Synergistic Effect of Permeable and Impermeable Cryoprotectants on the Survival and Hatching Rate of Sutchi Catfish (*Pangasius hypophthalmus*) Embryos Treated at Subzero Temperatures

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ABSTRACT- ***The sutchi catfish (Pangasius hypophthalmus) has been widely introduced for aquaculture industry in Asiatic countries. The cryopreservation of catfish embryos is important to facilitate stock enhancement programs. The*** **present study** ***was designed to determine the effect of combination of permeable and impermeable cryoprotectants on survival and hatching of catfish embryos aiming to understand the basic conditions for their conservation by vitrification. Sutchi catfish embryos at somites and optic cups were treated at room temperature, 4 ^{o}C, 0 ^{o}C and -20 ^{o}C, with impermeable sucrose as a factor of dehydration and membrane stability, separately or in different combination with glycerol, methanol (MeOH), ethylene glycol (EG), and propanediol (PG). The obtained results show that the catfish embryos are sensitive to low temperature and the hatching rate was significantly decreased for embryos treated at temperature below 0 ^{o}C. Catfish embryos are able to perform a partial dehydration in 0.5 M, 1.0 M and 2.0 M sucrose, respectively. Dehydration with 2.0 M sucrose has high protective effect for embryos treated at 0 ^{o}C. The combination of dehydration and MeOH, PG and EG at high concentration of more than 5.0 M can be used safely to keep embryos at 0 ^{o}C and -20 ^{o}C for short duration. In conclusion, present information related to cryobehavior and cryoprotectants was established for further study of vitrification in catfish embryos.***

Keywords- Catfish, Cryoprotectants, Embryos, Hatching rate

1. INTRODUCTION

Cryopreservation of gametes and embryos has been considered as an important tool for biodiversity conservation, contributing to develop fish industry. Although to date successful spermatozoa freezing has been reported for many fish species, no technique is available for fish embryo cryopreservation.

Great volume of yolk syncytial layer (YSL), low membrane permeability and fast development characterizing for fish embryos are the main obstacles to perform water dehydration and cryoprotectants penetration - two critical steps required for successful freezing. According to Hagedorn et al. (2004), slow freezing technique is not a suitable option for fish embryos since high ice nucleation temperature cannot be avoided. Actually, most studies on fish embryo freezing have been concentrated on vitrification using different approaches (Robles et al. 2003, Cabrita et al. 2006, Chen et al. 2005). Janik et al. (2000) and Robles et al. (2004) reported an approach to overcoming the low membrane permeability of fish embryo using electroporation or sonication. In addition, the use of microinjection as an effective way for the introduction

of cryoprotectants into embryos or for partial removal of yolk has also been reported (Hagedorn et al., 1997; Liu et al., 1999; Janik et al., 2000; Liu et al., 2001). The specific techniques such as incorporation of antifreeze proteins into the embryos (Robles et al. 2003, 2007), incorporation of aquaporin-3 into the plasma membrane (Hagedorn et al. 2002) and increasing of embryo permeability by dechorionation (Hagedorn et al. 1997) were tested, but failed to produce viable frozen thawed embryos (Lahnsteiner, 2008).

Management of dehydration and cryoprotectant penetration, control of cryoprotective toxicity are important requirements for embryo preservation by vitrification. Unequal or incomplete penetration may have lethal effects, but the presence of a high amount of cryoprotectant is also critical to embryo survival rate. Therefore, it is essential to develop techniques that allow using maximum concentration of cryoprotectants, but safe on embryo survival. Previous studies with mammalian models showed that the use of sucrose as impermeable cryoprotectant for partial dehydration can be successful methods for embryo conservation by rapid or ultra rapid freezing (Renard et al, 1984; Nguyen et al, 2000). Sucrose can be used as hypertonic impermeable cryoprotectant which improves the embryo survival rate at low temperature due to its both effects of removing of intracellular water and protecting membrane integrity. Recently, it has been reported that the combination of treatment of sutchi catfish embryos (*Pangasius hypophthalmus*) in medium containing 2.0 M sucrose and low temperature of 0^oC resulted in an integrated positive effect on the survival of embryos (Nguyen et al, 2012).

In this paper, we report the results of investigation on the synergistic effect of permeable and impermeable cryoprotectants and low temperature on the survival of sutchi catfish embryos. We provide baseline information regarding the tolerance of Sutchi catfish embryos at low temperature condition and the finding of the optimal combination of cryoprotectants available for further investigation on their cryopreservation by vitrification.

2. MATERIALS AND METHODES

2.1 Embryos

Sutchi catfish embryos were obtained after insemination from the fish farm and transported to laboratory for embryo incubation. Incubation was performed in a 30 L tank with recalculating fresh water system and air supply at 28 ± 1°C. Embryos were incubated under these conditions until somites stage which corresponds to approximately 18h after fertilization (Fig.1a). Selected embryos were then kept in Petri-dishes (50-100 embryos) in 10 ml fresh water at the same temperature for the next treatment.

2.2. Cryoprotectants

Four differents cryoprotectants: Glycerol (Gly), methanol (MeOH), ethylene glycol (EG), propanol (PG) and sucrose (Suc) were purchased from Prolabo- France. Solution of single cryoprotectant including sucrose 0.5 M, 1.0 M and 2.0 M; 1.0 M and 3.0 M for other permeable cryoprotectanst in water were prepared. in water. Mixture of sucrose 0.75 M with permeable cryoprotectant including EG 4.5 M – MeOH 0.7 M (VS1), EG 6.5 M (VS2) and PG 3.5 M - MeOH 2.0 M (VS3) were used as vitrification solutions without ice formation at low temperature.

2.3 Effect of Low Temperature

Embryos were exposed to filtered water and were kept at room temperature (28±1^{o}C), 4^{o}C, 0^{o}C and -20^{o}C, respectively for 60 min. After required exposure, embryos were thawed at room temperature, washed by three-step dilution with filtered water and transferred to culture in tanks with a recirculating water system with oxygen supply and controlled temperature (28 ± 1^{o}C) for further evaluation of the hatching.

2.4 Dehydration

Embryos were exposed to sucrose solution of different concentration of 0.5 M, 1.0 M and 2.0 M and kept at room temperature (28±1^{o}C) for investigation effect of dehydration on their survival and hatching capacity. To study the protective effect of dehydration at low temperature, embryos were exposed to sucrose solution and kept at 4^{o}C, 0^{o}C and -20^{o}C, respectively for 60 min. After required exposure, embryos were thawed at room temperature, washed by three-step dilution with filtered water and transferred to culture in tanks with a recirculating water system with oxygen supply and controlled temperature (28± 1^{o}C) for further evaluation of the hatching.

2.5 Toxicity of Cryoprotectants

Embryos were exposed to solution containing single or mixture of cryoprotectants and kept at room temperature for different duration of times (20, 60 or 120 minutes). Embryos were then washed by three-step dilution with filtered water and transferred to culture in tanks with a recirculating water system with oxygen supply and controlled temperature (28 ± 1^{o}C) for further evaluation of the hatching.

2.6 Protective Efficiency of Combination of Permeable and Impermeable Cryoprotectants

Embryos were exposed to concentration of mixture of sucrose and cryoprotectants at different concentration and were kept at 4°C, 0°C and -20°C, respectively for different duration of times. After required exposure, embryos were thawed at room temperature, washed by three-step dilution with filtered water and transferred to culture in tanks with a recirculating water system with oxygen supply and controlled temperature (28±1°C) for further evaluation of the hatching.

2.7 Incubation and Viability Assessment

After treatment, embryos were incubated in a recirculating water system with oxygen supply and controlled temperature (28±1°C) until 1-day larvae. Embryos were evaluated as viable when they revealed a normal development without signs of malformations, spontaneous movement, and a functional heartbeat and blood circulation. Hatching catfishes were assessed as survival when they had no signs of malformations and could move. Evaluations were made in a dissecting microscope at 4 and 10-fold magnification. The hatching percentage was counted as the number of hatching catfish in relation to the total number of embryos.

2.8 Statistical Analysis

The data were transformed as percentage with respect to control and are expressed as means ± SEM. Data were treated and analyzed using Microsoft Excel and one-way ANOVA software. Significant differences between treatments in the different experiments were detected using SNK (Student test) statistical test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Effect of Low Temperature on Survival and Hatching Rate

Our results of catfish embryos treated at room temperature (RT), 4°C, 0 °C and -20 °C for 60 minutes show that the embryos were well tolerated at 4°C; there was no significant different in hatching rate in comparison with embryos keeping at room temperature as control group (Table 1). Therefore, embryos were sensitive to 0°C and -20°C; the hatching rate was importantly decreased after treatment at 0°C (13,33 ± 1,93) and no embryos hatch after treatment at -20°C(Fig.1c).

Table 1: Effect of low temperature on the hatching rate of catfish embryos

Temp. (°C)	No. of embryos	No. of survival embryos	Survival rate (%)	No. of embryos hatched	Haching rate (%)
RT 28°	90	90	100.00 ± 0.00^{a}	88	97.78 ± 1.11^{a}
4	90	90	100.00 ± 0.00^{a}	85	94.11 ± 1.11^{a}
0	90	16	17.78 ± 1.11^{b}	12	13.33 ± 1.93^{b}
- 20	90	2	2.22 ± 1.11^{c}	0	0.00 ± 0.00^{c}

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column ($P < 0.05$).

3.2 Effect of impermeable cryoprotetants on Survival and Hatching Rate of Embryos Kept at Different Low Temperature

The added concentrations of sucrose to culture medium were used to study the possibility to increase the incubation time at low temperature to 2 h. The general trend of decreasing of the survival and hatching rate was observed when embryos were treated with low temperature in the presence of sucrose at concentration 0.5 M - 1.0 M (Table 2). Hatching rates were slightly decreased for embryos treated at 4°C (80% - 87% vs. 97% for control group); any embryos hatched after treatment at 0°C and -20°C. It is interesting to note that the increasing of sucrose concentration to 2.0 M resulted in the decreasing of the hatching rate for embryos treated at room temperature, but reversely increasing significantly the survival and hatching rate to more than 90% for embryos kept at 0°C(Fig.1d).

Table 2: Effect of sucrose on the survival and hatching rates of embryos treated with low temperature

Temp. (°C)	Sucrose concentration (M)	No. of embryos	No. of survival embryos	Survival rate (%)	No. of embryos hatched	Haching rate (%)
28	0.5	90	87	96.67 ± 1.93[a]	81	90.00 ± 0.00[a]
	1.0	90	87	96.67 ± 1.93[a]	85	94.44 ± 2.22[a]
	2	73	68	93.75 ± 6.25[a]	0	0.0 ± 0.00[b]
0	0.5	95	27	29.2 ± 22.4[b]	0	0.00 ± 0.00[b]
	1.0	92	44	46.8 ± 24.2[b]	0	0.00 ± 0.00[b]
	2.0	60	60	100.00 ± 0.00[a]	54	90.00 ± 0.00[a]
-20	0.5	90	0	0.00 ± 0.00[b]	0	0.00 ± 0.00[b]
	1.0	90	0	0.00 ± 0.00[b]	0	0.00 ± 0.00[b]
	2.0	90	0	0.00 ± 0.00[b]	0	0.00 ± 0.00[b]

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column (P < 0.05).

3.3 Effect of Treatment with Permeable Cryoprotectant on Survival and Hatching Rate of Embryos Kept at Different Low Temperature

For embryos treated at room temperature and at4^{o}C in the presence of glycerol, PG, MEOH and EG at concentration of 1.0 M, survival and hatching rates were not different compared to control groups (Table 3). Therefore, protective effect of cryoprotectant for embryos treated at 0^{o}C was observed only with PROH and MeOH. High hatching rate of more than 80% was kept in case of embryos treated in the presence of PG. However this protective effect was not observed when temperature was decreased to -20^{o}C.

Table 3: Effect of cryoprotectants on the survival and hatching rates of embryos treated with low temperature

Temp. (°C)	CPA	No. of embryos	No. of survival embryos	Survival rate (%)	No. of embryos hatched	Haching rate (%)
RT	Con.	90	90	100.00 ± 0.00[a]	88	97.78 ± 1.11[a]
	PG	90	90	100.00 ± 0.00[a]	84	93.33 ± 1.93[a]
	MeOH	90	90	100.00 ± 0.0[a]	86	95.56 ± 1.11[a]
	EG	90	90	100.00 ± 0.00[a]	84	93.33 ± 3.85[a]
	Gly	90	89	98.89 ± 1.11[a]	86	95.56 ± 1.11[a]
4	Con.	90	90	100.00 ± 0.00[a]	85	94.11 ± 1.11[ab]
	PG	90	89	98.89 ± 1.11[a]	87	96.67 ± 0.00[a]
	MeOH	90	90	100.00± 0.00[a]	78	86.67 ± 5.09[ab]
	EG	90	90	100.00 ± 0.00[a]	74	82.22 ± 4.01[b]
	Gly	90	90	100.00 ± 0.00[a]	84	93.33 ± 3.85[ab]
0	Con.	90	16	17.78 ± 1.11[c]	12	13.33 ± 1.93[c]
	PG	90	76	84.44 ± 7.78[b]	73	81.11 ± 7.78[b]
	MeOH	90	31	34.40 ± 19.50[c]	18	20.00 ±15.00[c]
	EG	90	0	0.00 ± 0.00[c]	0	0.00 ± 0.00[c]
	Gly	90	8	8.89 ± 4.84[c]	0	0.00 ± 0.00[c]
-20	Con.	90	2	2.22 ± 1.11[c]	0	0.00 ± 0.00[c]
	PG	90	0	0.00 ± 0.00[c]	0	0.00 ± 0.00[c]
	Met	90	26	28.9 ± 14.6[c]	0	0.00 ± 0.00[c]
	EG	90	5	5.56 ± 2.94[c]	0	0.00 ± 0.00[c]
	Gly	90	12	13.33 ± 6.94[c]	0	0.00 ± 0.00[c]

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column (P < 0.05).

When compare the maximum hatching rates, although there was no significant difference, it seems that the protective efficiency was in the order of PG > glycerol > MeOH > EG for embryos treated at 4 ^{o}C; PG > MeOH > EG, glycerol for embryos treated at 0^{o}C.

Table 4 presents the results of an attempt to treat embryos with cryoprotectants at concentration of 3.0 M for short duration of 20 minutes. For the embryos treated with PG and MeOH and at room temperature, the survival and hatching rate was not affected by increasing cryoprotectant concentration to 3.0 M. Although the important effect of cryoprotectant toxicity was observed in the case of embryos treated with glycerol and EG; any embryos from these groups were able to hatch. The results of treatment at 0^{o}C show also that EG and glycerol at concentration of 3.0 M are not capable to protect embryos (Fig.1b).

Table 4: Effect of treatment with cryoprotectants at 3.0 M concentration on survival and hatching rate

Temp. (°C)	CPA (3 M)	No. of embryos	No. of survival embryos	Survival rate (%)
RT	Con.	90	88	97.78 ± 1.11^{a}
	PG	84	78	92.44 ± 1.17^{abc}
	MeOH	94	91	96.32 ± 0.87^{ab}
	EG	78	0	0.00 ± 0.00^{d}
	Gly	79	0	0.00 ± 0.00^{d}
0	Con.	91	83	91.22 ± 1.06^{abc}
	PG	132	102	78.63 ± 2.63^{c}
	MeOH	66	52	77.89 ± 14.52^{c}
	EG	99	0	0.00 ± 0.00^{d}
	Gly	107	0	0.00 ± 0.00^{d}
-20	Con.	61	54	87.62 ± 2.62^{c}
	PG	61	53	86.89 ± 0.22^{cd}
	MeOH	64	57	88.93 ± 2.26^{bc}
	EG	99	0	0.00 ± 0.00^{d}
	Gly	107	0	0.00 ± 0.00^{d}

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column (P < 0.05).

3.4 Effect of Combination of Permeable and Impermeable Cryoprotectants on Hatching Rate of Embryos Kept at Room Temperature

The combined treatment of dehydration using 0.5 M - 1.0 M sucrose and cryoprotectants at concentration 1.0 M for 60 min do not effect on the survival and hatching rates of embryos treated at room temperature. Higher hatching rates were observed for all combinations of sucrose with glycerol, EG, PG and MeOH (Table 5).

Table 5: Effect of combination of dehydration and cryoprotectants on the survival and hatching rates of embryos kept at room temperature

CPA	SUC (M)	No. of embryos	No. of survival embryos	Survival rate (%)	No. of embryos hatched	Haching rate (%)
Con.		90	90	100.00 ± 0.00	89	98.89 ± 1.11^{a}
PG	0.5	90	89	98.89 ± 1.11	87	96.67 ± 0.00^{ab}
	1	90	90	100.00 ± 0.00	78	86.67 ± 1.93^{c}
MeOH	0.5	90	90	100.00 ± 0.00	87	96.67 ± 1.93^{ab}
	1	90	90	100.00 ± 0.00	87	96.67 ± 1.93^{ab}
EG	0.5	90	90	100.00 ± 0.00	87	96.67 ± 1.93^{ab}
	1	90	89	98.89 ± 1.11	82	91.11 ± 1.11^{bc}
Gly	1	90	90	100.00 ± 0.00	87	96.67 ± 1.93^{ab}
	1	90	90	100.00 ± 0.00	89	98.89 ± 1.11^{a}

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column (P < 0.05).

3.5 Effect of Combination of Permeable and Impermeable Cryoprotectants on Hatching Rate of Embryos Kept at 4 °C

As in the case of treatment at room temperature, the combined treatment of dehydration using 0.5 M - 1.0 M sucrose and cryoprotectants at concentration 1.0 M for 60 min don not effect on the survival and hatching rates of embryos treated at 4 °C. High hatching rates were observed for all combination with glycerol, EG, PG and MeOH (Table 5).

When compare the maximum hatching rates, the protective efficiency was in the order of PG > glycerol > MeOH > EG.

Table 6: Effect of combination of dehydration and cryoprotectants on the survival and hatching rates of embryos kept at 4 °C

CPA	SUC (M)	No. of embryos	No. of survival embryos	Survival rate (%)	No. of embryos hatched	Haching rate (%)
PG	0.5	90	90	100.00 ± 0.00	81	90.00 ± 1.92^{ab}
	1	90	90	100.00 ± 0.00	85	94.44 ± 1.11^{ab}
MeOH	0.5	90	90	100.00 ± 0.00	81	90.00 ± 1.92^{ab}
	1	90	90	100.00 ± 0.00	77	85.56 ± 5.88^{ab}
EG	0.5	90	90	100.00 ± 0.00	82	91.11 ± 1.11^{ab}
	1	90	84	93.33 ± 3.85	72	80.00 ± 0.00^{b}
Gly	0.5	90	87	96.67 ± 1.92	83	92.22 ± 1.11^{ab}
	1	90	85	94.44 ± 4.01	76	84.44 ± 1.11^{ab}

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column ($P < 0.05$).

3.6 Effect of Combination of Permeable and Impermeable Cryoprotectants on Hatching Rate of Embryos Kept at 0 °C

Table 7: Effect of combination of dehydration and cryoprotectants on hatching rate of embryos kept at 0°C

CPA	SUC (M)	No. of embryos	No. of survival embryos	Survival rate (%)	No. of embryos hatched	Haching rate (%)
Con.		90	16	17.78 ± 1.11	14	15.56 ± 1.11^{b}
PG	0.5	90	58	64.40 ± 32.30	50	55.6 ± 27.80^{ab}
	1	90	47	52.22 ± 4.01	21	23.30 ± 11.70^{b}
MeOH	0.5	90	58	64.40 ± 32.30	50	55.6 ± 27.80^{ab}
	1	90	72	80.00 ± 18.40	61	67.80 ± 9.00^{ab}
EG	0.5	90	87	96.67 ± 0.00	79	87.78 ± 2.94^{a}
	1	90	21	23.33 ± 1.92	9	10.00 ± 1.92^{b}
Gly	0.5	90	40	44.44 ± 2.94	11	12.22 ± 2.94^{b}
	1	90	40	44.44 ± 1.11	11	12.22 ± 1.11^{b}

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column ($P < 0.05$).

The protective effect of combined treatment of dehydration and cryoprotectants on survival and hatching rates was demonstrated more clearly for groups of embryos kept at 0°C. In comparison with the embryos kept at 0°C in water only

or in the presence of single cryoprotectant, the hatching rates of embryos treated with sucrose were increased and higher, particularly in the case of the combinations with sucrose 1 M (Table 7). The protective efficiency was changed to new order of MeOH > PG > EG > glycerol. Highest hatching rate of more than 87% was obtained in case of embryos treated with combination MeOH-Sucrose.

3.7 Effect of Combination of Permeable and Impermeable Cryoprotectants on Hatching Rate of Embryos Kept at -20 ^{o}C

The protective effect of combined treatment of dehydration and cryoprotectants on survival and hatching rates for groups of embryos kept at -20 ^{o}C was presented in table 8. Although the low survival rate was observed after thawing, no embryo was hatched in this group.

Table 8: Effect of combination of dehydration and cryoprotectants on hatching rate of embryos kept at -20 ^{o}C

CPA	SUC (M)	No. of embryos	No. of survival embryos	Survival rate (%)	No. of embryos hatched	Haching rate (%)
Con.		90	2	1.11 ± 1.11	0.	0.00 ± 0.00
PG	0.5	90	9	10.00 ± 5.09	0.	0.00 ± 0.00
	1	90	7	7.78 ± 4.01	0.	0.00 ± 0.00
MeOH	0.5	90	18	20.00 ± 10.20	0.	0.00 ± 0.00
	1	90	18	20.00 ± 10.20	0.	0.00 ± 0.00
EG	0.5	90	3	3.33 ± 1.93	0.	0.00 ± 0.00
	1	90	9	10.00 ± 5.09	0.	0.00 ± 0.00
Gly	0.5	90	5	5.56 ± 2.94	0.	0.00 ± 0.00
	1	90	2	2.22 ± 1.11	0.	0.00 ± 0.00

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column ($P < 0.05$).

The results obtained with the combined treatment of cryoprotectants and sucrose at concentration of 1.0 M show that the tested combinations are not able to protect embryos treated at temperature below 0^{o}C.

3.8 Effect of Treatment with High Concentration of Cryoprotectants on Survival and Hatching Rate

For the embryo kept at temperature below 0^{o}C, the loss of survival was demonstrated with lysed membrane and the modification of intracellular structure. The formation of ice crystal during keeping at low temperature may be one of most reasons for these damages. In order to avoid the effect of ice crystallization, embryos were treated with combinations of cryoprotectants with higher concentration. Table 9 presents the results of treatment at room temperature, 0^{o}C and -20^{o}C in the presence of mediums containing EG – MeOH (VS1), PG – MeOH (VS2) at concentration more than 5.0 M and EG more than 6.5 M (VS3), respectively. All of these mediums were able to keep at 0^{o}C and -20^{o}C without ice formation.

The obtained results show that the VS1, VS2 and VS3 combinations are safe for embryos treated at room temperature for short duration of times. No significant difference was observed in hatching rate between treated and control groups (87% to 92% vs. 97%, respectively). High protective effect was observed for the embryos treated at 0^{o}C and -20^{o}C (Fig.1e;1f). In addition, the survival and hatching rates were higher and significantly increased in comparison with control groups of the same temperature (79,1 % for the VS1 vs. 13,3% for the case of 0^{o}C; 92% for VS1, 98% for VS2 vs. 0%, in case of -20^{o}C).

In general, our results of treatment in catfish embryos at different low temperature conditions indicated that catfish embryos were sensitive to temperature below 0^{o}C. The survival and hatching rates show common trend of decreasing at low temperature which is in line with previous studies reported for other fish species embryos (Zhang et al. 2004, Dinney et al. 1996; Isayeva et al. 2004).

The protective effect of permeable cryoprotectant as MeOH, PG, glycerol and EG for embryo treated at temperature below 0^{o}C was limited and different depending on the type of cryoprotectant. The protective effect of sucrose at concentration of 2.0 M for embryo treated at 0^{o}C was high, while this effect was not observed for the concentration of 0.5 M and 1.0 M (Fig. 2). Since the sucrose is impermeable to embryo, this effect may due to the two important actions of sucrose: (i) the mobilization of intracellular water for partial dehydration; and (ii) the action on membrane stability. The difference in protective efficiency between 0.5 M - 1.0 M and 2.0 M sucrose show that partial dehydration is concentration dependent and that the level of partial dehydration is more important factor for embryo survival at low temperature.

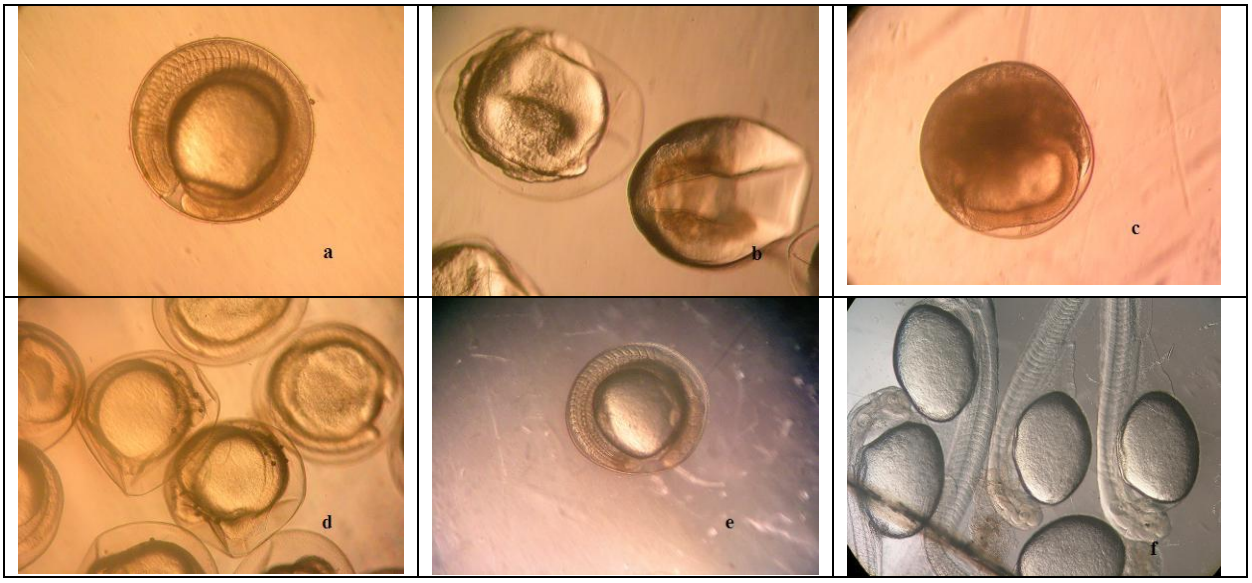

Figure 1: Effect of Low Temperature and Combination of Dehydration and Cryoprotectants on Survival and Hatching of Catfish Embryo (a) embryo at somites stage; (b) damaged embryo after treatment with cryoprotectant at high concentration; (c) damaged embryo after keeping at -20 ^{o}C; (d) embryo treated of dehydration in 2 M sucrose; (e) embryo after keeping in VS1 at -20 ^{o}C; and (f) hatched and developed embryo after keeping in VS1 at -20 ^{o}C.

Table 9: Effect of treatment vitrification medium on survival and hatching rate of catfish embryos

Temp. (°C)	Group	No. of embryo	No. of intact	Survival rate (%)	No. of hatched embryos	Hatching rate (%)
RT	Control	90	100	100.00 ± 0.00^{a}	88	97.78 ± 1.11^{a}
	VS1	34	34	100.00 ± 0.00^{a}	34	100.00 ± 0.00^{a}
0	Control	90	16	17.78 ± 1.11^{bc}	12	13.33 ± 1.93^{b}
	VS1	38	38	100.00 ± 0.00^{a}	38	100.00 ± 0.00^{a}
-20	Control	90	2	2.22 ± 1.11^{d}	0	0.00 ± 0.00^{b}
	VS1	50	47	95.00 ± 2.89^{a}	46	93.33 ± 4.41^{a}
RT	Control	90	90	100.00 ± 0.00^{a}	88	97.78 ± 1.11^{a}
	VS2	65	63	95.56 ± 4.44^{a}	60	91.67 ± 2.55^{a}
-20	Control	107	105	98.14 ± 1.19^{a}	96	89.02 ± 1.79^{b}
	VS2	80	79	96.67 ± 3.33^{a}	79	96.67 ± 3.33^{a}
RT	Control	90	90	100.00 ± 0.00^{a}	88	97.78 ± 1.11^{a}
	VS3	35	35	100.00 ± 0.00^{a}	35	100.00 ± 0.00^{a}

Data are presented as means ± SEM from three replicated experiments.
Values with different superscripts are significantly different in each column ($P < 0.05$).

Sucrose can protect embryo from cell membrane damage which can occurs during chilling as results of changes in their properties and integrity (Ghetler et al. 2005; Pang and Ge, 2002). The cell membrane shrinkage at temperature below freezing point is relative to the intracellular and extracellular ice formation and resulted in the destabilization of hydrophobic bounds and denaturation of proteins (Dinney et al. 1996).

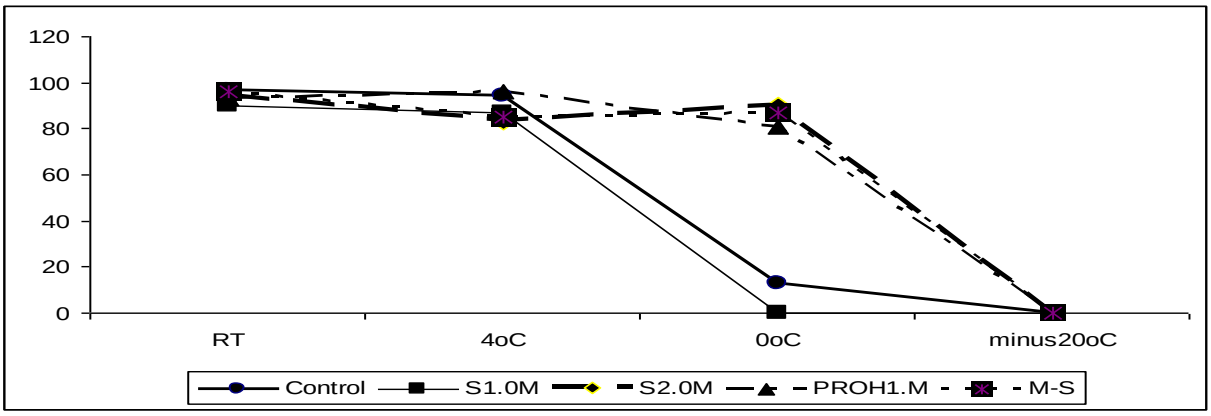

Figure 2: Cryobehavior and Effect of Dehydration and PG on Hatching Rate of Catfish Embryos at Low Temperature

Our study indicated the effect of positive integration of two types: permeable and impermeable cryoprotectants on the survival of catfish embryo. The hatching rates of embryo in groups treated with combination of dehydration and cryoprotectants were increased significantly in comparison with embryos treated with cryoprotectants alone (Fig. 3). The hatching rate for embryos treated with MeOH 1.0 M - sucrose 1.0 M was more than 87%, significantly higher than 20% in case of treatment with MeOH 1.0 M alone; or hatching rate of 24% for embryos treated with EG 1.0 M - Sucrose 1.0 M was significantly higher than embryos treated with sucrose 1.0 M alone (no hatching embryos).

In addition, our results shown that this effect of positive integration between dehydration and cryoprotectants was demonstrated more clearly in case of treatment of embryos at -20^{o}C where the potential of ice crystallization is higher. The observation of dynamic of embryo shrinkage during dehydration demonstrated that the transport across membrane may be only one direction from inside to outside and the penetration of permeable cryoprotectants was not happened. It is allowing to suggest that in the case of treatment of embryos at -20^{o}C in solution with higher concentration of protect embryos from damage. In this case, the formation of intra-embryonic ice can not be avoided. The duration of which embryos can tolerate low temperature without intracellular ice formation depends on the levelof partial dehydration, or in the function of concentration of sucrose in the combined solution.

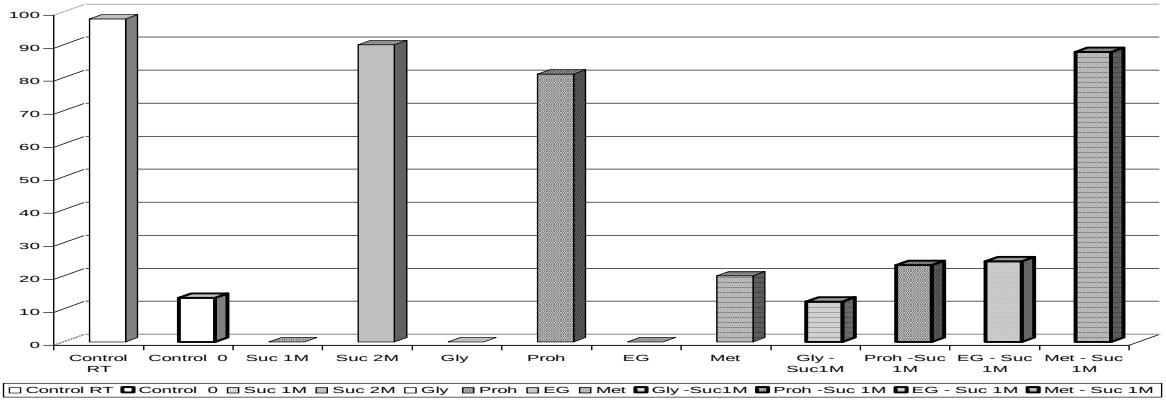

Figure 3: Effect of Single Cryoprotectant and The Combination with Sucrose on Hatching Rate of Catfish Embryos

In our study, the cryoprotectant toxicity and protective efficiency was changed after treated temperature as well as the type of combination of dehydration and cryoprotectants. For the combination with concentration in the limit of 1.0 M, they were in the order of PG > glycerol > MeOH > EG for embryos treated at 4^{o}C; PG > MeOH > EG, glycerol for embryos treated at 0^{o}C. In case of treatment with 3.0 M concentration, the order PG > MeOH > EG, glycerol was kept for all groups treated at room temperature, -0^{o}C and -20^{o}C.

Previous studies showed that the toxicity of our studied cryoprotectants is also species and stage dependent sensitivity. The change in toxicity order from methanol < Me2SO < glycerol in morula and half-epiboly stages to methanol < glycerol < Me2SO in the heartbeat stage was reported for teleost embryos exposure to cryoprotectants for 1h (Dinnyes et al, 1998). In case of flounder embryos, the toxicity was increased in the order of PG < MeOH < glycerol < EG and in consequence, the protective efficiency of each cryoprotectant, at -15^{o}C for a period of 60 min are in the sequence: PG > Me2SO approximately MeOH approximately glycerol > EG; and the best protection was in case of combination of methanol with any one of the other cryoprotectants (Zhang et al, 2005). Experiment of exposure embryos of Japanese whiting Sillago japonica for 20 min revealed that cryoprotectant toxicity was increased in the order of PG <

Me2SO < EG < MeOH < Gly (Rahman et al, 2008), while the toxicity for medaka (Oryzias latipes) embryos is in the order of PG < MeOH = DMSO < Gly < EG (Zhang et al, 2012).

In conclusion, the cryobehavior and cryoprotectant cryopreservation potential are varied depending on the species and stage of embryo development. Our results show that catfish embryos at somites stage are sensitive to low temperature and the hatching rate was decrease significantly for embryos treated at temperature below 0 ^{o}C. Partial dehydration with 2.0 M sucrose had a high protective effect for embryos treated at 0 ^{o}C. The combination of sucrose and MeOH, PG or EG at high concentration of more than 5.0 M can be use for safely keeping embryos at 0 ^{o}C and -20 ^{o}C for short duration. Further study is being made, aiming to evaluate the potentials of these findings for the development of the catfish embryo preservation by vitrification.

4. ACKNOWLEDGEMENTS

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