International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 1 (2014) pp. 8-17**
http://www.ijcmas.com

Original Research Article

Effect of various cryoprotectants in the preservation of *Brachionus plicatilis* embryo and its role in expression profile of heat shock protein (HSP70)

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A B S T R A C T

Cryopreservation of the rotifer *Brachionus plicatilis* embryo was experimented with variety of cryoprotectants for the purpose of long term storage. In the present study, rotifer embryos were experimented using various cryoprotectants (CPAs) such as dimethyl sulphoxide (DMSO), glycerol, methanol, ethylene glycol and propane diol. Of these, cryoprotectants used DMSO showed maximum survivability when compared with other cryoprotectants at 10% concentration. In addition, effect of various CPAs and its role in expression profile of heat shock protein (HSP) was monitored using western blot and RT-PCR analysis. The result clearly depicts that CPA has drastic effect on the expression profile of HSP. Interestingly, an increased expression of HSP70 in embryos treated with ethylene glycol, methanol and glycerol was observed. On the contrary there was low level expression of HSP was found in the samples treated with propane diol and DMSO. Thus, the expression of HSP demonstrated the level of stress experienced by embryos treated with various CPAs. Altogether, the results conclude that HSP gene can be used as a biomarker in the selection of CPAs for preservation of biological samples.

K e y w o r d s

Heat shock protein; Cryo-protectants; DMSO; Toxicity; *Brachionus plicatilis*

Introduction

Maintenance of monogonont rotifers in hatcheries and their desirable clones would require considerable routine effort for finfish and shellfish larval rearing. King *et al.* (1983) and Koehler (1967) have studied the possibility of cryopreserving the rotifer with minimal survival for the first time. Rotifers provide a complex system with a number of distinct osmotic compartments with extracellular aqueous solution and intracellular content of the cell. Earlier studies have been reported the

difficulties in preservation of fully differentiated organisms and organs might be a consequence of this complex histological architecture and differentiated multicellularity (Newton and Subramoniam, 1996). Although successful cryopreservation of mammalian embryos have been achieved and studied indepth, cryopreservation information of the invertebrates is rather limited (King *et al.* 1983; Toledo *et al.* 1989).

Cryoprotectants used should be a safe biological material against freezing damage, which can be a toxic and hindrance in revival. It has also been shown that the extent of toxicity of various CPAs differs with respect to the particular biological sample (Fahy, 1984). As a former, Shaluei *et al*. (2013) revealed the importance of permeable and non-permeable CPAs concentration and equilibration period for designing optimized cryo-media for preservation of goldfish embryos. CPAs provide protection from cold and hot shock treatments to prevent cell dehydration. In addition, mechanism of CPA toxicity, distraction of trans membrane ionic pumps, enzymes, generate toxic formaldehyde, disruption of the mitochondrial transport system, dissolving DNA structure, metaboloic activity and cytoskeleton proteins have been the proposed factors of injury to cells (Bonner and Klibanov, 2000; Zampolla *et al*., 2011; Spikings *et al*., 2012).

Organism must be able to adapt with a constantly changing external environment for survival. One of the mechanisms that a cell use to respond to adverse environmental conditions is the synthesis of stress protein, which to act as a molecular chaperones mainly assist in maintaining protein integrity. The stress proteins have widely been employed as biomarkers for a wide variety of aquatic organisms (Werner and Nagel, 1997). In particular, *B. plicatilis* has been demonstrated to be an ideal system for both toxicity assay and stress studies related (Cochrane *et al.*, 1991; Wheelock *et al.,* 1999).

King *et al*. (1983) suggested a range of 9-12% DMSO for the cryopreservation of monogonont rotifer. Stress protein studies have been documented in rotifers exposed to different stresses such as heavy metals (Cochrane *et al.* 1991), chemically dispersed oil (Wheelock *et al.*, 1999), and span extension (Kaneko *et al.*, 2002). Moreover, nutritional stress and temperature in *Plationus patulus* (Cecchine and Snell, 1999) and arsenite stress in *Cladoceran* (Chen and Sillett, 1999) have also been reported. Similarly, Wheelock *et al* (1999) found increased level of HSP expression at elevated temperatures in *B. plicatilis*. Although, several studies have reported the effect of CPAs in various animal models, there is no clear evidence to exhibit CPAs toxic effect in the organisms used for cryopreservation. To best of our knowledge, this is the first report to give a concrete evidence that CPA cause toxic effect which results in the expression profile of HSP. In the present study, effects of various CPAs in correlation with expression profile of HSP in rotifer embryo have been documented.

Materials and Methods

Test organism

Brackish water rotifer *B. plicatilis* was used as the model organism. It was identified and isolated by following the method of Ruttner-Kolisko (1974).

Rotifers were fed with *Chlorella vulgaris* at a density of $2x10^6$ cells/ml and the pH was maintained at 6.5±0.5. The experimental temperature for the culture of rotifers was 25°C with 30‰ of salinity was maintained under laboratory conditions (Hirayama and Kusano, 1972). Rotifer embryos were normally attached to the body of adults. Embryos were separated by vortex mixing for 10-15 min and the culture was left for 10 min to allow the separated embryos to settle (Parez *et al.* 1988).

Cryoprotectants

Five well-known low molecular weight permeating CPAs such as dimethyl sulphoxide (DMSO), methanol, ethylene glycol, propane diol and glycerol were purchased from Sigma Aldrich & Co (St Louis, USA) and methanol from SRL (Mumbai, India).

Cryoprotectant toxicity assessment

The cryoprotectant toxicity study was carried with various CPAs at the concentration of 10% (v/v) for 20 min equilibration time in artificial seawater. Isolated embryos were transferred individually by using a Pasteur pipette into the CPAs medium at room temperature (25°C) (Bedding *et al.,* 2000). For complete penetration of the CPA 10%concentration and 20min equilibration period was maintained (Toledo *et al.,* 1989). After exposure, the embryos were rinsed with culture medium (seawater 30‰) to remove the CPA (six replicates /n-50). Survival was expressed by the number of embryos that provided neonates swimming actively divided by the number of embryos recovered after 8-24 hr (hatching period) of BOD incubation. Unhatched embryos were scored and the percentage of mortality was calculated, in control 100% of hatching was obtained without CPA (King *et al.,* 1983).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from CPAs treated rotifers by following the TRIZOL method (Qiagen, Hilden, Germany). Two micrograms of total RNA was used for cDNA synthesis. Primers used were F1 (5'-GGATCATCAA(C/T) GA GCCCACGGC-3') and R1 (5'-AACA AGAGCATCAACCC(C/A/G) GACGAG GC-3') for HSP70 as described by Kaneko *et al* (2002). PCR was carried out for 3 min at 94°C followed by 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 90s. The final extension step was performed at 72°C for 5 min. Each of the 100µL reaction mixtures contained 40pmol of forward and reverse primers approximately 1µg first strand cDNA synthesized from total RNA of rotifers, 20nmol dNTP mixture, 10µL of 10X PCR buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 15mM $MgCl_2$, 0 01% (w/v) gelatin) and 1U Ex *Taq* DNA polymerase. PCR products were separated on 1% agarose gels and stained with ethidium bromide. The intensity and expression of the product was observed and compared with β-actin which served as an internal standard.

Western blot analysis

After CPAs toxicity assessment, the embryos were isolated, homogenized, and the total protein content was determined by Bradford assay (1976). Samples (20µg) were boiled for 10 min in SDS-PAGE sample buffer (Laemmli, 1970), centrifuged and loaded in a 10% SDS-

polyacrylamide gel. Electrophoresis was carried out at 50 mA for 4 hr. Electroblotting of the proteins upon the supported nitrocellulose was performed for 90 min at 25V/ 130mA as described by Towbin *et al* (1979). After transfer, the membrane was removed and the protein was visualized using Ponceau S. The membrane was blocked in blocking buffer for 1 hr at room temperature and washed with washing buffer, and subsequently incubated with the primary antibody mouse monoclonal HSP 70 antibody (1:3000) for overnight at 4^{o}C. The membrane was washed in washing buffer twice and incubated with a secondary antibody (alkaline phosphatase conjugated antibody) (1:1000) for 2 hr at room temperature. The membrane was once again washed and then incubated with DAB for 10-15 min at room temperature in the dark. After color development the membrane was washed twice with distilled water to stop the DAB reaction. Expression of the product was observed and compared with β-actin which was used simultaneously as an internal control. The blots were transformed to digital format using a scanning densitometer (Bio-Rad,USA).

Statistical analysis

The percentage of survival and expression of stress protein was subjected to Arc sin transformation (T= arcsin √p), followed by ANOVA to determine the variation of survival and expression of HSP 70 in rotifers changed significantly with CPAs treatment.

Results and Discussion

The protective role of CPAs has been described in terrestrial invertebrates (Baust and Morrissey, 1977). It is generally known that DMSO is the most commonly used CPA for the cryopreservation of fish sperm and provides better protection at concentration between 5 and 25 %. In the present study, it was determined that 10 % concentration of DMSO was found suitable for the preservation of rotifer embryo than other CPAs. The patent of the CPA toxicity is dependent on the type of CPAs, concentration, equilibration time and temperature during preservation (Fahy, 1984: Edyta and Szurek, 2011).

The results clearly indicated that low survival rate was obtained with glycerol (30.33±2.5%) in comparison with DMSO (74.66±4.70%). Other CPAs such as methanol (65.33±2.5 %), propane diol (51.66±2.5 %) and ethylene glycol (40.66±2.5 %) resulted in a minimal survival rate of rotifer embryos respectively (Figure.1). However, survival of rotifer embryos using the methanol, propane diol, ethylene glycol and glycerol was significantly lower when compared with the DMSO ($P< 0.05$). Meryman *et al.,* (1997) has reported that CPAs such as glycerol, glucose, polyhydric alcohol and DMSO are known to function as effective cryoprotective agents during low temperature and in freezing conditions.

Although several studies on CPA toxicity have been show clear evident as an effective protectant its mechanism of action still remains poorly understood. Hydrophobic interactions between CPAs and proteins and the extent of hydrogen binding between CPAs and water molecules (Arakawa *et al*., 1990; Fahy *et al*., 2004) have been proposed as major cause for CPA toxicity. Nevertheless, it has also been shown that CPAs change the intracellular pH which cause intracellular Ca_2^+ release (Damien *et al*., 1990), and induce formaldehyde formation in cryopreservation medium (Karran and Legge, 1996). Further, it has been

questioned whether CPA toxicity is related to osmotic stresses that occur during addition and removal of CPAs. (Huang *et al*., 2006; Fahy, 1984). In addition, Pedro *et al*., (2005) clearly stated that PROH permeates into mouse oocytes faster than DMSO and EG, and thus causes less osmotic stresses among the tested CPAs. In our study, it is clear that DMSO and methanol at 10% concentration prove to be optimal for successful cryopreservation of rotifer embryos. Whereas other CPAs resulted in high mortality possibly due to the enhanced permeability provided by these CPAs which had an adverse effect on rotifer embryos.

On the other hand, there is an evidence that biological samples exposed to various CPAs during the course of preservation do experience the stress conditions. In few reports animals exposed to metals, hormones, pesticides and effluents cause genetically controlled tolerance to stressors. For example, by the induction of HSPs (Sanders, 1993; Janz *et al.,* 1997; Duffy *et al.,* 1999; Iwama *et al.,* 1999). While others have concluded that the threshold for HSP induction can be modified by acclimation and acclimatization. Heat shock proteins are known to play a pivotal role in protein homeostasis and the cellular stress response within cells (Lindquist, 1986). Dietz and Somero (1993) demonstrated that the summer-acclimatized *Gillichthys* sp. fish had significantly higher levels of HSP 90; there is strong evidence suggesting that HSPs have a critical role in adapting to such environmental change. In rotifers, Cochrane *et al*. (1991) found that HSP 58 was induced as a response to copper and tributyltin stress.

After the rotifer subjected to various CPAs, the embryos were analyzed for expression of HSP using SDS-PAGE (10%) followed by western-blot analysis. Immunoblot analysis of the rotifers revealed a single fraction at the proximal part of the membrane with a molecular weight of 70 kDa.

The detection of HSP70 by western blot revealed an increased expression of HSP70 in embryos treated with ethylene glycol, methanol, and glycerol compared to the DMSO and propane diol. Surprisingly, expression profile of HSP70 treated with DMSO and methanol showed a decrease in the HSP70 expression level which was almost similar to that of the control (Figure. 2a). The relative percentage of HSP70 expression was evident in untreated groups of rotifers when compared with β-actin (control). Statistical analysis clearly indicated the variation in the expression of HSP70 was significant in all CPAs ($P<0.05$). However, the level of expression in propane diol was comparable to the DMSO (Figure. 2b). The expression of HSP70 protein in the western blot analysis was further confirmed by HSP70 gene expression in RT-PCR analysis (Figure. 3). The differential expression of HSP70 was noticed (579 bp) in rotifers exposed to the CPAs. Among them ethylene glycol, methanol and glycerol showed elevated HSP70 gene expression in rotifer, whereas moderate expression level was observed in propane diol and DMSO. Therefore, based on all the above results it is clear that 10% of DMSO found to be more suitable to preserve the rotifer embryos. In addition, expression studies using western blot and RT-PCR analysis also emphasized the same by showing the low level expression of HSP70 using the DMSO. However, results were in *vice versa* for other CPAs resulting in higher level HSP70 expression and showed high mortality in the rotifer embryos.

Figure.1 Survival of *B. plicatilis* embryo exposed to 10% concentration of CPA with 20 min equilibration time (mean±SD of 50 no. of embryos for in each group-five replicates)

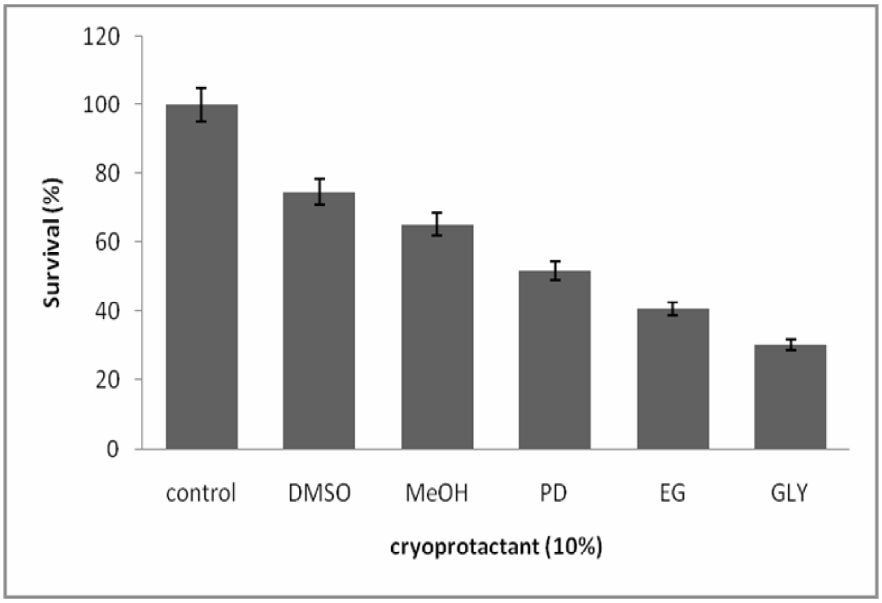
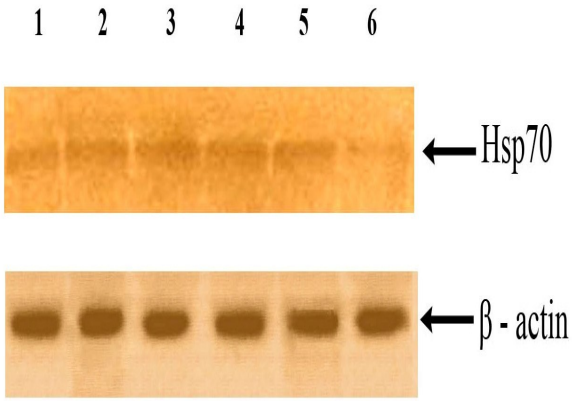


Figure.2a Western blot analysis of rotifer embryos with different CPAs



Supernatant separated on SDS-PAGE and electroblotted to a nitrocellulose membrane (0.45µM). The membrane was probed using mouse monoclonal Hsp70 antibody. Developed membrane probe shows single band with an estimated molecular weight of ~70 kDa

Lane 1. DMSO 2. Methanol 3. Propanediol 4. Ethylene glycol 5**.** Glycerol 6. Control (normal embryo)

Figure.2b *Hsp70* gene expression profile of rotifer embryo with different CPAs treated groups compared with β–actin as internal control.

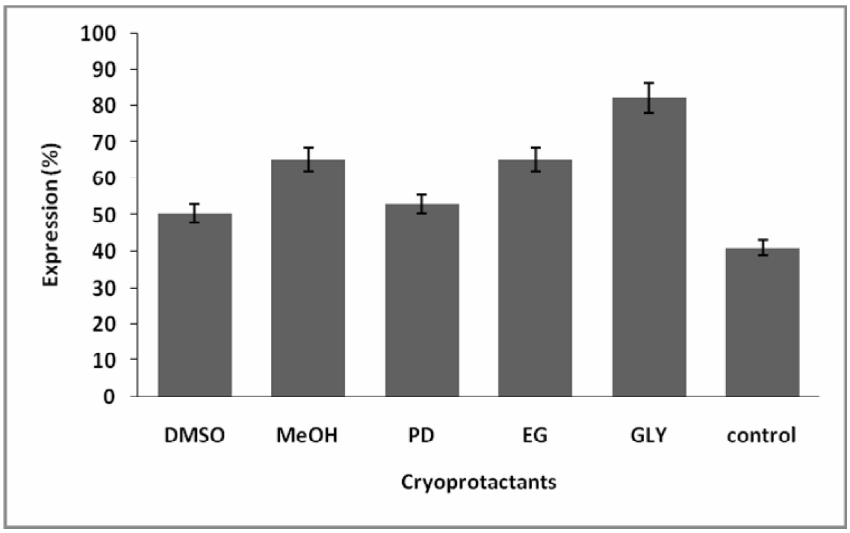
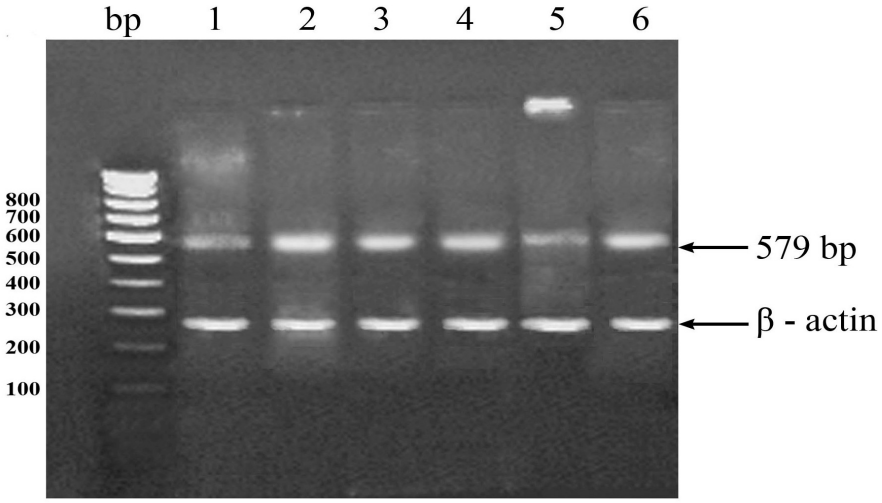


Figure.3 RT-PCR expression profile of *Hsp70* gene of rotifer embryo with different CPAs with β–actin as internal control



Amplification of Hsp70 gene expression in 1% agarose gel electrophoresis. The expected amplicon size of 579bp gene in rotifers subjected to different CPAs at 20 min equilibration time. (bp - Ladder 1000 bp

Lane 1. DMSO 2. Ethylene glycol 3. Glycerol 4. Methanol 5. Propanediol 6. Control (without CPA)

In conclusion, the results obtained in the present study gives us a strong evident that CPA and their concentrations are very crucial in order to obtain high recovery rate. On the other hand, the present investigation revealed minimal expression of HSP70 in rotifers exposed to methanol and DMSO compared to other treated groups. Thus, stress protein expression can be used as biomarkers for selection of suitable CPAs for embryo preservation. This study could serves as platform for the cryobiologist to employ and adopt a protocol for an effective cryopreservation of any biological samples. Further, this study certainly would have revolutionary impact not only in preservation of endangered species but also to retain and nurish the genetic resources.

Acknowledgement

Financial assistance from DST, Government of India (SR/SO/As-03/2003) and UGC- DRS-II are gratefully acknowledged. Thanks to Dr. V. Murugan (late) GE Lab, Anna University for his assistance in molecular studies.

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