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CRYOPRESERVATION OF PRIMARY CELL CULTURES OF MARINE INVERTEBRATES

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Summary

Primary cell cultures obtained from somatic and larval tissues of bivalve molluscs and from embryos of sea urchins were frozen to –196ºC by two-step freezing using 10% dimethyl sulfoxide (DMSO) or/and trehalose (3-30mg/ml) as cryoprotectants. We estimated both cell viability and the RNA synthetic activity after freeze-thaw. Total lipid extracts from the tissues of echinoderms examined as possible cryoprotective agents demonstrated a weak cryoprotective capacity. Mussel lipid extract was found to possess a considerable cryoprotective activity. Cryoprotective capacity of tested lipids correlated with their thermotropic behaviour. DMSO + trehalose combination was shown to be a favourable cryoprotectant and sea urchin blastula cells the most freezing-tolerant cells.

Keywords: Cryopreservation, primary cell culture, mollusc, sea urchin, trehalose, total lipids, phase transition

INTRODUCTION

It has been well established that the cells of a wide variety of organisms may be successfully cryopreserved. Nevertheless, there have been very few studies on freezing tolerances in the cells of invertebrates. Single examples of cryopreservation of primary cell cultures of invertebrates, insects (16, 19) and molluscs (22), have been reported. Development in methods of cryopreservation permits the removal of season and geographic limitations of investigations on sea animals, helps to establish cryobanks of rare and disappearing species and would be a useful tool in cell technology of marine bioactive materials.

Sperm cells of marine invertebrates and fishes are successfully preserved in liquid nitrogen (2, 4, 20, 38). However, cryopreservation methods described for embryos and larvae of these animals are still inadequate in maintaining the structure and activity of embryos after freeze - thawing (2, 4, 12, 21, 34, 38). Post-thaw development in cryopreserved embryos often showed abnormalities in structure and colour, which were difficulty to analyse quantitatively (11). Cultured cells of marine invertebrates could be used as a model system in studies on cryoprotection and help to choose an optimal cryoprotectant quickly and estimate quantitatively the chilling injury.

The main site of damage during freeze-thaw cycles is the bilayer structure of membranes (15). During freezing and thawing, cell damage is due to the destructive action of the

concentrated salt solutions to which the cells are exposed when water is removed as ice. As a result of cold shock, lipids as well as some membrane proteins are lost from mammalian spermatozoa (3, 26, 28). The protective effect of phospholipids against cold shock in mammalian spermatozoa is due to a "loose" interaction of lipid structures with the plasma membrane of the cells (36). Phospholipids of marine invertebrates could be used for cell cryopreservation, because their crystalline to liquid crystalline phase transitions lie mainly in the low temperature range (from –50º to 0ºC) (30, 35).

However, not only lipids, but also the carbohydrates could significantly influence membrane integrity. It has been reported earlier that OH-groups of the carbohydrate moiety of the antifreeze proteins have a structuring effect on water (9), and the high carbohydrate content (57%) of antifreeze proteins of certain Antarctic fishes is necessary for the ice-formation-inhibiting activity (8). Among many tested carbohydrates, trehalose showed maximal stabilizing effect (5) reducing mobility of water molecules connected with carbohydrates. At physiological concentrations, this stabilizing effect is a property of trehalose not shared by other carbohydrates (6). In addition, trehalose is one of a number natural cryoprotectants (29).

In this study we used dimethyl sulfoxide (DMSO) as a main cryoprotectant and tested the compounds with potential cryoprotective properties both carbohydrate and lipid nature. The advantage of DMSO over other cryoprotectants was shown for sea urchin embryos (2, 21), for carp embryos (38), and barnacle larvae (12). In our earlier study, we have shown that DMSO was better than glycerol as a protectant for mollusc cells (22). Carbohydrates were found to reduce distinctly toxic effects of the main cryoprotectants and to promote cell integrity (5). The aim of the present study is the search for optimal cryoprotectants for marine invertebrate cells.

MATERIAL AND METHODS

Materials

Molluscs (*Mytilus trossulus* and *Mizuchopecten yessoensis*) and sea urchins (*Strongylocentrotus nudus*) were collected in Vostok Bay of the Sea of Japan for experiments. Primary cell cultures from gills and muscles of molluscs, and from larvae of molluscs and sea urchins were used as a model. The embryonic material was obtained by artificial fertilization. Spawning was induced by thermal shock or an electric shock (8 - 12 V). The embryos were placed in closed tanks with UV-sterilised seawater (17ºC). Molluscan larvae at the trochophore stage (24-30 h after fertilization) and sea urchin larvae at the blastula and gastrula stages (16 h and 24 h after fertilisation, respectively) were collected onto a finely-meshed gauze, washed in Ca^{2+}- and Mg^{2+}-free artificial sea water (CMFSS) containing antibiotics (100 IU:100 μg/ml penicillin:streptomycin, Serva, Feinbiochemical, Heidelberg, Germany), then twice washed in sterile sea water with the same antibiotics.

Lipid preparation

Total lipids were extracted from tissues of the most accessible species of marine invertebrates in the Sea of Japan: echinoderms (sea urchin *Strongylocentrotus intermedius* and starfish *Asterias amurensis*) and mussel (*Crenomytilus grayanus*). The tissues of whole animals were homogenised, and lipid extraction was performed by the chloroform-methanol procedure according to Folch *et al*. (17). Aliquots of total lipid extracts were mixed with heated culture medium (60-70ºC) for 1-2 min and then the mixture was treated by ultrasound (at 22 kHz for 1-2 min). Lipid emulsions were stable for 3-4 days at 4ºC.

Primary cell cultures

Primary cell cultures were obtained as described earlier (23). In brief, the tissues and embryos were dissociated with 0.125% collagenase (produced from the liver of the crab *Paralithodes camtschatica* in the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia) during 2.0-2.5 hr at 10-12^{0}C for adult animals and 20-30 min for larvae. Dissociated cells were washed several times in sea water with antibiotics added (penicillin G, 500 U/ml and gentamycin, 40 mg/ml, Gibco Laboratories, Grand Island, NY, USA), and then a Leibovitz medium (Flow Laboratories, Irvine, Scotland) modified by Odintsova and Khomenko (23) was added to give a count of 2-40 x 10^6 cell/ml. The nutrient medium (1100 mOsmol) was supplemented with 2% fetal calf serum (Gibco Laboratories, Grand Island, NY, USA), insulin (5 mg/L, Sigma Chemical Co., St.Luis, MO, USA), α-tochopheryl-acetate (1.75 mg/L, Sigma Chemical Co., St.Luis, MO, USA), glucosamine (50 mg/L, Sigma Chemical Co., St.Luis, MO, USA), glutamine (100 mg/L, Serva, Feinbiochemical, Heidelberg, Germany) and gentamycin (40 mg/L, Sigma Chemical Co., St.Luis, MO, USA).

Freezing of cell suspension

To investigate the role of cryoprotectants in preventing damage to the embryonic and somatic cells of the bivalve molluscs and the sea urchins, several cryoprotectants were tested. Effects of trehalose (3-30 mg/ml) and lipid extracts (LE) from tissues of some marine invertebrates were examined both in complex with, and without, major cryoprotectant - (DMSO - Sigma Chemical Co., St.Luis, MO, USA) at a final concentration of 10%. It has been reported earlier that DMSO (at a final concentration of 5-10%) is toxic at room temperature for cells and tissues of various animals (4), and therefore, all procedures were carried out in an ice water bath, and all solutions were cooled to 10^{0}C. All cryoprotectant solutions were prepared in a modified Leibovitz medium (L-15 M). Aliquots of cell suspension (0.33 ml) were transferred into 2 ml-polypropylene sterile ampoules (Corning, NY, USA) and then the growth medium or cryoprotectant solutions (1 ml) were added gradually. Lipid emulsion was added into the cell suspension to the final concentration of 0.085-0.15% by volume (this concentration was chosen after preliminary experiments in which the effect of lipid extracts on cell viability was tested in the concentration range of 0.037-0.5% - data not shown). The samples were maintained in an ice water bath for an equilibration period of 15 min prior to freezing. Cooling to -196ºC was performed by two-step freezing (22), at first the cell suspension was rapidly cooled to -28ºC at a rate of 6-8ºC/min by immersion in an ethanol-glycerol mixture in thermostatic circulator (LKB, Bromma, Sweden) for 15 min and then by plunging in liquid nitrogen for storage.

Thawing of cell suspension

After storage in liquid nitrogen for 1-30 days, the ampoules were put into a water bath at 20-22ºC at continuous mixing. Immediately after thawing, the content of ampoules was transferred into cooled sterile centrifuge tubes. A dilution of the cryoprotectant in the sample medium was made by a gradual washing with 10 volumes of sterile sea water at 0ºC. Samples were centrifuged for 5 min at 1000g, washed by fresh seawater and finally the cells were resuspended into 0.4 ml of the growth medium. The thawed cells were cultivated at 17ºC for 12-48 h onto 24 multi-well plates (Corning, NY, USA). Cell viability was estimated by simultaneous staining with fluorescein diacetate (a marker for live cells)–propidium iodide (a marker of dead cells - 18) or by the trypan blue exclusion test. There were no significant differences in cell viability determined by different staining methods. The cell viability was monitoring at once and for 24 h after thawing. We estimated the number of viable, dead and damaged cells. The number of damaged cells was given by the difference between initial cell number (before freeze-thaw) and the total number of viable and dead cells after freeze-thaw.

All results were compared with those for control unfrozen cells of the original primary cultures. Each experiment was carried independently at least three times, and assays were performed in triplicate.

(H^3)-uridine incorporation

The level of RNA synthesis (the level of (H^3)-uridine incorporation) was determined as described (24). The thawed cells were plated at 5 x 10^5 cells per well, cultivated at 17^0C for 24 h and then the labelled precursor (^{3}H-uridine, 10 μCi/ml, specific activity 0.7 Ci/mmol, Amersham, Buckinghamshire, UK) was added to the cell suspension for 24 h. The contents of the wells were quantitatively deposited on the membrane filters (0.45 μm, Hemapol, Prague, Czechoslovakia). Additionally, the wells were washed twice with CMFSS containing EDTA (15 mM). To remove material that non-specifically binds to the cells, the filters were washed in 10% ice-cold trichloroacetic acid (10 ml) followed by ethanol and put into vials with 5 ml of a toluene scintillator. Radioactivity was measured on a LC-30 counter (Intertechnique, France). Experiments were run in triplicate.

Statistical analysis

Results are presented as means ± SEM. The data were analysed by pairwise comparisons (control versus all treatments) using Student's t-test (with Sidak′s correction for multiple comparisons) (32). The 0.05 level was selected as the point of minimal statistical significance in all analyses.

Differential scanning calorimetry (DSC)

DSC analysis was used to investigate the crystal - liquid crystal phase transitions of tested lipids, as well as to define the melting temperature of the solutions of DMSO (10%) and/or trehalose (3 –30 mg/ml). Vacuum dried lipid samples of approximately 10 mg were sealed into pans and placed in a DSC-2M differential scanning calorimeter (Experimental design bureau, Puschino, Russia). Lipid samples were either heated or cooled at 16°C per min in temperature range between -100°C and 80°C at a sensitivity of 5 mW. Aliquots of the culture medium and its mixtures with 10% DMSO and/or trehalose (3 –30 mg/ml) were melted and cooled at a sensitivity of 40 mW. The peak in the heat capacity was recorded as the transition temperature. The temperature range was calibrated by using naphthalene, mercury and indium as described (31).

RESULTS

Table 1 shows the number (in %) of viable, dead and damaged cells in mollusc primary cultures of somatic and larvae cells after freeze-thaw. Viability of control unfrozen cells of the original primary cultures was 90-95% both for larval cultures and cultures from adult mollusc tissues. After freeze-thaw cell viability fell sharply: the majority of the cells (60-95%) were destroyed. The most freezing-tolerant cells of the molluscs are gill cells. In the presence of DMSO the number of viable cells increased to 20-30% in primary culture of gills whereas that it was in the range of 3-10% in larval or muscle primary cultures. Cell viability of mussel larval cells after the thawing was actually the same irrespective of cryoprotectants used. For the larval cells of *M. yessoensis* trehalose, when used alone, gave only a low cell survival, but in combination with DMSO it gave an increased viability (Table 1).

The incorporation level of (H^3)-uridine in primary cell culture of the mussel *Mytilus trossulus* after freeze-thaw is presented on Fig. 1. The essential differences of the cell condition after DMSO and other cryoprotectants were observed in primary cell cultures from mussel larvae (Fig. 1A). It should be noted that the incorporation level of (H^3)-uridine in the

Table 1. The viability of mollusc cells after freeze-thaw and cultured at 17ºC for 24 h

Object / cryoprotectants	Viable cells (%)	Dead cells (%)	Damaged cells (%)
***Mytilus trossulus* Trochophore cells**			
without cryoprotectants	2.6 ±0.55	4.2 ±0.22	93.2±2.8
DMSO	5.0 ±0.35*	34.5 ±1.73	60.5±1.81
DMSO + trehalose (3 mg/ml)	5.0 ±0.35*	33.5 ±1.68	61.5±1.85
DMSO + trehalose + mussel LE	5.0 ±0.34*	21.5 ±1.08	73.5 ±2.2
trehalose (3 mg/ml)	2.9 ±0.2	4.4 ±0.22	92.7 ±2.7
trehalose + mussel LE	6.5 ±0.46**	6.5 ±0.33	87.0 ±2.61
trehalose + sea urchin LE	3.15 ±0.22	1.75 ±0.09	95.1±2.8
***Mytilus trossulus* Gill cells**			
without cryoprotectants	5.5 ±0.42	3.7 ±0.15	90.8 ±2.7
DMSO	20.0 ±1.4**	15.0 ±0.75	65.0 ±1.95
DMSO + trehalose (3 mg/ml)	24.4 ±1.7**	10.6 ±0.53	65.0 ±1.9
DMSO + trehalose + mussel LE	30.1 ±2.1**	10.4 ±0.5	59.5 ±1.8
trehalose (3 mg/ml)	7.5 ±0.52*	2.0 ±0.1	90.5 ±2.55
trehalose + mussel LE	5.9 ±0.40	4.3 ±0.22	89.8 ±2.7
trehalose + sea urchin LE	4.8 ±0.33	3.2 ±0.15	92.0 ±2.7
***Mizuchopecten yessoensis* Trochophore cells**			
without cryoprotectants	2.7 ±0.21	1.8 ±0.33	95.5 ±2.84
DMSO	8.5 ±0.6**	2.9 ±0.15	88.6 ±2.7
DMSO + trehalose (3 mg/ml)	8.3 ±0.55**	2.8 ±0.14	88.9 ±2.51
DMSO + trehalose + mussel LE	10.3 ±0.71**	4.2 ±0.2	85.2 ±2.45
trehalose (3 mg/ml)	3.15 ±0.22	1.75 ±0.11	95.0 ±2.59
trehalose + mussel LE	3.3 ±0.23	1.3 ±0.06	95.4 ±2.81
***Mizuchopecten yessoensis* Muscle cells**			
without cryoprotectants	1.1±0.55	1.3 ±0.60	97.6 ±2.83
DMSO	3.6 ±0.25**	1.0 ±0.06	95.4 ±2.82
DMSO + trehalose (3 mg/ml)	8.3 ±0.59**	2.7 ±0.13	89.0 ±2.3
DMSO + trehalose + mussel LE	10.2 ±0.7**	4.6 ±0.21	85.2 ±2.25
trehalose + mussel LE	3.3 ±0.25**	1.3 ±0.07	95.4 ±2.83

LE = Lipid extract, * $p < 0.05$; ** $p < 0.01$.

control (unfrozen) cells was almost 2-fold higher than that in cells protected by 10% DMSO, DMSO + trehalose combination or trehalose alone. Mussel lipid extract was found to possess a considerable cryoprotective activity for mussel cells, especially at the presence of trehalose. The incorporation of (H^3)-uridine in the larval cells protected by trehalose + mussel lipid extract combination amounted to that of unfrozen cells. There was no advantage between any cryoprotectants used for gill cells (Fig.1B). Thawed gill cells demonstrated a minor fall of the RNA synthesis in comparison with control (unfrozen) cells**.** In cells frozen without any cryoprotectants (only in the growth medium), the RNA synthesis was almost unappreciable (data not shown).

The ratio between viable, dead and damaged cells in sea urchin embryonic primary cultures is presented in Table 2. Viability of control unfrozen cells of both blastula- and gastrula-derived cultures was 95-98%.

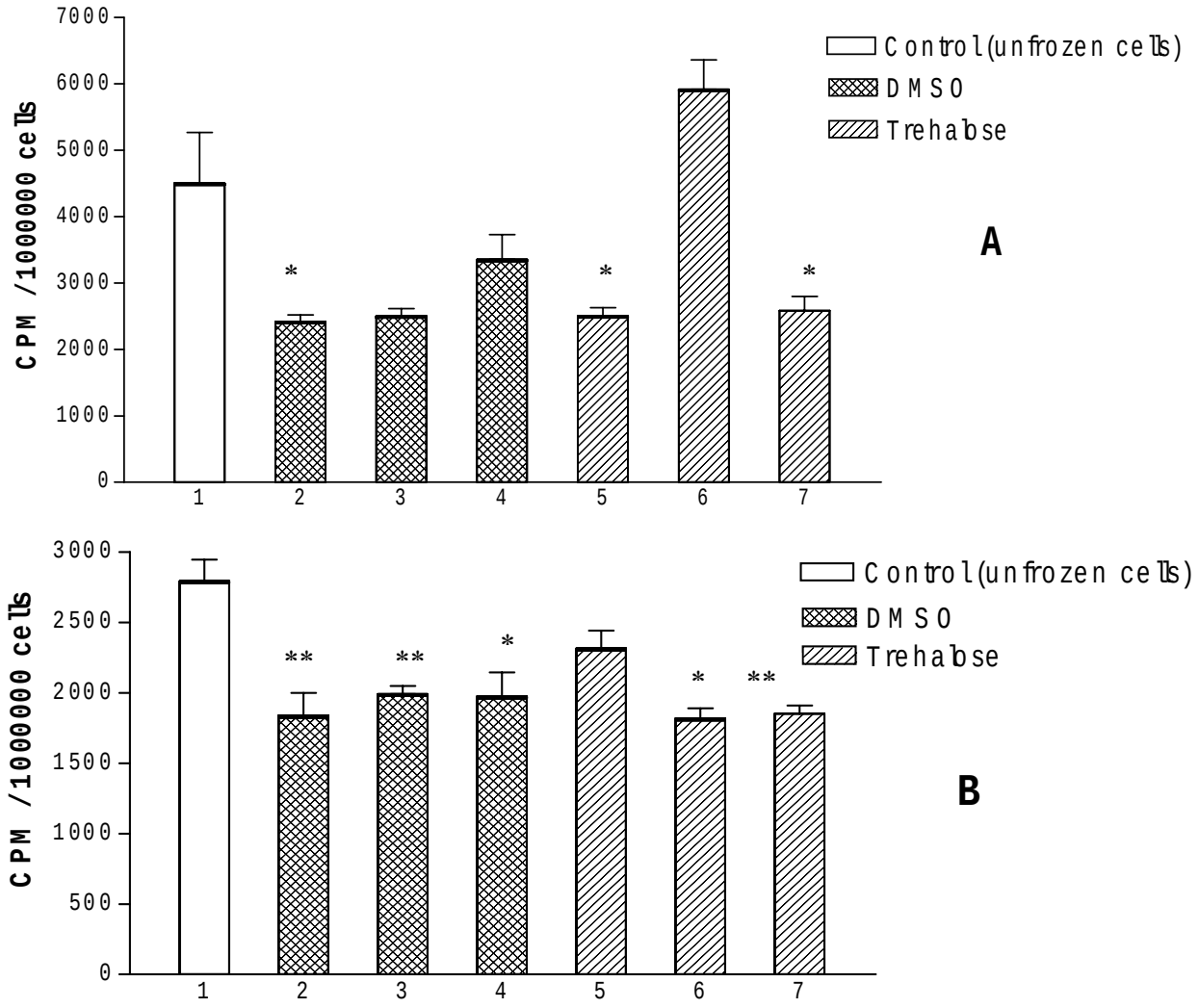

Figure 1. (H^3)-uridine incorporation in the cells of the mussel *Mytilus trossulus* after freeze-thaw in primary culture: A – trochophore cells; B – gill cells. Treatment key:- Unfrozen cells (1); cells frozen with DMSO (2); DMSO + trehalose (3); DMSO + trehalose + total lipid extract from the mussel (4); trehalose (5); trehalose + total lipid extract from the mussel (6); trehalose + total lipid extract from tissues of the sea urchin (7). Results expressed as radioactive counts per minute in $1x10^6$ cells. The thawed cells were cultivated at 17°C for 48h. * $p < 0.05$; ** $p < 0.01$.

On the whole, the sea urchin cells are more freezing-tolerant than the molluscan cells. But for all that, the survival of gastrula cells of the sea urchin was higher than that of blastula cells after cryopreservation (Table 2). Nevertheless, the majority of the cells was destroyed after freeze-thaw: 35-90% in blastula-derived culture and 35-70% in gastrula-derived culture. The essential differences of the cell viability after DMSO and trehalose cryoprotection were observed in blastula cells. The addition of trehalose to the major cryoprotectant (DMSO) intensified the cryoprotective effect in embryonic sea urchin cells of both developmental stages. Total lipid extracts from the tissues of echinoderms examined as possible cryoprotective agents demonstrated a weak cryoprotective capacity for gastrula cells and had toxic effect for blastula cells.

The incorporation level of (H^3)-uridine in embryonic sea urchin cells in blastula-derived culture protected with DMSO or trehalose was almost the same as in the control (unfrozen)

Table 2. The viability of the embryonic cells of the sea urchin *Strongylocentrotus nudus* after freeze-thaw and cultured at 17°C for 24h

Object / cryoprotectants	Viable cells (%)	Dead cells (%)	damaged cells (%)
Blastula cells			
without cryoprotectants	2.6 ±0.18	17.4 ±0.87	80.0 ±2.7
DMSO	8.6 ±0.6**	18.8 ±0.97	72.6 ±2.15
DMSO + 10% FCS	13.1 ±0.9**	52.3 ±2.63	34.6 ±1.05
DMSO + trehalose (3 mg/ml)	16.5 ±1.2**	21.9 ±1.1	61.6 ±1.8
DMSO + trehalose (30 mg/ml)	3.4 ±0.2*	7.1 ±0.3	89.5 ±2.7
DMSO + trehalose (3 mg/ml) + sea urchin LE	2.6 ±0.18	27.0 ±1.3	65.2 ±2.0
DMSO + trehalose (3 mg/ml) + starfish LE	1.6 ±0.1**	25.4 ±1.27	73.0 ±2.17
trehalose (3 mg/ml)	20.3 ±1.5**	17.4 ±0.87	62.3 ±1.9
trehalose (3 mg/ml) + sea urchin LE	14.4 ±1.2**	14.7 ±14.7	70.9 ±2.2
trehalose (3 mg/ml) + starfish LE	6.8 ±0.49**	22.8 ±1.15	70.4 ±2.19
trehalose (16 mg/ml)	19.6 ±1.5**	21.3 ±1.1	59.1 ±1.8
trehalose (30 mg/ml)	18.9 ±1.47**	19.9 ±1.0	61.2 ±1.9
Gastrula cells			
without cryoprotectants	12 ±1.05	20.5 ±1.91	67.5 ±2.45
DMSO	18.7 ±1.46*	36.6 ±1.83	44.7 ±2.3
DMSO + 10% FCS	10.3 ±0.72	22.8 ±1.15	66.9 ±2.1
DMSO + trehalose (3 mg/ml)	32.2 ±2.5**	12.8 ±0.65	55.0 ±2.03
DMSO + trehalose (30 mg/ml)	20.9 ±1.4**	22.3 ±1.15	56.8 ±1.7
DMSO + trehalose (3 mg/ml) + sea urchin LE	25.3 ±1.8**	37.4 ±2.0	37.3 ±1.8
DMSO + trehalose (3 mg/ml) + starfish LE	21.8 ±1.55**	24.6 ±1.6	53.6 ±1.9
trehalose (3 mg/ml)	20.4 ±2.04*	20.7 ±1.61	58.9 ±2.1
trehalose (3 mg/ml) + sea urchin LE	15.7 ±1.1	39.3 ±2.0	45.0 ±2.1
trehalose (3 mg/ml) + starfish LE	21.6 ±1.5**	37.7 ±1.8	40.7 ±1.82
trehalose (16 mg/ml)	22.2 ±1.7**	23.05 ±1.8	54.75 ±1.6
trehalose (30 mg/ml)	23.3 ±1.72**	41.34 ±2.2	35.36 ±1.7

LE = lipid extract, * $p < 0.05$; ** $p < 0.01$.

cells (Fig. 2A). The RNA synthesis was much lower in the sea urchin cells of the later stages of development (Fig. 2 B): this level in frozen cells in gastrula-derived culture was more than 2-fold lower of that in the control cells. It should be noted that the synthetic activity was reduced with the increase of trehalose concentration. The similar trend was observed both in blastula- and gastrula-derived cultures. Cell freezing without cryoprotectants resulted in almost total loss of cell functional activity (data not shown).

Echinoderm lipid extracts were found to have no positive effect in the presence of trehalose both for mollusc and sea urchin cells (Figs. 1 & 2). Indeed, these extracts, as well as 10% fetal calf serum (Fig. 2), resulted in significant decrease in the RNA synthesis in sea urchin cells after freeze-thaw at the presence of 10% DMSO.

Thermograms of tested total lipids, DMSO and trehalose are presented in Fig. 3. The transition ranges were for mussel lipid extracts from –80 to 10°C, for sea urchin from –60 to 40°C, and for starfish from –45 to 45°C (Fig. 3 A). Practically, the thermogram of mussel total lipids is located at temperature below 0^0C. Peak maximum temperature for mussel lipid extract was –12°C. Whereas, the substantial part of thermograms of both echinoderms is

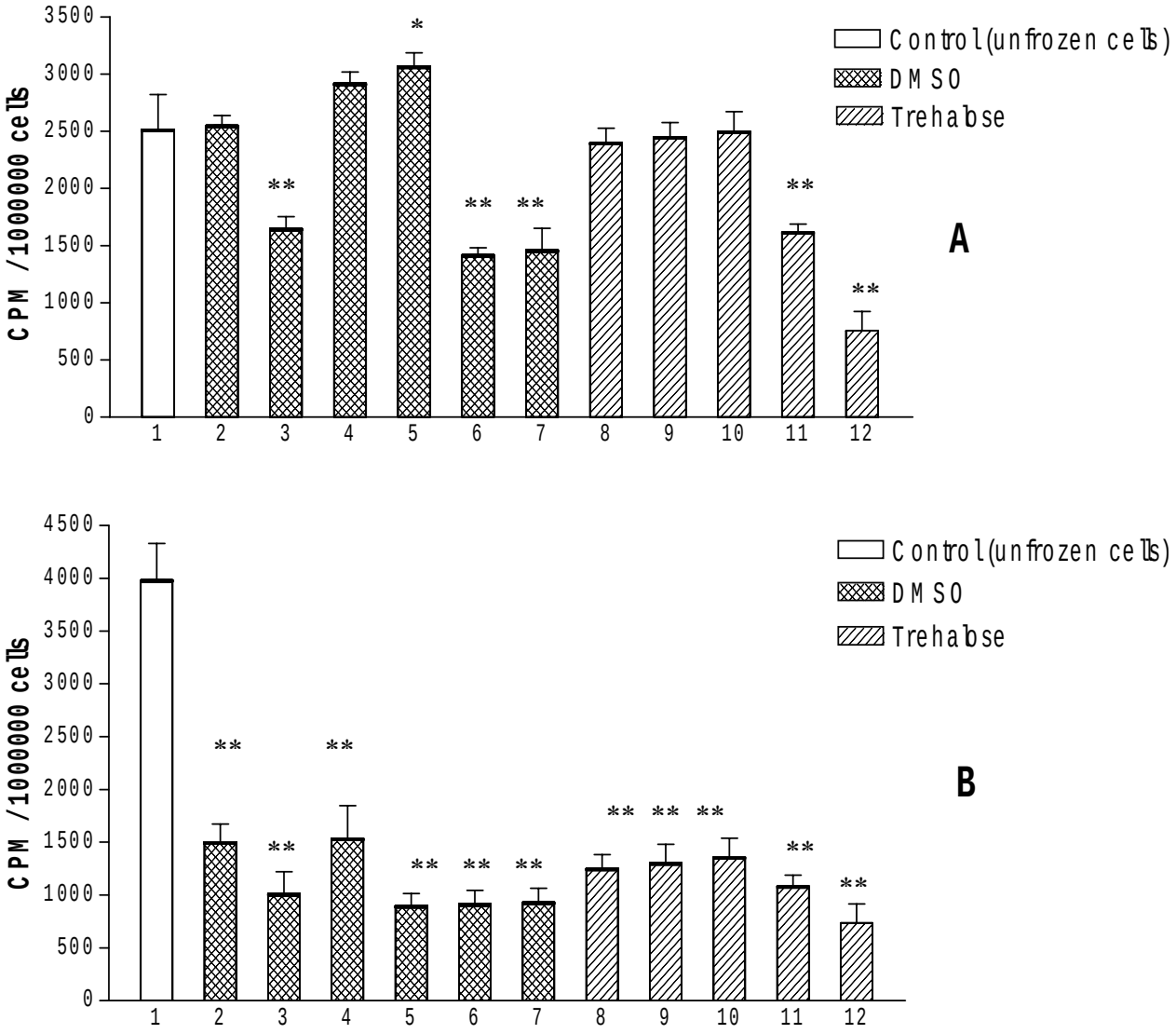

Figure 2. (H^3)-uridine incorporation in sea urchin embryonic cells after freeze-thaw: A - in blastula-derived culture; B – in gastrula-derived culture. Treatment key:- Unfrozen cells (1); cells frozen with DMSO (2); DMSO + 10% FCS (3); DMSO + trehalose, 3 mg/ml (4); DMSO + trehalose, 30 mg/ml (5); DMSO + trehalose (3mg/ml) + total lipid extract from the sea urchin *S. intermedius* (6); DMSO + trehalose (3 mg/ml) + total lipid extract from starfish *A. amurensis* (7); trehalose, 3 mg/ml (8); trehalose (3 mg/ml) + total lipid extract from the sea urchin *S. intermedius* (9); DMSO + trehalose (3 mg/ml) + total lipid extract from the starfish *A. amurensis* (10); trehalose, 16 mg/ml (11); trehalose, 30 mg/ml (12). Results expressed as radioactive counts per minute in $1x10^6$ cells. The thawed cells were cultivated at 17°C for 48 h. * $p < 0.05$; ** $p < 0.01$.

recorded at temperature above 0°C. The peak temperature for maximum phase change for starfish lipid extract was 4°C. Sea urchin lipid extract thermogram had a complex profile characterised by two peaks. The main peak was detected at –10°C, and the other was at 20°C. DSC thermograms of the melting of the culture medium and its mixtures with 10% DMSO

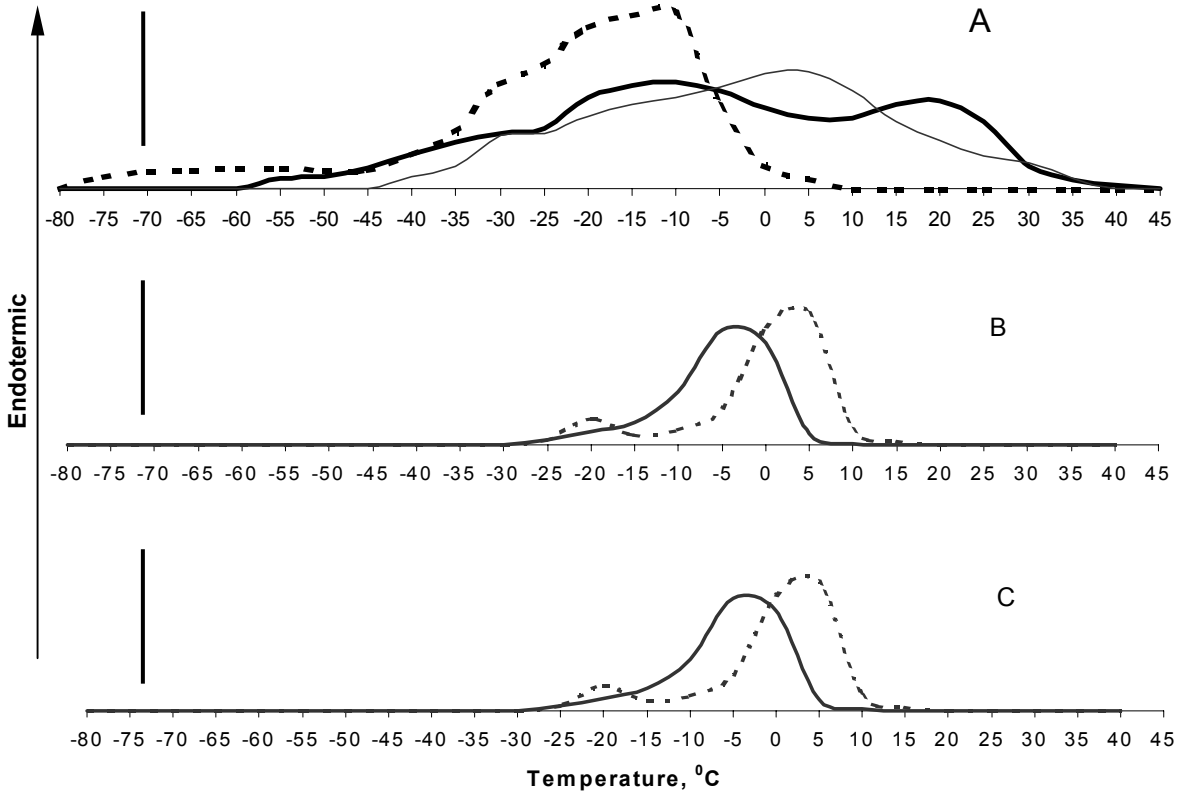

Figure 3. A. Thermograms of total lipids extracted from *C. grayanus* (- - -), *S. intermedius* (▬), *A. amurensis* (—).
B. Freezing medium L-15 M +10% DMSO (▬), L-15 M + 3 mg/ml trehalose (- - -)
C. Freezing medium L-15 M + 10% DMSO + 30 mg/ml trehalose (▬), L-15 M alone (- - -)
Vertical bar represents 1 mW (A), 20 mW (B) and 20 mW (C). Scanning rate was 16 °C /min. Samples were scanned at least three times.

and/or trehalose (3-30 mg/ml) are shown on Fig. 3B & C. The DMSO thermogram is mainly located at temperatures below 0°C with peak maximum temperature at -5°C. The addition of trehalose (3-30 mg/ml) to the culture medium did not influence either the temperature of the melting (2°C) or the thermogram profile. Additionally, trehalose in combination with DMSO did not change any thermodynamic parameters of the mixture melting.

DISCUSSION

We carried out the estimation of complex influence of various cryoprotectants on cell viability and the RNA synthesis in the mollusc and sea urchin cells depending on cell type, animal species and developmental stage.

There were no essential differences in viability of somatic cells frozen with these cryoprotectants. However, the synthetic activity evaluation for which a high level of functional integrity is necessary showed that the RNA synthesis was lower in cells protected with trehalose alone then in those protected with DMSO + trehalose combination.

Larval cells of different developmental stages had various freezing tolerances. The cell survival of the later stages was obviously superior to that of the early stages. So, for scallop larval cells the veliger was shown to be the most freezing-tolerant developmental stage (22).

However, in sea urchin embryonic cells the level of RNA synthesis in gastrula-derived culture fell significantly after freeze-thaw while the incorporation level of (H^3)-uridine of frozen cells in blastula-derived cultures was almost the same as in the control (unfrozen) cells. Earlier, it was shown that during the cultivation (without freeze-thaw) the level of RNA synthesis was reduced greatly in gastrula-derived cultures but not in blastula-derived cultures (25). For comparison, it is interesting to note that the most freeze-tolerant developmental stage of the sea urchins was reported to be blastula stage (20).

In this work we showed that the majority of the cells (60-95%) were destroyed after freeze-thaw. It is known that cell damage during freezing is not connected with the cell's ability to endure storage at very low temperatures (less than -180°C). Rather, it is explained by the lethality of an intermediate zone of temperature (-15 to -60°C) that a cell must traverse twice (12). Grishenko with colleagues suggest that after freeze-thaw only the most stable cells keep their viability (14). Probably, liquid crystalline lipids capable of repairing the damaged sites or to interfere with damage of cell membranes during freeze-thaw. Chilling susceptibility of animal oocytes and spermatozoa was shown to be related to lipid profile in animal gametes and conformed to lipid phase transitions of their membranes (1, 27, 28). Perhaps, cryoprotective properties of lipids may also correlate with their phase transition temperatures. We tested the extracts of total lipids differed by their thermotropic behaviour. The cryoprotective ability of mussel total lipids differed sharply from that of echinoderm lipids. This result correlated with essential differences in the crystalline to liquid crystalline phase transitions of these lipid extracts (Fig. 3). So possibly, protective function of mussel total lipids is the best because their liquid crystalline state will be maintained between -15°C and -60°C, whereas the major part of total lipids from echinoderms will have undergone phase transition at these temperatures. Hence, not only lipid phase transitions in cell membranes define the cold shock damage (36), but also cryoprotective properties of exogenous lipids depend upon their thermotropic behaviour. As shown by Sanina and Kostetsky (31), the main reason for changes in thermotropic behavior of marine invertebrate phospholipids seems to be the decrease of saturated/polyunsaturated fatty acid rations.

The relative specificity of the bonding between phosphate head groups of phospholipids and trehalose may be an important factor in the ability of trehalose to replace water around the head group of a phospholipid and thereby to stabilise membranes (6). Carbohydrates, especially sucrose, raffinose and trehalose, are often used to stabilize liposomal membranes during freeze-drying (7, 33, 37). We evaluated several concentrations of trehalose for the cryopreservation of marine invertebrate cells. When trehalose was used alone, high concentrations lowered the incorporation level of (H^3)-uridine in the sea urchin cells. When trehalose was used in combination with DMSO, there were no any significant differences in cryoprotective ability of this cryoprotective mixture and DMSO alone. This fact correlated with the capacity of DMSO, in contrast with trehalose, to decrease the temperature of the melting of the culture medium (Fig. 3 B). Usually, the freezing of the cells is conducted using 0.15-0.25 M trehalose in combination with DMSO (4, 7, 10, 21). These concentrations are 2-3-fold higher than in our experiments.

Interestingly, the greatest cryoprotective effect of mussel total lipids was revealed for embryonic cells in comparison with somatic cells. The embryonic cells of *M. trossulus* protected with complex cryoprotectant (DMSO + trehalose + mussel lipid extract) had more intensive RNA synthesis than the cells protected with only DMSO or trehalose. These results are coincident with the data of Crowe et al. (6) about specific interaction of trehalose with lipids.

In conclusion, our results suggest that DMSO + trehalose combination and mussel lipid extract can be used as a cryoprotective agent for marine invertebrate cells.

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