# Effect of Cryoprotectants on walking catfish (*Clarias batrachus*) embryos

**Abdur Rahman Khan**

**Roll No: MS-170619**



**Fisheries and Marine Resource Technology Discipline**

**Khulna University, Khulna**

**Bangladesh**

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# Effect of Cryoprotectants on walking catfish (*Clarias batrachus*) embryos

**A Thesis**

By

**Abdur Rahman Khan**

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**Approved as to the style and content by**

(Signature of the Head)

**Dr. Muhammad Abdur Rouf**

Professor

Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna-9208, Bangladesh

**July, 2019**

# Declaration

## Effect of Cryoprotectants on walking catfish (*Clarias batrachus*) 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)

**Abdur Rahman Khan**

Examination Roll No: MS-170619

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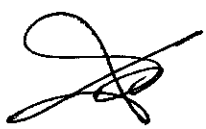

(Signature of the Supervisor)

**Dr. Muhammad Abdur Rouf**

Professor

Fisheries and Marine Resource Technology Discipline

Khulna University, Khulna-9208, Bangladesh

**July, 2019**

**DEDICATED TO**

**MY**

**BELOVED**

**PARENTS**

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# ABSTRACT

The purpose of this study was to examine the toxicity of cryoprotectant (CPA) on the embryos of walking catfish (*Clarias batrachus*), a small-sized fresh water fish which may constitute a suitable experimental material for the development of cryopreservation methods for walking catfish embryos. This study also observed the suitability of ultrasound in embryo cryopreservation studies. Exposure of morula, six-somites and tail elongation stages embryos to 10%, 15%, 20% and 25% solutions of propylene glycol (PG), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethylformamide (DFA) and ethylene glycol (EG) for 20 min revealed that CPA toxicity for walking catfish embryos increased in the order of PG < DMSO < MeOH < EG < DFA. Twelve mixture solution, prepared based on the toxicity of single CPA were used at different concentration to test the toxicity to embryos. Mixture solution, MS1 (mixture of 15% PG, 10% DMSO, 10% EG, 5% MeOH, 5% DFA and 55% YRS solution), MS7 (mixture of 20% PG, 10% DMSO, 10% EG, 5% MeOH, 0% DFA and 55% YRS solution) and MS10 (mixture of 25% PG, 10% DMSO, 10% EG, 5% MeOH, 0% DFA and 50% YRS solution) were found less toxic to embryos than other mixture solutions. Embryos exposed to ultrasound in mixture solutions (MS1, MS7 and MS10) for 2 min could be effective for cryopreservation studies of walking catfish embryos. This study also proved that tail elongation stage was more tolerated to CPA solution than other developmental stages. However, further study is needed to investigate the use of these protocols when combined with cooling or vitrification of this high valued catfish species.

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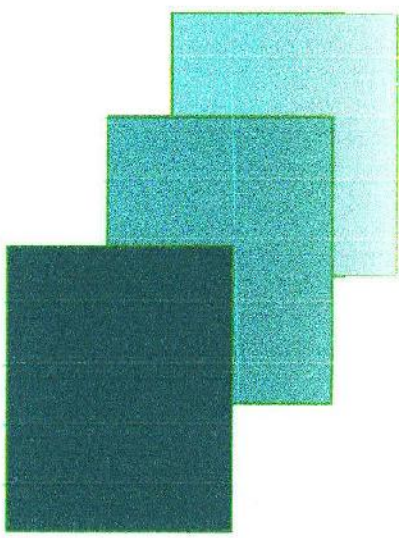
# Chapter 1

# INTRODUCTION



# Chapter-1

# INTRODUCTION

## 1.1 Background of the study:

The worldwide natural fish population is declining due to overexploitation and anthropogenic changes in the environment and therefore, it is necessary to develop gene banks for wish fish resources (Harvey, 2000). Besides, the global growth of intensive aquaculture has further necessitated the search for efficient and effective means of conserving fish gametes (Donaldson, 1997). Based on the report of the red data book of the "International Union for Conservation of Nature and National Resources, 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). Cryopreservation 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.

Fish embryo cryopreservation could play a vital role not only for aquaculture production but also for the conservation of endangered species. However, successful cryopreservation of fish embryos through commonly used impregnation protocols has never been achieved, most probably due to the insufficient impregnation with cryoprotectants (CPA) before freezing. 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 pursuit 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.*, 2004; Cabrita *et al.*, 2006; 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.*, 1996; Zhang and Rawson, 1996).

Methods for the cryopreservation of fish sperm have been developed for numerous species of inland water and marine fish. On the other hand, although tremendous efforts have been undertaken in the last decades, cryopreservation of eggs or embryos has made little progress. In 1989, Zang *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 but their success proved impossible later by Edashige *et al.* (2006). After long intervals, Tian *et al.* (2015), Keivanloo and Sudagar (2016) and Tian *et al.* (2018) reported successful fish embryos after freeze-cooling with varying degree of successes. A recent study conducted by Khosla *et al.* (2017) reported promising results with embryos microinjected with CPAs and gold nanorods that allowed rapid internal warming when excited by a laser pulse and thus mitigated devitrification during thawing. Therefore, it is necessary to verify the protocols they used for the cryopreservation of the species they used for native fish species observed in Bangladesh.

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 dechorionation, 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; Silakes and Bart, 2010) and marine whiting (Rahman *et al.*, 2013) while no such studies have been conducted for any freshwater common

carp fish species. In this regards, this study was examined the suitability of ultrasound for the cryopreservation of freshwater walking catfish (*Clarias batrachus*) embryos in Bangladesh.

Bangladesh is a land of river and possesses a vast water body that contains a huge number of fresh water fish species. Many species have reached at critically low levels and this likely to cause irreversible genetic bottlenecks in Bangladesh. This is particularly important in the farming of walking catfishes because their wild brood or seed collection is reduced due to environmental changes, pollution, over fishing and genetic introgression between populations as a result of anthropogenic introductions.

Walking catfish (*Clarias batrachus*) is a popular species and have a price in Bangladesh with promising importance for aquaculture. In Bangladesh, generally it is known as magur falls under the order Siluriformes and family Claridae (Rahman *et al.*, 2011). Walking catfish can be found in a variety of habitats, but they are most commonly encountered in muddy or swampy water of high turbidity (Courtenay *et al.*, 1974).Warm, stagnant, often hypoxic waters such as muddy ponds, canals, ditches, swamps and flooded prairies are common habitat for this fish. It is also found in lowland streams, swamp and rice fields. Except for occasional foraye to the surface for gulps of atmospheric air, this species spends most of its time on or near the substrate (Rahman *et al.*, 2011). Once it was common in nature, but the fish has recently become scarce because of many adverse changes in their natural habitats. For this reason, this species is now critically endangered in Bangladesh. In this context, the main goal of the study was to examine the toxicity of various permeable CPAs to walking catfish (*Clarias batrachus*) Embryos. The present study was also designed to assess the suitability of impregnation protocols on different embryonic developmental stages.

## 1.2. Objectives of the Study:

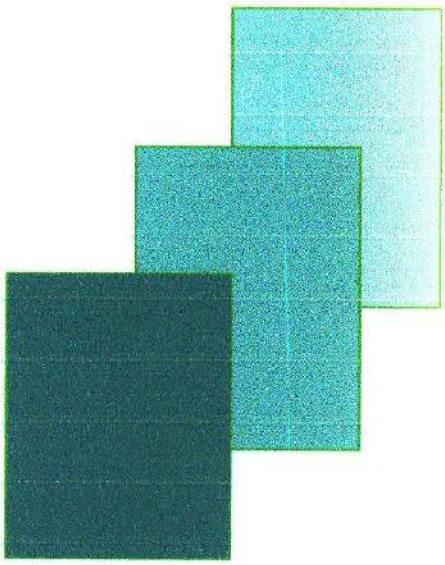
In this study, the embryos of walking catfish (*Clarias batrachus*) were chosen due to the fact that this species can spawn in captive condition, produce numerous eggs and finally short embryonic development. The main objective of this study is to develop suitable impregnation protocols for the cryopreservation of walking catfish larvae.

**Specific objectives:**

- To examine the toxicity of CPA single and mixture solutions to the fish embryos at different developmental stages.
- To observe the toxicity of ultrasound in the presence or absence of CPA mixture solution.

# Chapter 2

# LITERATURE REVIEW



# Chapter-2

## LITERATURE REVIEW

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. 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 developed only for sperm cells and isolated blastomeres (early cells of the embryos) of some species, while cryopreservation protocols of embryos have been developed for few species but still in experimental phases. This study reviews the outcomes of fish embryo cryopreservation studies published elsewhere.

Zhang *et al.* (1993) studied on Cryopreservation of pre-hatch embryos of zebrafish (*Brachydanio rerio*) and used five cryoprotectants - methanol, DMSO, glycerol, ethanediol and sucrose to investigate the toxic level of each cryoprotectant. Embryos were exposed to a range of cryoprotectant concentrations for 30 min at room temperature. Post-heart beat stage embryos were more resistant to cryoprotectants than early embryonic stage and gradual stepwise addition did not reduce toxicity. Heart beat stage embryos survived 2 M methanol at room temperature for up to 5 hours, whilst DMSO and ethanediol were toxic after 3 and 1 hour exposure respectively.

Adam *et al.* (1995) conducted a study on effect of cryoprotectants on activity of selected enzymes in fish embryos. In this study, Rosy barb (*Puntius conchonius*) and zebra fish (*Brachydanio rerio*) embryos at cleavage, epiboly, and closure of blastopore stages were exposed to $Me_2SO$ 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 $Me_2SO$. Zebra fish embryos were more resistant to cryoprotectant enzymatic denaturation than rosy barb embryos.

Suzuki *et al.* (1995) carried out a study in relation between toxicity of cryoprotectant DMSO and its concentration in several fish embryos (medaka, pejerrey, rainbow trout and carp). The average DMSO lethal concentration of each embryo, giving a cumulative mortality of 50%,

and its variance were determined. It was concluded that the tolerance to DMSO becomes higher in the order of carp, rainbow trout, pejerrey, and medaka.

Zhang T. and Rawson D.M. (1996) studied on the feasibility of cryopreservation by vitrification of intact zebrafish (*Brachydanio rerio*) embryos at the 6-somite (12-h) and heartbeat (27-h) developmental stages. The vitrification characteristics and the toxicity of cryoprotectants methanol, $Me_2SO$ , ethanediol, glycerol, acetamide, propane-1,2-diol, and butane-2,3-diol, and their mixtures (supplemented with polyethylene glycol 400) were examined prior to embryo vitrification studies. Result showed that propane-1,2-diol and methanol to be the least toxic to zebrafish embryos. The results demonstrated that although the chorion of the embryos was permeable to water and cryoprotectants, the vitelline membrane permeability to cryoprotectant was low.

Ahammad *et al.* (1998) studied on effect of different concentrations of cryoprotectants and extender on the hatching of Indian major carp embryos (*Labeo rohita, Catla catla*, and *Cirrhinus mrigala*) stored at low temperature. Rohu, catla and mrigal 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, dimethyl sulfoxide and glycerol separately as well as together with a single concentration of sucrose (0.5 M). The hatching rate of embryos was significantly higher when sucrose was added to methanol when compared with methanol alone.

Cabrita *et al.* (2003) carried out a research on the effect of different cryoprotectants and vitrificant solutions on the hatching rate of turbot embryos (*Scophthalmus maximus*) by using dimethyl sulfoxide, methanol or ethylene glycol in concentrations ranging from 0.5 to 6M for periods of 10 or 30 min at two developmental stage (tail bud and tail bud free). They found that embryos tolerated higher concentrations of dimethyl sulfoxide than other cryoprotectants.

Cabrita *et al.* (2003) studied on the effect of different treatments on the chorion permeability to DMSO of turbot embryos (*Scophthalmus maximus*). They used several concentrations of pronase (E, type XIV of *Streptomyces griseus*) and chlorine solutions ($Ca(OCl)_2$ and $Na(OCl)_2$) from 0.01% to 0.0001%) and the embryo viability was recorded. Result showed that DMSO concentration inside the chorion was stage and CPA concentration dependent. It

also concluded that Pronase and chlorine solutions were non-toxic to embryos but it did not improve the DMSO permeation inside the chorion.

Bart *et al.* (2003) studied on survival of zebrafish, *Brachydanio rerio* (Hamilton-Buchanan), embryo after immersion in methanol (40-60%) in the presence or absence of ultrasound with implications to cryopreservation. Older embryos were more resistant to cryoprotectant toxicity and ultrasound-induced mortality. The high concentration of methanol (60%) was more toxic to embryos than the low concentration (40%).

Ahammad *et al.* (2003) performed a study about hatching of common carp (*Cyprinus carpio* L.) embryos stored at 4 and −2 °C in different concentrations of methanol and sucrose. The hatching performance of common carp (*Cyprinus carpio* L.) embryos was examined after 12–72-h storage at 4 and −2 °C using different concentrations of sucrose, methanol (MeOH, or varying concentrations of methanol in 0.5 M sucrose. It was found that 0.5 M sucrose showed the maximum survival (41±1% (12 h) to 11±1.5% (72 h)) at 4 °C. No survival was observed at −2 °C with any concentration of sucrose. The combination of 1.5 M (4.8%) methanol and 0.5 M (17.10%) sucrose produced the best results among all the concentrations tested at both temperatures.

Ahammad *et al.* (2003) conducted study about stage-dependent hatching responses of rohu (*Labeo rohita*) embryos to different concentrations of cryoprotectants and temperatures. Hatching performances of three embryonic stages of post fertilization rohu (*Labeo rohita*) (9-, 12-, and 15-h) were examined after treatment with various concentrations (0.5–4.5 M) of two cryoprotectants (methanol and propylene glycol) supplemented with 0.1 M trehalose. Among three embryonic stages 12-h stage showed better results in trehalose treatment than sucrose. Among all concentrations of trehalose tested 0.1 M gave the maximal survival rate of the rohu embryos.

Ahammad *et al.* (2004) studied on toxicity of protectants to embryos of silver carp (*Hypophthalmichthys molitrix*) after cold storage at different storage time periods. The hatching success of silver carp embryos was examined after 12–24 h of storage at 4°C. The protectants used were propylene glycol (PG), sucrose and different concentrations of PG, with 0.25 M sucrose in each. Result showed that lower concentration of sucrose, PG and the mixture of lower concentration of PG and Sucrose gave better hatching performance.

Chen S.L. and Tian Y.S. (2005) conducted a study on cryopreservation of flounder (*Paralichthys olivaceus*) embryos by vitrification. Their initial finding was that propylene glycol (PG) and methanol (MeOH) were less toxic to embryos than dimethylformamide (DMF) or dimethyl sulfoxide ($Me_2SO$), whereas ethylene glycol (EG) and glycerol (Gly) were toxic to all tested embryos. The final finding was that embryos at the tail bud stage exhibited greater tolerance to vitrification than embryos at other stages.

Zhang *et al.* (2005) carried out a study on toxicity and protective efficiency of cryoprotectants to flounder (*Paralichthys olivaceus*) embryos. In this purpose they used five commonly used cryoprotectants—dimethyl sulfoxide ($Me_2SO$), glycerol, methanol (MeOH), 1, 2-propylene glycol (PG) and ethylene glycol (EG). They found that the toxicity to flounder embryos of the five cryoprotectants is in the following sequence: PG < MeOH < $Me_2SO$ < glycerol < EG ($P < 0.05$. Result also concluded that Methanol combined with any one of the other cryoprotectants gave the best protection.

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.

Cabrita *et al.* (2006) conducted a preliminary study 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. They found that DMSO-based solutions were better tolerated by seabream embryos than EG-based solutions. From their study we can understood that the absence of a proper incorporation of cryoprotectants prior to vitrification is the main problem that must be overcome. This procedure should be optimized in order to avoid ice crystal formation inside embryo compartments.

Ding *et al.* (2007) studied on the vitrification of red sea bream (*Pagrus major*) embryos and wanted to observe 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 on post-thaw hatching rates of fish embryos. In results, they found that 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. Among nine cryoprotectant mixtures, the combination of 20% DMSO + 10% PG + 10% MeOH had the lowest toxicity after exposure for 10 min or 15 min.

Wang *et al.* (2008) studied on ultrasound enhanced methanol penetration of zebra fish (*Danio rerio*) embryos measured by permittivity changes using impedance spectroscopy. Zebra fish embryos at 50% epiboly stage were exposed to 2 M methanol for 25 min before ultrasound treatment for 5 min at 22°C. 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.

Rahman *et al.* (2008) examined the 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 ($Me_2SO$), dimethylformamide (DFA), ethylene glycol (EG), and glycerol (Gly) in artificial sea water (ASW; 33 psu) for 20 min revealed that PG was the most suitable cryoprotectant. Overall, the results of toxicity and permeability suggest that PG, MeOH, and EG could be useful for the development of CPA solutions for whiting embryos.

Xiao *et al.* (2008) studied on the effect of cryoprotectants on hatching rate of red seabream (*Pagrus major*) embryos. They use 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 by immersing heart beat embryos for testing the effect of that cryoprotectant on hatching rate. They found that the hatching rate of the embryos treated with permeable cryoprotectants decreased ($P < 0.05$) 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.

El-Battawy and Linhart (2009) studied on Cryopreservation of Common Tench (*Tinca tinca*) Embryos. In that study they used methanol and glycerol at the concentration of 10% and 20% for periods of 20 min. They found that 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.

Sawitri Silakes and Amrit N. Bart (2010) studied on Ultrasound enhanced permeation of methanol into zebrafish, Danio rerio, 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 embryos. Dechorionated embryos had the highest relative difference in methanol levels between ultrasound and controls. Although, ultrasound significantly affected survival rate, survival was generally high (64±2% to 100%).

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Lin *et al.* (2011) studied on permeability of DMSO in embryos of red seabream by capillary electrophoresis and wanted to investigate the permeability of DMSO to red seabream (*Pagrus major*) embryos by capillary electrophoresis and the effects of DMSO concentrations (5%- 40%, v/v) and immersion times (10, 30 and 60 min) on hatching rate. They found that the internal DMSO concentrations were positively related with the external concentrations and exposure times, while the hatching rate was negatively related. In all groups, when hatching rate was >50%, the internal DMSO concentration was < 2%, which was still insufficient for successful cryopreservation.

Huang *et al.* (2011) conducted a study on the effect of cryoprotectant toxicity on embryos of the Chinese mitten crab, *Eriocheir sinensis* (*Decapoda, Brachyura*). Five stages of embryonic development (cleavage, blastula, gastrula, eyed stage, and heart-beating stage embryos corresponding to 7, 14, 20, 26, and 31 days post-spawning) were chosen and exposed to various cryoprotectants, namely methanol (MeOH), propylene glycol (PG), dimethyl sulfoxide (DMSO), and dimethylformamide (DMF), at three concentrations (10, 15, 20%) for an experimental period of 30 min. at culture temperature (16°C). Result concluded that higher survival percentage was observed in heart beating stage embryos than in gastrula and eyed stage embryos. PG and DMF were relatively less toxic as compared to DMSO and MeOH. Cleavage and blastula embryos were very sensitive to cryoprotectant concentrations above 10%.

Shaluei *et al.* (2013) observed the toxicity of different concentrations (5, 10, 15, 20 and 25 for 5, 15 and 30 min.) of permeable and non-permeable cryoprotectants (DMSO, MeOH, PG, Gly, and EG) of goldfish (*Carassius auratus*) embryos. The viability of the embryos after the treatment was found in the sequence of PG<DMSO<MeOH<EG-Gly.

Hong *et al.* (2013) conducted a study on the effect of combinations of sucrose and cryoprotectants on the survival of catfish embryos (*Pangasidae hypophthamus*) at low temperatures (4, 0 and -20 °C) for short-term storage. Embryos of somites and optic cups were exposed to different combinations of sucrose with methanol, 1.2-propylene glycol and ethylene glycol at four concentrations. They found that the catfish embryos are sensitive to sub-zero temperatures and the combined treatment of 0.5 M sucrose and 1 M propylene glycol can be used to protect catfish embryos from damages caused by low temperature (0 °C and -20 °C).

Keivanloo and Sudagar (2013) performed a study to observe the feasibility on vitrification of Persian Sturgeon (*Acipenser persicus*) Embryos. Six vitrificant solutions (V1-V6) were tested using a stepwise incorporation protocol where three are permeable and three are non-permeable cryoprotectant. The hatching rate of embryos that had been exposed to the vitrificant solutions was analyzed and the highest hatching rate was obtained with exposure to V1.The obtained results establish that cryopreservation of Persian sturgeons embryos by vitrification is possible.

Sharifuddin and Azizah (2014) carried out a preliminary study on cryopreservation of snakehead (*Channa striata*) embryos. Embryos at morula and heartbeat stages were selected and incubated in 1 M dimethyl sulfoxide ($Mc_2SO$), 1 M ethylene glycol (EG), 1 M methanol (MeOH) and 0.1 M sucrose solutions at different temperatures for a period of time. Their results showed that there was a significant decrease of hatching rate in both developmental stages following exposure to $4^0$C and $2^0$C at 1 h and 3 h exposure in each treatment. Heart beat stage was more tolerant against chilling at $2^0$C for 3 h exposure in $Me_2SO$ followed by MeOH, sucrose and EG.

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% and15% 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. Among the cryoprotectants used it was also observed that DMSO proved better than Glycerol as cryoprotectant for both the samples.

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 common carp 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. It also found that higher concentration is toxic than lower.

Tian Y *et al.* (2015) studied on the effects of cryopreservation on the survival rate of the seven-band grouper (*Epinephelus septemfasciatus*) embryos. Six small molecule cryoprotectants (PG, MeOH, Gly, DMF, DMSO and EG) and four macromolecular cryoprotectants (glucose, fructose, sucrose and trehalose) were used to determine the embryo toxicity levels. Results showed that PG and MeOH was showing higher survival rate of the embryos and embryos of tail bud stage exhibits higher hatching rates than embryoid body formation stage and the somite stage.

Rahman *et al.* (2015) conducted a research for 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, propylene glycol (PG), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethylformamide (DFA), and ethylene glycol (EG). They found that hatching rates decreased with increasing cryoprotectant concentrations and toxicity to olive barb embryos varied in the order of PG < EG < MeOH < DMSO < DFA.

Keivanloo S and Sudagar M. (2016) performed a study on cryopreservation of Persian sturgeon (*Acipenser persicus*) embryos by six DMSO-based vitrificant solutions (V1–V6) contained DMSO + permeable cryoprotectants + nonpermeable cryoprotectants to choose

proper vitrificant solutions and temperature for thawing. Their study concluded that cryopreservation of Persian sturgeon (*Acipenser persicus*) embryos by DMSO-based vitrificant solutions is possible.

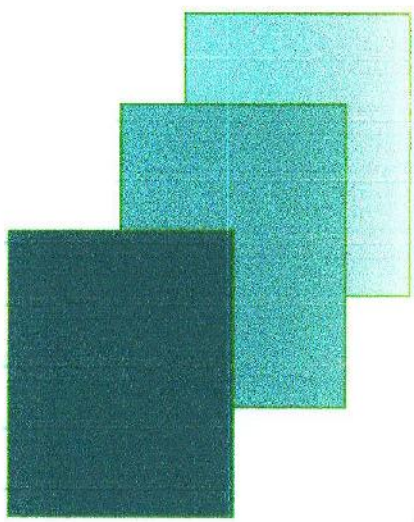
K. Khosla *et al.* (2017) studied on gold nanorod induced warming of Zebrafish embryos from the cryogenic state enhances viability. Zebrafish embryos can attain a stable cryogenic state by microinjection of cryoprotectants followed by rapid cooling, but the massive size of the embryo has consistently led to failure during the convective warming process. After conducting the whole process they conclude that nanoparticle-based warming process could be applied to the storage of fish, and with proper modification, can potentially be used for other vertebrate embryos

Rahman *et al.* (2017) studied on the effects of ultrasound on permeation of cryoprotectants into Japanese whiting *Sillago japonica* embryos to promote the incorporation of CPAs in two different embryonic developmental stages (somites and tail elongation. 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.

Tian Y.S. *et al.* (2018) conducted a study on the effect of vitrification solutions on survival rate of cryopreserved *Epinephelus moara* embryos. In this study, Embryos in stages 10 pairs somite (10S), 18 pairs somite (18S), 22 pairs somite (22S), tail-bud (TB), embryo twitching (ET) and pre-hatch (PH) were treated with five-step equilibrium penetration in 40% PMG3T vitrification solution, which contained 15.75% 1,2-propylene glycol, 10.50% Methanol, 8.75% Glycerol and 5.00% Trehalose. Result showed that 18S, 22S, TB and ET stage embryos had higher survival rates and were more tolerant to the vitrification solution and also found that higher concentration of the vitrification solution led to significantly lower embryonic survival rate ($P < 0.05$). The survival rate of cryopreserved embryos was 5.15%, the normal development rate was 1.31%, and the malformation rate was 3.66%.

# Chapter 3

# MATERIALS AND METHODS



# Chapter-3

# MATERIALS AND METHODS

## 3.1. Brood Collection:

Mature healthy broods of walking catfish, *C. batrachus* (Figure 1) were collected from the natural sources before the advent of monsoon and maintained in tanks (500 L) until experiment. Broods were fed commercial pellets at a maintenance level of 5% of their body weight daily. All experiments were conducted at the laboratory of Fish Physiology and Breeding, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna. For this experiment, required number of females and males ranged from 130 to 190 g for male and 100 to 160 g for female were used for artificial insemination.

**Figure.1**: Walking Catfish (*Clarias batrachus*).

### 3.1.1. Taxonomic Classification:

Local Name: Magur

Common Name: Walking Catfish

Scientific Name: *Clarias batrachus*

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Siluriformes

Family: Clariidae

Genus: Clarias

Species: *Clarias batrachus* **Linnaeus, 1758**

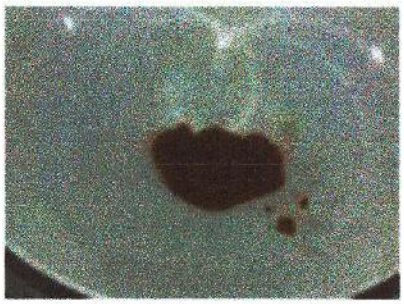
### 3.2. Induced breeding and Embryos collection:

Gravid males and females were selected for induced breeding where females having round and bulging abdomen and button-shape genital papilla and males look slender having an elongated and pointed papilla. Both the sexes were given injection of Ovaprim at the rate of 1.0 ml/ kg body weight to females and 0.5ml/kg body weight to males. The testis of male fishes were dissected out after a latency period of 16-18 hours, macerated with the help of pestle and mortal after pooling all the testes for getting milt and immediately diluted with 0.9% saline (NaCl solution).

The eggs from all the females were stripped out immediately after the preparation of milt suspension in a dry tray. Collected eggs and sperm were mixed thoroughly with a soft and clean feather and then it was shaken gently to ensure effective fertilization (Figure 2). Fertilized eggs were washed several times with fresh water and incubated in a tank until the desire stage to occur.

Hormone injection

Collecting testis

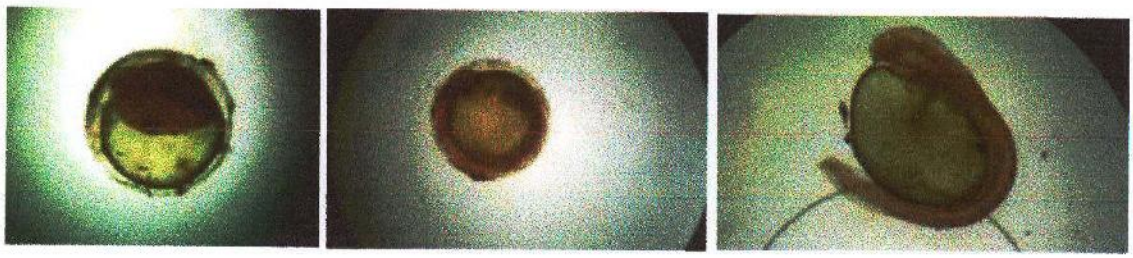


Eggs after Stripping female

Mixing of milts and Eggs

**Figure. 2:** Different stages of induced breeding of walking catfish (*Clarias batrachus*).

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 (4 × resolution).



Morula stage Six Somite stage Tail elongation stage

**Figure.3**: Different developmental stages of *Clarias batrachus* embryos.

### 3.3. Solution Preparation:

Fish Ringer solution was used for the incubation of embryos to monitor survival after exposure to cryoprotectant solutions. In this study, Yamamoto Ringer Solution (YRS) was treated as fish ringer solution that also used for the preparation of cryoprotectant solutions. The composition of Ringer solution is presented in table 1 (Yamamoto, 1939).

**Table.1**: Composition of Yamamoto Ringer Solution (YRS).

| Chemicals | Concentrations (%) |
|---|---|
| NaCl | 0.75 |
| KCl | 0.02 |
| $CaCl_2$ | 0.02 |
| $NaHCO_3$ | 0.002 |

### 3.4. Toxicity of Solutions with a Single Cryoprotectant (CPA) to Embryos:

Five cryoprotectants, dimethylformamide (DFA), propylene glycol (PG), methanol (MeOH), ethylene glycol (EG) and dimethyl sulfoxide (DMSO) at various concentrations were used to evaluate the toxicity of solution to Walking Catfish embryos. Each cryoprotectant was diluted with Yamamoto Ringer Solution (YRS) (0.75 % NaCl, 0.02 % KCl, 0.02 % $CaCl_2$, and 0.002 % $NaHCO_3$ adjusted to pH 7.3 with $NaHCO_3$) to obtain 10, 15, 20, and 25% cryoprotectant solutions (v/v) (Table 1). Embryos at morula, six-somites, and tail elongation stages were

exposed to 10 ml of 10–25 % of CPA solutions in plastic containers (petri dishes) for 20 min at temperature 28°C approximately. Immediately after exposure, embryos were carefully washed with YRS for about 10 min and transferred to the mechanized plastic bowls which contain small iron sieve for hatching purposes until hatching. Hatched larvae were handled using a large-mouthed plastic dropper. Hatching rates were the indication of embryo viability. Each treatment consisted of five replicates with approximately 50 embryos per replicate. Embryos not receiving any cryoprotectant exposure will be considered as controls. Experimental protocols are briefly summarized in the following flowchart (Figure 4).

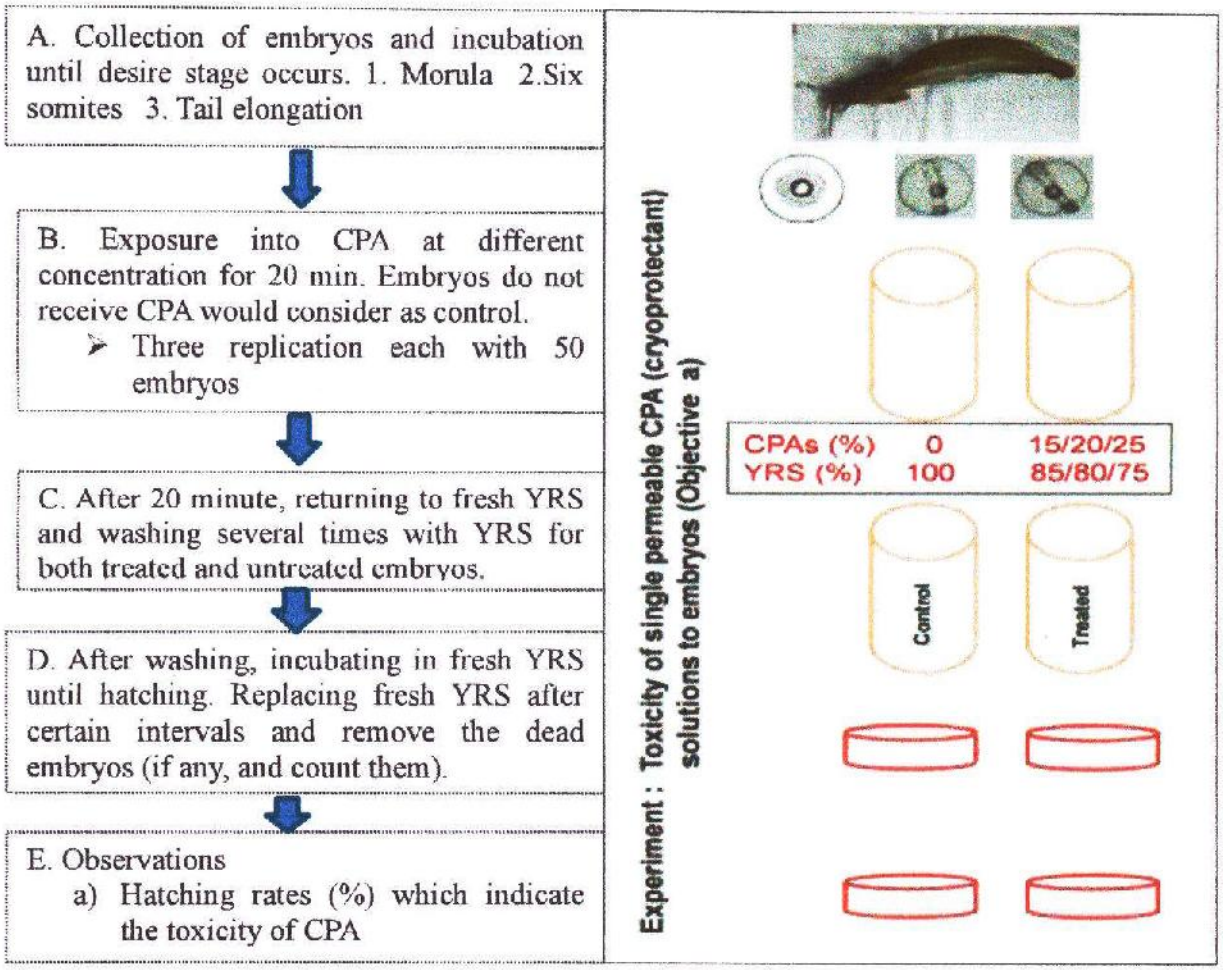



**Figure.4:** Flow chart of experimental protocols.

**Table 2:** Composition of Single CPA (cryoprotectant) solution. Propylene glycol (PG), Dimethyl sulfoxide (DMSO), Ethylene glycol (EG), Methanol (MeOH) Dimethyl formamide (DFA).

| CPA Solutions | Chemicals (%) | YRS (%) |
|---|---|---|
| 10% CPA | 10 (PG / EG/ DMSO/ DFA/ MeOH) | 90 |
| 15% CPA | 15(PG / EG/ DMSO/ DFA/ MeOH) | 85 |
| 20% CPA | 20(PG / EG/ DMSO/ DFA/ MeOH ) | 80 |
| 25% CPA | 25(PG / EG/ DMSO/ DFA/ MeOH ) | 75 |

**3.5. Toxicity of mixture CPA solutions and impregnation protocols to embryos:**

Four to five CPAs were used for the preparation of twelve mixture solutions. The composition and concentration of each mixture solution is shown in Table 3. Concentration and type of cryoprotectants were selected based on the toxicity of single CPA to embryos. The toxicity of the CPA mixture solutions was tested during stepwise impregnation (5 steps of 2 and 3 min) of embryos of tail elongation stage at room temperature. The five steps were represented increases in CPA concentration of 20% per step until 100%. Embryos were then incubated in hatching unit for the observation of survival after several washing with freshwater. Five replicates, each consisting of approximately 50 embryos were used for each treatment. Untreated embryos were considered as controls.

**Table 3**: Composition of the mixture solutions

| Mixture solution(MS) | PG (%) | DMSO (%) | EG (%) | MeOH (%) | DFA (%) | YRS (%) |
|---|---|---|---|---|---|---|
| **MS1** | 15 | 10 | 10 | 5 | 5 | 55 |
| **MS2** | 15 | 15 | 10 | 5 | 5 | 50 |
| **MS3** | 15 | 15 | 15 | 5 | 5 | 45 |
| **MS4** | 20 | 10 | 10 | 5 | 5 | 50 |
| **MS5** | 20 | 15 | 10 | 5 | 5 | 45 |
| **MS6** | 25 | 15 | 15 | 5 | 5 | 35 |
| **MS7** | 20 | 10 | 10 | 5 | 0 | 55 |
| **MS8** | 20 | 15 | 15 | 5 | 0 | 45 |
| **MS9** | 20 | 20 | 20 | 5 | 0 | 35 |
| **MS10** | 25 | 10 | 10 | 5 | 0 | 50 |
| **MS11** | 25 | 15 | 15 | 5 | 0 | 40 |
| **MS12** | 25 | 20 | 20 | 5 | 0 | 30 |

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

### 3.6. Effect of ultrasound on Embryos with Laboratory water and CPA mixture solutions:

For examining the time effect of ultrasound, embryos of tail elongation stage were exposed to ultrasound in laboratory water for 2, 3, 4, 5, 6 and 7 min with continuous pulse. Each treatment consists of four replications and each replication consisted of approximately 50 embryos. In second trial, embryos at tail elongation stages were exposed to ultrasound for 2 and 3 min in the presence of CPA mixture solutions. As it was seen that that tail elongation stage is more tolerant, this stage was chosen for ultrasound treatment. After ultrasound treatment embryos were transferred for hatching operation to get the survival rate or hatching rate. Embryos which did not receive any ultrasound and CPA solutions were considered as control.

**Figure 5:** Ultrasound device.

**Figure 6:** Embryos treatment system in ultrasound

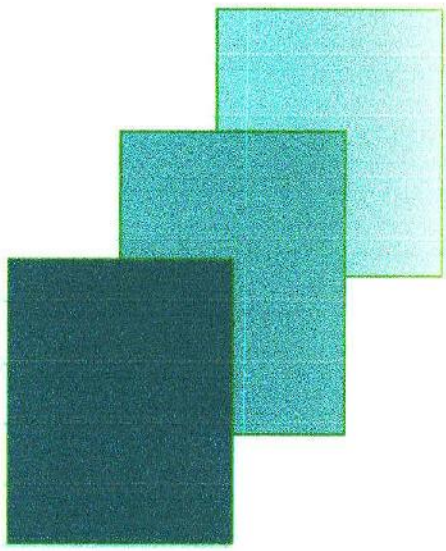


### 3.7. Data Analysis:

Data analysis was carried out by using Microsoft Excel and SPSS software. All numerical results were represented as mean±SD. Shapiro–Wilk Test were done to check for the heterogeneity of variance of the data to make sure that the data was 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

# RESULTS

## 4.1. Toxicity of single cryoprotectant solutions to *Clarias batrachus* embryos:

Hatching rates of embryos of walking catfish (*Clarias batrachus*) treated with the six cryoprotectant solutions varied markedly with the type and concentration of cryopretectants (Figs.7-9). Embryos at morula stage were less tolerant to the CPAs than the other two stages. The results showed that all the CPA has a significant differences (Tukey test, $P<0.05$) with control group in all developmental stages. Exposure to MeOH, EG and DFA even at the lowest concentration (10%), significantly reduced the hatching rates of all developmental stages (Figs.7-9). No embryos hatched after treatment with 15%, 20% or 25 % of DFA, 20 or 25 % of EG, 20 or 25% MeOH. Embryos exposed to PG were the least affected followed by those exposed to DMSO, MeOH, EG and DFA in this order. Hatching rate decreased significantly (Tukey test, $P<0.05$) when embryos were exposed to increasing concentration of most CPAs.

The embryos at the tail elongation stage exposed to 15% EG solution had about 21% hatching rates in sharp contrast to six-somite stage and morula stages showing 12.44% and 0% hatching rate, respectively (Figs.7-9). CPAs at 10% concentration for 20 min were not toxic to walking catfish embryos at six-somite stage and tail elongation stage except DFA but in Morula stage. Result showed that higher hatching rate was found in case of PG, DMSO and MeOH. No significant difference was found between concentration of PG-20% and MeOH 10% at morula stage and between DMSO 15% and MeOH 10% at six-somite stage. In case of tail elongation stage no significant difference were found between concentration of PG-15% and DMSO 10%. Higher hatching rate (46.68%) was found at tail elongation stage at 10% concentration of propylene glycol (Fig. 9). PG and DMSO were the best CPAs tolerated by the embryos at 20 min exposure, as the hatching rate was significantly higher than those obtained for MeOH, EG and DFA at the same concentration (Figs.7-9).

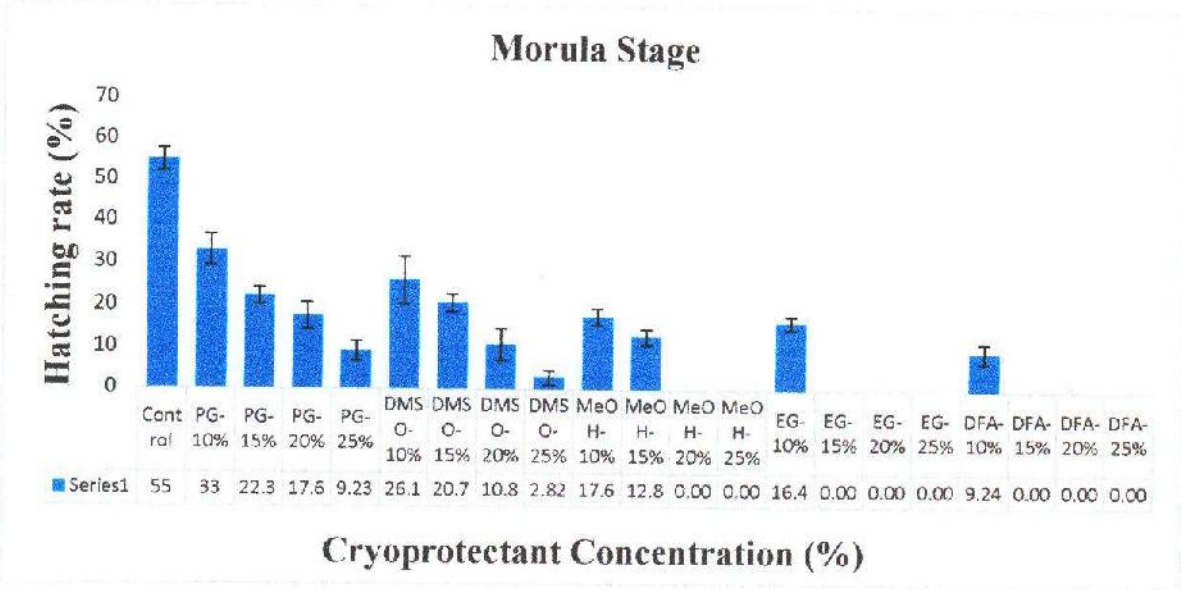



**Figure 7:** Hatching rates (%) of walking catfish (*Clarias batrachus*) embryos at morula stage exposed to different cryoprotectants and cryoprotectant concentrations for 20 min. Data shown as mean and standard deviation of five replicates with approximately 50 embryos each. DFA- Dimethylformamide; PG- Propylene glycol; MeOH- Methanol, EG- Ethylene glycol, Gly- Glycerol, and DMSO- Dimethyl sulfoxide.

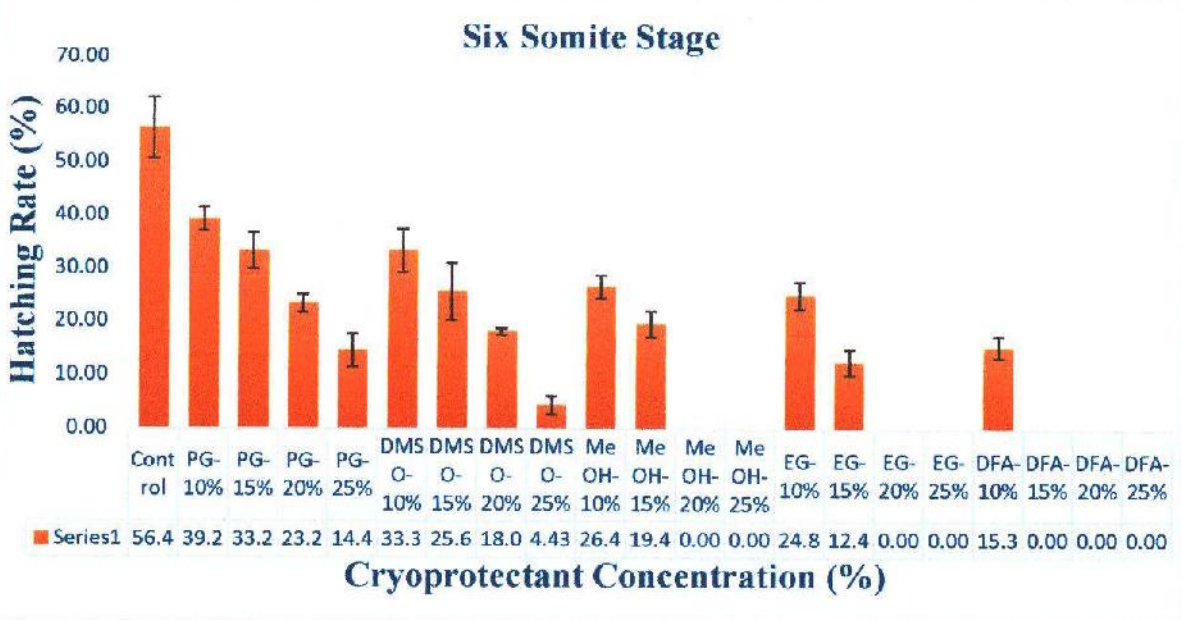



**Figure 8:** Hatching rates (%) of walking catfish (*Clarias batrachus*) embryos at six somite stage exposed to different cryoprotectants and cryoprotectant concentrations for 20 min. Data shown as mean and standard deviation of five replicates with approximately 50 embryos each. DFA- Dimethylformamide; PG- Propylene glycol; MeOH- Methanol, EG- Ethylene glycol, Gly- Glycerol, and DMSO- Dimethyl sulfoxide.

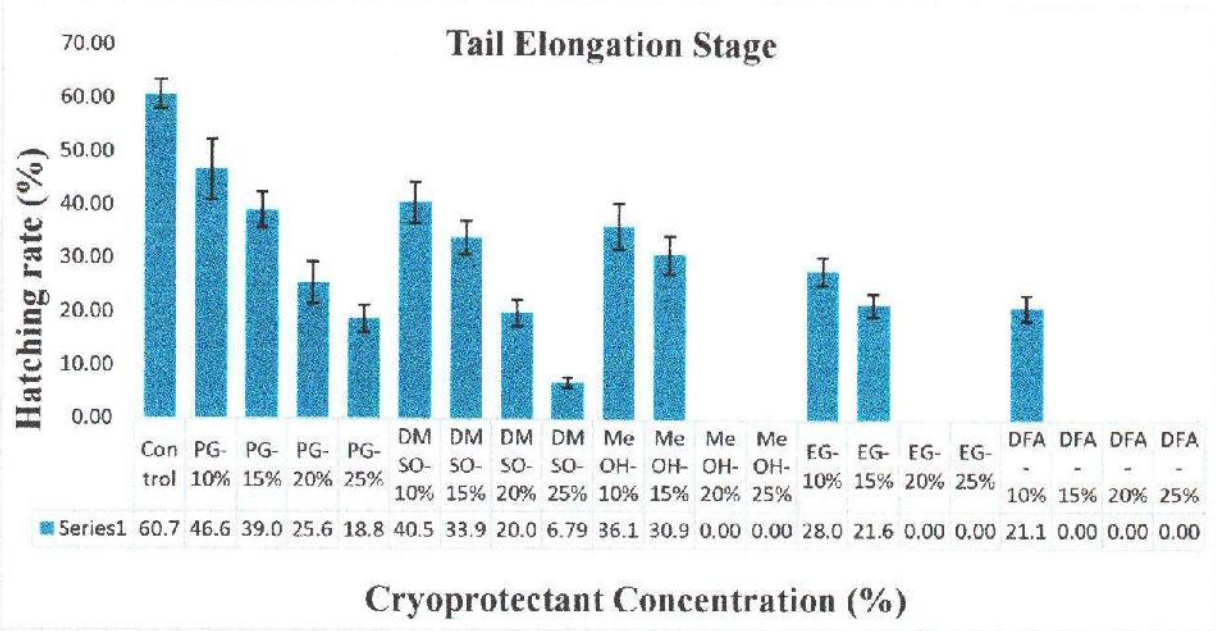



**Figure 9:** Hatching rates (%) of walking catfish (*Clarias batrachus*) embryos at tail elongaton stage exposed to different cryoprotectants and cryoprotectant concentrations for 20 min. Data shown as mean and standard deviation of five replicates with approximately 50 embryos each. DFA- Dimethylformamide; PG- Propylene glycol; MeOH- Methanol, EG- Ethylene glycol, Gly- Glycerol, and DMSO- Dimethyl sulfoxide.

### 4.2. Toxicity of mixture CPA solutions and impregnation protocols to *Clarias batrachus* embryos:

It was found that the permeation of mixture solution into the embryos of walking catfish at tail elongation stage in five steps of 2 min were higher than those impregnated in four steps of 3 min nevertheless of CPA solution (Fig. 10). The result showed that significant reduction (Tukey test, $P<0.05$) in hatching rate compared to the control was observed when embryos were treated with mixture solution (Tukey test, $P<0.05$).

During treatment of embryos with mixture solution (MS), the survival rate of tail elongation embryos permeated with cryoprotectant mixture solutions (5 steps of 2 min) were sharply reduced to one-third for MS1 (39%), MS7 (40%) and MS10 (37%) than that of control (60%) while CPA solutions MS2, MS4, MS5, MS8 and MS11 gave hatching between 9% and 22%. In contrast, no embryos survived after exposure to mixture solutions of MS3, MS5, MS6, MS9, MS11 and MS12 (Fig. 10) in case of five steps of 3 minute treatment while solutions of MS1, MS2, MS4,MS7, MS8 and MS10 gave hatching rates of about 26, 12, 5, 25,11, and 16%, respectively.

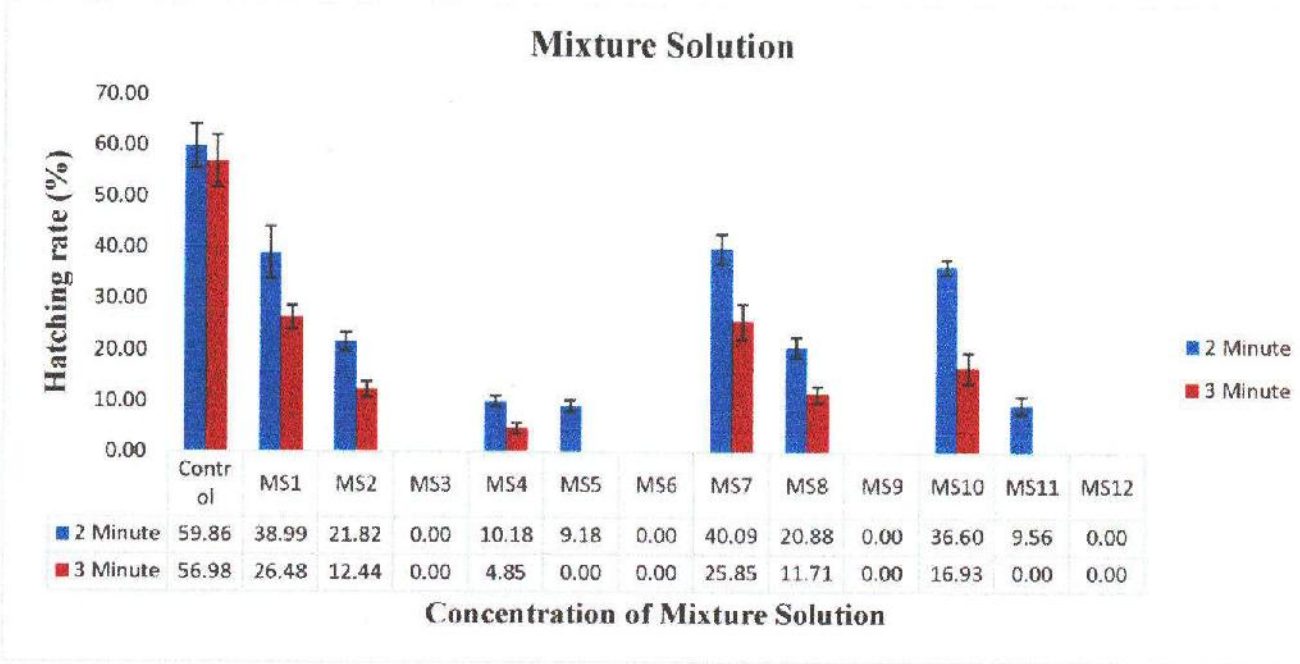



**Figure 10:** Hatching rates of Walking Catfish (*Clarias batrachus*) embryos exposed to different cryoprotectant mixture solutions and impregnation protocols. Data shown as mean and SD of 5 replicates with approximately 50 embryos each.

### 4.3. Effect of ultrasound in water and CPA mixture solutions:

Figure 11 describes the effect of ultrasound in water under various exposure times. Hatching rates of tail elongation embryos exposed to ultrasound in water were decreased gradually with increasing exposure time and varied significantly (Tukey's test, $P<0.05$) than that of embryos did not expose ultrasound. Embryos exposed to ultrasound in ultrasound for 2 min have had higher hatching rate than exposure of embryos into ultrasound in water for 3, 4 and 5 min. Hatching rates of embryos exposed to ultrasound in water for 2, 3, 4, and 5 min was found as about 48, 40, 20, and 8%, respectively. No embryos were survived after 6 and 7 min of exposure into ultrasound in water (Fig 11).

On the other hand, embryos were found more sensitive to ultrasound in the presence of CPA mixture solutions than ultrasound without CPA mixture solutions. Hatching rates of tail elongation embryos exposed to ultrasound in CPA mixture solutions reduced sharply from the respective controls (Tukey's test, $P<0.05$) with increasing exposure time (Fig 12). CPA mixture solution MS1, MS7 and MS10 exposed for 2 min after ultrasound only gave hatching rates about 4.8, 7.4 and 6%, respectively. Experiment resulted that other CPA mixture solutions were found toxic to the embryos and data did not show as none of the embryos survived after treatment.

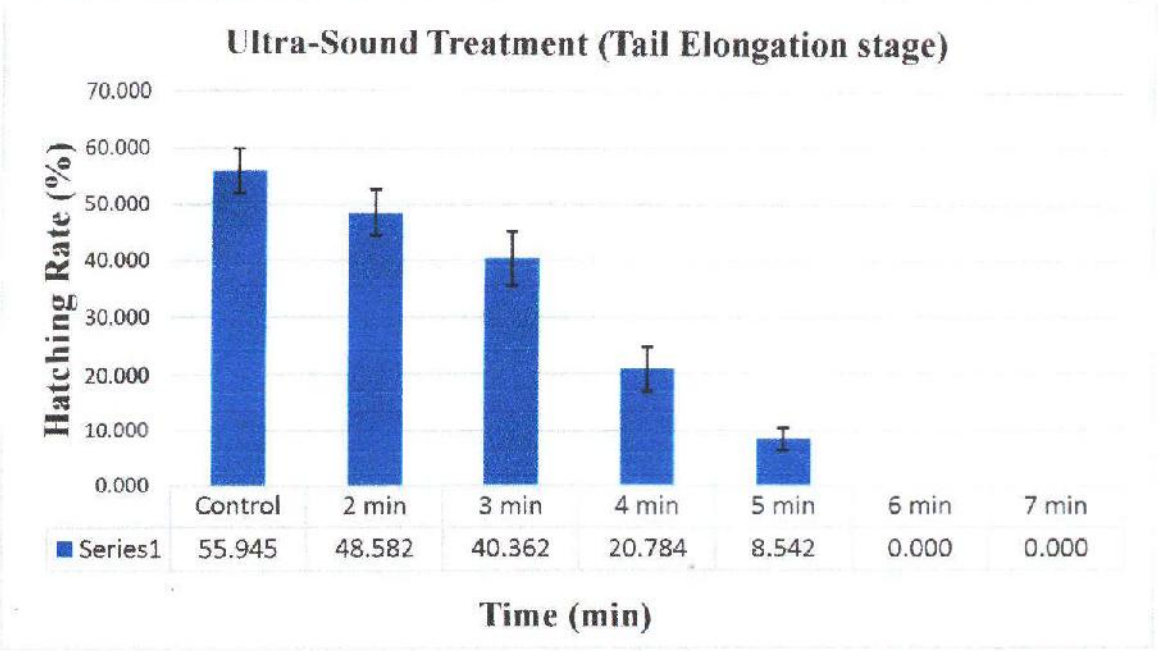



**Figure.11:** Hatching rates of Walking Catfish (*Clarias batrachus*) embryos exposed to laboratory water within ultrasound. Data shown as mean and SD of 4 replicates with approximately 50 embryos each.

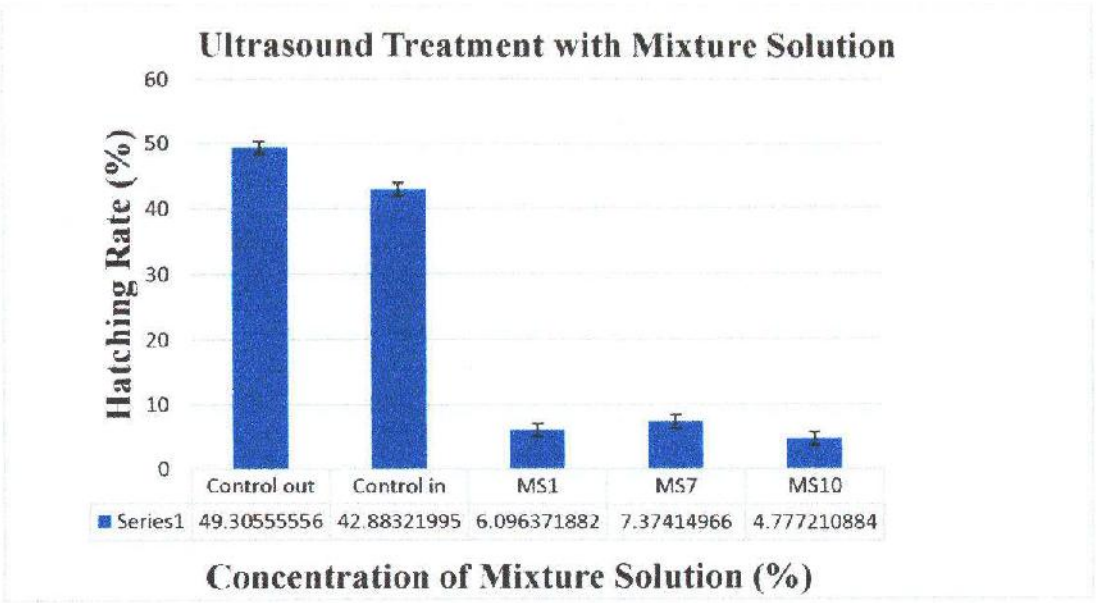
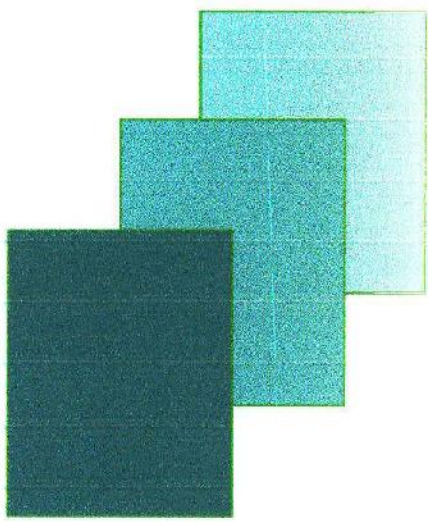



**Figure 12:** Hatching rates of Walking Catfish (*Clarias batrachus*) embryos exposed to mixture solution with ultrasound. Data shown as mean and SD of 3 replicates with approximately 50 embryos each.

Chapter 5

# DICUSSION



# Chapter-5

## DISCUSSION

The application of cryopreservation technique in case of fish embryos is a challenging task due to some factors including their size and physical complexity which affect the penetration of cryoprotectants and water, and toxicity of cryoprotectants. Vitrification, high CPA concentration and sharp freezing, could be a promising tool to minimize the effect of cell injury like damage of yolk, ice formation into the fish embryo cell. However, high cryoprotectant concentrations of CPA are toxic to embryos for long term exposure and therefore, cavitation level of ultrasound could be promising to improve the CPA permeability sharply and minimize the toxic effects.

Successful cryopreservation technology depends on the optimization of several steps from the selection of a suitable cryoprotectant to the post-thawing care of the embryos, and its development is not a straightforward process as most of the steps involved are interdependent. The choice of a suitable cryoprotectant treatment, for example, must take into consideration its permeability and toxicity to the embryos, physicochemical properties, and ultimately its ability to minimize or restrict the formation of ice crystals during cryopreservation because the toxicity of cryoprotectant varies species to species even among different developmental stages within the same species. Because of diversification of cryoprotectants toxicity over the species-specificity 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 during cryopreservation.

The present study used 10, 15, 20, and 25% of five cryoprotectants PG, MeOH, EG, DFA and DMSO to evaluate the toxicity of three developmental stages of walking catfish (*Clarias batrachus)* embryos. The results revealed that PG was the least toxic among them followed by DMSO, MeOH, EG and DFA. The hatching rate of the embryos treated with PG was higher than those obtained from other four cryoprotectants at the same concentrations and exposure time. Results for other fish species show some parallels but also marked differences with those for *Clarias batrachus* embryos. Janik *et al.* (2000) reported that PG produced a better survival rate following microinjection of cryoprotectants into zebrafish embryos. In addition to PG, DMSO and MeOH were also well tolerated by the *Clarias batrachus* embryos. The effect of DMSO and EG was species-dependent in fish; DMSO was better

tolerated in turbot embryos (Cabrita *et al.*, 2003) but poorly tolerated in gilthead seabream embryos (Cabrita *et al.*, 2006), whereas EG was better tolerated in gilthead seabream embryos, but not tolerated in embryos of turbot (Cabrita *et al.*, 2003), flounder (Zhang *et al.*, 2005) or sea perch (Tian *et al.*, 2002).

On the other hand, studies on gilthead sea bream (*Sparus aurata*) showed that EG was the least toxic to embryos among four CPAs (EG, DMSO, MeOH, and PG) (Cabrita et al., 2003). For permeable cryoprotectants, the effects of the three factors and their interactions on the hatching rate were all significant. Cabrita *et al.* (2006) also reported that cryoprotectant concentration and exposure time significantly influenced the hatching rate of gilthead seabream embryos. In the present study, concentration was the most significant factor. The concentration effect was mainly related to the change of osmotic pressure and toxicity of the cryoprotectant. Duration of exposure was the second most significant factor. With more prolonged exposure, concentrations that embryos could tolerate decreased significantly, consistent with previous reports in gilthead seabream embryos (Cabrita *et al.*, 2006) and turbot embryos (Cabrita *et al.*, 2003). Although the cryoprotectant effect was the lowest among the three factors, it was still highly significant.

From previous study about successful use of cryoprotectants, it has been found 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.*, 2008; Suzuki *et al.*, 1995 and Cabrita *et al.*, 2003). Cryoprotectant tolerance to embryos is also stage-dependent (Robertson and Lawrence 1988) 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.

Embryos impregnated with high concentration of single cryoprotectant often seem to be lethal which could be reduced by using the mixture of different cryoprotectants in vitrifying solutions. Such vitrifying solutions not only reduce the osmotic damage of embryos (Rall *et al.*, 1987) but also reduce the toxic effect of any compound from the chemicals (Chao and Liao, 2001). For example, embryos at the tail elongation stage of walking catfish embryos impregnated with 20% PG solution gave hatching rates about 25.61% while embryos impregnated with high concentration of mixture solution composed of 20% PG, 15% DMSO,

15% EG and 5% MeOH (MS8) given almost similar hatching success (20.88%) hatching rate with 5 steps-2 min treatment.

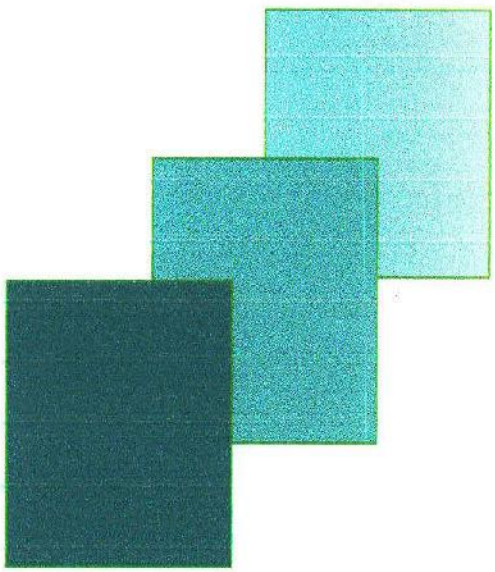
Step-wise increments in cryoprotectant concentrations during both cryoprotectant loading and removal are widely used to reduce the cryoprotectants toxicity and osmotic stress on the embryos (Chen and Tian, 2005;, Cabrita *et al.*, 2003; Gwo, 2000). As the cryoprotectant concentration outside the cells rises it leads to the loss of water from the cells. This 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). In this study, hatching rates of walking catfish embryos diverse with time incorporation: five-step of 2 min addition performed better than five-step of 3 min addition.

The main requirement of successful ultrasound treatment is to increase the cryoprotectant delivery into the embryos without compromising their viability. It is reported that low frequency high intensity ultrasound is much more effective than higher frequencies for transportation of proteins and other large molecules (Wang *et al.*, 2008). In this study, fixed frequency was used with different exposure times (1-7 min) for walking catfish embryos. Hatching rates of embryos in water decreased with increasing exposure times and further reduced when embryos were exposed to ultrasound in the presence of CPA mixture solution. Similar results were obtained by Rahman *et al.*, 2017; Wang *et al.*, 2008; Silakes and Bart, 2010. The reduction of survival could be the passive uptake CPA uptake by ultrasound medicated embryos. In this study, CPA uptake by the embryos was not quantified. Working with marine whiting embryos, Rahman *et al.* (2017) repoted such uptake patterns, similar to Silakes and Bart (2010) and Wang *et al.* (2008) for zebrafish embryos.

This study examined the toxicity and the suitability of impregnation protocols, optimum ultrasound conditions and different embryo developmental stages for the embryos of walking catfish species. However, further studies are required to observe the effectiveness of these conditions for final embryos cryopreservation studies of walking catfish species.

Chapter 6

# CONCLUSION



# Chapter-6

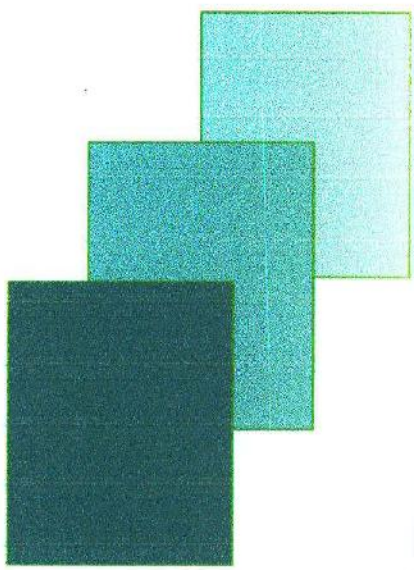
## CONCLUSION

In conclusion, this study outlines cryopreservation experiments with the embryos of walking catfish, a fresh water fish that spawns small eggs. Walking catfish may be advantageous as a model for cryopreservation studies because it is both small and prolific, and because it's developmental speed is very short. The findings of this study provide important basic information on suitable CPAs, their concentration, and impregnation protocol with applicability for embryos of this species and perhaps for other species as well.

Hatching rates decreased with increasing cryoprotectant concentrations and PG was found less toxic to embryos followed by DMSO, MeOH, EG, and DFA. Tail elongation stage was more tolerant to CPA solutions than morula and somites stages. Embryos (somites) tolerated better a five step than a four step impregnation. Mixture solution, MS1, MS7, and MS10 could be a option for impregnation protocols. Embryos tolerated well ultrasound in mixture solutions for two min. These results should be very useful for designing optimized cryoprotectant solutions and establishing freezing protocols for the cryopreservation of embryos from this freshwater fish.

Chapter 7

# REFERENCES



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