

## Toxicity tolerance of oyster embryos to selected cryoprotectants

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### Abstract

With the aim of establishing cryopreservation protocols for oyster (*Crassostrea gigas*) embryos, toxicity of single or combined cryoprotectants to oyster embryos was investigated. In experiments on the toxicity tolerance of oyster embryos, four conventional cryoprotectants, namely, acetamide (A), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG), with concentrations from 1 to 5 M were used to test the toxicity tolerance of oyster embryos at eight different developmental stages. On a molar-equivalent basis, DMSO appeared to be less toxic to PG, EG and acetamide in general. Oyster embryos were tolerant to low concentrations of all cryoprotectants tested in the range from 1M to 2 M for all developmental stages. Early stage embryos were more vulnerable to high concentration (4 M and 5 M) cryoprotectants tested than late stage embryos. Experiments were subsequently performed to study the combined effects of DMSO and acetamide adding trehalose or glucose in reducing toxicity to 4-hour oyster embryos. The use of trehalose or glucose reduced toxicity of high cryoprotectant concentrations. Survival was higher when DMSO was combined with acetamide than when using DMSO or acetamide alone at high concentration (5 M). By adding trehalose to combined 5 M DMSO and acetamide (total concentration) an average survival rate of  $67 \pm 7\%$  was obtained in contrast to  $8 \pm 5\%$  or  $2 \pm 1\%$  survival when testing with 5 M DMSO or 5 M acetamide, respectively.

**Keywords:** Toxicity, cryoprotectant, oyster embryos, cryopreservation.

*Etude de la tolérance d'embryons d'huître à la toxicité de cryoprotecteurs.*

### Résumé

La toxicité de cryoprotecteurs utilisés seuls ou combinés est étudiée pour définir des protocoles de congélation d'embryons d'huîtres. Des expérimentations de tolérance à la toxicité ont porté sur quatre cryoprotecteurs conventionnels, l'acétamide A, le diméthyl sulfoxyde (DMSO), l'éthylène glycol (EG) et le propylène glycol (PG), à des concentrations de 1 à 5 M testés à huit stades différents de développement. Sur une base d'équivalent molaire, le DMSO apparaît être moins toxique que PG, EG et l'acétamide en général. Les embryons d'huîtres tolèrent tous les cryoprotecteurs testés à faible concentration (1 et 2 M) pour tous les stades de développement. Les premiers stades embryonnaires sont plus vulnérables que les derniers aux concentrations élevées (4 et 5 M de cryoprotecteurs). Des expérimentations ont été conduites pour tester les effets du DMSO et de l'acétamide en ajoutant à des embryons d'huîtres de 4 heures, du tréhalose ou du glucose pour en réduire la toxicité. L'utilisation de tréhalose ou de glucose réduit la toxicité des fortes concentrations en cryoprotecteurs lorsque le DMSO est combiné à l'acétamide, la survie est plus élevée que lorsque le DMSO ou l'acétamide sont utilisés seuls à 5 M. L'addition de tréhalose à du DMSO combiné à l'acétamide à la concentration totale 5 M, permet d'obtenir un taux moyen de survie de  $67 \pm 7\%$  comparé à  $8 \pm 5\%$  avec le DMSO (5 M) ou à  $2 \pm 1\%$  avec l'acétamide (5 M).

**Mots-clés :** Toxicité, cryoprotecteur, embryon d'huîtres, congélation.

## INTRODUCTION

Preservation of desirable shellfish strains can be accomplished by either one of the following approaches: (1) the shellfish in one's pond, (2) distributing the shellfish to several farms to reduce the mortality risk, or (3) maintaining cryopreserved gametes. Of these strategies, cryopreservation of gametes has several potential advantages because it is less bulky and labour-consuming and more durable in the future. Literature on the cryopreservation of shellfish embryos is limited to a few species such as marine mussel (*Choromytilus chorus*) and blue mussel (Gallardo *et al.*, 1988; Toledo *et al.*, 1989). For other aquatic animals, cryopreservation of embryos is limited to sea urchin (Asahina and Takahashi 1978, 1979) and rotifera (Okamoto *et al.*, 1987; Toledo *et al.* 1991). Several reports on successful freezing of viable fish eggs are interpreted as supercooling *e.g.* freezing down to  $-55^{\circ}\text{C}$  in rainbow trout (Zell, 1978) and to  $-35^{\circ}\text{C}$  in goldfish (Liu *et al.*, 1993).

Addition of cryoprotectants (CPA) is a necessary step in cryopreservation procedures, whether it is accomplished by conventional two-step method or vitrification approach. Because of the high concentration of cryoprotectants required in the cryopreservation procedure, survival of the cryopreserved embryos could be greatly affected by the application of cryoprotectants. The apparent toxicity of the cryoprotectants is dependent on the type and concentration of CPA, the temperature and duration of exposure (Fahy *et al.*, 1984; Steponkus *et al.*, 1991). As a preliminary step of establishing a successful cryopreservation protocol for oyster embryos, the specific objectives of this study were to investigate the appropriate developmental stage of oyster embryos in view of their toxicity tolerance to the selected cryoprotectants, to choose pertinent cryoprotectant for the cryopreservation of oyster embryos, and to test the effect of combined cryoprotectants on reducing the apparent toxicity to oyster embryos.

## MATERIAL AND METHODS

In experiments on the toxicity tolerance of oyster (*Crassostrea gigas*) embryos to selected CPAs, oyster embryos at different stages were obtained from a population of fertilized eggs from 60 to 240 minutes at a 30-minute interval after artificial fertilization in sea water of  $34\pm 2\%$  salinity at a temperature of  $27\pm 1^{\circ}\text{C}$ . Hundreds of oyster embryos pooled from at least two females and two males were placed in each Petri dishes for various treatments. Four conventional cryoprotectants, namely, acetamide (A), dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG), with concentrations from 1 to 5 M (mol/l) were used to test the toxicity tolerance of oyster embryos at seven different developmental stages. All the cryoprotectants were of the highest

available purity form Sigma Chem. Corp., St. Louis, MO, USA. Addition of cryoprotectants was carried out at  $27\pm 1^{\circ}\text{C}$ . Embryos were directly exposed to the desired concentrations of each treatment and 5 min equilibration time was allowed before returning to sea water of  $34\pm 2\%$  salinity to unload the cryoprotectant. Experiments on the use of combined cryoprotectants and addition of trehalose or glucose were performed using 240-min embryos to study the effects of toxicity reduction. Living embryos were determined 10 hours after fertilization by counting the embryos with active rotary motion. All survival rates indicated in this study were normalized to the untreated control in each experiment. Normally more than 80% of artificially fertilized embryos reached their late D-trochophore stage with rotary motion in the experiments. Each group of the sub experiment was tested in triplicate and at least 100 oyster embryos were counted in each sample.

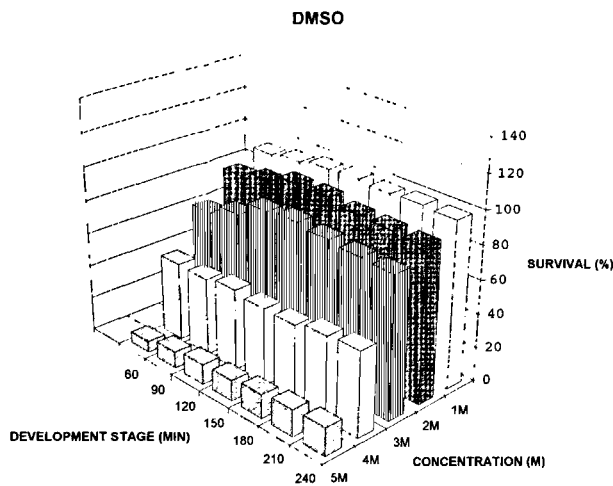
For experiments testing the toxicity tolerance of oyster embryos of individual cryoprotectant, the effect of cryoprotectant concentration and embryo developmental stage on the survival of oyster embryos was analyzed by linear regression of separate effects on embryos survival and tested with *F* statistic. For the experiments on the combined effect of cryoprotectants on the embryo toxicity tolerance, differences among treatment means were compared using Duncan's multiple range test. A probability (*p*) less than 0.05 was considered significant.

## RESULTS AND DISCUSSION

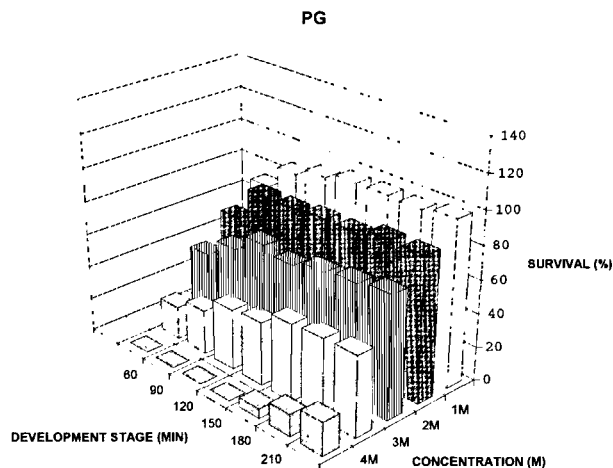
### Toxicity of individual cryoprotectant

As shown in *figure 1* using survival rate of oyster embryos as an index, toxicity of DMSO tended to increase as the concentration of DMSO increased from 1 to 5 M. The developmental stages were based on time courses roughly representing 4-cell, 16-cell, 64-cell, early morula, late morula, early D-trochophore, and late D-trochophore stages. *Figure 1* shows that there was no significant difference of survival between the embryos treated with DMSO below 3 M. Notable decrease in survival occurred when concentration of DMSO was above 4 M. There was no significant difference on toxicity tolerance among embryonic stages when the concentration of DMSO was below 4 M ( $F=111.5$ ,  $p<0.001$ ).

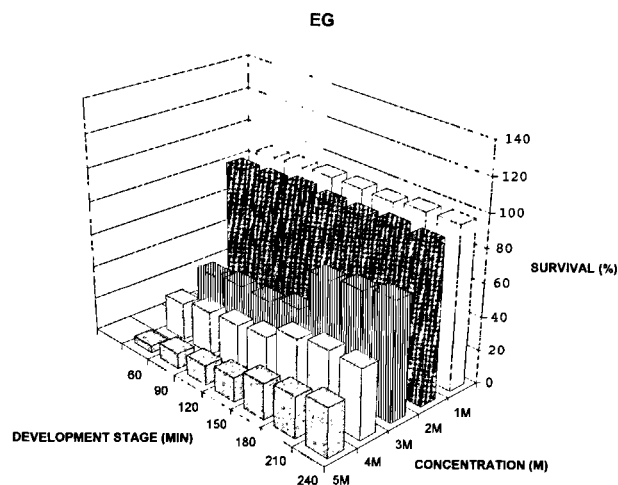
Ethylene glycol was comparatively toxic to oyster embryos based on the survival rates at seven stages treated with EG of five concentrations, *i.e.* 1, 2, 3, 4, and 5 M (*fig. 2*). There was no notable decrease in survival at concentrations of 1 and 2 M. However, a transition from high survival to low survival was observed when the concentration reached 3 M, especially for the early stage embryos. A statistical test revealed that no significant difference was found between the embryonic stages when linear regressions



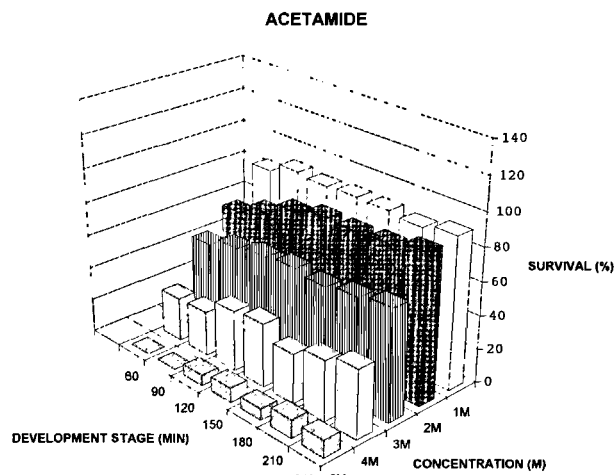
**Figure 1.** – Survival rate of oyster embryos at different developmental stages treated with **DMSO** at different concentrations (1, 2, 3, 4, and 5 M).



**Figure 3.** – Survival rate of oyster embryos at different developmental stages treated with **propylene glycol** at different concentrations (1, 2, 3, 4, and 5 M).



**Figure 2.** – Survival rate of oyster embryos at different developmental stages treated with **ethylene glycol** at different concentrations (1, 2, 3, 4, and 5 M).



**Figure 4.** – Survival rate of oyster embryos at different developmental stages treated with **acetamide** at different concentrations (1, 2, 3, 4, and 5 M).

were performed on all the survival data ( $F=1.37$ ,  $p>0.005$ ). However, there was significant difference between cryoprotectant concentrations ( $F=217.05$ ,  $p<0.001$ ). At higher concentration (*e.g.* 3 M, 4 M and 5 M), late stage embryos (240-min) appeared to have significantly higher survival rate than the 60- and 90-min early stage embryos.

Figure 3 shows the survival data of oyster embryos subjected to various concentrations of PG at various embryonic stages. Significant transition from high survival rate to low survival rate occurred at the concentration of 4 M. In contrast to DMSO and EG, PG seemed to be more toxic to early stage oyster embryos at high concentrations. For embryos treated with 5 M PG there were no survivals for the early stage embryos. This is similar to the trend as demonstrated in figures 1 and 2 that late stage embryos were more tolerant to cryoprotectant toxicity.

Survival of oyster embryos treated with acetamide was similar to PG as shown in figure 4. Embryos treated with 5 M acetamide resulted in relatively low survivals. Linear regression tests revealed that developmental stage was a significant factor on embryo survival for embryos treated with 2, 3, 4 and 5 M acetamide. Similar to the other three selected cryoprotectants, late stage embryos appeared to be more tolerant to acetamide at high concentration.

Based on the results from figures 1 to 4, it emerges that in general DMSO is less toxic to oyster embryos than the other three selected cryoprotectants. Significant decrease in survival occurred when the cryoprotectant reached 4 M or 5 M. The factor of developmental stage on the toxicity tolerance became more apparent when embryos were treated with high concentrations of 4 M or 5 M.

### Effect of combined cryoprotectants

Judging from the survival data of oyster embryos treated with the four selected cryoprotectants at various developmental stages, it was therefore assumed that embryos at 240-min stage rather than that of other earlier stages should be selected for further investigation in order to establish a successful cryopreservation protocol. Our preference for using embryos of the stage was based on results from a series of accumulated toxicity tolerance tests in this study. Since the species-specific changes occurred in low temperature tolerance of the eggs of four marine fish during embryonic development as indicated by Sasaki *et al.* (1988), species-specific difference in shellfish should be further studied in the future.

Arakawa and Timasheff (1982) indicated that carbohydrate functions to stabilize protein and thus protects cell membranes. Addition of trehalose or glucose to cryoprotectant solution has been shown to be beneficial in increasing survival for several biological systems (Ramløv and Westh, 1992; Karow *et al.*, 1992). A series of experiments was therefore designed and performed to test the effect on reduction cryoprotectant toxicity by adding trehalose or glucose. Figure 5 shows the effect of adding trehalose or glucose to acetamide. In the figure, groups A, B and C represent acetamide concentration of 3, 4 and 5 M in the combined CPAs, respectively. There was no significant difference within groups A, B and C. For group C, either single acetamide or acetamide

Table 1.

Group	A (M)	AT (A+T) (M)	AG (A+G) (M)
A	3	3+0.09	3+0.09
B	4	4+0.12	4+0.12
C	5	5+0.15	5+0.15

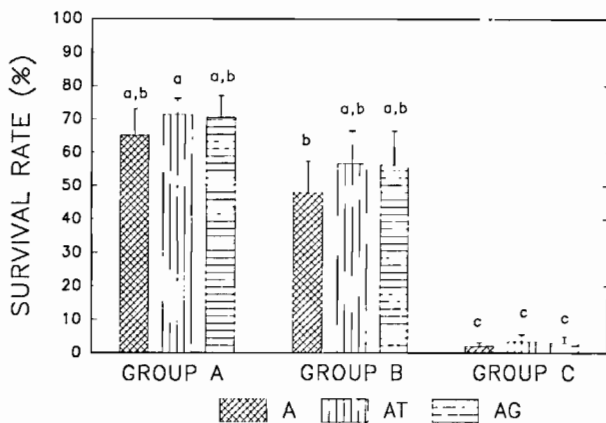


Figure 5. – Survival rate of oyster embryos treated with single or combined cryoprotectants of major acetamide (A), and minor trehalose (T) or glucose (G).

combined with trehalose or glucose resulted in very low survival.

Addition 0.09, 0.12 or 0.15 M trehalose and glucose, respectively, to 3, 4 or 5 M DMSO raised the survival rate to a range from 2% to 10% more as shown in figure 6. However, the increase in survival was not significant. In comparison to group C of figure 5, survival rates of embryos were higher for the DMSO-based group C of figure 6. This is consistent with the previous presentation the DMSO was less toxic to oyster embryos at high concentration.

Table 2.

Group	D (M)	DT (D+T) (M)	DG (D+G) (M)
A	3	3+0.09	3+0.09
B	4	4+0.12	4+0.12
C	5	5+0.15	5+0.15

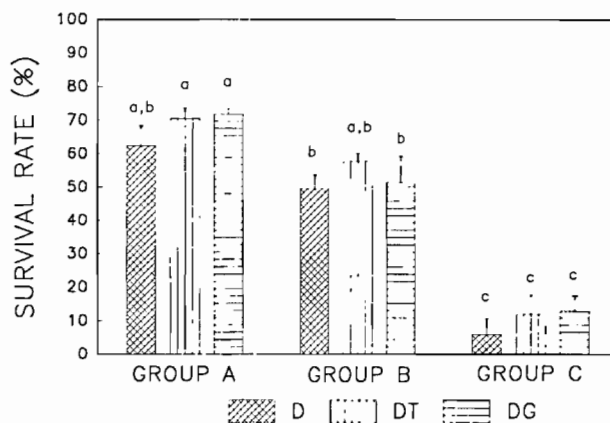


Figure 6. – Survival rate of oyster embryos treated with single or combined cryoprotectants of major DMSO (D), and minor trehalose (T) or glucose (G).

Mere acetamide at a concentration of 5 M resulted in significantly low survival rate of 2% while 3 M acetamide yielded an average survival rate of 65%. DMSO alone showed decreasing survival rate from about 62 to 8% as the concentration increased from 3 to 5 M. Combining DMSO and acetamide resulted in higher survival, on a molar-equivalent basis, especially at high concentration (5 M) as one may compare group C in figures 5, 6 and 7, where very low survival was obtained using DMSO or acetamide alone at a total concentration of 5 M. This corresponded with the observation made by Crowe *et al.* (1990). Fahy (1984) reported improved toxicity of kidney slices frozen and thawed in the presence of DMSO when a portion of DMSO was replaced by acetamide, formamide or urea. It was interpreted as reflecting a specific reduction of cryoprotectant toxicity in the frozen state related to direct molecular interaction between DMSO and amides.

Table 3.

Group	A (M)	D (M)	AD (A+D) (M)
A	3	3	1.5+1.5
B	4	4	2.0+2.0
C	5	5	2.5+2.5

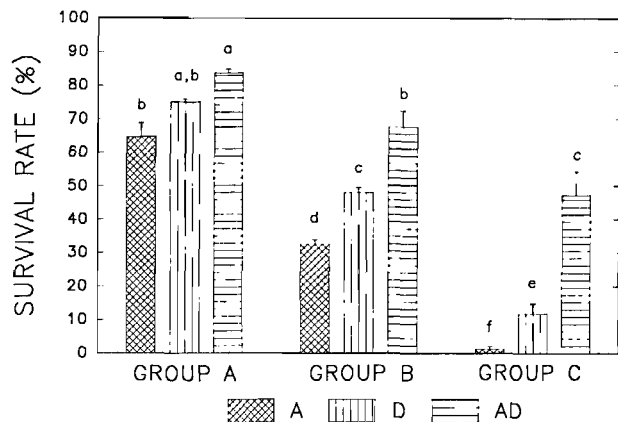


Figure 7. – Survival rate of oyster embryos treated with single or combined cryoprotectants of acetamide (A), and DMSO (D).

Combining the three CPAs may be more functional in increasing survival rate, based on the original concentration of the two major CPAs. Figure 8 illustrates that further addition of 0.15 M trehalose or 0.15 M glucose (into combined CPAs of acetamide and DMSO) increased significantly the survival rate to 33 or 27% more, respectively, than a combination of 2.5 M acetamide and 2.5 M DMSO alone (group C).

An overall evaluation of positive effect of trehalose and glucose in figures 5 to 8 corresponded to the findings of Crowe *et al.* (1990) and Fahy *et al.* (1984). Further study on the practical application of trehalose or glucose in protecting oyster embryos during cryopreservation should, therefore, be made.

### CONCLUSION

Four different cryoprotectants at a range from 1 to 5 M were tested against various developmental stages of oyster embryos to investigate the toxicity tolerance. On a molar-equivalent basis, DMSO appeared to be less toxic to EG, PG and acetamide. Oyster embryos were tolerant to low concentrations of all cryoprotectants tested in the range from 1 to 2 M

Table 4.

Group	AD (A+D) (M)	ADT (A+D+T) (M)	ADG (A+D+G) (M)
A	3	1.5+1.5+0.09	1.5+1.5+0.09
B	4	2.0+2.0+0.12	2.0+2.0+0.12
C	5	2.5+2.5+0.15	2.5+2.5+0.15

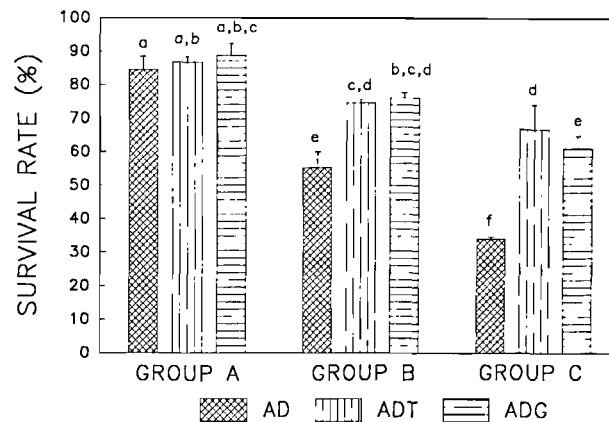


Figure 8. – Survival rate of oyster embryos treated with single or combined cryoprotectants of major acetamide (A), DMSO (D) and minor trehalose (T), glucose (G). The same indices on each bar indicate absence of a significant difference between treatments.

for all developmental stage. This is beneficial for the application of conventional two-step cryopreservation during the loading step before freezing. However, if vitrification is the approach to be taken for the cryopreservation of oyster embryos, early stage embryos appeared to be vulnerable to the toxicity of high concentration (4 M and 5 M) cryoprotectants tested. Therefore, using late stage oyster embryos as a first attempt in setting up cryopreservation protocol should be a reasonable choice.

Effect of using trehalose or glucose in the reduction of cryoprotectant toxicity to oyster embryos was demonstrated in this study. The effect is especially enhanced when high cryoprotectant concentrations, which are required for vitrification, were applied. Again, on a molar-equivalent basis, higher survival was observed when DMSO is combined with acetamide than using DMSO or acetamide alone at high concentration (5 M). By adding trehalose to combined DMSO and acetamide, even higher survival of  $67 \pm 7\%$  was obtained. This result revealed the possibility of using vitrification as an alternative approach to cryopreserve oyster embryos.

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