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Theriogenology 70 (2008) 1086–1092
Theriogenology
www.theriojournal.com

Effect of cryoprotectants on hatching rate of red seabream (*Pagrus major*) embryos

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Received 26 January 2008; received in revised form 29 April 2008; accepted 6 June 2008

Abstract

The objectives were to investigate the effect of cryoprotectants on the hatching rate of red seabream embryos. Heart-beat embryos were immersed in: five permeable cryoprotectants, dimethyl sulfoxide (DMSO), glycerol (Gly), methanol (MeOH), 1,2-propylene glycol (PG), and ethylene glycol (EG), in concentrations of 5–30% for 10, 30, or 60 min; and two non-permeable cryoprotectants: polyvinylpyrrolidone (PVP), and sucrose (in concentrations of 5–20% for 10 or 30 min). The embryos were then washed and incubated in filtered seawater until hatching occurred. 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. At a concentration of 15% and 30 min exposure, the hatching rate of the embryos immersed in PG was 93.3 ± 7.0% (mean ± S.D.), however, in DMSO, EG, Gly, and MeOH, it was 82.7 ± 10.4, 22.0 ± 5.7, 0.0 ± 0.0, and 0.0 ± 0.0%, respectively. Hatching rate of embryos treated with PVP decreased ($P < 0.05$) with the increase of concentration and exposure time, whereas for embryos treated with sucrose, there was no significant decrease in comparison with the control at the concentrations used.

Keywords: Red seabream; Embryo; Cryoprotectant; Toxicity; Permeable and non-permeable

1. Introduction

Although huge efforts have been made in embryo cryopreservation for over 50 years [1–5], successful fish embryo cryopreservation has not been achieved. Fish embryos are difficult to cryopreserve, due to their complex multi-compartmental system, high water content of water, sensitivity to chilling, large amount of egg yolk, and low membrane permeability [6,7]. It is well known that the cells are subjected to a series of cryoinjuries, including pH fluctuation, cold shock, ice formation, and cryoprotectant toxicity [8]. Although cryoprotectants play a protective role in embryo cryopreservation, such as minimizing cell damage associated with ice formation [9,10], most of the commonly used cryoprotectants are toxic [11–13]. The nature of the toxicity includes denaturing cellular

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0093-691X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2008.06.028

proteins and reducing pre-freezing cell viability [14], which limits the protective capability of these agents, since cryoprotectants with higher concentrations would cause damage to embryos and significantly reduce the hatching rate. Therefore, determining the effects of cryoprotectants on fish embryos is important for developing appropriate cryopreservation protocols.

Several studies of the effects of cryoprotectants on embryos have been reported in carp [15], flounder [16], and gilthead seabream [17]. In these studies, cryoprotectant toxicity changed during embryo development and was species-specific; therefore, for each species, it is necessary to perform preliminary studies to determine the effects of cryoprotectants.

Red seabream (*Pagrus major*) is an important marine species with commercial value in China. Some studies have been done on red seabream embryos in preservation protocols, including assessment of extra- and intracellular ice formation and cryoprotectant tolerance in various stages of embryo development [5,18]. We found that Hank's solution was the best extender, and heart-beat stage embryos were more tolerant of this cryoprotectant. However, more information regarding cryoprotectant tolerance is needed.

The objectives of the present study were to investigate the effects of various cryoprotectants, and exposure times, on red seabream embryos (at the heart-beat stage), for designing optimal cryoprotectant solutions and appropriate duration of exposure, to minimize toxicity and maximize protection for cryopreservation of red seabream embryos. Five permeable cryoprotectants, dimethyl sulfoxide (DMSO), glycerol (Gly), methanol (MeOH), 1,2-propylene glycol (PG), ethylene glycol (EG) and two non-permeable cryoprotectants, polyvinylpyrrolidone (PVP), and sucrose, were tested. For permeable cryoprotectants, embryos were exposed for 10, 30, and 60 min, whereas for non-permeable cryoprotectants, the duration of exposure was 10 and 30 min.

2. Materials and methods

2.1. Fish breeding and embryo collection

Sexually mature red seabream (8 females and 12 males; body weight, 3–4 kg) were reared in a 12 m^3 concrete tank (temperature: 16–18 °C) with filtered seawater (changed twice a day) and a supply of pumped air. The fish were fed cooked mussel twice a day. The photoperiod was fixed at $L{:}D = 16$ h:8 h. Naturally fertilized embryos were collected in the early morning and incubated in filtered seawater at 18 ± 1 °C in a small plastic barrel. Embryos at the heart-beat stage (heart rate, 60–90 beats/min; approximately 36 h after fertilization) were used. The developmental stages of the embryo were determined morphologically using a light microscope (Nikon-YS100, Japan).

2.2. Solutions

Five permeable cryoprotectants (DMSO, Gly, MeOH, PG and EG) and two non-permeable cryoprotectants (PVP and sucrose) were used in the following experiments. The cryoprotectants were diluted in Hank's solution [19] (8 g/L NaCl, 0.4 g/L KCl, 0.14 g/L $CaCl_2$, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $MgCl_2{\cdot}6H_2O$, 0.06 g/L $Na_2HPO_4{\cdot}12H_2O$, 1 g/L glucose, and 0.35 g/L $NaHCO_3$) to corresponding concentrations (v/v). DMSO was purchased from Sigma–Aldrich China Inc., Shanghai, China; all other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

2.3. Effects of cryoprotectants on embryos

For permeable cryoprotectants, embryos were exposed to each cryoprotectant at concentrations of 5, 10, 15, 20, 25, and 30% (v/v) for 10, 30, or 60 min, respectively, at room temperature. For non-permeable cryoprotectants, the concentrations used were 5, 10, 15, and 20% (v/v), and the exposure times were 10 and 30 min. For each test, approximately 50 embryos were exposed to 10 mL of each cryoprotectant solution for the designated time.

After immersion, the embryos were first removed from the cryoprotectant solution using a nylon mesh, and then were carefully washed three times with fresh seawater in the nylon mesh. Then, the embryos were transferred to a 100 mL beaker containing 80 mL fresh seawater, to allow embryos to incubate and hatch (seawater was changed 1 h later). Control groups were incubated in filtered seawater at room temperature. For each concentration and exposure time, the experiment was performed three times with different batches, and each batch had its own control group. The toxicity of the cryoprotectant was assessed by the hatching rate, which was calculated as the percentage of hatched larvae (48 h after fertilization) in relation to the total number in each group.

2.4. Statistical analysis

Percentage data were normalized through arcsine transformation and analyzed by ANOVA, with significant

differences located with the Student–Newman–Keuls (SNK) test. Statistical significance was judged at the level $P < 0.05$. For permeable and for non-permeable cryoprotectants, three-way ANOVA analysis was carried out respectively, with cryoprotectant, concentration and exposure time as the main effects (the model included their interactions). Furthermore, the importance sequence was obtained by comparing their Eta squared values. Statistical analysis was carried out using SPSS software (SPSS Inc., Chicago, IL, USA) and results were expressed as mean ± S.D.

3. Results

3.1. Effects of permeable cryoprotectants

The hatching rate of embryos treated by permeable cryoprotectants for 10 min is shown (Fig. 1). After exposure to most of the cryoprotectant solutions for 10 min, the hatching rate of embryos showed no significant decrease compared to control (Fig. 1a). However, the hatching rate of embryos exposed to ≥25% DMSO, ≥25% Gly, ≥20% MeOH, and ≥25% EG was lower ($P < 0.05$) than that of control (Fig. 1b–d).

The hatching rate of embryos treated by permeable cryoprotectants for 30 min is shown (Fig. 2). There was no significant decrease in the hatching rate of embryos exposed to 5 or 10% DMSO, 5% Gly, 5, 10% MeOH, 5–20% PG, and 5 or 10% EG in comparison with that of control (Fig. 2a). However, the hatching rate of embryos exposed to 15% DMSO, 10% Gly and 25 or 30% PG decreased ($P < 0.05$) compared to the control, but still exceeded 75% (Fig. 2b–c) The hatching rate of the embryos showed large reduction (most were reduced to zero) after exposure to ≥20% DMSO, ≥15% Gly, ≥15% MeOH, and ≥15% EG (Fig. 2d).

The hatching rate of embryos treated by permeable cryoprotectants for 60 min is shown (Fig. 3). The hatching rates of embryos exposed to cryoprotectants of low concentration (5 or 10% DMSO, 5% Gly, 5% MeOH, 5 or 10% PG, and 5% EG) showed no significant decrease compared to control (Fig. 3a). However, for all other cryoprotectant solutions, hatching rates were all lower ($P < 0.05$) than that of control (Fig. 3b–e). No embryos survived to hatching after exposure to ≥20% DMSO, ≥10% Gly, ≥15% MeOH, 30% PG, and ≥15% EG.

All main effects, including cryoprotectant (a), concentration (b), and exposure time (c), as well as their interactions, were significant ($P < 0.001$) in relation to hatching rate of red seabream embryos (Table 1). Furthermore, their importance sequence was $b > c > a > a*b*c > b*c > a*b > a*c$. The hatching rate of embryos treated with 5% PG for 10 min was significantly higher than others.

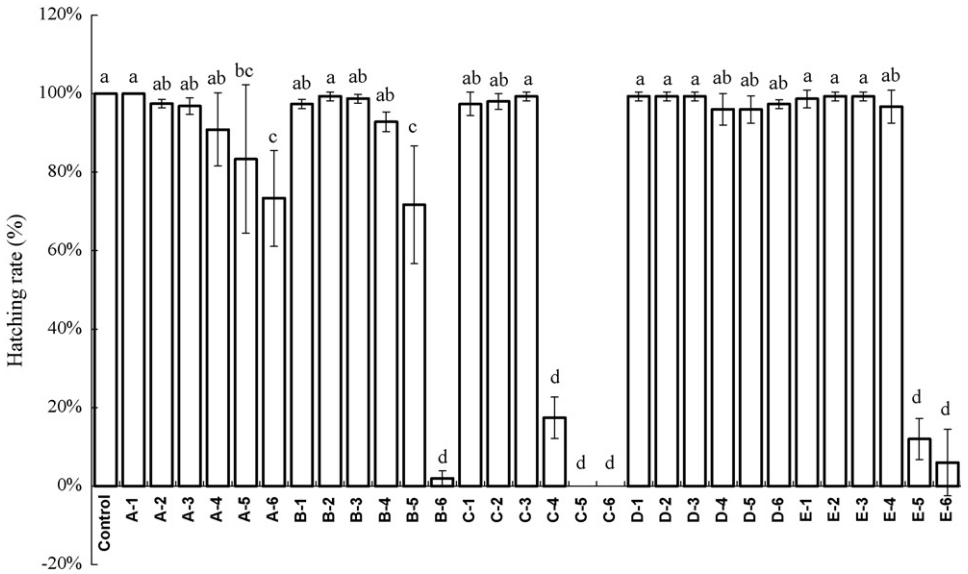

Fig. 1. Mean (±S.D.) hatching rates of red seabream embryos exposed to permeable cryoprotectants for 10 min (three replicates, with approximately 50 embryos/replicate for each cryoprotectant). In the abscissa, A–E represent DMSO, Gly, MeOH, PG, and EG, respectively, whereas 1–6 represent cryoprotectant concentrations of 5–30% (in increments of 5%). Columns without a common letter (a–d) differed ($P < 0.05$).

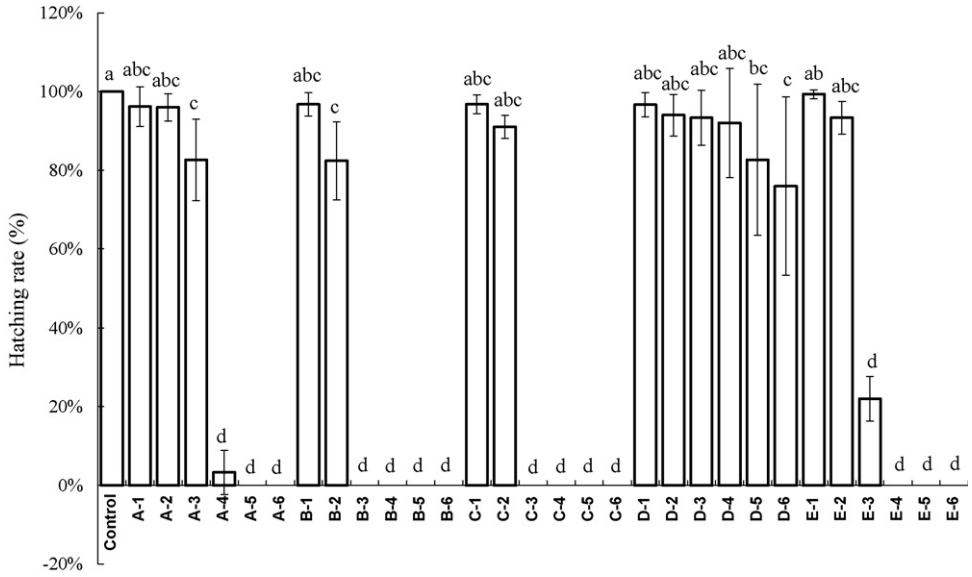

Fig. 2. Mean (±S.D.) hatching rate of red seabream embryos exposed to permeable cryoprotectants for 30 min (three replicates, with approximately 50 embryos/replicate for each cryoprotectant). In the abscissa, A–E represent DMSO, Gly, MeOH, PG, and EG, respectively, whereas 1–6 represent cryoprotectant concentrations of 5–30% (in increments of 5%). Columns without a common letter (a–d) differed ($P < 0.05$).

3.2. Effects of non-permeable cryoprotectants

The hatching rate of embryos treated by non-permeable cryoprotectants for 10 and 30 min are shown (Table 2). In this experiment, embryos were generally tolerant to non-permeable cryoprotectants, since none of the concentrations tested reduced the hatching rate to zero, except for 15 and 20% PVP with an exposure time of 30 min (Table 2). The hatching rate of embryos treated with PVP decreased ($P < 0.05$) as the treatment time and cryoprotectant concentration increased, whereas the embryos treated with sucrose had no significant decrease compared with the control at the concentrations analyzed (Table 2).

All main effects, including cryoprotectant (a), concentration (b), and exposure time (c), as well as

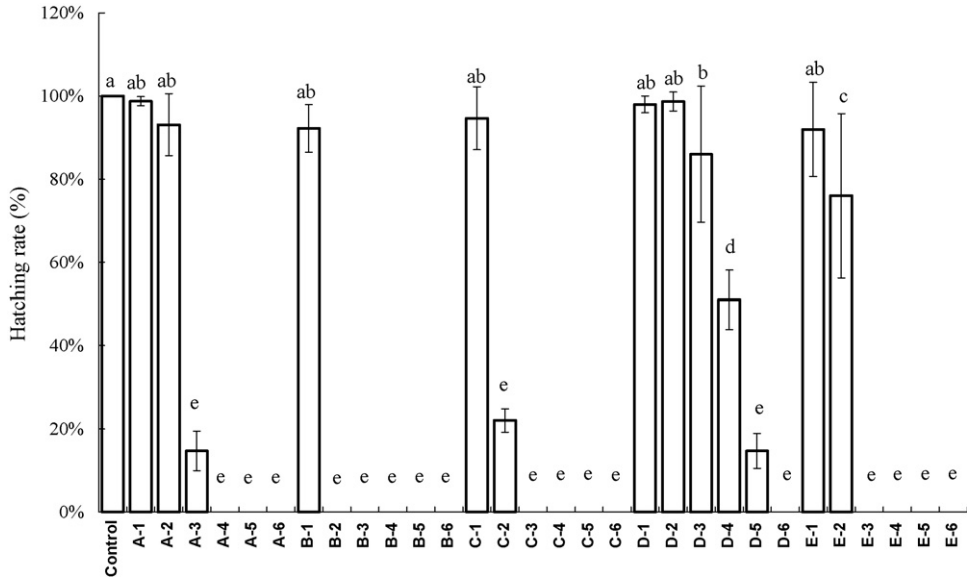

Fig. 3. Mean (±S.D.) hatching rate of red seabream embryos exposed to permeable cryoprotectants for 60 min (three replicates, with approximately 50 embryos/replicate for each cryoprotectant). In the abscissa, A–E represent DMSO, Gly, MeOH, PG, and EG, respectively, whereas 1–6 represent cryoprotectant concentrations of 5–30% (in increments of 5%). Columns without a common letter (a–e) differed ($P < 0.05$).

Table 1
Statistical output for the effects of various permeable cryoprotectants on hatching rate of red seabream embryos

Source of variation	d.f.[a]	Hatching rate		
		Mean square	F-Snedecor	Eta squared
a	4	3.566	157.914**	0.788
b	5	9.509	421.024**	0.925
c	2	9.909	438.747**	0.838
*a*b*	20	0.220	9.743**	0.534
*a*c*	8	0.194	8.593**	0.288
*b*c*	10	0.638	28.234**	0.624
*a*b*c*	40	0.307	13.599**	0.762

Main effects were cryoprotectant (*a*), concentration (*b*), and exposure time (*c*).
[a] Degrees of freedom.
** $P < 0.001$.

Table 3
Statistical output for the effects of non-permeable cryoprotectants on hatching rate of red seabream embryos

Source of variation	d.f.[a]	Hatching rate		
		Mean square	F-Snedecor	Eta squared
a	1	8.801	603.902**	0.936
b	3	1.129	77.434**	0.850
c	1	0.140	9.591***	0.190
*a*b*	3	1.334	91.511**	0.870
*a*c*	1	0.538	36.942**	0.474
*b*c*	3	0.145	9.965**	0.422
*a*b*c*	3	0.072	4.932***	0.265

Main effects were cryoprotectant (a), concentration (b), and exposure time (c).
[a] Degrees of freedom.
** $P < 0.001$.
*** $P < 0.01$.

their interactions, were significant ($P < 0.05$) in relation to hatching rate of red seabream embryos (Table 3). Furthermore, their importance sequence was $a > a*b > b > a*c > b*c > a*b*c > c$.

4. Discussion

4.1. Effects of permeable cryoprotectants

Hatching rate of the red seabream embryos treated with permeable cryoprotectants decreased in association with increases in cryoprotectant concentration and exposure time. Although embryos tolerated low cryoprotectant concentrations or short durations of exposures, increased concentrations or exposures significantly decreased the hatching rate compared with controls. These results were consistent with previous studies [2,16,17].

In the present experiments, PG was the least toxic permeable cryoprotectant, followed by DMSO and EG, whereas Gly and MeOH were the most toxic. In that regard, PG is a commonly used cryoprotectant in cryopreservation of fish embryos. 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. There were similar results in studies of embryos from sea perch [20] and flounder [16]. In addition, Janik et al. reported that PG produced a better survival rate following microinjection of cryoprotectants into zebrafish embryos [21].

In addition to PG, DMSO and EG were also well tolerated by the red seabream embryos. Even after immersion in 10% DMSO and EG for 60 min, the hatching rate of embryos still exceeded 75%. The effect of these two cryoprotectants was species-dependent in fish; DMSO was better tolerated in turbot embryos [2], but poorly tolerated in gilthead seabream embryos [17], whereas EG was better tolerated in gilthead seabream embryos, but not tolerated in embryos of turbot [2], flounder [16], or sea perch [20].

In the present study, Gly and MeOH were the worst tolerated by the red seabream embryos. Embryos exposed to these two cryoprotectants did not tolerate concentrations >10%, even at 30 min exposure. Although Gly was the first cryoprotectant used in sperm cryopreservation [22], it was not tolerated by embryos of many marine species, such as flounder [16], zebrafish [11], oyster [23], and penaeid shrimp [24].

Table 2
Mean (±S.D.) hatching rates of red seabream embryos exposed to non-permeable cryoprotectants

Cryoprotectant	Duration of exposure (min)	Cryoprotectant concentration (%)				
		Control	5	10	15	20
PVP	10	99.3 ± 1.2^{a}	98.7 ± 1.1^{ab}	91.4 ± 6.0^{bc}	49.2 ± 24.68^{de}	10.2 ± 7.2^{e}
	30	99.3 ± 1.2^{a}	98.7 ± 1.2^{ab}	43.2 ± 13.8^{de}	0.0 ± 0.0^{e}	0.0 ± 0.0^{e}
Sucrose	10	99.3 ± 1.2^{a}	98.0 ± 0.0^{ab}	97.4 ± 1.1^{ab}	99.3 ± 1.15^{a}	99.3 ± 1.2^{a}
	30	99.3 ± 1.2^{a}	100.0 ± 0.0^{a}	98.0 ± 2.3^{ab}	98.5 ± 1.00^{ab}	100.0 ± 0.0^{a}

Within a row, values without a common letter (a–e) differed ($P < 0.05$).

Since MeOH is one of the most permeable cryoprotectants [25,26], it would be present inside embryos at a higher concentration than other cryoprotectants. In previous studies, MeOH was relatively nontoxic to embryos of penaeid shrimp [27], flounder embryos [16], zebrafish [11], and Indian major carp [28]. However, MeOH was more toxic than other cryoprotectants on the embryos of black tiger shrimp [29], consistent with species-specific differences.

For permeable cryoprotectants, the effects of the three factors and their interactions on the hatching rate were all significant. Cabrita also reported that cryoprotectant concentration and exposure time significantly influenced the hatching rate of gilthead seabream embryos [17]. 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 [17] and turbot embryos [2]. Although the cryoprotectant effect was the lowest among the three factors, it was still highly significant.

4.2. Effects of non-permeable cryoprotectants

Although the mechanism of protection by large polymers is unclear, the addition of non-permeable cryoprotectants has been adopted in embryo freezing. Non-permeable cryoprotectants are good inhibiters of ice crystal formation [30–33] and are essential for reducing the toxicity of high concentrations of permeable cryoprotectants [2,31].

In the present study, we tested the effects of PVP and sucrose for red seabream embryos. Overall, they were less toxic than permeable cryoprotectants at the concentrations used. The toxicity of sucrose was very low, with no significant difference in hatching rate between control embryos and those treated with 5–20% sucrose. However, in the study of turbot embryos at G stage, the hatching rate of embryos treated with 20% sucrose deceased significantly [2]. In red seabream embryos, sucrose produced a better hatching rate compared to PVP. Therefore, we inferred that sucrose is a good non-permeable cryoprotectant for cryopreserving red seabream embryos.

In conclusion, we systematically analyzed five permeable and two non-permeable cryoprotectants to determine their effects on the hatching rate of red seabream embryos. For all cryoprotectants except sucrose, the hatching rate decreased significantly with the increase of concentration and duration of exposure. Cryoprotectant, concentration, duration of exposure, and their interactions, were statistically significant in relation to hatching rate of red seabream embryos. We inferred that PG and sucrose would be good options for cryopreservation of red seabream embryos. These results should be very useful for designing optimized cryoprotectant solutions and establishing freezing protocols for the cryopreservation of embryos from this marine fish. However, ‘‘absolute toxicity’’ for the cryoprotectants is not the only relevant criterion for the cryopreservation; more important is the protective efficiency of the cryoprotectants. Therefore, further study on the freezing and thawing process in cryopreservation of red seabream embryos is required.

Acknowledgement

This work was supported by National Natural Science Foundation of China (No. 30571427).

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