologists due to the good results obtained in sperm cryopreservation (Rana, 1995), other cryoprotectants are now becoming popular for embryo cryopreservation due to cryoprotective effectiveness of different fish embryos.

It is well known that cryoprotectant tolerance varies with the type and concentration of the CPA, the impregnation protocol, as well as the species of the embryos (Routray et al., 2002; Rahman et al., 2008b; Suzuki et al., 1995; Cabrita et al., 2003 and Magnus et al., 1995). Cryoprotectant tolerance to embryos is also stage-dependent (Robertson and Lawrence 1988; Urbanyi et al., 1997) and younger stages are generally less tolerant to cryoprotectant solutions than older ones (Adam et al., 1995; Magyary et al., 2000 and Bart 2000). Our toxicity test on the developmental stages demonstrated that tail elongation stage were more tolerant to cryoprotectant solutions than 6-somites and morula stages.

On the other hand, Chen and Tian (2005) reported that flounder embryos between four somites and tail bud stage had more tolerant to vitrifying solutions than those of other stages. Similar results were also obtained with zebrafish embryos (Zhang et al., 1993). Cabrita et al. (2003) reported that cryoprotectant toxicity of turbot embryos was lower at tail bud stage than at tail bud free stage which is in accordance with the findings of the present study. They further noticed that embryos at tail bud free stage had a more permeable chorion, because chorion permeability increases with development stage (Cabrita et al., 2003) and hatched prematurely when they were subjected to the cryoprotectants at high concentrations. This premature hatching was due to the high osmotic pressure of solutions, which produced excessive dehydration and rupture of the chorion.

This stage-dependent CPA tolerance must be considered in future studies on the cryopreservation of silver carp embryos but it will be obviously counterproductive to choose less permeable developmental stages to maximize survival if it means less CPA content after impregnation. Hence, it is likely that success will come from the use of combinations of several CPAs as

opposed to high concentrations of a single one (Rahman et al., 2008b) and by performing impregnation and actual cryopreservation faster, before the CPAs become toxic to the cells (Routray et al., 2002).

Impregnation with high concentration of single cryoprotectant often appears to be lethal which could be minimized by using the mixture of different cryoprotectants in vitrifying solutions. Such mixture solutions also reduce the osmotic damage of embryos (Rall et al., 1987): predict that the solution will be less toxic because the concentration of any one component is decreased (Chao and Liao, 2001). Alternative approach to reduce the cryoprotectant toxicity and osmotic stress of embryos is to incorporate cryoprotectant gradually in a step-wise fashion (Gwo, 2000; Cabrita et al., 2003 and Chen and Tian, 2005). The addition of cryoprotectant to the cells in solutions causes the release of water from the cells as the concentration equilibrium is maintained. The dehydration of cells can be lethal if it occurs too rapidly and therefore, cryoprotectant solutions should be added to the medium in a series of increasing concentrations (Leibo et al., 1974). Therefore, most recent studies followed step-wise embryo impregnation prior to super cooling. In this study, twelve cryoprotectant mixture solutions composed of either four or five CPAs were used for the embryos (tail elongation stage) of silver carp. Embryos tolerated well 10 min (five steps of 2 min) than 15 (5 steps of 3 min). In case of silver carp, cryoprotectant mixture solutions, CS2, CS3, CS5, CS6, CS8, CS9, CS10, CS11, and CS12 gave hatching rates between 7 to 48% for 10 min steps wise impregnation while CS3, CS5, CS6, CS11, and CS12 gave hatching rates between 6 to 13% for 15 min steps wise impregnation. Effective ultrasound treatment requires the increased cryoprotectant delivery into the embryos without compromising their viability. It is reported that low frequency high intensity ultrasound (conditions promoting collapse cavitation) is much more effective than higher frequencies for transportation of proteins and other large molecules (Wang et al., 2008; William et al., 2004). Silakes and Bart (2010) and Wang et al. (2008) optimized ultrasound conditions (24-47 KHz, 175-480 V, 2-5 min exposure) for improving the methanol permeation into zebrafish embryos. In this study, fixed frequency was used with different exposure times (1-8 min) for silver carp embryos. Hatching rates of embryos decreased with increasing exposure times.

Decreasing in survival rates could be the reason of higher CPA uptake by embryos. In this study, CPA uptake was not quantified by the embryos. Literature suggested that embryos of Japanese whiting increased DMSO level when the embryos were treated with ultrasound (Rahman et al.,

2017). Similar findings were also recorded by Silakes and Bart (2010) and Wang et al. (2008) for zebrafish embryos. Again, Rahman et al. (2013) also reported that whiting embryos further improved the DMSO uptake (about 36%) by embryos when the embryos were pre-treated with CPA solution following ultrasound treatment. For this reason, embryos viability reduced to about half when the embryos were pre-exposed to CPA mixture solution prior to ultrasound treatment in the same solution for silver carp. Pre-exposure with DMSO might equilibrate the water and CPA movement between intra-embryonic and extra-embryonic fluid (Wang et al., 2008).

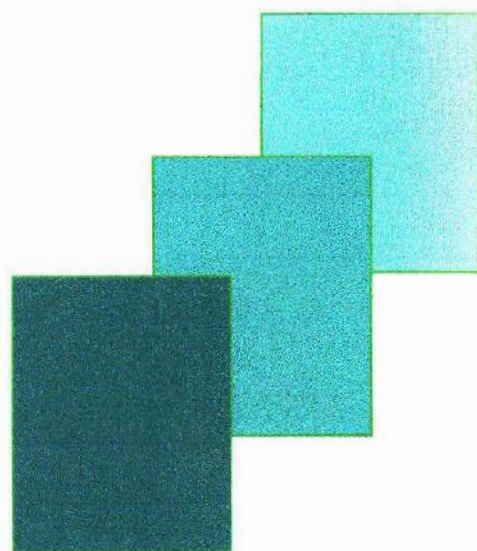
Next, using the best ultrasound condition, embryos at tail elongation stage were drawn into net cage and then plunged into liquid nitrogen for about 1 hour. Immediately after thawing, embryo survival and their morphology were analyzed. Exposure of embryos to CPA mixture solution in the presence or absence of ultrasound did not response well and very few embryos showed intact morphology after thawing. However, these intact embryos failed to develop further during their incubation. Several embryo deformities including, chorion rupture, protuberances of the yolk, yolk darkness were also observed after thawing. Intra-embryonic ice formation inside the embryos indicates that cryoprotectant penetration has not been sufficient to achieve vitrifying conditions. Similar phenomenon were also noticed by other studies (Liu et al. 1998; Lin and Chao 2000; Robles et al., 2003; Cabrita et al., 2006; Edashige et al., 2006; Ding et al., 2007; El-Battawy and Linhart 2009; Rahman et al., 2017 and 2013).

This study also examined the effect of the developmental stage of the embryos on the probability of freezing injury during cooling. Tail elongation silver carp embryos consistently had the lowest rates of morphological abnormalities after freeze-thawing across all CPA solutions tested. Morula stage was found highly sensitive to freezing as none of the intact embryos were evident after thawing. Embryo morphology after freeze-thawing was also observed by Rahman et al. (2008) for marine whiting embryos. They determined that that whiting embryos at the somites and tail elongation stages were more tolerant to the CPAs than those at the gastrula and pre-hatching stages.

Cryopreservation of fish embryos is an effective means not only establish for gene-banking endangered species but also develop for sustainable aquaculture production. This study examined the toxicity and the suitability of impregnation protocols, optimum ultrasound conditions and different embryo developmental stages for the embryos of silver carp species.

Chapter 6

CONCLUSION



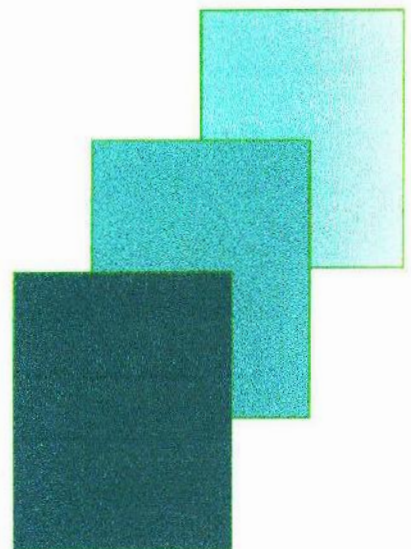
CHAPTER- 6

CONCLUSION

This study outlines the results of cryopreservation experiments with the embryos of silver carp. The observation of the morphology and clearness of the embryos after attempted vitrification revealed that CPA mixture solutions CS3, CS6, and CS12 appeared to be promising in terms of tolerance (intact embryos) by the embryo and prevention of ice formation. It also showed that the tail elongation stage appears to be the most tolerant to chilling injury. However, embryos treated with CPA solution under the best conditions determined in this study did not resume development after attempted vitrification. Previously Rahman et al. (2008 and 2011) observed that embryos dehydrated with sugar followed by exposure to CPA in $\text{CaCl}_2/\text{MgCl}_2$ improved the permeation greatly. Routray et al. (2002) reported that thermal treatment also improved the permeation. Embryos pre-treated with simple sugar and slats at thermal manipulation before ultrasound could improve sufficient CPA permeation to prevent cryoinjuries during cryopreservation require further investigation.

Chapter 7

REFERENCES



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