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Cryopreservation of Crassostrea gigas vesicular cells: Viability and metabolic activity

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Abstract

Cryopreservation is widely used for long-term conservation of various tissues, embryos or gametes. However, few studies have described cryopreservation of invertebrate primary cell cultures and more particularly of marine invertebrate somatic cells. This technique would however be of great interest to facilitate the study of various metabolic processes which vary seasonally. The aim of the present study was to develop a protocol for cryopreservation of *Crassostrea gigas* vesicular cells. Different parameters were adjusted to improve recovery of cells after freezing. The most efficient cryoprotectant agent was a mix of Me_2SO, glycerol, and ethylene glycol (4% each). The optimal cooling rate was -1 °C min^{-1} down to −70 °C before transfer into liquid nitrogen. In these conditions the percentage of viable cells reached 70% of the control. The glucose metabolism of thawed cells was evaluated using radioactive glucose as a tracer. Immediately after thawing, glucose uptake involving membrane transporters was greatly reduced (24% of control) whereas glucose incorporation into glycogen was less affected (68% of control).

Keywords: *Crassostrea gigas*; Oyster; Mollusc; Cryopreservation; Vesicular cells; Glycogen metabolism

Introduction

Growth and reproductive activity of the oyster *Crassostrea gigas* are mainly supported by glycogen reserves stored in vesicular cells, constituting the storage tissue. This storage tissue is located in the labial palps, the mantle, the gonadal area, at the base of the gills and around the digestive gland [3]. Vesicular cells have been the focus of many studies because of their involvement in energetic support of gametogenesis [2, 16, 20, 29, 43]. Suspensions of isolated vesicular cells were used for investigation of metabolic activity by *in vitro* bioassays (^{14}C Glucose uptake and its incorporation into glycogen) [4]. This *in vitro* approach demonstrated a clear seasonality in glucose metabolism in oysters, inversely correlated with the reproductive cycle, with glycogen storage in autumn and mobilisation of reserves in spring to support gonadal development. *In vivo* experiments in which labelled glucose was injected into whole animals confirmed the seasonal variations of glycogen metabolism [21]. The seasonal cycle of accumulation and mobilisation of storage products is not strictly correlated with availability of food. It is probably heavily influenced by endocrine regulation in response to the high energy demand associated with reproduction and growth in summer. Identification of the factors involved in this regulation as well as characterisation of the regulatory mechanisms are among our main research objectives. However, this requires testing and comparison of the biological effects of extracts or of potentially active substances from living vesicular cells with metabolic activity oriented both towards the build up of reserves (autumn / winter) and towards mobilisation of reserves (spring / summer). Until present, these effects were by necessity tested on freshly isolated cells at 6 month intervals, but it is obviously problematic to compare the results of totally independent experiments, particularly in animals which exhibit high levels of variation both between individuals and inter-annually. For this reason we envisaged cryopreservation of reference batches of metabolically characterised vesicular cells in liquid nitrogen as a complementary approach which would allow simultaneous testing of regulatory factors in strictly identical experimental conditions. In this manner, analytical biases associated with, for example, variable receptivity of target cells, can be reduced. The feasibility of cryopreservation of these vesicular cells was the object of the present study.

Cryopreservation was developed mainly for long term storage of vertebrate cells, tissues and embryos. Few studies have reported successful of cryopreservation of molluscan cells. Of these, most involve germinal cells or embryos (**Table 1**); spermatozoa were successfully cryopreserved in *Crassostrea tulipa*, *Crassostrea virginica*, *Crassostrea gigas* and cryopreservation has been tested on oocytes of *Crassostrea gigas*. Embryos and larvae from various species including *Crassostrea virginica* and *Crassostrea gigas* have also been the subject of attempts at cryopreservation, but several authors have underlined the difficulty of maintaining structure and activity of embryos after thawing [41, 6, 7, 33, 27]. Concerning differentiated somatic cells, *Crassostrea virginica* and *Pecten maximus* cardiac cells were successfully cryopreserved as were dissociated mantle cells and hemocytes from the gastropod *Haliotis tuberculata*. For these molluscan cell types, several cryoprotectant agents (CPAs) were used separately or in combination (ethylene-glycol (EG), dimethylsulfoxide (Me_2SO) and/or glycerol (G)). The respective efficiency of CPAs seems to be cell type- and/or species-dependent. The freezing protocol also strongly influenced final cell recovery. The cooling rate is one of these critical parameters: slow cooling of cells can reduce the risk of intracellular ice formation. Around the critical subzero temperature, cells are exposed to increased concentrations of solutes that may become toxic and therefore this critical temperature zone should be passed as quickly as possible. However, within the same

temperature range, cells require a sufficient time to reduce water content in order to prevent ice formation. The combination of these two factors will determine the optimal cooling rate in different cryoprotectant agents [30]. Before transfer into LN, cell water content must be low enough to inhibit intracellular ice formation (IIF). Subsequently, the solute and /or the CPA concentration must be sufficiently high to facilitate vitrification on rapid cooling. Therefore, cells must be progressively cooled avoiding ice formation in supercooled internal solutions during transfer to LN. The importance of determining a suitable temperature at which living samples can be transferred into LN without major damages has been demonstrated in a study of cryopreservation of ragworm larvae [36].

Cell recovery from deep freezing is estimated using different tests like the Trypan blue permeation test, the MTT reduction assay, evaluation of protein content (Bradford) or DNA content (Burton test). Recovery of physiological capacities is crucial in cryopreservation processes and the use of *in vitro* tests allowing measurement of specific metabolic activity is of great importance in this respect. Functional tests based on metabolic activities have occasionally been applied for somatic cells. These tests involved measurement of radioactive precursor incorporation: ^{3}H-leucine, ^{3}H-thymidine or ^{3}H-uridine to evaluate protein, DNA or RNA synthesis respectively [26, 35, 39, 40].

The present study aimed to define a protocol convenient for freezing oyster vesicular cells with high post-thaw viability levels as estimated by the MTT reduction test. Moreover, we investigated the performance of specific functional properties of these cells by *in vitro* measurement of glucose uptake and glycogen synthesis.

Material and methods

Animals

Three-year-old Pacific oysters (*Crassostrea gigas*) were obtained from a commercial oyster farm in Saint-Vaast-la-Hougue (Normandy, France). Oysters were always used within 24 hours of reception.

Preparation of vesicular cell suspensions

Oysters (36 animals) maintained in ice during dissection were opened and thoroughly rinsed with sterile seawater. The labial palps containing vesicular cells were dissected, rinsed three times in sterile seawater and then once in Leibovitz culture medium [Leibovitz L15 (1.5%; NaCl 340 mM, KCl 50mM, Hepes 20mM, pH 7.4, 1100 mOsm, filtered on a Millipore 0.22µm filter] supplemented with antibiotics (streptomycin 70µM, penicillin 170µM). The dissociation was performed as previously described by Berthelin *et al.* [3]. Briefly, tissues were minced with scissors and cells were dissociated by mechanical stirring associated with enzymatic treatment (collagenase 0.1% wt/vol). Cells were then filtered through a 100µm nylon mesh and rinsed in Leibovitz medium containing antibiotics. They were then pelleted by centrifugation (80g, 30 min., 15°C) before re-suspending in fresh culture medium at the chosen cell concentration.

Freezing of cells

Cell suspensions ($50x10^6$ cell mL^{-1}) were pippetted into 1.8 ml cryotubes (0.6 ml per tube). An equal volume of cryoprotectant solution was added at room temperature using the following stepwise additions in order to reach for each cryoprotectant agent the specific 1x final concentrations deteilled in **Table 2** : 0.20 x, 10 min ; 0.40 x, 5 min ; 0.65 x, 5 min ; 1 x, 10 min. Cryoprotectant solutions were prepared by diluting CPA in modified Alsever's solution (MAS: sodium citrate 27 mM, EDTA 12 mM, sodium chloride concentration was adjusted so in order to prevent cells from being exposed to an osmolarity exceeding 2.2 $Osm.kg^{-1}$) according to **Table 2**. Samples were then submitted to freezing procedures using a controlled-rate cooler (IMV-CryoBioSystem, L'Aigle, France). The freezing protocols included three phases: a stepwise first step cooling from ambient temperature to the melting temperature of each considered cryoprotectant solution, a seeding procedure, and a second stepwise cooling down to the final holding temperature before transferring into LN.

A series of controls was set up in order to measure the toxicity of each CPA and the effect of cryoprotectant treatment on frozen cell viability. Three different cooling rates were applied to the cells: $-1°C.min^{-1}$, $-3°C.min^{-1}$ and $-5°C.min^{-1}$ (classically used for mollusc somatic cells [26, 40]). Samples were transferred to liquid nitrogen at various final temperatures: -30°C, -50°C or -70°C.

Thawing of cells

After storage periods ranging from two days to seven days, samples were extracted from LN and thawed by immersing in a 37°C water bath until the last ice crystal disappeared (approximately 90 sec). Cryoprotectant agents were then diluted in MAS using the following stepwise decreasing concentration (initial 1x concentration for each CPA was reported in **Table 2**): 0.8 x, 3 min ; 0.6 x, 3 min ; 0.4 x, 5 min, 0.1 x, 10 min). Cells were then

centrifuged (80g, 30 min at 15°C), the supernatant was discarded and the pellet was re-suspended in sterile Leibovitz medium containing antibiotics.

MTT reduction assay

A test based on the mitochondrial enzymatic reaction with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT (Sigma) as a substrate and adapted to molluscan cells [32, 12] was used to evaluate cell viability. Five hundred µl of cells suspension ($5x10^6$ cells per ml) were incubated in a 24 well plate with 1/10 (vol:vol) MTT solution (1,2 mM in culture medium) for 24 hours at 15°C. An equal volume of isopropanol containing 0.04 N HCl was then added to each well and after 30 min agitation, absorbance was measured at a wavelength of 570 nm, with a reference at 630 nm. Each value is the mean of three replicates and results were analysed using a Student's test.

Glucose uptake into vesicular cells

Five hundred µl of cell suspension were incubated in 24 well culture plates with 50 µl [U-^{14}C]glucose (0.5 µCi) (MP-Biomedicals) and 50 µl of unlabelled D-glucose (0.5 mM in final concentration). After three hours incubation at 15°C, ^{14}C-glucose uptake was stopped by addition of 300µl of 20mM glucose. Cells were centrifuged (80 g, 10 min at 15°C), rinsed three times in Leibovitz medium containing glucose (20mM), then dispersed in 200 µl perchloric acid (15% vol-vol in distilled water). The radioactivity was measured using a scintillation counter (Packard®) with internal quenching correction

Controls were stopped immediately after labelled glucose addition and negative controls without cells or without tracer were also performed. Each value is the mean of six replicates and results were analysed using a Student's test.

[U-^{14}C]glucose incorporation into glycogen of vesicular cells

This protocol was previously described by Berthelin *et al*. [3] for the same cell type. Briefly, 500 µl of cell suspensions were incubated in 5 ml sterile tubes with 50 µl of [U-^{14}C]glucose (0.5 µCi) (MP-Biomedicals) and 50 µl of unlabelled D-glucose (at a final concentration of 0.5 mM). After 20 hours incubation at 15°C, cells were centrifuged (10000 g, 10 min). 300 µl of the glycogen-containing supernatant were precipitated with ethanol (with 10mg of unlabelled glycogen as a carrier) and the glycogen was recovered by centrifugation (2500 g for 10 min). After 3 washes in absolute ethanol containing D-glucose (0.1 M), the pellet was air dried and resuspended in 500µl distilled water before counting using a scintillation counter (Packard®).

Standards were stopped immediately after radioactive glucose addition and negative controls without cells or without tracer were also performed. Each value is the mean of six replicates and results were analysed using a Student's test.

Results

Toxicity of CPA treatments

Cell viability (MTT reduction assay) was measured in unfrozen dissociated cells previously treated with each cryoprotectant solution in order to evaluate their putative harmful effects. Results were expressed in % viability relative to untreated cells. As shown in **Figure 1,** MTT values varied according to the CPA. The maximal cell loss was observed with glycerol (MTT activity decreaed by 46.9 ± 1.6 %). The toxic effect of Me_2SO (D) and ethylene glycol (E)

treatments was less important (36.5 ± 0.4 % and 28.8 ± 0.5 % respectively) whereas Me2SO-glycerol-ethylene glycol (DGE) appeared significantly less harmful (20.3 ± 1.2 %) ($p<0.05$).

Thawed cell recovering

Cell recovery after thawing was estimated by the ratio of cells surviving after freezing and thawing (MTT test) in comparison with CPA-treated cell viability before freezing. **Figure 2** illustrates the respective ability of the four CPA treatments to prevent cells from cooling injury: glycerol 10% was the most efficient CPA in this respect (91.5±5.2 %) whereas the other cryoprotectant solutions were slightly less efficient (recovery rate ranging from 63.7±4.0 % for DGE to 72 ± 0.64% with EG)

Effects of cooling rate

Three different cooling rates, low (-1°C.min^{-1}), intermediate (-3°C.min^{-1}) and high (-5°C.min^{-1}) were applied during the freezing procedure using DGE (the least toxic cryoprotectant solution) and glycerol (the most efficient cryoprotectant) down to a temperature of transfer into liquid nitrogen fixed at -50°C. Results are presented in **Figure 3** with unfrozen cells resuspended in MAS as the control. For DGE treatment, the low cooling rate (-1°C.min^{-1}) induced a significant improvement in cell recovery reaching 84.4±10.4 % . The recovery of cells subjected to the other cooling rates was significantly lower (57.6±9.5 % and 57.1±9.1 % respectively for -3°C/min and for -5°C/min). With glycerol treatment, whatever the cooling rate, the percentage of cell survival never exceeded 53 %.

Effects of the final cooling temperature before plunging into LN

Vesicular cells treated with DGE were cooled (-3°C.min^{-1}) down to three distinct temperatures before transferring into LN. The effect on cell recovery of each transfer temperature is presented in **Figure 4** with unfrozen cells treated with MAS as the control. The lowest transfer temperature (-70°C) led to the greatest recovery (68.5±2.2 %) whereas cell viability decreased at -50°C (51.4±2.9 %) and even more so at -30°C (34.8±6.5 %).

Metabolic activity of cryopreserved vesicular cells

Glucose uptake and glucose incorporation into glycogen were compared before and after freezing of vesicular cells in the optimal conditions presented above (DGE treatment, cooling rate of -1°C.min^{-1} down to -70°C). Results are expressed in percentage of metabolic activity relative to unfrozen untreated cells (F**igure 5**). The capacity of glucose uptake was significantly reduced after thawing (24.3± 19.7 % of the initial activity for surviving cells) whereas incorporation seemed to be less affected (69.8±29.2 %). Cell viability versus the MAS control (62.1±2.3 %) confirmed the expected value.

Discussion

In order to understand the glycogen metabolism of the oyster *Crassostrea gigas* in relation with seasonal reproductive activity, we have developed a vesicular cell enrichment procedure based on moderate enzymatic dissociation of labial palps. Such cell suspensions have permitted *in vitro* measurement of ^{14}C-glucose uptake (Hanquet-Dufour *et al.*, in prep) and incorporation into glycogen [4]. Nevertheless the difficulty in undertaking studies of seasonal variations of glycogen storage and metabolism led us to envisage the banking of homogenous pools of vesicular cells in LN.

Little information is available about cryopreservation of marine invertebrate somatic cells, however it is widely accepted that the addition of cryoprotectant agents is essential to

protect cells from freezing injury. The concentrations of CPAs required in cryopreservation procedures may cause severe toxic effects to cells. For molluscan somatic cells, different CPAs have been tested. Among these agents, glycerol, Me_2SO and ethylene glycol were the most commonly used and the most efficient CPA concentration was close to 10% [10, 26, 34, 39, 40]. We therefore tested the ability of these CPAs to protect vesicular cells from cryoinjury. Combinations of two or more CPAs are assumed to be less toxic than a single-CPA solution because the concentration of any one component is then decreased [8]. Moreover, it is known that the toxic activity of Me_2SO can be decreased by acetamide [15]. Indeed, in embryos of *Crassostrea gigas*, biochemical toxicity of Me_2SO has been mitigated by dimethylacetamide [6]. In order to define a cryoprotectant solution as harmless as possible, a combination of three CPAs (4 % vol-vol of each in final concentration) was tested.

Since toxicity of CPAs is known to vary with cell type, a necessary preliminary step in order to define a successful cryopreservation protocol was to determine the tolerance to the selected cryoprotectant treatments. Using the metabolic MTT test to estimate cell viability, we first compared the effect of cryoprotectant agents on dissociated cells without freezing. Cell viability varied from 53 % with G to 80 % with DGE. Toxicity was significantly different between cryoprotectant treatments and glycerol was the most harmful, as was previously observed with *Crassostrea gigas* spermatozoa [23]. As expected, DGE was found to be the least toxic treatment for vesicular cells. Chao *et al.* [6] reported a significant reduction in toxicity to oyster embryos by the addition of glucose. Carbohydrates such as sucrose and especially trehalose have also been shown to protect cell membranes during cooling [44]. These disaccharides were successfully used for the cryopreservation of spermatozoa of the Pacific oyster [48, 1]. However, addition of glucidic substances would interfere with glycogen metabolism and therefore this method was not used in this study.

In the second step of our study, the cryoprotective efficiency of each treatment (the ratio of survival rates after freeze-thawing and in unfrozen controls, both after CPA treatment) was compared. The results revealed that glycerol was significantly more effective than the three other treatments. However, when compared to untreated control cells, the survival rate dramatically decreased to 48.6±2.8 % and was in fact lower than DGE treatment (50.8±19.0 %) which was significantly less toxic. The relatively low survival rates are not surprising given that cell viability was estimated with a metabolic test based on mitochondrial activity. Poncet *et al.* [40] observed strong variations in *Haliotis tuberculata* hemocyte viability according to the estimation method and showed that the survival percentages obtained with the MTT test (ranging from 30% to 75%) were generally lower than those obtained by protein or DNA content tests.

The optimal cooling rate should be a compromise between sufficient cell dehydration to prevent IIF and limited solute effects and pH variations. Optimizing cooling rate is essential in order to obtain the best cell survival. With DGE as the cryoprotectant treatment, the survival of vesicular cells is strongly influenced by the cooling rate. The slowest rate (-1°C.min^{-1}) led to a final recovery rate reaching 84.4±10.2 % compared to the unfrozen control, significantly higher than when cooled at -3°C.min^{-1}or -5°C.min^{-1} ($p<0.05$). These data are in agreement with the assumption that slow cooling is required for cells having a low surface/volume ratio [13]. Such a cooling rate has also been applied to heart cells of *Pecten maximus* [26] and to haemocytes of *Haliotis tuberculata* [40].

The lower the temperature before transferring into LN, the more dehydrated the cell should be. Therefore this parameter may greatly influence the cryopreservation success, as shown by Cheng *et al*. [10] on heart cells of the oyster *Crassostrea virginica*. In our case, the optimal temperature of transfer in liquid nitrogen was the lowest (-70°C). This is in accordance with the transfer temperature used for the cryopreservation of other molluscan somatic cells, such as heart cells of *Pecten maximus* [26] or of *Crassostrea gigas* [10].

The optimal conditions for cryopreservation of vesicular cells were found to be: cryoprotective treatment consisting of a combination of Me_2SO (4%), glycerol (4%) and ethyleneglycol (4%), a cooling rate of -1°C.min^{-1} down to -70°C before transferring into LN. In these conditions, the survival rate estimated by the MTT reduction test was about 70% (taking into account cell mortality and loss of cells during thawing and CPA-diluting procedures). However, cell viability varied slightly through the year (from 60 to 75%) probably due to the physiological state of vesicular cells which appeared to be more sensitive when they were larger and fully loaded with glycogen reserves.

The metabolic activity of the cryopreserved vesicular cells was also investigated by measuring cellular uptake of glucose and glucose incorporation into glycogen. Glucose uptake into surviving cells was drastically affected (only 24.3% of initial activity) by the freeze-thawing. This loss of activity suggests that cryopreservation affected the organisation of the cell membrane and disturbed the functioning of glucose transporters just after thawing. It would be interesting to verify if this disorganisation is definitive (glucose uptake was measured over 3 hours just after thawing) or if glucose uptake could recover after a period in standard culture conditions. The addition of trehalose (which may stabilize cell membranes) to the cryoprotective solution may also be considered. The fact that glucose incorporation into glycogen (measured over a 24-hour period) of thawed cells was less affected (68% of the initial activity) may confirm the hypothesis of temporary damage of transporters. Glycogen synthesis involves intracellular enzymes that may not be disturbed to the same extent as membrane components.

Our results confirm the possibility of successfully cryopreserving *Crassostrea gigas* somatic cells. However, this technique may not be suitable for investigating seasonal variations in glycogen metabolism given the reduction of glucose uptake after thawing.

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Table 2: Composition of the different cryoprotectant solutions

Designation	Cryoprotectant agent	Dilution in MAS
D	Me_2SO	10%
G	Glycerol	10%
E	Ethylene glycol	10%
DGE	Me_2SO	4%
	Glycerol	4%
	Ethylene glycol	4%

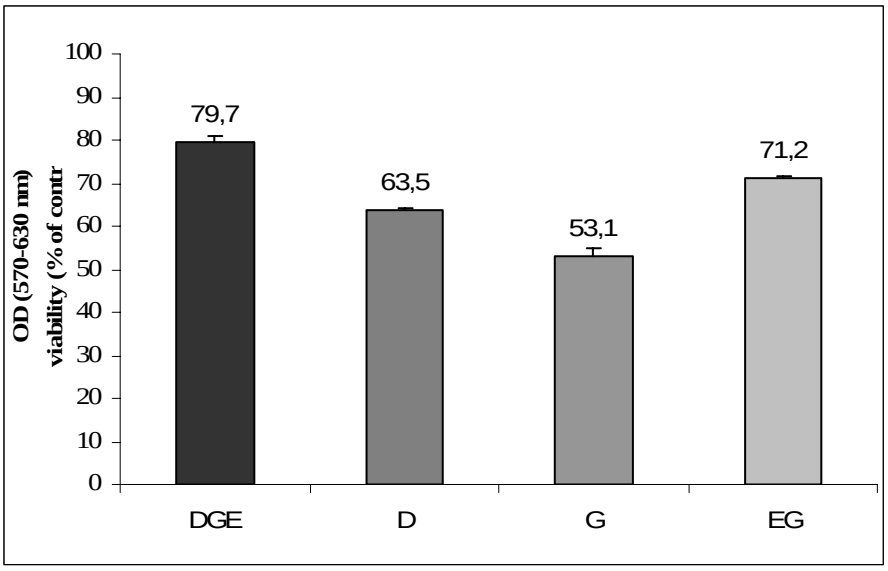

Figure 1: Toxicity of CPA treatments (cooling at the temperature rate of 3°C/min until -50°C before transfer into LN) estimated by MTT reduction assay in treated unfrozen cells visa untreated control (expressed in percentage of control). Each data point represents the means +/- SD of 3 replicates.

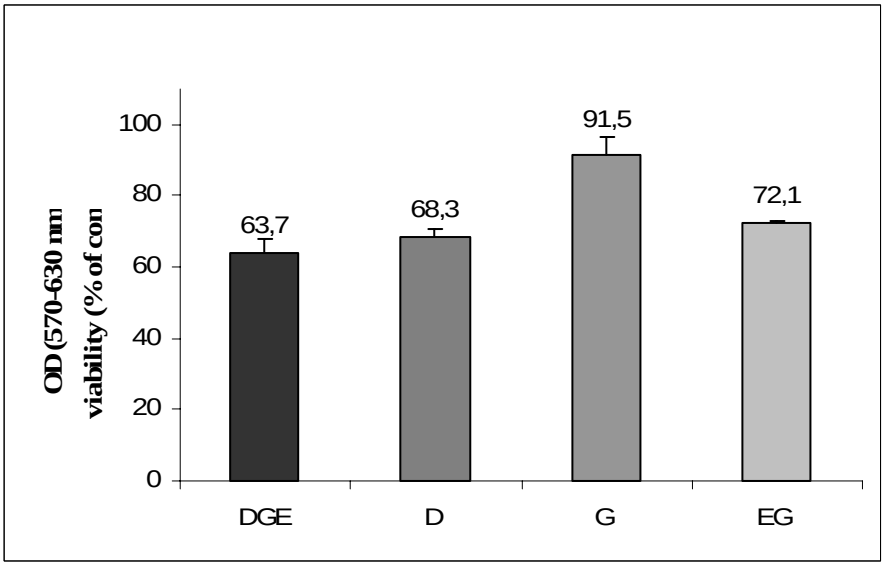

Figure 2: Cell recovery after thawing.For each CPA treatment, cells were cooled at 3°C/min until -50°C before transfer into LN. The efficiency was estimated by MTT reduction assay after freezing and thawing (expressed in percentage of CPA treated unfrozen cells). Each data point represents the means +/- SD of 3 replicates.

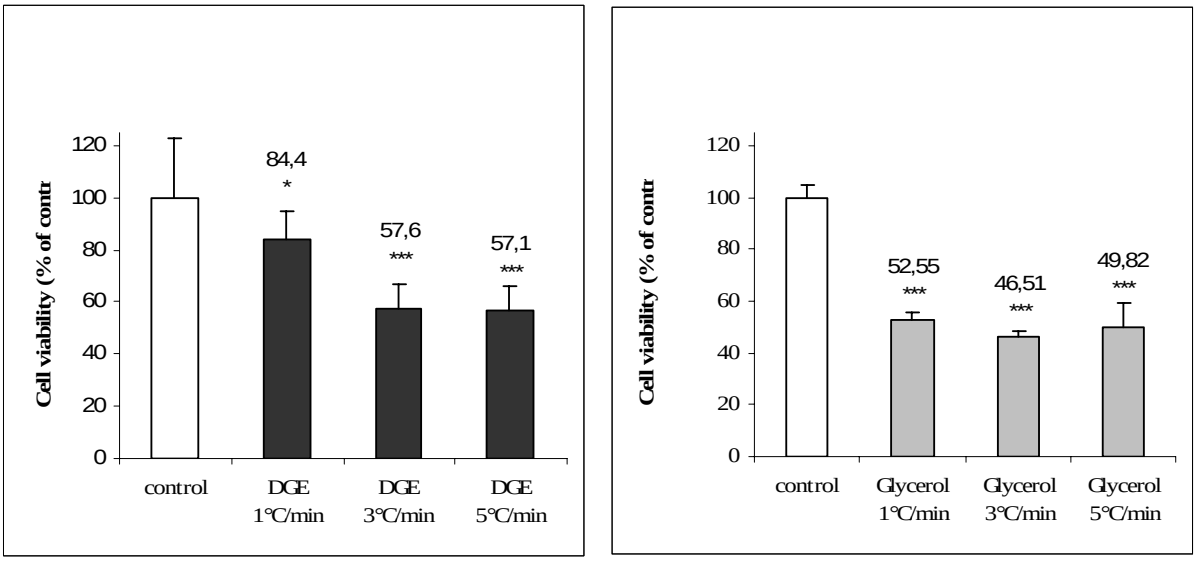

Figure 3: Effect of the cooling rate (-1; -3; -5°C/min)using (a) DGE or (b) Glycerol as CPA . Cell viability was estimated with the MTT reduction assay and expressed as % of unfrozen MAS treated cells. Samples were cooled down to -50°C before transfer into LN.Each data point represents the means +/- SD of 5 replicates. Significant differences from control cell were indicated: * P<0.05; ** P<0.01; *** P<0.001 (student test).

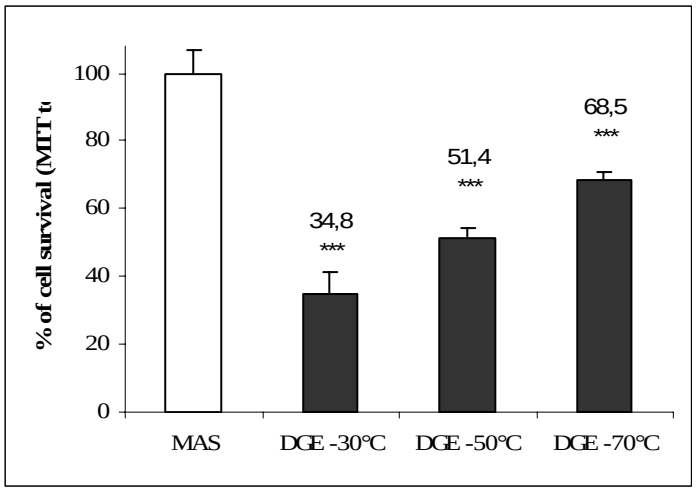

Figure 4: Effect of final cooling temperature before transfer into LN with DGE as CPA. Three temperatures were compared (-30°C, -50°C and -70°C) at the intermediate cooling rate -3°C/min and survival was estimated according to the MTT reduction assay. Each data point represents the means +/- SD of 5 replicates. Significant differences visa MAS treated cells were indicated: * P<0.05; ** P<0.01; *** P<0.001 (Student test).

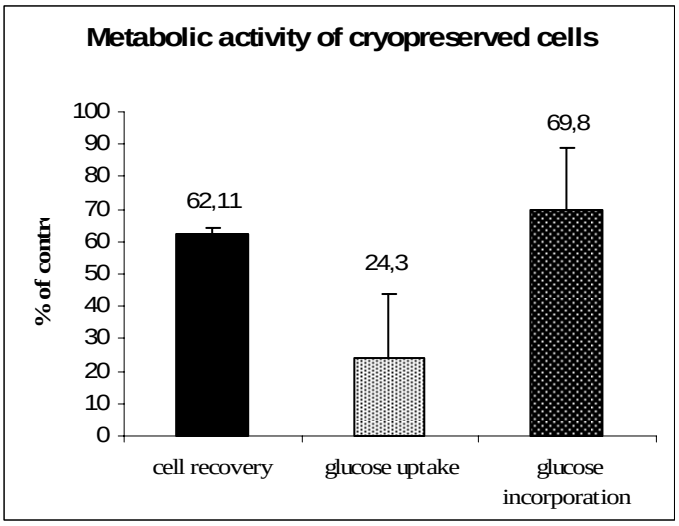

Figure 5: Metabolic activity of thawed cells (-1°C/min, DGE treatment, transfer into LN at -70°C) estimated by glucose uptake into vesicular cells and glucose incorporation into glycogen. Results were expressed in % of untreated cells activity. Each data point represents the means +/- SD of 5 replicates

Species	cell type	CPA	test of viability or cell activity	Reference
Crassostrea tulipa	spermatozoa	Me_2SO	fertility	[50]
Crassostrea virginica	larvae	Propylene glycol	survival, growth	[37]
	spermatozoa	Propylene glycol	fertility	[38, 51, 22]
		Me_2SO		
	heart cells	Me_2SO, Glycerol, Propylene glycol	MTT	[10]
Crassostrea gigas	spermatozoa	Me_2SO	fertility	[23, 48, 49]
			eosine-nigrosine	[24]
		Me_2SO, Trehalose	fertility	[1, 31]
		Ethylene glycol		[25]
		Propylene glycol		[5]
		Glycerol	comet assay	[17]
	spermatozoa 2n	Me_2SO	fertility	[14]
	oocytes	Me_2SO	fertility	[9]
		Glycerol		[33]
	embryos	Propylene glycol, Glycerol	viability	[19]
		Ethylene glycol, Me_2SO	embryonic development	[6]
		Me_2SO, Ethylene glycol		[28]
		Me_2SO		[7]
		Propanediol, Methanol		[42]
		Sucrose		[33, 41]
	larvae	Me_2SO	development	[33]
			viability	[48]
Pinctada fucata	larvae	Me_2SO	viability	[11]
Pecten maximus	heart cells	Me_2SO	Trypan blue, Bradford	[26]
			[^{3}H] thymidine incorporation	
			[^{14}C] leucine incorporation	
	embryos	Ethylene glycol	viability	[42]
		Propanediol, Methanol		
		Me_2SO, Glycerol, sucrose		
Ruditapes philippinarum	embryos	Ethylene glycol	viability	[42]
		Propanediol, Methanol		
		Me_2SO, Glycerol, Sucrose		
Mizuchopecten yessoensis	primary culture	Me_2SO, Glycerol,	Trypan blue	[34]
	embryo cells	Trehalose	[^{3}H]-uridine incorporation	[35]
	digestive gland cells			
Mytilus trossulus	primary culture	Me_2SO	[^{3}H]-uridine incorporation	[34]

	mantle, gills, muscles			[35]
Mytilus edulis	embryos	Me_2SO	viability	[45]
Meretrix lusoria	embryos	Me_2SO	development	[7]
Haliotis tuberculata	mantle cells hemocytes	Me_2SO, Glycerol Ethylene glycol	MTT, Bradford, Burton test, alkalin phosphatase activity [^{3}H]-Leucine incorporation	[39] [40]
Haliotis diversicolor	spermatozoa	Me_2SO, Glucose Me_2SO	fertility	[46] [18]

Table 1: Cryopreservation and molluscan species: cell type, nature of cryoprotectant agent (CPA) and test of viability or cell activity.