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CELL BIOLOGY

Freezing Tolerance of Sea Urchin Embryo Pigment Cells[1, 2]

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Abstract—Various stresses, including exposure to cold or heat, can result in a sharp increase in pigmentation of sea urchin embryos and larvae. The differentiation of pigment cells is accompanied by active expression of genes involved in the biosynthesis of naphthoquinone pigments and appears to be a part of the defense system protecting sea urchins against harmful factors. To clarify numerous issues occurring at various time points after the cold injury, we studied the effect of shikimic acid, a precursor of naphthoquinone pigments, on cell viability and expression of some pigment genes such as the *pks* and *sult* before and after freezing the cultures of sea urchin embryo cells. The maximum level of the *pks* gene expression after a freezing–thawing cycle was found when sea urchin cells were frozen in the presence of trehalose alone. Despite naphthoquinone pigments have been reported to possess antioxidant and cryoprotectant properties, our data suggest that shikimic acid does not have any additional cryoprotective effect on freezing tolerance of sea urchin embryo pigment cells.

Keywords: cell culture, gene expression, quinone pigments, sea urchin, shikimic acid

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INTRODUCTION

Invertebrate animals have developed a variety of defense reactions. One of them is pigmentation, due to which foreign organisms can be encapsulated and melanized with the participation of some secondary metabolites (polyketide compounds) and specific enzymes (polyphenol oxidases). From simple invertebrates to complex living beings such as human, pigmentation is regulated by genetic and environmental factors. Sea urchin embryos and larvae are highly influenced by their environment and can be used as bioindicators in ecotoxicological studies [11, 16, 17]. Sea urchin pigment cells are developed from secondary mesenchymal cells at the mesenchymal blastula stage [10] and manifest a behavior similar to that of macrophages, thus confirming the involvement of pigment cells in the defense system of larval sea urchins [8]. During gastrulation, these cells begin synthesizing pigment granules containing carotenoids and naphthoquinone pigments, echinochrome and spinochromes [7]. Three groups of genes, playing a key role in the biosynthesis of naphthoquinone pigments, were reported to be expressed specifically in pigment cells: the gene cluster of polyketide synthetases (*pks*), the sulfotransferase gene (*sult*), and the family of genes encoding flavin-containing monooxygenases (*fmo*) [3, 6].

An effective cryopreservation protocol for sea urchin cells, including three-step freezing with a low cooling rate (1–2°C/min) and a combination of nonpenetrating and penetrating cryoprotectants, was developed previously [14]. Using a trypan blue exclusion test, it has been demonstrated that our methods of cryopreservation allow providing a high percentage of viable cells, up to 75%. The level of expression of some genes associated with the induction of pigment differentiation (*Sipks* and *Sisult* genes) in the cells after freezing was found to be much lower than that in intact cells of the sea urchin *Strongylocentrotus intermedius*. Nevertheless, no clear correlation between the expression of genes involved in pigment differentiation and the type of cryoprotectant was detected.

In this study, we have attempted to clarify this question and to understand the principles of functioning of cell genomic apparatus after cryopreservation by using shikimic acid (ShA), a precursor of naphthoquinone pigments [5]. As it was shown previously, an addition of ShA to zygotes and embryos of sea urchins led to an increase in the level of expression of the *pks* and *sult*, the genes involved in pigment differentiation [2]. A ShA concentration above 0.5 mM caused a

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slowdown or cease of embryos' growth; no effect was detected at a concentration of ShA below 0.1 mM. Additionally, the data for cultivated sea urchin cells showed that the presence of 0.5 mM ShA in a nutrient medium did not have any effect on the level of the *sult* gene expression, but raised significantly the level of expression of the *pks* genes. The increased expression of this group of genes had previously been observed during incubation of sea urchin embryos with marine bacteria from the nine genera tested [9]. The present study was undertaken in order to examine changes at the level of expression of the studied genes involved in pigment differentiation in sea urchins after freezing–thawing in the presence of ShA.

MATERIALS AND METHODS

Animals and Embryo Cell Culture

Adult sea urchins (*S. intermedius*) were collected from the Peter the Great Bay (Sea of Japan). The animals were rinsed with UV-sterilized filtered seawater (SW); spawning was induced via intracoelomic injection of 0.5 M KCl (0.2–0.7 mL per animal). Eggs from different females were artificially fertilized using the pooled sperm from different males; zygotes were placed in tanks filled with UV-sterilized SW, incubated at 17°C, and harvested at the mesenchymal blastula stage (at 16 h post-fertilization) [2].

Sea urchin embryos were collected on a fine 30-μm nylon mesh and dissociated into single cells with 0.25% collagenase at 17°C (for 20–30 min), as described previously [2, 14]. Sterile SW, supplemented with 2% fetal calf serum (FCS), was added to cell suspension to obtain concentration of $15–20 \times 10^6$ cells/mL. All reagents were purchased from Sigma Chemical (St. Louis, United States).

Freezing and Thawing of Cells

The resulting cell suspension that contained all cell types (0.6 mL) was transferred to sterile 2-mL polypropylene cryotubes (TPP, Switzerland), with subsequent gradual addition of 1.2 mL of cryoprotective solutions (cooled to 4°C). The cryoprotective solutions were prepared with sterile 32‰ SW and contained either only non-penetrating cryoprotectants such as disaccharide trehalose (Tr, at a final concentration of 15 mg/mL) or various combinations of penetrating (dimethyl sulfoxide, Me_2SO, and ethylene glycol, EG) and non-penetrating cryoprotectants (at the final concentrations of 1 M and 15 mg/mL, respectively). In addition, ShA was present in some cryoprotective mixtures. The samples were then kept in an ice water bath for equilibration during 10 min prior to freezing. Freezing to −196°C was performed by the three-step procedure [14].

After storage in liquid nitrogen for 20 days, the cryotubes were placed into a bath with circulating 30°C water until the ice was completely melted. The thawed samples were gradually (during 3–5 min) diluted ten-fold in sterile SW at 0°C with gentle shaking, centrifuged (5 min at 1000 g), washed once with SW, and re-suspended in 0.6 mL of SW supplemented with 2% FCS for cultivation at 17°C in 24-well plates (Nunc, Denmark). The cells that had undergone freezing–thawing and the control unfrozen cells were incubated for 24 h at 17°C. Cell viability was estimated using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, based on measurements of the metabolic activity of mitochondrial enzymes as described in [14].

Pigment Differentiation and Quantitative Real-Time PCR (qRT-PCR) Analysis of Pigment-Specific (pks and sult) Genes

After 24 h cultivation, the total RNAs were extracted from sea urchin cells using the Yellow Solve reagent (Clonogen, Russia) and treated with DNase I (Sileks, Russia) to remove genomic DNA. The first strand of cDNA was synthesized using the reverse transcription system (Sileks) with 1.5 μg of total RNAs as a template in a 50 μL reaction volume. The PCR reactions were performed in an iCycler thermocycler (Bio-Rad, United States) by the following protocol: one cycle at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 50°C for 15 s, and 72°C for 35 s, with the final extension cycle at 72°C for 10 min. TaqMan qRT-PCR was performed using the established protocol [2, 14]. The amplification procedure included one cycle at 95°C for 2 min, followed by 50 cycles at 95°C for 10 s and 62°C for 25 s. The TaqMan PCR assays were performed in an iCycler thermocycler equipped with an iQ5 Multicolor RT-PCR detection system (Bio-Rad). The data were analyzed using the iQ5 Optical System Software v.2.0 and presented in relative units. The *S. intermedius* actin gene (GenBank accession number DQ229162) and the ubiquitin gene (LOC754856) were used as the endogenous controls to normalize variance in the quality and amount of cDNA in each qRT-PCR experiment. The expression of two from the three groups of genes in sea urchin pigment cells—the *pks* and *sult* genes (GenBank accession numbers JF809814.1 and JF809813.1, respectively)—was analyzed. The data from five qRT-PCR independent experiments were summarized. The primers and TaqMan probes for actin, ubiquitin, and the *pks* and *sult* genes, used in qRT-PCR, had been described previously [2, 9, 14].

Statistical Analysis

Each experiment was conducted independently at least five times, and all the assays were performed in triplicate. The results were subjected to one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test with the use of MS Excel 2013

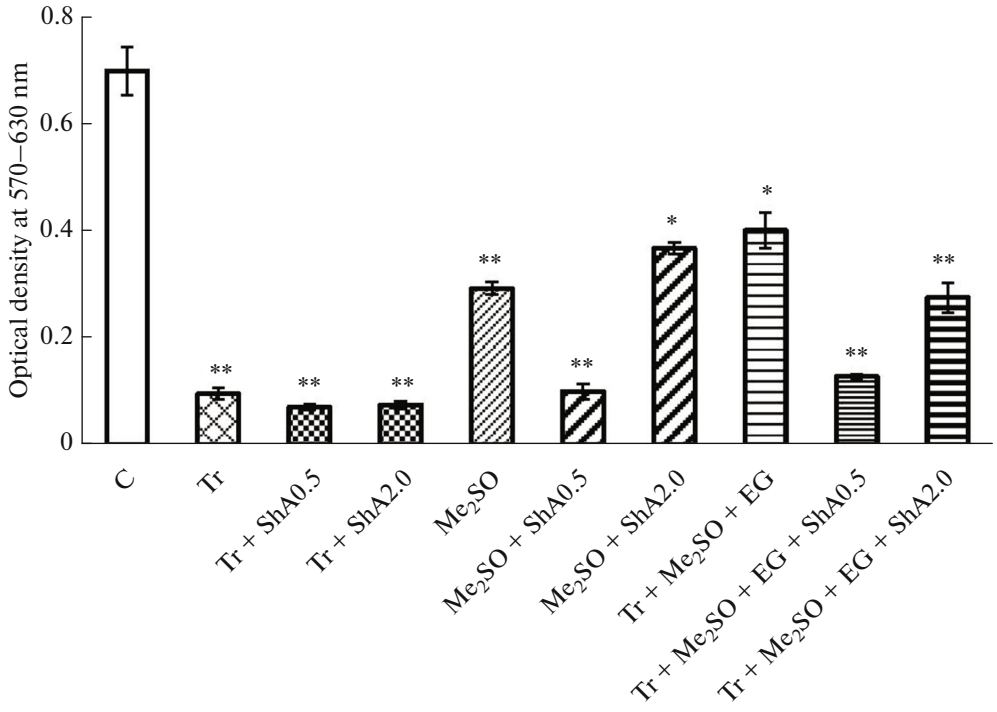

Fig. 1. Cell viability before and after freezing-thawing (24 h), as shown using the MTT assay, in blastula-derived cell cultures of the sea urchin *S. intermedius*. Treatment key: C, control unfrozen cells; cells frozen with: Tr, trehalose (15 mg/mL); Tr and shikimic acid (ShA, 0.5 mM); Tr and ShA (2.0 mM); Me_2SO, dimethyl sulfoxide (5%); Me_2SO and ShA (0.5 mM); Me_2SO and ShA (2.0 mM); Tr, Me_2SO and EG; Tr, Me_2SO, EG and ShA (0.5 mM); Tr, Me_2SO, EG and ShA (2.0 mM). Significance levels are $*p < 0.05$ and $**p < 0.01$.

software (Microsoft, United States). A p-value < 0.05 was selected as the statistical significance threshold for all the analyses.

RESULTS AND DISCUSSION

Assessment of Viability of Sea Urchin Embryo Cells after a Freezing–Thawing Cycle

An assessment of cell viability after freezing–thawing using the MTT assay revealed significant variations in cell viability depending on the type of cryoprotectants (Fig. 1). The number of viable cells found after freezing with the non-penetrating cryoprotectant, trehalose, was almost seven times as low as that of the control unfrozen cells. A higher level of cell viability (to 40–65%) was detected when the mixture of penetrating and non-penetrating cryoprotectants was used. These results are confirmed by other studies [2, 15, 17]. The addition of ShA greatly increased cell viability, depending on its concentration, but only when Me_2SO was used alone as a cryoprotector. In other cases, when only non-penetrating cryoprotectors or the cryoprotective mixture containing Me_2SO, EG and Tr, were used, the addition of ShA resulted in a significant decrease of cell viability.

We suggest that these effects can be connected with the changes which occur in the structure of ShA and influence the intracellular redox processes. The penetrating cryoprotectant Me_2SO is an effective oxidant for primary and secondary alcohols. The structure of ShA includes three secondary hydroxyl groups, which, after interaction with Me_2SO, can be oxidized to ketone groups. This probably results in a higher level of cell viability in sea urchins. During freezing–thawing, the non-penetrating cryoprotectants (for example, trehalose) is supposed to block ShA that passes into cell and prevent its oxidation even in the presence of penetrating cryoprotectors (Me_2SO and EG).

The Level of Expression of Genes Associated with Induction of Pigment Differentiation in Sea Urchin Cells Before and after Freezing in the Presence of ShA and Without It

The levels of expression of some genes, associated with the induction of pigment differentiation in sea urchin cells, differed significantly before and after cryopreservation (Fig. 2). The maximum level of the *pks* gene expression in sea urchin embryo cells after a freezing–thawing cycle was recorded when cells were frozen in the presence of trehalose alone (Fig. 2a). The addition of ShA (0.5–2.0 mM) resulted in a significant

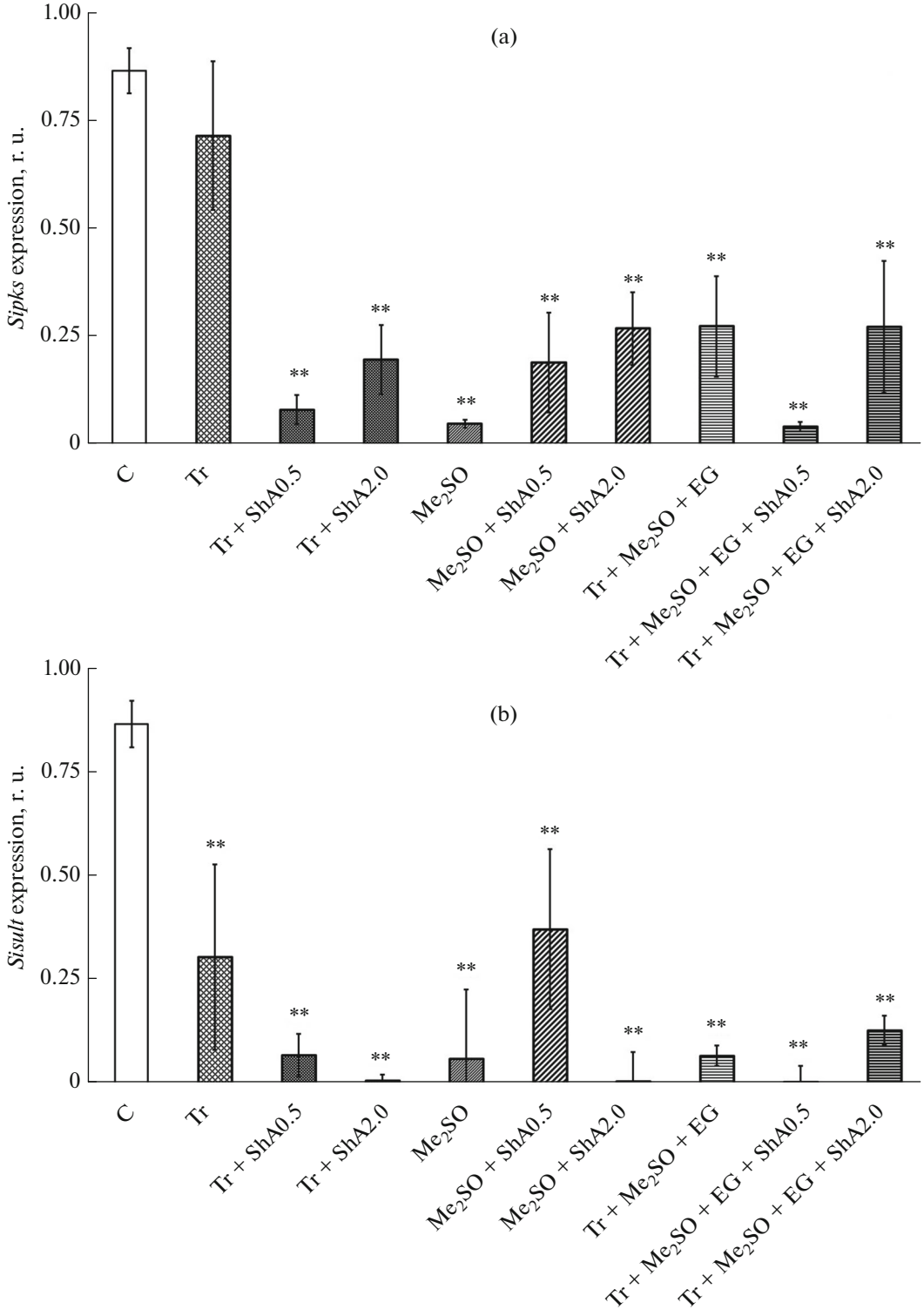

Fig. 2. Levels of expression of the *Sipks* (a) and *Sisult* (b) genes, determined using qRT-PCR, in a blastula-derived cell culture before and after freezing–thawing in the presence of different cryoprotectants (24 h-cultivation). Treatment key: C, control unfrozen cells; cells frozen with: Tr, trehalose; Tr and shikimic acid (ShA, 0.5 mM); Tr and ShA (2.0 mM); Me_2SO, dimethyl sulfoxide (5%); Me_2SO and ShA (0.5 mM); Me_2SO and ShA (2.0 mM); Tr, Me_2SO and EG; Tr, Me_2SO, EG and ShA (0.5 mM); Tr, Me_2SO, EG and ShA (2.0 mM). The data are the mean (± SEM) of three independent experiments (*$p < 0.05$); "r.u.", relative units.

reduction of both this level and cell viability, whereas 2.0 mM ShA in the presence of the penetrating cryoprotectors was shown to increase the expression of the *pks* genes. For another pigment gene (*sult*), no clear correlation between the expression level and the type of cryoprotectant was found (Fig. 2b). Despite ShA is an important precursor in the biosynthesis of naphthoquinone pigments, which have been reported to exhibit antioxidant and cryoprotectant properties [12, 13], the results of this study show the lack of any additional cryoprotective effect of ShA on freezing tolerance of pigment cells of sea urchin embryos.

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