International Journal of Agricultural Technology 2020 Vol. 16(4): 1047-1062
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Investigation of appropriate cryoprotectants to be used in the cryopreservation of Thai walking catfish (*Clarias macrocephalus*) embryo

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Wankanapol, A. and Vera Cruz, E. M. (2020). Investigation of appropriate cryoprotectants to be used in the cryopreservation of Thai walking catfish (*Clarias macrocephalus*) embryo. International Journal of Agricultural Technology 16(4): 1047-1062.

Abstract The appropriate cryoprotectant used in the cryopreservation of Thai walking catfish (*Clarias macrocephalus*) embryo as investigated. The Thai walking catfish embryo at somites developing stage was exposed to each cryoprotectant solution. The results showed that 40% PG + 5% egg yolk was the least toxic to Thai walking catfish embryo at 40 min exposure time. It was followed by GLY, DMSO and MeOH at 40% + 5% egg yolk, respectively. The permeability is conversely correlated with PG which was the least toxic and had the the lowest permeability while MeOH was the most toxic and highest permeability to Thai walking catfish embryos. Unfortunately, the embryos were not recovery after thawing. The cryoinjuries include ice crystal formation, cold shock and thermal shock might lead to the viability damage.

Keywords: Cryoprotectants, Cryopreservation, Thai walking catfish, Embryo

Introduction

Thai walking catfish (*Clarias macrocephalus*) is a native freshwater fish of Thailand and Southeast Asian countries. It is a main ingredient for some Thai dishes (Na-Nakorn, 2004) and is also relatively prefered by Thai consumers compared to the other walking catfish species (Srisuvantach and Thangtrongpiros, 2005). On the contrary, it grows slowly leading to lower yield per unit area and with a long culture period. Moreover, the destruction of the environment including community expansion, waste water from households and the use of agricultural chemicals affected their natural habitats and lead them to be assessed by the IUCN red list species status as “near threatened” since 2011 (Vidthayanon and Allen, 2011). The new genetic gain to develop their traits by cross breeding was performed in order to be able to cope with the changing requirements and future demands in breeding and selection. The

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hybrid walking catfish was made by cross breeding male African sharptooth catfish (*Clarias gariepinus*) and female Thai walking catfish (*Clarias macrocephalus*). The advantages of the hybrid include higher growth performance, greater preference meat quality and better disease resistance. Since 2003, Thailand statistics showed that the total Thai walking catfish production was increasing continuously and reached over 10^5 tons which started from this year. Moreover, the 2 top ranking freshwater aquaculture fish production was recorded every year until 2017 (Department of Fisheries, 2017).

Cryopreservation is an important technique in the retention of the genetic resource and possible restocking depending on the ability to conserve relevant material (Bart, 2000). While cryopreservation using fish sperm has been relatively successful, limited studies have been conducted on the fish oocytes and embryos and most of them have not been completely successful as it appears to be formidable constraints to the development of suitable methods. Thai walking catfish eggs are big, thick, demersal and have slow embryonic development. For the success for embryo cryopreservation, there is a necessity to remove the water from the individual cells. If water is left, it forms crystals when frozen. These crystals act like knives and cut inside cells through the outer layer or membrane. The embryo will not survive if disruption has occurred. In order to avoid the formation of the water crystals, a cryoprotectant is added which replaces most of the water inside the embryo and the embryo can safely withstand the drastic reduction in temperature required for cryogenic storage.

This study aimed to identify appropriate cryoprotectant to be used for cryopreservation of embryos of Thai walking catfish (*Clarias macrocephalus*). It attempted to provide primary methods required to preserve catfish embryos for a long period of time that would be a reliable method of conservation, source of genetic material for scientific and aquaculture purposes as well as for the preservation of pure strains. Thus, Thai walking catfish could be an alternative model for the study of cryopreservation of freshwater commercial fish embryos.

Materials and methods

Fish culture and embryo collection

Thai walking catfish broodstock were cultured and maintained at the Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Thailand. Male and female broodstocks with sizes ranging from 250 to 300 g were stocked in seperated 2 m^3 circular tank at 5 fishes m^{-3}. The fishes were fed

with commercial feed (with 30% crude protein) twice a day at 3% of the body weight. Before artificial breeding, no feeding for one day was done. The pregnant females were collected and injected with buserelin acetate hormone at the rate of 30 µg kg^{-1} combined with domperidone at 10 mg kg^{-1}. The males were collected and injected with buserelin acetate hormone at15 µg kg^{-1} combined with 10 mg kg^{-1} domperidone. Modified dry method was used for fertilizing eggs and incubated in fine mess net in square tank until they reached somite embryonic development stage. To avoid infection and to maintain the fertilized eggs, they were incubated and received diluted fish ringer solution containing traces of malachite green, as a prophylactic agent, combined with 2 ppm of $KMnO_4$ for disinfection. Embryos with size 1 mm were selected for use in the study. Embryo developmental stages were determined using stereomicroscope (Nikon, Tokyo, Japan) at 40x magnification. The embryos at the segment period particularly somites developmental stage were used.

Preparation of cryoprotectant solutions

There is a wide range of cryoprotectants being used in fish embryo cryopreservation but this experiment focused on the four commonly used which were expected to be suitable for embryo cryopreservation. Distilled water was used for the preparation of cryoprotectant solutions and for incubation of embryos to monitor survival after toxicity trials. The four permeable cryoprotectant agents (CPAs) evaluated were dimethylsulfoxide (DMSO), glycerol (GLY), methanol (MeOH) and propylene glycol (PG). The 5% (v/v) egg yolk was designed to combine with each previous cryoprotectants as non permeable cryoprotectant. Embryos were exposed to 100 ml of each cryoprotectant at concentrations of 40% (v/v) combined with 5% egg yolk in distilled water for 40 minutes at temperature 35˚C. After the preparation of the solutions, the osmolality and pH of each solution was measured in mmol kg^{-1} unit. Immediately after exposure, the embryos were rinsed in washing solution, a 0.125 M sucrose solution (1042 mmol kg^{-1}) for cryoprotectant removal. Twice for 10 minutes and incubated in glass petri dishes containing distilled water. After incubation, viable embryos were counted for computation of the survival rate (SR). Moreover, morphological changes after their immersion were evaluated under the microscope. In terms of toxicity to CPA, assay on the sensitivity of embryos individual CPA was examined. The survival of the embryos was assessed by their ability to hatch into live fry. The hatching rate of embryos was used to determine the toxicity of the cryoprotectant by counting the number of hatched fry relative to the total number of incubated embryos.

The permeability of cryoprotectants

The Thai walking catfish embryoes were incubated in 40% of each cryoprotectant solution (PG, GLY, DMSO and MeOH) combined with 5% egg yolk for 40 minutes before impregnation. The permeability of each CPA to catfish embryos was determined using High Performance Liquid Chromatography (HPLC) for MeOH and Gas Chromatography (GC) for PG, GLY and DMSO analyses. Permeability assessment was expressed as the concentration of each CPA inside the whole embryo. The HPLC analysis was done using Agilent 1100 series HPLC Value system, (Agilent Technologies, USA) the conditions and details were followed properly. The Zorbax SB-C 18 3.5µm 4.6 x 100 mm column was used in HPLC technique at 1.0 mL min^{-1} flow rate. The detector used was Diode array detector (DAD) at 210 nm wave length. The injection volume was 20 µL. The mobile phase was H20: Methanol (90:10) and the total analysis was 6 min. In the case of GC analysis using Agilent 6890 series Gas Chromatography (Agilent Technologies, USA), the conditions and details were follows as follows: The HP-5 (length 30 m, id (mm) 0.25, film (µm) 0.25, max temp 280) was the column used in GC technique at 1.0 mL min^{-1} flow rate. The initial temperature of the oven temperature was 50˚C, held for 4 min and was increased at a rate of 15˚C min^{-1} up to 180˚C, then 180˚C up to 230˚C at a rate of 7˚C min^{-1}. The flame ionization detector (FID) was the detector used and run on split operation mode. The 2 µL was the injection volume using helium as a carrier gas and the total analysis was 23 min.

Osmolality and freeze control assessment

Osmolality Osmolality measurement was done using Cryoscopic Osmometer OSMOMAT® 030 model developed by Gonotec GmbH, Germany.

Freeze control system was employed using temperature control software (Cryogenesis™ V5 for Windows program) and programmable temperature controller (cryochamber and cryobath; CL-3300 model, CryoLogic Pty.Ltd., Australia).

Cryopreservation method

After exposure of the embryoes in the least toxic cryoprotectant solution, the temperature was decreased slowly (cooling rate) at 0.2˚C min^{-1} using freeze control system controlled by Cryogenesis program Version 5 until it reached final a temperature at -50˚C and the embryo was stored in liquid

nitrogen (LN_2). After 3 hours the embryo was thawed at 40 °C temperature and viability was evaluated.

Statistical analysis

Survival data results were expressed as mean ± SEM. The statistical significance of differences between means was analyzed using one way analysis of variance (ANOVA) followed by Duncan's new Multiple Range Test (DMRT) as the post hoc test. All data were analyzed using SAS software version 9.1. Differences were considered as statistically significant at a probability value of $P < 0.05$.

Results

Cryoprotectant toxicity analysis

The composition, osmolality and pH of each cryoprotectant solution are summarized in Table 1. Generally, PG 40% + EY 5% attained the highest osmolality followed by GLY 40% + EY 5%, DMSO 40% + EY 5% and MeOH 40% + EY 5% obtained the lowest with 3870, 3210, 3060 and 2640 mmol kg^{-1}, respectively. As to the pH of cryoprotectant solutions, the MeOH attained the highest pH compared to the others wherein PG, GLY while DMSO obtained the pH of 8.40, 7.83, 7.76 and 6.94, respectively.

Table1. Composition, osmolality and pH of the cryoprotectant solutions used in the experiment

Cryoprotectant Solution	Concentration (%)	Osmolality (mmol kg^{-1})	PH
Propylene Glycol (PG) + EY	40 + 5	3870	7.83
Glycerol (GLY) + EY	40 + 5	3210	7.76
Dimethyl sulfoxide (DMSO) + EY	40 + 5	3060	6.94
Methanol (MeOH) + EY	40 + 5	2640	8.40

The toxicity of each cryoprotectant solution in combination with EY at 40% + 5% concentration levels in somite embryo developmental stage exposed for 40 minutes is indicated in Figure 1. Comparing the four cryoprotectant solutions, PG obtained the highest survival rate followed by GLY, DMSO and lastly MeOH attained the lowest with 63.14 ±1.57, 38.24 ±2.36, 36.72 ±0.98 and 1.93 ±0.66 percent, respectively. Moreover, there was a highly significant survival rate ($P<0.01$) among the different cryoprotectant solution (F =63.57).

This result implied that PG was the least toxic to the embryos followed by GLY, DMSO. MeOH was the most toxic to the embryos at somite developing stage.

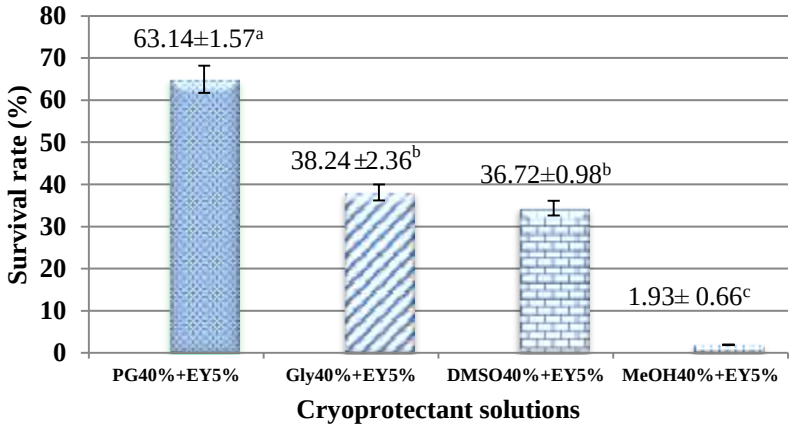

Figure 1. Mean survival rate (%) of Thai walking catfish embryo at somite development stage exposed to four cryoprotectant solutions at 40 minutes equilibration time

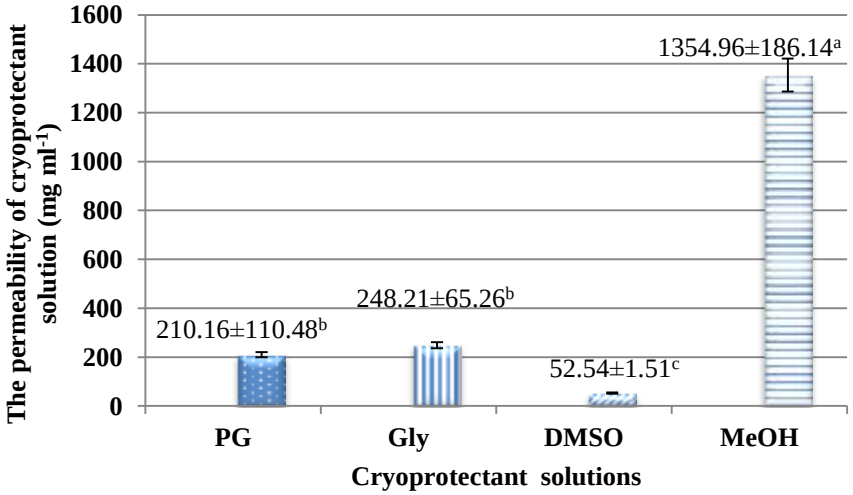

Figure 2. Mean of the permeability of cryoprotectant solution residue in Thai walking catfish embryo at somite development stage exposed with four cryoprotectant solutions at 40 minutes

Permeability assessment

High Performance Liquid Chromatography and Gas Chromatography techniques were used to analyse the internal concentration of cryoprotectant solution residue in embryo as illustrated in Figure 2. There were exactly different permeability patterns of the various cryoprotectant solutions moreover there was highly significant ($P<0.01$) difference between permeability of each cryoprotectant solution into Thai walking catfish embryo with $F = 78.73$. In the case of somites stage embryos 40%MeOH (1354.96±186 mg ml^{-1}) attained the highest relative uptake after exposure for 40 minutes followed by 40%GLY (248.21±65.26 mg ml^{-1}), 40%PG (210.16±110.48 mg ml^{-1}) while 40%DMSO (52.54±1.51 mg ml^{-1}) obtained the lowest relative uptake.

Cryopreservation of Thai walking catfish embryo

After exposure to 40% PG + 5%EY using the least toxic treatment with cooling rates of 0.2 ˚C min^{-1} until a final temperature of -50 ˚C was attained using Cryogenesis program version 5, the Thai walking catfish embryos were plunged into liquid nitrogen (LN_2). Unfortunately, the results in this study showed no recoveries of live embryos after thawing in all conditions examined. The morphological changes during thawing is shown in Table 2. With the temperature increasing, the ice thawed from the outside solution to the embryo and the egg yolk was the last to thaw.

Table 2. Mean viability of Thai walking catfish embryo at somites development stage exposed to 40% PG + EY 5% within 40 minutes

Viability of Thai walking catfish embryo after thawing	**Normal (%)**	**Broken (%)**	**Total (%)**
40% PG + EY 5% within 40 minutes	55.67 ± 2.39	44.33 ± 4.51	100

As to the morphological change observed, the diameter of the embryos were the same in size before and after plunging into LN_2. The color of catfish embryos before freezing in LN_2 was brighter than after freezing and thawing. After thawing, the cells were dead. The number of catfish embryos which maintained normal morphology was 55.67 ± 2.39 percent while the other, 44.33 ± 4.51 percent were broken after few minutes after thawing. The embryonic structure particularly the compartment barriers which consisted of chorion and vitelline membrane were damaged because of immediate melting. The most common damages observed in thawed embryos were changes in embryo compartments and yolk sac, yolk darkness, protuberance of the yolk, loss of

yolk mass and shrinkage of the yolk with increased perivitelline space, chorion rupture and tissue degradation.

Discussion

Cryoprotectant toxicity, their concentrations and embryonic development stage

Four permeable cryoprotectants (PG, GLY, DMSO and MeOH) were selected because are commonly used. Moreover, the non permeable ones (Egg Yolk) was selected in combination with them to protect the catfish embryo from outside solutions while permeable ones protect it from the inside solutions. The survival rate of the embryos treated with 40%PG was higher than those obtained from the other three CPAs at the same concentrations and exposure time. In this regard, PG was found to be the least toxic to Thai walking catfish embryo and this CPA is commonly used in the cryopreservation of embryos in many fish species. This result was similar with the results of Tian *et al.* (2003), Zhang *et al.* (2005), Chen and Tian (2005) and Rahman *et al.* (2008). While in other case, Zhang and Rawson (1996) and Pillai *et al.* (2001) demonstrated that MeOH and GLY were less toxic to the others fishes and shrimp embryos. DMSO, another common cryoprotectant, has been successfully used in the cryopreservation of gametes and embryos in a number of vertebrate and invertebrate species. The present study show that it was more toxic than PG and GLY. This is probably due to its molecular weights as the toxicity of cryoprotectant solution is related to its molecular weights (Subramoniam and Arun, 1999). However, the substantial differences reported between species emphasize the need to test cryoprotectant solution tolerance on a species to species basis. The type of cryoprotectant solution used varies widely among species and sometimes within one species a cryoprotectant solution is successfully used in one study and is found to be unsuited in another study with the same species (Hiemstra *et al.*, 2005). The different cryoprotectant solutions may differ widely in terms of cell membrane permeability and also may affect the membrane permeability to water. These parameters greatly affect the velocity of dehydration and these are important in the optimal range of cooling rates.

The concentration of PG combined with EY at a ratio of 40%: 5% was prepared at a higher concentration than those used in many previous studies. The results showed that survival of walking catfish embryo was lower than 50%. But among the cryoprotectant solutions evaluated in this study PG had the highest survival rate and this agrees with Wankanapol (2012) that higher

cryoprotectant solution concentration are more toxic to tilapia embryo and PG was the least toxic cryoprotectant solution than the other three. Moreover, Chen and Tian (2005), Vuthiphandchai *et al.* (2005), Zhang *et al.* (2005), Cabrita *et al*. (2006), Edashige *et al.* (2006) and Xiao *et al*. (2008) also found that toxicity of cryoprotectant solution increases with concentration and long exposure times. Under these conditions, cellular proteins can be denatured and pre-freezing viability was reduced. Effective toxicity depends on permeation rate of cryoprotectant solution, the concentration required for it to be effective, the temperatur, and the duration of exposure. A slowly permeating but less toxic cryoprotactant solution is therefore needed. Information on comparative toxic effects of the most commonly used cryoprotectant solutions on fish embryos is lacking.

The cryoprotectant solution concentration was the most significant factor that effect survival and was mainly related to the change of osmotic pressure and toxicity. In this study, the exposure time of 40 minutes to cryoprotectant solution was higher compared with many studies and induced higher mortality as stated by Dong *et al*. (2004) who found out that toxicity of cryoprotectant solution was also exposure time which was within the range 5 to 30 minutes. This was observed to be the optimal exposure period for single cell cryopreservation. It agrees with the studies in fishes by Bart (2000), Pillai *et al*. (2001), Dong *et al.* (2004), Xiao *et al.* (2008) and shrimp by Vuthiphandchai *et al*. (2005) that the longer exposure periods, within the range of 5 to 30 min, resulted in higher mortality. This fact was probably because all of these cryoprotectant solutions produced deleterious effects on the structure of the embryo, reducing hatching. This means that exposure time becomes an important factor with regards to cryoprotectant solution toxicity when the concentration increases. However, Arun and Subramoniam (1997) recommended that longer exposure period (>30 min) be used to obtain survival for larger biological entities such as shrimp embryos. Duration of exposure was the second most significant factor.

The somite embryonic development stages supported the studies of Chao and Liao (2001) and Hiemstra *et al*. (2005) who emphasized that the success of cryopreservation is dependent on the stage of embryo. Moreover, Urbanyi *et al*. (1997) reported that tolerance of fish embryos to CPA depends on the developmental stage, more mature embryos generally being more resistant than embryos at the earlier developmental stages. Furthermore, Cabrita *et al.* (2006) stated that toxicity resistance to CPA increases during embryonic development and species specific. Possibly the early embryo stages have higher lipid content and polar organization of the ova than the later embryo stages (Hiemstra *et al*., 2005). This ws also reported by Bart (2000) who found that

somites stage of zebrafish (*Brachydanio rerio*) was better to tolerate CPAs especially MeOH than epiboly stage. Additionally, Ahammad *et al.* (2003) also pointed out that selection of particular developmental stage depends on chill sensitivity. It has been reported in brown trout (*Salmo trutta*) (Maddock, 1974), rainbow trout (*Oncorhynchus mykiss*) (Haga, 1982), fathead minnows (*Pimephales promelas*) (Cloud *et al*., 1988), zebrafish (Zhang and Rawson, 1996), gold fish (*Carassius auratus*) (Liu *et al*., 1993), common carp (*Cyprinus carpio*) (Dinnyes *et al*., 1998) and ornamental carp (Magnus *et al*., 1995) that postgastrulation developmental stages are the least sensitive to chilling injury and early development stages are highly sensitive to chilling injury. Futhermore, Lahnsteiner (2008) also found that the early ontogenetic developmental stages of zebrafish (*Danio rerio*) were more sensitive to CPA exposure than the advanced stages. Chen and Tian (2005) also found out that tail bud stage of flounder (*Paralichtys olivaceus*) was the most resistant stage to the toxicity of all CPAs examined whereas pre hatching stage was more sensitive than tail bud, somites and gastrula stages. Higaki *et al*. (2009) reported that embryos of zebrafish at blastrula and gastrula stages had high sensitivities to CPA toxicity. Also, the study of Cabrita *et al.* (2006) found that developmental stage of gilthead seabream influenced the hatching rates significantly with tail bud free stage embryos less sensitive to inner CPA. Moreover, the embryonic stage was also a critical factor in the various biological systems because of the main physical difference of each embryo developmental stage where the difference in size, lipid content or amount of yolk and multiple compartment barrier including the thick chorion, become the major obstacles. The different Thai walking catfish embryonic developmental stages also have different sizes, varying/ large amount of yolk and complex structure of the embryonic body, e.g., yolk syncytial layer and vitelline/plasma membrane. These varying characteristics of the different stages of the embryo inhibited the uniform penetration of CPA. This may be the best reason why different stages had different tolerance to the CPA. The smaller size embryo with lower amount of yolk and reduced highly complex, multiple compartment barrier gave positive response in terms of tolerance to CPA. This finding supports the reason that the later developmental stages had higher tolerance limit to toxicity than the early stages. Although the early stages of Thai walking catfish embryos have lower survival rate than those in the later stages, they still have some advantages for preservation because their membranes are less complex with higher permeability to CPAs than later stage embryos (Zhang *et al*., 2005). Some studies focused on early stage development such as Kusuda *et al*. (2002) who studied chum salmon (*Oncorhynchus keta*) blastomere (morula and blastrula stage) and found that it can survive after thawing. Moreover,

Rahmann *et al.* (2008) also indicated that Japanese whiting embryos at pre-hatching stage were less tolerant to CPAs than those in the gastrula and somites stages. On the other hand, Babiak *et al.* (2008), using rainbow trout blastoderm, found that this stage can be successfully cryopreserved, particularly the smaller blastomeres. In the present study, it can be concluded that the latest stage of embryo is the most appropriate stage for cryopreservation than the early stages. Therefore, it is important to consider more specifically the following: embryo size, membrane permeability, chilling sensitivity, yolk size and complexity of the embryos in choosing a suitable embryo stage for cryopreservation studies. Futhermore, compared with the other fish species, Thai walking catfish eggs have a bigger size and being demersal eggs, they may have slightly different characteristics compared with the eggs of other fish species.

The permeability of each CPAs

The results of HPLC and GC analyses from this study were sufficient to quantify the exact amount of the CPAs inside a whole embryo but it did not provide information on the distribution of CPA in highly compartmentalized embryos (e.g. a large yolk, perivitelline space, the developing blastoderm and vitelline membrane). The results point to an intriguing correlation between relative permeability of the various CPAs and their toxicity. For instance, in this study, lowest embryo survival rate was associated with highest permeability of CPA (i.e. MeOH) whereas PG and GLY with moderate permeability were associated with the highest and second highest survival rates while DMSO with lowest permeability was associated with the second lowest survival rate. The CPAs permeation indicated that the permeability of a cryoprotectant is directly related to its molecular weight and density (Subramoniam and Arun, 1999 and Harvey and Ashwood-smith, 1980). This is shown in MeOH which was rated to be the best CPA in terms of permeability in the walking catfish embryo and DMSO, the lowest. Large size and structure of walking catfish embryos are known to lessen permeability hence the possibility for internal ice formation is high. This is similarly with Rahman *et al*. (2008) who also found low embryo survival associated with high permeability for MeOH. The degree of CPA permeation is inversely related to its molecular weight. The results of this study provide broad support to this assumption. Nevertheless, the dynamics of CPA permeation is also related to the presence of structural (membrane) barrier in the embryos, as can be inferred from the report of Hagedorn *et al.* (1997b) that MeOH was able to permeate the entire zebrafish embryo whereas DMSO did not. Thus, it might be worth investigating to what extent the differences in CPA toxicity reported among

fish species can be explained by the presence of specific structure barrier to particular CPA rather than toxicity itself. Ahammad *et al*. (2003) stated that due to the high molecular weight and dihydric nature of PG it is possible that PG could not penetrate the vitelline membrane of the fish embryo, but protected it from outside. Although the mechanism by which polymer protects fish embryos is not known, the size or configuration of the molecule may have an important role. It is possible that membrane lipid may form hydrogen bonds with polymers externally, resulting in protection of the embryo from the outside. Due to its relatively low molecular weight, MeOH on the other hand penetrated more easily. Furthermore, the effectiveness of MeOH was a function of temperature, implying that MeOH penetrated into the embryo much more rapidly at high temperatures. On the other hand, the differences in permeability between membranes and envelope probably provided different concentrations of various CPAs in each embryo compartment within the whole embryo (Cabrita *et al*., 2006), yolk sac (Magnus and Lubzens, 1995) or perivitelline space (Hagedorn *et al*., 1997c). Since measurement of various CPAs was from whole embryos, it is possible that barrier other than the chorion could interfere with various CPA uptake if CPA entry is located only in a small compartment, such as perivitelline space. There was also possibility that maximum permeability of the CPA was reached at that level.

The viability of Thai walking catfish embryos after plunge in liquid nitrogen

After thawing the embryos cryopreserved in LN_2, there was no survival in all of the embryonic developmental stages, an indication that Thai walking catfish embryos are one of the most vulnerable and still hard to preserve embryos under sub zero temperatures. It was observed that there were two terms of Thai walking catfish embryo which determines viability such as high amount of yolk and membrane disruption. Embryos are really hard to cryopreserve compared to sperm although the potentially detrimental effects of dehydration and ice had been minimized in the present study by high concentration of PG (40%), maintaining appropriate storage temperatures and controlling the rewarming rate. The catfish embryos were still influenced by chilling or cryoinjuries particularly pre freezing and post thawing. This is probably due to the fact that although PG in its highest concentration (40%) was the most suitable CPA for catfish embryos, however, the permeabililty of PG was still important to consider because it was found that small amount of PG inside the embryo may not be adequate for dehydration particularly in this species of fish. Moreover, the ice crystal formation was the main obstacle because the osmotic balance between water and PG did not reach equilibrium

time although the exposure time of 40 min in this study was designed to be the longest compared with many studies. This finding is consistent to that of Leung and Jaemison (1991) that cellular death is caused by dehydration, increased concentration of saline and physical destruction of cell themselves. The ice crystal formation (Brockbank *et al.,* 2003) results in an osmotic imbalance possibly by ice formation which occured while freezing because the water in the embryo cell may not have been replaced by PG. Another obstacle was the size of the embryos, including the amount of yolk and compartment barrier, because they are big in size (1 mm in this study) and large amount of yolk compared with the other species used in many researches. This condition may interfere in the penetration of PG into the whole embryo cell that is related to the ice formation phenomenon during slow freezing (Hagedorn *et al*., 1997b). There is insufficient information on the kinetics of CPA permeation into membrane bound compartment. The chorion and inner membrane might be preventing water and solute movement in and out of the embryo (Robles *et al*., 2003). However, the cold shock also might damage the embryo (Lillicrap, 2010) Glycoproteins, the outer layer of the chorion, are possibly responsible for the prevention of the transfer of aqueous material into and out of the embryo. However, these are contrary to the finding of Zhang and Rawson (1995) that chorion and perivitelline fluid are controlling the osmotic influx of chemicals in fish embryos. The osmoregulation in fish is related to the movement of water content, that is, it keeps the fish's fluids from becoming too diluted or too concentrated for maintenance of an optimal, constant osmotic pressure in their body by osmosis. In the present study, after exposing Thai walking catfish embryo to PG, the higher osmotic pressure at 40% PG solution, the more water tends to move out of the embryo but perhaps 40%PG was not adequate to replace all of the water inside a whole embryo. Actually, osmoregulation in embryo is different compared with the adult fish because in the adult fish osmoregulation is performed by the skin, gill and kidneys (Romer and Parsons, 1977). In contrast, the embryo does not have these organs, however it might be regulated by chorion and their compartment barriers. Furthermore, because of the difference in osmotic pressure inside and outside the embryo, the water continues to move out of the cells as long as the imbalance in salt concentration remains (Chao and Liao, 2001). The cells dry up as freezing proceeds affecting viability of the cells.

The 40%PG combined with 5%EY was found to have the lowest toxicity to Thai walking catfish embryo at somite development stage followed by GLY, DMSO and MeOH at the same concentration and exposure time. Unfortunately, their embryo would not survive after freezing in LN_2. It might be effected by cryoinjuries particularly cryoprotectant toxicity, ice crystal formation and cold

shock. Thai walking catfish embryo is hard to preserve at sub-zero temperatures.

Acknowledgements

This work was supported by Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai, Thailand.

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(Received: 23 September 2019, accepted: 20 June 2020)