

TOXIC EFFECTS OF CRYOPROTECTANTS ON OYSTER GAMETES AND EMBRYOS: A PRELIMINARY STEP TOWARDS ESTABLISHING CRYOPRESERVATION PROTOCOLS

Giovanni Sansone¹
Iracema A. Nascimento^{2,3}
Maria Bernadete N. L. Leite^{2,3}
Milena Maria Sampaio de Araújo²
Solange A. Pereira²
Ana Maria Mariani²

ABSTRACT

Aquaculture development is dependent on continuous seed production, regardless of the spawning season. Cryopreservation can therefore, be a valuable tool for achieving seed availability. The benefits of cryopreservation have been demonstrated for mammals, fish and, some achievements have been reported for invertebrates; however, cryopreservation has not yet contributed significantly to oyster rearing. The literature on this topic demonstrates that an optimal cryopreservation method developed for one species is not always applicable to another. One of the reasons is that there are differences among species in the toxicological responses of gametes and embryos to cryoprotectants. The aim of this study was to determine the toxic effects of cryoprotectants dimethylsulfoxide (Me2SO), glycerol (G), ethylene glycerol (EG), propylene glycerol (PG) and methanol (MET) on *Crassostrea rhizophorae* gametes and embryos. Gametes (oocytes and spermatozoa) and embryos (trochophores) were exposed to a range of concentrations of each cryoprotectant for 10, 20 and 30 minutes. Based on the EC₅₀-24h values there were no significant differences ($p > 0.05$) among the exposure times in toxic effects to either gametes or embryos. The trochophores were relatively resistant to cryoprotectant exposure, while oyster gametes became increasingly susceptible to the cryoprotectants as concentration levels were increased. Critical values (EC₅₀-24h) of cryoprotectants were markedly different for gametes and embryos. For gametes, both G and MET were more toxic (EC₅₀-24h respectively of 3.46 and 4.52% for oocytes, and 2.07 and 11.21% for spermatozoa) than EG, PG and Me2SO. However, PG (EC₅₀-24h = 23.56%) and EG (EC₅₀-24h = 45.18%) were more toxic for trochophores than Me2SO (EC₅₀-24h = 54.25) or MET (EC₅₀-24h = 55.63%). These results show the importance of previous toxicological studies for cryoprotectant selection as a preliminary step towards establishing cryopreservation protocols.

Key words: cryoprotectants, toxic effects, cryopreservation protocols, oyster gametes, trochophores.

RESUMO

O desenvolvimento da aquicultura é dependente da produção contínua de sementes; entretanto as desovas naturais ocorrem em períodos limitados. A criopreservação, desta forma, pode ser uma ferramenta valiosa para se conseguir a disponibilidade das sementes. Os benefícios da criopreservação foram demonstrados para mamíferos e peixes, e algumas experiências foram relatadas para invertebrados, inclusive bivalvos. Para ostras, foi demonstrado que um método otimizado de criopreservação desenvolvido para uma espécie, nem sempre é aplicável aos gametas ou aos embriões de outra. Uma das razões é que há diferenças nas respostas toxicológicas aos crioprotetores. O objetivo deste estudo foi determinar os efeitos toxicológicos dos crioprotetores dimetilsulfóxido (ME2SO), glicerol (G), etileno glicerol (EG), propileno glicerol (PG) e metanol (MET) em gametas e embriões de *Crassostrea rhizophorae*. Gametas

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¹ Dipartimento di Fisiologia Generale ed Ambientale, Università degli Studi di Napoli Federico II. Via Mezzocannone, 8-80134, Napoli Italy.

² Instituto de Biologia, Universidade Federal da Bahia. Campus Universitário de Ondina, CEP 40170-290, Salvador, Bahia, Brasil.
E-mail: iracema@ufba.br

³ Faculdade de Tecnologia e Ciências. Av. ACM, 3015, Iguatemi, CEP 41820-021 Salvador, Bahia, Brasil.
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(ovócitos e espermatozoides) e os embriões (trocóforas) foram expostos por 10, 20 e 30 minutos a uma escala crescente de concentrações destes crioprotetores. Nenhuma diferença significativa ($p > 0.05$) foi encontrada entre os períodos de exposição, tanto para os gametas como para os embriões. Entretanto, enquanto trocóforas foram relativamente resistentes à exposição, os gametas de ostra foram crescentemente mais susceptíveis aos crioprotetores à medida que os níveis de concentração foram aumentados. Os valores de CE_{50-24h} dos crioprotetores foram marcadamente diferentes para gametas e embriões. Para gametas, G e MET foram mais tóxicos (CE_{50-24h} respectivamente de 3.46 e 4.52% para ovócitos e 2.07 e 11.21% para espermatozoides) do que o EG, o PG ou o ME2SO. Para as trocóforas, PG ($CE_{50-24h} = 23,56\%$) e EG ($EC_{50-24h} = 45,18\%$) apresentaram maior toxicidade que ME2SO e MET. Os resultados mostram a extrema importância da avaliação toxicológica de crioprotetores como etapa preliminar para o estabelecimento de protocolos de criopreservação.

Palavras-chaves: crioprotetores, efeitos tóxicos, protocolos da criopreservação, gametas de ostra, trocóforas.

INTRODUCTION

The commercial cultivation of the mangrove oyster *Crassostrea rhizophorae* is increasing along the Northeast Brazilian coast (NASCIMENTO, 1983, 1994). However, it seems unlikely that sufficient natural spat will be available to support the growing demand. In spite of this species spawns throughout the year (NASCIMENTO, 1991a, 1991b), the availability of seeds is concentrated into two annual peaks (March/April and September/October). Therefore, the conditioning of brood stock and hatchery practices are necessary in order to obtain seeds throughout the year. These processes are dependent on high cost facilities. The cryopreservation of gametes or embryos appears to be a cheaper and less time-consuming alternative.

For the purpose of seed production, the thawing of cryopreserved material must follow the cryopreservation. This process involves a series of complex and dynamic physical and chemical alterations, which are associated with temperature changes. These changes may be drastic, causing the formation of ice crystals that in turn causes cellular death, especially during the cooling and freezing processes (HARVEY and ASHWOOD-SMITH, 1982). The addition of certain chemicals (cryoprotectants) can minimize such cell damages (LEUNG, 1991; LEUNG and JAMIESON, 1991). Capacity of penetration into cells and low toxicity are the most important properties inherent to these substances. However, both properties are associated with biological characteristics and may vary largely among organisms. Moreover, toxicological effects vary according to chemical concentration and the exposure time. Therefore, in order to select the best cryoprotectant for a particular application, it is important to determine the concentration and exposure time that minimize toxicological effects.

Techniques for the cryopreservation of gametes and embryos of several vertebrate species are already described (LEIBO and MAZUR, 1978; STOSS, 1983; FAHNING and GARCIA, 1992; RALL, 1993; POLLARD and LEIBO, 1994). Similar work has been successfully developed for a few species of aquatic invertebrates, (ASAHINA and TAKAHASHI, 1978; TOLEDO et al., 1989). However, reproducible techniques still need to be refined for bivalves (BOUGRIER and RABENOMANANA, 1986; TOLEDO et al., 1989; RENARD, 1989, 1991; RENARD and COCHARD, 1989; GWO, 1994; CHAO et al., 1997; PANIAGUA-CHAVEZ and TIERSCH, 2001). Different protocols for cryopreservation of oyster spermatozoa have been suggested for species such as *C. gigas*, *C. cucullata*, *C. iredalei* and *C. tulipa* (YANKSON and MOYSE, 1991). The important variables in these cryopreservation protocols were the selection of cryoprotective diluents and their concentration and exposition time. All these variables influence the cryoprotectant's capacity to cause toxic effects.

The aim of this study is to determine the toxicity of different cryoprotectants to *Crassostrea rhizophorae* gametes and embryos in order to contribute to the establishment of cryopreservation protocols.

MATERIALS AND METHODS

Oysters were collected from Barra dos Carvalhos (South of Bahia), an area free from industrial or domestic waste, then brought to the laboratory, cleaned of fouling organisms, and kept overnight in filtered and aerated sea water, at the same salinity level ($S = 28$) and temperature ($26 \pm 1^\circ\text{C}$) observed in the field. Gametes were obtained by stripping the oysters. Oocytes and sperm were placed separately into 2-L

glass beakers containing glass fiber (GF/C) filtered and sterilized seawater. Oocyte density in the suspension was determined by counting three samples, and the density was then adjusted to 10^4 oocytes/L (dos SANTOS and NASCIMENTO, 1985).

Part of each gamete suspension was reserved to generate embryos (from unexposed gametes), utilized as control in the experiments; the other part was used to provide gametes for the exposure tests. These consisted in exposing the gametes for 10, 20 and 30 minutes to the cryoprotectants dimethylsulfoxide (Me2SO); glycerol (GLY); ethylene glycerol (EG); propylene glycerol (PG) and methanol (MET), diluted (v/v) in sea water, at concentrations of 0 (control), 5, 10, 15 and 20%. The exposed oocytes were fertilized by unexposed sperm and vice-versa. Fertilization was done by adding 2 ml of a concentrated (turbid) sperm suspension to the oocyte suspension. One hour after fertilization, the suspensions were checked at light microscopy for the presence of cells in division. The embryos, from both origins, were counted and distributed at a concentration of 10/ml in triplicate test tubes containing seawater and left for 24h at room temperature ($26 \pm 20^\circ\text{C}$). After this period, 0,5 ml of buffered formalin (4%) was added to the test tubes and the material was examined for abnormalities. The numbers of normally and abnormally developed embryos were counted.

Trochophores were obtained 8h after the fertilization of unexposed gametes. They were counted, kept at a density of 10/ml and exposed to the different cryoprotectants at concentrations (v/v) in seawater of 0 (control), 5, 10, 15 and 20%. Exposure times of 10, 20 and 30 minutes were used. After the exposure time, the trochophores were retained in plankton net (36 μm size), washed, and kept in seawater ($S = 28$; 26°C). This test was also run for 24h. The trochophores were preserved by the addition of formalin to the test tubes and the abnormal and normal D-shaped larvae were then counted under microscope. Responses to the different treatments were recorded as the percentage of embryos failing to develop (or developing abnormally) in relation to the maximum number of D larvae expected ($n = 100$), in the absence of any treatment (NASCIMENTO et al., 2000a; NASCIMENTO et al. 2000b).

The percentage of abnormalities was calculated as the % net risk, according to FINNEY (1971)

$$\% \text{ net risk} = \frac{\% \text{ abnormal in treatment} - \% \text{ of abnormal in control}}{100 - \% \text{ of abnormal in control}} \times 100$$

Based on the data of % net risk for each cryoprotectant, the $\text{EC}_{50-24\text{h}}$ values were calculated using the Trimmed Spearman-Kärber Method (HAMILTON et al., 1977) and the values for different exposure periods (10, 20 and 30 minutes), were arc-sin transformed and compared by ANOVA using the SPSS, (SOKAL and ROHLF, 1981). No differences ($p > 0.05$) were observed. Consequently, the values for the different exposure periods were jointly considered for each cryoprotectant to calculate the final $\text{EC}_{50-24\text{h}}$ values, by using the regression method.

Based on the EC_{50} values, multiple range tests using the SNK (SOKAL and ROHLF, 1981) have allowed for the comparison of which cryoprotectants could produce the most or the least toxic effects on *C. rhizophorae* gametes and trochophores.

RESULTS

The data related to the effects of the cryoprotectants on *C. rhizophorae* gametes and embryos are presented in Table 1. For spermatozoa (Fig. 1), Glycerol (G) and methanol (MET) were very toxic, showing values of $\text{EC}_{50-24\text{h}}$ of 2.07 and 11.21% respectively. These differed significantly ($p < 0.05$) between themselves and from the other three cryoprotectants (Me2SO, EG and PG), showing $\text{EC}_{50-24\text{h}}$ values respectively of 15.30, 17.89 and 18.09%.

Similar responses were obtained for oocytes (Fig. 2). Glycerol (G) and methanol (MET) presented significantly ($p < 0.05$) higher toxicity ($\text{EC}_{50-24\text{h}}$ values of 3.46 and 4.52% respectively) than EG, PG and Me2SO, which had $\text{EC}_{50-24\text{h}}$ values of 12.04, 16.53 and 18.82% respectively, and did not differ from each other.

The trochophores were shown to be more resistant than the gametes to the exposure of all cryoprotectants utilized (Fig. 3), by presenting higher $\text{EC}_{50-24\text{h}}$ values. Furthermore, they presented, in comparison to gametes, a very different response pattern of relative sensitivity. Glycerol was the least toxic to the embryos (with a value of $\text{EC}_{50-24\text{h}}$ that was so high that it could not be calculated by Trimmed Spearman Karber or by PROBIT analysis). The $\text{EC}_{50-24\text{h}}$ values for Me2SO and MET were not significantly different ($p > 0.05$) from each other. The $\text{EC}_{50-24\text{h}}$ values for propylene glycerol (PG) and ethylene glycerol (EG) were lower (23.56 and 45.18% respectively) than for Me2SO (54.25%) and MET (55.63%), both of which were less toxic for the trochophore stage.

DISCUSSION AND CONCLUSIONS

The cells and embryos exposed to the cryoprotectants shrink by losing water and there is an influx of the cryoprotectant until equilibrium is reached (GWO, 1994). The re-expansion and survival of the cells are dependent on factors such as the permeability of the membrane to the cryoprotectant, and its toxicity. The first factor is directly related to exposure time and is determined by cell physiology and the surface-to-volume ratio (FAHY, 1984). Consequently, the efficiency of the cryoprotectant is associated with the rate of cell penetration, which is dependent on the nature of the cryoprotectant and the resistance offered by hatching envelopes or cellular membranes. Primarily, due to the complex interactions between these factors, cryopreservation methods cannot necessarily be applied inter-specifically between embryos and larvae (ROBERTSON and LAWRENCE, 1987; ROBERTSON et al., 1988). With regard to cryopreserved sperm cells of oysters species, evidence has also been found of inter-specific differences in viability, due not only to the concentration of Me2SO, but also to exposure time (YANKSON and MOYSE, 1991). Both factors are related to toxicity.

Cryoprotectants can suppress most cryoinjuries but when used at higher concentrations, most of them become toxic to biological material (LEUNG, 1991). The chemical and physical properties of most cryoprotectants are listed by NASH, 1966 (cited by LEUNG, 1991). Of these, water solubility and low toxicity are the most important for cryopreservation purposes.

Dimethylsulfoxide (Me2SO) inhibits catalase and peroxide activity. Glycerol is often found to be the least toxic cryoprotectant to most biological materials. The cell membrane, however, is often not very permeable to glycerol and hence, takes a relatively long time to equilibrate with the cell osmolarity. The large difference between the permeability of water and glycerol causes volume effects during the introduction and removal of this cryoprotectant (SCHNEIDER and MAURER, 1983). Unlike glycerol, the permeability of Me2SO is not markedly affected by low temperatures and is, therefore, the most widely used permeating cryoprotectant.

Ethylene glycerol and propylene glycerol decrease the polarity of the aqueous phase and change the partition of hydrophobic molecules between the cell membrane and the external phase, causing dehydration of the phospholipid bilayer and possible membrane damage (LEUNG, 1991). The cell membrane is

generally highly permeable to methanol, but it is generally considered the most toxic cryoprotectant.

For gametes, this research showed that the highest EC_{50-24h} values for the used cryoprotectants was 19%. This value represents the concentration that causes an acute toxic effect on 50% of the exposed gametes or embryos. According to FRESHNEY (1987) the cryoprotectants can be effective when used at concentration levels between 5 and 15%. However, at this range, some proportion of toxic effects is always expected for gametes cryopreservation (VAN der HORST et al., 1985). This has been noticed by KUROKURA et al., (1990) who suggested that the fertilizing capacity of the preserved *C. gigas* semen was 10^{-3} of that of fresh semen. In the case of oysters, this low fertilizing capacity could be compensated for, by increasing the amount of semen for insemination up to a concentration of 10^6 sperm/egg (SANTOS and NASCIMENTO, 1985).

The present research supports data from previous authors (CHEN et al., 1989; RANA et al., 1992; GWO, 1994) who reported that the trochophore stage was more resistant to cryoprotectants than earlier embryo stages or gametes. In fact, in this study, the EC_{50-24h} for trochophores treated with PG, Me2SO or MET was found to range from 23.56 to 55.63%. This helps to explain why the cryopreservation of oyster embryos is generally more successful than oyster gametes, whose oocytes present a high level of difficulty to be cryopreserved (CHEN et al., 1989; RANA et al., 1992).

Some previous findings (KUROKURA et al., 1990; GWO, 1994) showed that oyster gametes and embryos became increasingly susceptible to the cryoprotectants as the concentration of the cryoprotectant was increased and the equilibration time was lengthened. In the case of sperm cells, malformation of the acrosome has been noticed by slow cooling in the presence of cryoprotectants (KUROKURA et al., 1990). The exposure time varies with the cryopreservation protocols and is mostly related to the nature of the cryoprotectant and the test species. However, in most of these protocols a time of 10 to 30 minutes is considered favorable (LEUNG, 1991). In terms of exposure time the present research does not show significant differences in effects within a range of 10 to 30 minutes. However, critical values (EC_{50-24h}) of cryoprotectants were markedly different for gametes and embryos. For gametes, both G and MET were more toxic (EC_{50-24h} respectively of 3.46 and 4.52% for oocytes, and 2.07 and 11.21% for

spermatozoa) than EG, PG and Me2SO. However, PG (EC_{50} -24h = 23.56%) and EG (EC_{50} -24h = 45.18%) were more toxic for trochophores than Me2SO (EC_{50} -24h = 54.25, G or MET (EC_{50} -24h = 55.63%).

The results found here provide not only toxic indicator levels (EC_{50} -24h values) for G, Me2SO, PG, EG and MET but also permitted a comparison of effects between five of the most commonly used cryoprotectants. For gamete cryopreservation, Me2SO, PG and EG shall be preferably used, since they were the least toxic among the cryoprotectants (average EC_{50} -24h values varying from 12.04 to 18.82). For trochophore, a different toxicity trend was shown, methanol and glycerol were the least toxic and shall be selected as cryoprotectants. These results show the importance of previous toxicological studies for cryoprotectant selection as a preliminary step towards establishing cryopreservation protocols.

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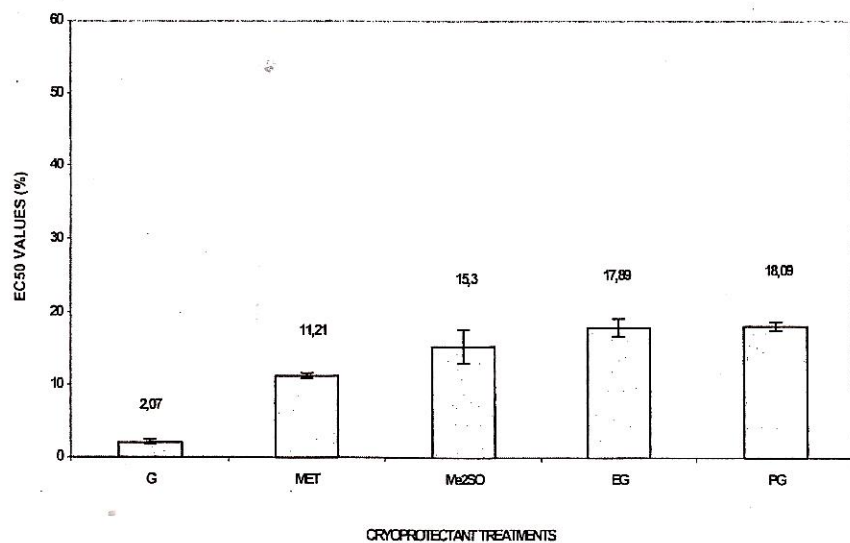
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TABLE 1 – EC₅₀-24h (%) mean values and other statistical data for *C. rhizophorae* early embryos and trochophores exposed to cryoprotectants

Cryoprotectant	Unexposed oocytes × Exposed spermatozoa					Unexposed spermatozoa × Exposed oocytes					Trochophores originated from unexposed gametes				
	CE50 Values	CE50 Means Values	SD	Lower 95% Conf. Limit	Upper 95% Conf. Limit	CE50 Values	Ce50 Means Values	SD	Lower 95% Conf. Limit	Upper 95% Conf. Limit	CE50 Values	Ce50 Means Values	SD	Lower 95% Conf. Limit	Upper 95% Conf. Limit
Glycerol	2.14 1.69 2.39	2.073	0.355	1.192	2.955	1.51 4.85 4.01	3.456	1.737	-0.859	7.773	-	-	-	-	-
Methanol	11.20 11.60 10.83	11.210	0.385	10.253	12.167	7.74 3.27 2.56	4.523	2.808	-2.453	11.500	56.05 55.52 55.33	55.633	0.373	54.706	56.560
Me2SO	16.71 16.58 12.61	15.300	2.331	9.510	21.090	20.48 20.45 15.53	18.820	2.849	11.741	25.899	60.90 47.30 54.56	54.253	6.805	37.347	71.160
Etylene Glycerol	16.52 18.24 18.91	17.890	1.233	14.827	20.953	15.54 11.00 9.58	12.040	3.113	4.306	19.774	45.00 45.48 45.06	45.180	0.261	44.530	45.830
Propylene Glycerol	18.77 17.97 17.55	18.096	0.619	16.557	19.636	15.75 18.63 15.21	16.530	1.839	11.962	21.098	23.26 24.08 23.35	23.563	0.449	22.446	24.681

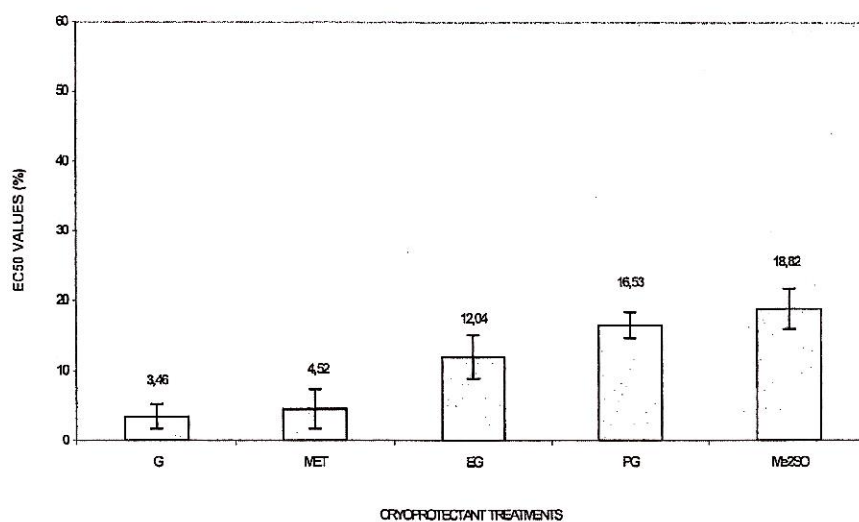


MULTIPLE RANGE TEST

Values united by dashed line are not significantly different ($p > 0.05$)

TREATMENTS	G	MET	Me2SO	EG	PG
MEANS	2.07	11.21	15.30	17.89	18.09

Fig. 1. Means, standard deviations and multiple range comparison test (SNK) results based on EC_{50} -24h (%) values for *C. rhizophorae* spermatozoa, exposed to glycerol (G), methanol (MET), dymethylsulfoxide (Me2SO), etylene glycerol (EG) and propylene glycerol (PG).

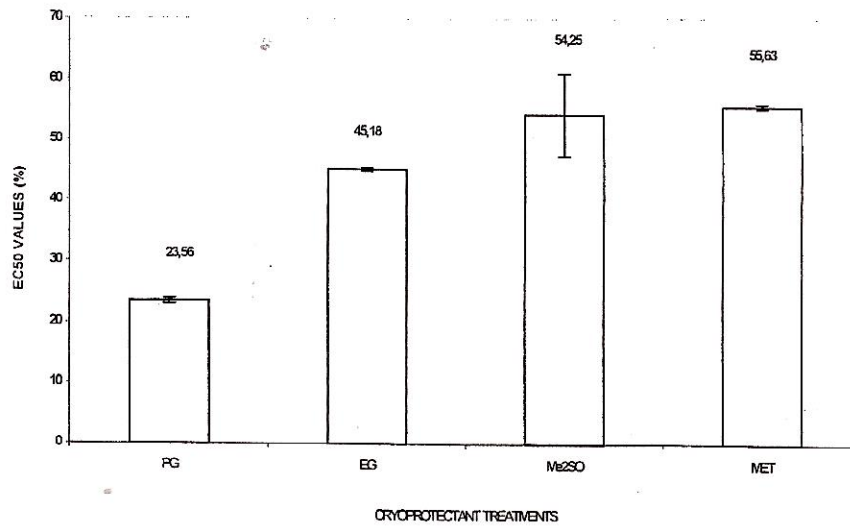


MULTIPLE RANGE TEST

Values united by dashed line are not significantly different ($p > 0.05$)

TREATMENTS	G	MET	EG	PG	Me2SO
MEANS	3.46	4.52	12.04	16.53	18.82

Fig. 2. Means, standard deviations and multiple range comparison test (SNK) results based on EC_{50} -24h (%) values for *C. rhizophorae* oocytes, exposed to glycerol (G), methanol (MET), dymethylsulfoxide (Me2SO), etylene glycerol (EG) and propylene glycerol (PG).



MULTIPLE RANGE TEST

Values united by dashed line are not significantly different ($p > 0.05$)

TREATMENTS	PG	EG	Me2SO	MET
MEANS	23.56	45.18	54.25	55.63

Fig. 3. Means, standard deviations and multiple range comparison test (SNK) results based on EC_{50-24h} (%) values for *C. rhizophorae* trochophore, exposed to methanol (MET), dimethyl sulfoxide (Me2SO), ethylene glycerol (EG) and propylene glycerol (PG).