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Toxicity of Selected Cryoprotectants to the First Zoeal Stages of Giant Freshwater Prawn *Macrobrachium rosenbergii* (de Man)

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Abstract

The toxicity of various concentrations of dimethyl sulphoxide (DMSO), methanol, glycerol and ethylene glycol (EG) were assessed at 20°C on the first zoeal stages of the giant freshwater prawn *Macrobrachium rosenbergii, Palaemonidae.* In addition, the toxicity of one permeating DMSO, glycerol, methanol and ethelene glycol along with two nonpermeating cryoprotectants polyvinyl pyrrolidone (PVP at 0.25%) and sucrose (0.1 M) were also assessed. Larvae were exposed to different concentrations of cryoprotectant (CP) solutions for 20 and 40 min at 20°C. Direct observation of morphology and motility was used to assess the survival of cryoprotectant treated zoeal stages. Methanol was found to be the most toxic followed by DMSO. Ethylene glycol and glycerol were comparatively less toxic. The larvae could be safely exposed to these cryoprotectants up to 10% v/v for 20 min at 20°C. It could also be observed that the combinations of cryoprotectants were relatively less toxic.

Introduction

Macrobrachium rosenbergii, the largest natantian is gaining wider attention as a suitable candidate for freshwater aquaculture in India. At present the major constraint in the expansion of prawn culture is lack of sufficient seed supply. Availability of cryopreserved larvae would greatly help in facilitating stock distribution, in weight reduction and in coping with time limitations on shipments. So far little work has been done on the

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cryopreservation of gametes/embryo of invertebrates. However, there are only a few reports on successful cryopreservation of crustacean embryo/larvae (Baust and Lawrence 1977; Newton and Subramaniam 1993; Simon et al. 1994) and molluscan embryo (Toledo et al. 1989; Renard 1991).

A successful cryopreservation of any cell type requires that the cell lose water through osmotic dehydration during cooling to the point where crystallization of the remaining intracellular water does not cause any irreparable damage. Cryoprotectants minimize cellular injury during cryopreservation by decreasing the amount of intracellular ice present at any given time. However they themselves can cause cellular injury by osmotic trauma and many are extremely toxic. Thus as a basis of research on cryopreservation of embryo/larvae, information on relative toxicities of cryoprotectants is of key importance.

Limited studies have been done on the relative toxicities of different cryoprotectants on a few species (Robertson et al. 1988; Renard and Cochard 1989; Newton and Subramoniam 1993; Chao et al. 1994) but none on *M. rosenbergii*. This paper reports data on the toxicity of some of the commonly used cryoprotectants on the first zoeal stages of giant freshwater prawn *Macrobrachium rosenbergii*.

Materials and Methods

Berried females bearing grey eggs (late embryonic stage) were collected from the broodstock rearing facility of the Central Institute of Freshwater Aquaculture, Bhubaneswar, India and maintained in the laboratory in 10‰ saline media. The hatched out larvae were used for the experiments. The cryoprotectants used in the study were glycerol, ethylene glycol, dimethyl sulphoxide and methanol at concentrations of 4, 7, 10 and 20% (v/v). In addition, a combination of one permeating (glycerol, methanol, ethylene glycol, DMSO each at 4, 7, 10 and 20%) with two nonpermeating cryoprotectants (sucrose and polyvinyl pyrrollidone at 0.1 M and 0.25% w/v respectively) were also assessed for their toxicity.

The cryoprotectant solutions were prepared in filtered and well aerated 10‰ saline media. All the experiments were carried out at 20°C in a BOD incubator. For each concentration of cryoprotectants three replicates of 20 larvae each were used. The larvae were cooled to 20°C in 100 ml of 10‰ saline media and an equal volume of double concentrations of cryoprotectants was added serially to reach the final concentration in three steps. The equilibration periods (exposure period) used were 20 and 40 min respectively. Once the equilibration period is over the cryoprotectants were serially diluted in a three step dilution process and survival was estimated 24 h after transfer to the fresh media. Motility and appendage movement were used as the criteria for survival.

The results were compared by two-way ANOVA on Arcsin transformed data. Means were separated using the least significant difference (LSD) test (Steel and Torrie 1980).

Results

Data on the survival of prawn larvae (Ist zoeal stage) treated with different concentrations of cryoprotectants for different exposure periods indicated that the percentage of survival decreased as the concentration was increased and the exposure period was lengthened. Statistical analysis revealed that for all the CP's tested, both concentration and exposure period had significant effects on the percentage of survival of prawn larvae. Furthermore the significant interaction observed between concentration and equilibration period for all the CP's tested indicated that the influence of concentration differ for both 20 and 40 min exposures.

Among the CP's tested glycerol was the least toxic to prawn larvae. The larvae could be safely exposed up to 10% v/v (1.35 M) for both 20 and 40 min. However, a significant reduction in survival ($p<0.01$) could be observed when the larvae were exposed to 20% (2.69 M) (Fig.1). Exposure for 40 min at 20% was found to be more toxic than 20 min exposure (Fig. 2).

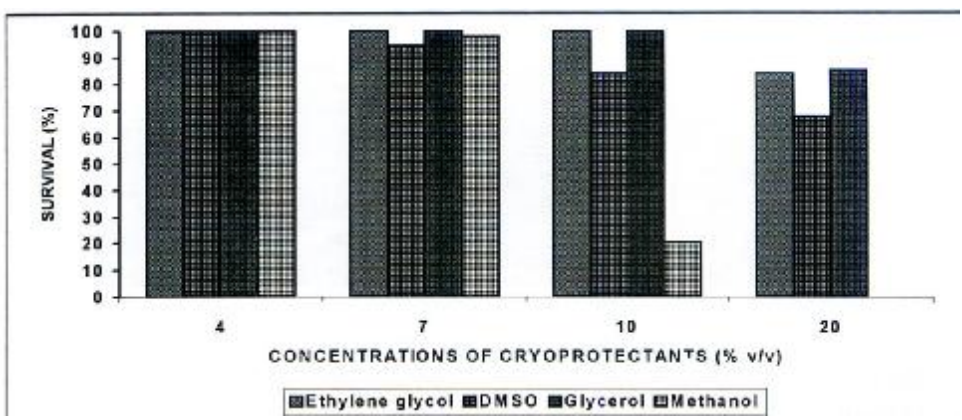

Fig. 1. Survival of giant prawn larvae exposed to various concentrations of cryoprotectants for 20 min at 20^{0}C.

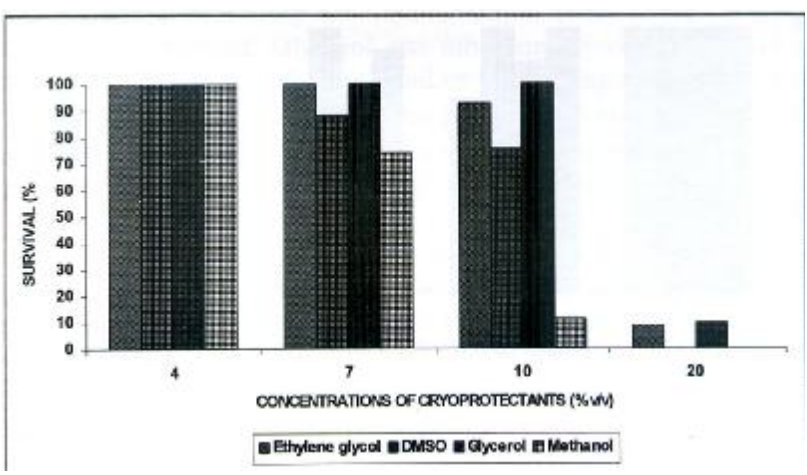

Fig. 2. Survival of giant prawn larvae exposed to various concentrations of cryoprotectants for 40 min at 20^{0}C.

Significantly similar results could be observed when the larvae were exposed to glycerol combined with sucrose and PVP, except that a significant reduction in survival rate could be observed following 40 min exposure at 10% combined with PVP and sucrose (Figs. 3 and 4).

Ethylene glycol was less toxic to prawn larvae. The larvae could be exposed up to 10% (1.79 M) for 20 min without any significant reduction in percentage of survival (Fig. 1). Exposure for 20 min at 20% (3.6 M) as well as 40 min exposure to both 10 and 20% resulted to significant reduction in survival rate ($p<0.01$) (Fig. 2). Ethylene glycol combined with sucrose and PVP were even less toxic to prawn larvae. No significant reduction in survival could be observed when the larvae were exposed for 20 min to 20% (Fig. 3). However, exposure for 40 min to 20% EG in combination with PVP and sucrose resulted to a significant reduction in survival rate ($p<0.01$) (Fig. 4).

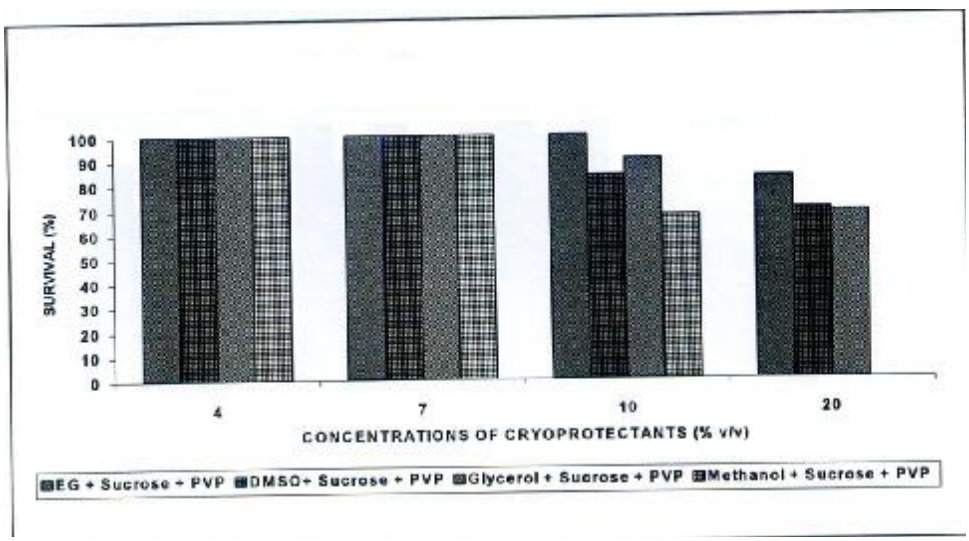

Fig. 3. Survival of giant prawn larvae exposed to various concentrations of combined cryoprotectants for 20 min at 20^0C.

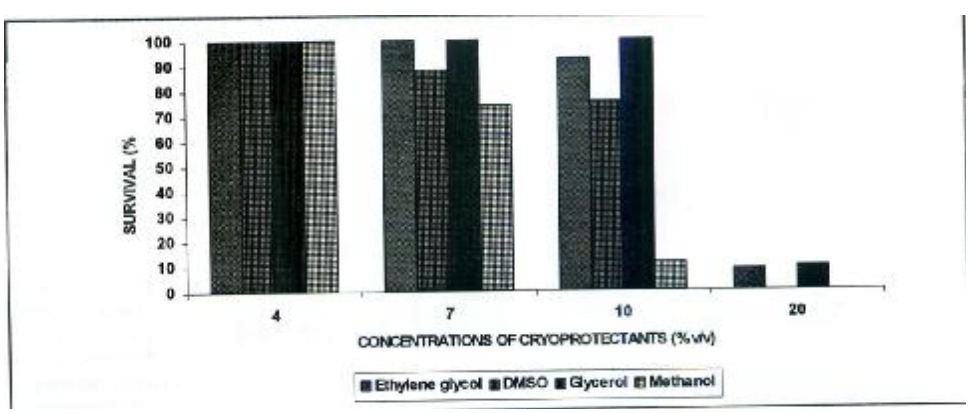

Fig. 4. Survival of giant prawn larvae exposed to various concentrations of combined cryoprotectants for 40 min at 20^0C.

DMSO was comparatively more toxic to prawn larvae. Even at the lowest concentration tested (4%; 0.56 M) the larvae were found to be less active and at 7(0.99 M), 10(1.40 M) and 20% (2.82 M) there was progressive decline in survival (Fig. 1). Exposure to 20% for 40 min was found to be lethal for the larvae (Fig. 2). DMSO in combination with PVP and sucrose was found to be less toxic to the larvae. The larvae were active and swam in column following an exposure for 20 and 40 min up to 7% DMSO with PVP and sucrose. No reduction in the survival rate could be observed following an exposure for 20 min to both 10 and 20%. However, the larvae were weak and stayed mostly at the bottom of the container making only feeble movements. A significant reduction in survival could be observed when the larvae were exposed for 40 min to both 10 and 20%.(Fig. 4)

The prawn larvae were most sensitive to methanol. Larvae tolerated an exposure to 4% (1.0 M) for 20 and 40 min without any significant reduction in survival. Exposure to 7% (1.75 M) for 40 min significantly reduced survival (Fig. 2). Drastic decline in survival could be observed following exposure to 10% (2.5 M) for both 20 and 40 min. All larvae were instantly killed in 20% (5.0 M) methanol. Methanol in combination with sucrose and PVP was less toxic. Larvae could be safely exposed to 7% for both 20 and 40 min. However, exposure to 10% for 20 and 40 min resulted to a significant reduction in survival ($p<0.01$) (Figs. 3 and 4). The larvae could not tolerate an exposure to 20% methanol even in combination with PVP and sucrose.

Discussion

The concentration of cryoprotectants, the mode of its addition and dilution as well as the duration and temperature of exposure are critical steps according to experience from mammalian embryo preservation (Wittingham 1980). Since various species show varied responses to different cryoprotectants it is essential to study the individual species tolerance.

Among the cryoprotectants studied methanol was found to be most toxic to prawn larvae. Reduction in survival rate could be observed following an exposure to a relatively low concentration (7%). DMSO was the next most toxic cryoprotectant. Glycerol and ethylene glycol were relatively less toxic. The larvae could be safely exposed to these compounds up to a concentration of 10% for 20 min. An equilibration period of 20 min was found to be less toxic than 40 min. It could be seen that equilibration period becomes an important factor with regard to cryoprotectant toxicity when the concentration of CP's exceeded 10%.

The study showed that the larvae became increasingly susceptible to cryoprotectants as the concentration was increased and the time of immersion was lengthened. Cryoprotectant induced injury can be considered to be either biochemical and/or osmotic (Renard and Cochard 1989). Though the exact nature of biochemical injury is not known, it is shown that the injury increases with exposure time. It could also be observed in the present study that CP toxicity increases with time, especially when the concentration was high

(>10%). Osmotic injury results from changes in cell volume which may be induced as a result of cryoprotectant incorporation and/or removal (Renard and Cochard 1989).

Earlier studies have shown that osmotic damage can be minimized by slow incorporation and/or removal of the CP. Thus, care was taken in the present study to minimize osmotic injury by incorporating and removing the cryoprotectants slowly in a three-step dilution procedure.

Varied degrees of toxicity in different species of animals were also shown in earlier studies. Ben-Amotz and Rosenthal (1981) reported that Atlantic herring embryos could withstand a two hour equilibration in 1.5 M DMSO at 5°C whereas glycerol and EG were toxic at the same molality. Harvey et al. (1983) found that 1 M DMSO did not affect percentage hatch of zebra fish embryos exposed for up to two hours, whereas glycerol was highly toxic. DMSO (2M) and methanol (2M) were least toxic to the red drum embryos when compared to glycerol, EG, sucrose and artificial seawater (Robertson et al. 1988).

Among these, glycerol was intermediate in toxicity and sucrose was most toxic on a molar equivalency basis. Renard and Cochard (1989) while examining the toxicity of cryoprotectants such as methanol, EG, propylene glycol, DMSO, glycerol and sucrose on the bivalve embryos found that methanol was less detrimental to embryo development than the progressive EG, propylene glycol, DMSO, glycerol and sucrose. Subramoniam and Newton reported that glycerol and formamide were extremely toxic to the embryos of penaeid prawn *Penaeus indicus* above 5% v/v. Other CP's studied such as acetamide, methanol and DMSO were relatively less toxic and EG was the least toxic. Results of these studies clearly indicate that toxicity of CP's is highly species specific.

The protective effect of multiple cryoprotectant systems has been described in *Artemia salina* (Baust and Lawrence 1980). The authors observed an overall potentiation of survival with multiple cryoprotectant system. Sucrose, a nonpermeating cryoprotectant in combination with a permeating agent has shown to afford protection during freezing of mouse embryos in liquid nitrogen (Takahashi and Kanagawa 1985). Bast and Lawrence (1980) opined that optimal cryoprotection may be afforded by a synergistic combination of cryoprotectants of different actions (intracellular and extracellular) coupled with equilibration at low temperature. The present study also shows that the multiple cryoprotectant system is less toxic to the larvae when compared to the single cryoprotectant system.

Conclusion

In summary, the study revealed that EG and glycerol at concentrations < 10% v/v could be safely used as cryoprotectants in the cryopreservation work of early larval stages of *M. rosenbergii*. Besides, all the multiple cryoprotectants system studied (<10% v/v) could also be used in the cryopreservation work.

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