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Cryopreservation of mantle dissociated cells from *Haliotis tuberculata* (Gastropoda) and postthawed primary cell cultures☆

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Abstract

Dissociated mantle cells from the gastropod mollusc *Haliotis tuberculata* were cultured after a freezing–thawing procedure using either 10% dimethyl sulfoxide (Me_2SO) or 10% glycerol (Gly) as a cryoprotector. The survival rate of 2-day-old cultured cryopreserved cells after thawing, based on analysis of DNA and protein contents, was nearly 80% in comparison with 2-day-old cultured fresh cells. Cells thawed after cryopreservation exhibited the maintenance of all tested physiological activities. Metabolic activity (measured by the MTT test) and the activity of alkaline phosphatase (a plasma membrane-bound enzyme) were not decreased in comparison to those in cultured fresh cells. In addition, cryopreserved cultured cells maintained a physiological stimulation ability in response to treatment with growth factors. These results taken together represent one of the most convincing demonstrations of the survival and of the recovery of intact functional activities of molluscan cells after a freeze–thawing procedure. Our results suggest that in the future primary cultures of cryopreserved mantle cells will be able to be used for fundamental research, in toxicity tests, or in the field of biotechnology.

Keywords: Mollusc; Mantle cells; Cryopreservation

Cryopreservation of mammalian cells or tissues is widely used for fundamental or applied research as well as biotechnology. Many attempts have been made to establish long-term primary cell cultures of marine molluscs, and some suitable models have been successfully used to investigate a number of cellular activities, including cell differentiation or signaling [9,12,21], molecule synthesis [14,17,20], and environmental toxicological effects [1,16]. Despite recent progress, to date no continuous cell lines from marine molluscs have been reported [15]. At the present time therefore, cryopreservation for long-term storage of molluscan cells remains the most desirable alternative to collection of living cells. Cryopreservation also provides the following advantages: it allows banking of cells from a number of animals, it

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facilitates the transport of cells between different centers, allowing sufficient time for large sets of complementary investigations, and it may provide a source of standardized biological material.

Among molluscan cells, mantle cells from the nacreous mollusc *Haliotis tuberculata* have been shown to be a useful experimental model for investigation of the in vitro synthesis of extracellular matrix components, such as collagen and proteoglycans, and possibly for improving knowledge about biomineralization and shell building [14,17–19]. Taking into account that collagen and proteoglycans are commonly used as biomaterial, molluscan mantle cells may be of particular interest in the field of fundamental research and also for biotechnology. These cells may also represent an interesting model for investigating the toxicity of chemicals and metals which are contaminants of the environment.

Cryopreservation of molluscan mantle cells could open up new opportunities for many other investigations. Among marine molluscs, cryopreservation has been conducted with a few cell types. Small mantle pieces from pearl oyster [6] or dissociated mantle cells from mussel [13] have been preserved in liquid nitrogen. Successful cryopreservation was also performed with oyster embryos [10] or heart cells from *Pecten maximus* [8]. The experiments reported in these studies were undertaken to investigate the development of protocols for cryopreservation. The two typical compounds used as cryoprotectants, namely, glycerol (Gly) and dimethyl sulfoxide (Me_2SO), were specifically examined. Cell attachment or viability and measurement of some functional capacities, such as DNA, RNA, protein, or lipid syntheses, were determined before and after cryopreservation [8,13]. However, questions remain regarding how molluscan cell enzymatic activity and physiological responses are affected by the freezing and thawing processes. The purpose of this paper was to extend our knowledge by quantifying the degree of cryopreservation-induced injury on molluscan mantle cells. With this in view, we conducted a series of studies in order to evaluate: (1) the viability (DNA and protein contents); (2) metabolic activity (MTT test and incorporation of [^{3}H]leucine); (3) enzymatic activity (alkaline phophatase, ALP, activity); and (4) physiological stimulation as estimated by protein synthesis in response to treatment with growth factors, before and after cryopreservation of mantle cells from the gastropod mollusc *H. tuberculata*.

Material and methods

Animal

Adult wild abalones, 10–12 cm in shell length, were gathered from the northern Cotentin peninsula (France). They were acclimated to laboratory conditions for a few weeks before experimentation in natural, continuously aerated seawater regulated at 15 ± 3 °C. Animals were fed with freshly collected local algae (*Palmaria palmata* and *Laminaria digitata*).

Cell dissociation

Mantle cells were dissociated as described previously by Poncet et al. [14]. A preliminary decontamination step involved twice washing freshly removed mantles with sterile 199–Hanks' medium modified by addition of 250 mM NaCl, 10 mM KCl, 25 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM Hepes, and 10 mM glutamine, pH 7.4, and supplemented with antifungal and antibacterial substances (10 μg/ml streptomycin, 60 μg/ml penicillin, 35 μg/ml polymixin, 70 μg/ml bacitracin, 2 μg/ml amphotericin B, and 8 μg/ml nystatin). Tissues were then cut into small pieces and treated twice with dissociating medium containing 0.1% protease (w/v) and 0.1% collagenase (w/v) for 90 min. Dissociated cells were filtered through a 100-μm nylon mesh. They were then washed in modified 199–Hanks' medium supplemented only with penicillin and streptomycin (culture medium).

Freezing of cells

Cell suspensions were divided into three aliquots. Each of them was centrifuged at 4 °C for 10 min at 280*g*. The first resulting pellet was resuspended in ice-cold culture medium in order to attain a density of approximately 2.5×10^6 cells/ml. The second one was similarly resuspended in culture medium supplemented with 10% Me_2SO (v/v), whereas the third one was resuspended in culture medium containing 10% glycerol (v/v). As described by Le Marrec-Croq et al. [8], cell suspensions were rapidly centrifuged at 4 °C, and pellets were placed in a polystyrene pack in a −80 °C freezer for 12 h. Aliquots were then plunged into liquid nitrogen. The same procedure was carried out using a similar cell suspension, but, instead of being frozen, pellets were resuspended in culture medium in order to make up

controls. Cells were stored at −196 °C for a period of between a few days and several weeks.

Thawing of cells

Samples were placed in a water bath at 25 °C until the last ice crystal disappeared and immediately pellets were resuspended in 6 ml of culture medium. Cells were then centrifuged for 10 min at 280*g* and one more time resuspended in culture medium in order to remove cryoprotectants.

Primary cultures of mantle cells

Mantle cells were cultured as described previously by Poncet et al. [14]. Control and thawed cells were both plated at 1.5×10^5 cells/cm^2 in either 9.4-cm^2 culture flasks or 1.9-cm^2 culture dishes with culture medium (approximately 1 ml/10^6 cells). Cultures were maintained at 15 °C for 2 days in a humidified incubator in darkness.

MTT reduction assay

Cell viability was evaluated by the MTT (tetrazolium salts) reduction assay [11] adapted to molluscan cell cultures [4,9]. As described elsewhere, 10% (v/v) of the MTT stock solution (5 mg MTT/ml of PBS) was added to 1.9-cm^2 culture dishes. After a 24-h incubation, in order to dissolve the converted formazan, an equal volume of isopropanol containing 0.04 N HCl was added to each culture. Absorbance was then measured at a wavelength of 570 nm, with a reference at 630 nm.

Evaluation of protein content

Protein content was estimated using Bradford's method [2]. Cells (about 3×10^5 cells/dish) were scraped 2 days after seeding. After washing with 50 mM Tris buffer, pH 7.4, the cell suspension was ultrasonicated with 800 μl of Tris buffer. Samples were then clarified by a 1500*g* centrifugation for 20 min at 4 °C. The supernatant was collected and 200 μl was fourfold diluted with distilled water and completed with addition of 200 μl of protein assay kit (Bio-Rad, France). Total protein amount was determined by absorbance measured at a wavelength of 595 nm, with a BSA gradient as standard.

Estimation of DNA content

Burton's test [3] was used to determine the DNA content of 2-day-old mantle cell cultures. Cells were scraped and centrifuged. The resulting pellets were dissolved in a solution containing 5% perchloric acid, 2% diphenylamine, and 0.004% acetaldehyde. Samples were then stored for 24 h in darkness at room temperature, and DNA concentration was estimated by absorbance at 595 nm. A gradient of calf thymus DNA concentration was used as the standard.

ALP activity

Supernatants were obtained, as described above, by washing and subsequent ultrasonication and centrifugation of cultured cells. An equal volume of 1.5 M Tris buffer, pH 10, supplemented with 5 mM *p*-NPP (*para*-nitrophenyl phosphate, Sigma) as substrate, 1 mM $ZnCl_2$, and 1 mM $CaCl_2$ was added to each sample. They were then incubated at 37 °C for half an hour, and the reaction was stopped with 0.5 N NaOH. The reduction rate of *p*-NPP to *p*-NP (*para*-nitrophenol), revealing the ALP activity, was evaluated by the increase in absorbance at 410 nm, with a panel of *p*-NP concentrations as standard.

Incorporation of [^{3}H]leucine

Abalone cell cultures were supplemented with [^{3}H]leucine (NEN, Life Science Products, 179 Ci/mmol) corresponding to a final concentration 0.8 μCi/ml. Cultures were performed in the absence or the presence of bovine insulin (5 μM final concentration) or human IGF-I (0.01 μM final concentration). As described [9], after 24 h of culture at 15 °C, leucine incorporation was stopped by precipitation with an equal volume of ice-cold trichloroacetic acid (10%). Cultures were then scraped and centrifuged at 3000*g* for 10 min. The resulting pellets were washed twice with 10% TCA in order to evacuate the remaining free labeled leucine and dissolved in heated 0.3 M KOH. The radioactivity contained in each sample was then measured and controls were performed by TCA addition to cultures immediately after [^{3}H]leucine addition.

Statistical analysis

The significance of the difference between mean values was estimated using the Student's *t* test. Each experiment was repeated at least three times and for one experiment the means were calculated from triplicates.

Results

Effects of cryoprotective treatment on control cell cultures

Cell viability and metabolic and enzymatic activities were measured in unfrozen control cell cultures previously treated with Me_2SO or Gly in order to assess potential harmful effects of each of these cryoprotectors. As shown in Table 1, MTT reduction (OD 570 nm/ref.630 nm $= 0.133 \pm 0.004$ for control cells) and ALP activity (24.6 ± 1 nmol *p*-NP/μg protein·hour for control cells) are only slightly and not significantly reduced by 10% Gly (0.131 ± 0.002 for MTT reduction and 22.9 ± 1.1 nmol *p*-NP/μg protein·hour for ALP activity). The effect of 10% Me_2SO was even less important (0.122 ± 0.003 for MTT reduction and 20.8 ± 1.3 nmol *p*-NP/μg protein·hour for ALP activity). Me_2SO (10%) and Gly (10%) induced a slight but not significant increase in the protein content (respectively, 5.11 ± 0.56 and 4.78 ± 0.16 μg/dish) compared to control levels (4.67 ± 0.14 μg/dish). It can therefore be assumed that neither 10% Gly nor 10% Me_2SO treatment acts as a toxic agent to cultured abalone mantle cells.

Evaluation of cell viability after thawing

In comparison with control cell cultures, it appears that after thawing an important part of the DNA content was retained in 10% Gly treated cells (67% in comparison with control cells) (Fig. 1). An even higher rate of preservation was attained with 10% Me_2SO as cryoprotectant (76% in comparison with control cells). Very similar results were obtained regarding whole culture protein content (4.7 ± 0.1 μg/dish for the control; 3.5 ± 0.1 and 3.2 ± 0.2 μg/dish for cells frozen, respectively, with 10% Me_2SO and 10% Gly). Thus high levels of viability seem to be retained during the freeze–thawing cycle in 10% Gly and even more in 10% Me_2SO.

Evaluation of cell activity after thawing

The MTT assay reflects the activity of mitochondrial deshydrogenases in cultured cells. Fig. 2 reveals that after thawing, cryoprotected cells may recover a large part of this activity. In such tests, a better cryoprotective effect is provided by Me_2SO (85% of the control activity) and 80% with 10% Gly-treated cells. ALP activity, expressed as an amount of *p*-NP formed per protein concentration and per hour, is higher after thawing than that in control unfrozen cells. Thus, cells protected with 10% Me_2SO yield 26.8 ± 0.6 nmol *p*-NP/μg protein·hour compared to 24.6 ± 1.0 nmol *p*-NP/μg protein·hour for control cultures, and this rate reaches 36.4 ± 2.2 nmol *p*-NP/μg protein·hour with Gly-treated frozen cells. Cell activity thus seems to be largely preserved after thawing on employing 10% Gly or 10% Me_2SO as cryoprotectant.

Effects of insulin and IGF-I after thawing in 2-day-old cultures

Protein synthesis was evaluated in cell cultures by the rate of incorporation of free labeled leucine. In control cells, the addition of insulin (5 μM) to the culture medium induced a substantial increase in protein synthesis (167 ± 5 dpm/dish for treated cells versus 74 ± 12 dpm/dish for control cells). This augmentation in protein synthesis is even more important with IGF-I (0.01 μM) (464 ± 41 dpm/dish). The ability of cultured cells to be stimulated in this way appears to be completely preserved after a freeze–thawing procedure in the presence of either Gly or Me_2SO. After insulin stimulation, the rate of [^{3}H]leucine incorporation reached, respectively, 295 ± 101 and 231 ± 18

Table 1
Protein content (μg/dish), MTT assay (OD 570 nm/ref. 630 nm) and ALP activity (nmol *p*-NP/μg protein·hour) in fresh control cell cultures or after treatment with cryoprotectors (10% Me_2SO or 10% Gly)

	Protein content (μg/dish)	MTT assay (OD 570 nm/ref. 630 nm)	ALP activity (nmol *p*-NP/μg protein·hour)
Control	4.67 (±0.14)	0.133 (±0.004)	24.6 (±1.0)
10% Me_2SO	5.11 (±0.56) NS	0.122 (±0.003) NS	20.8 (±1.3) NS
10% Glycerol	4.78 (±0.16) NS	0.131 (±0.002) NS	22.9 (±1.1) NS

Note. Cells were seeded at 3×10^5 cells per dish and grown for 2 days at 15 °C in modified Hanks'–199 medium. Each data point represents the mean ± standard deviation of triplicate cultures. NS, nonsignificant difference from control cells.

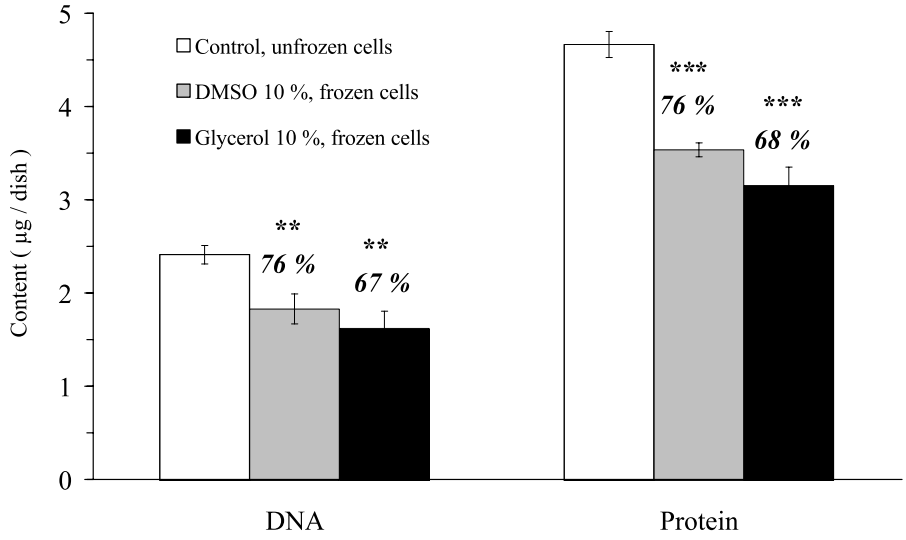

Fig. 1. Evaluation of cultured mantle cell viability after thawing. Control (unfrozen cells) or frozen cells (10% Me_2SO or 10% Gly) were seeded at 3×10^5 cells per dish and grown for 2 days at 15 °C in modified Hanks'–199 medium. DNA (µg/dish) and protein (µg/dish) contents were determined as described under Material and methods. Each data point represents the mean ± standard deviation of triplicate cultures. Significant differences from control cells at $P < 0.01$ (**); $P < 0.001$ (***). Italic values indicate the percentages of DNA and protein contents in comparison with those of the respective control.

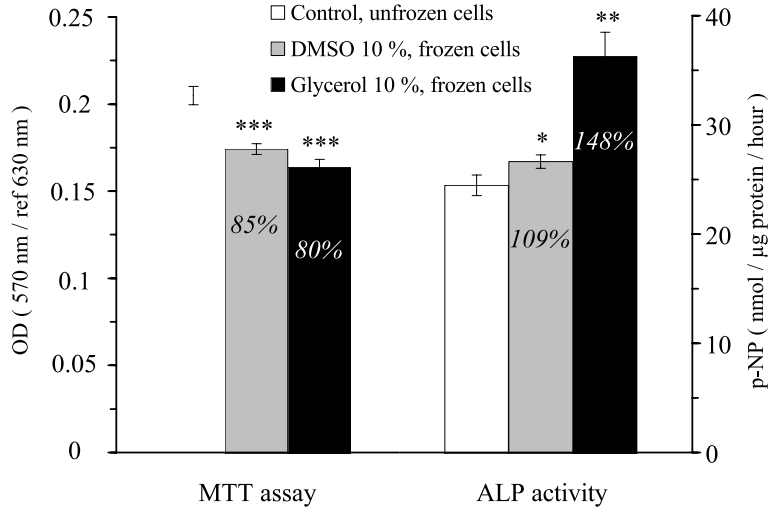

Fig. 2. Evaluation of cultured mantle cell activity after thawing. Control (unfrozen cells) or frozen cells (10% Me_2SO or 10% Gly) were seeded at 3×10^5 cells per dish and grown for 2 days at 15 °C in modified Hanks'–199 medium. MTT assay (OD 570 nm/ref. 630 nm) and ALP activity (*p*-NP nmol/µg protein·hour) were determined as described under Material and methods. Each data point represents the mean ± standard deviation of triplicate cultures. Significant differences from control cells at $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***). Italic values indicate the percentage of MTT values or ALP activities in comparison with control values.

dpm/dish. On the other hand, the use of IGF-I provided results in line with those of previous studies. A treatment with IGF-I induces a [^{3}H]leucine incorporation rate of 546 ± 52 dpm/dish for Me_2SO-protected cells and 553 ± 203 dpm/dish for Gly-protected cells. Thus, the

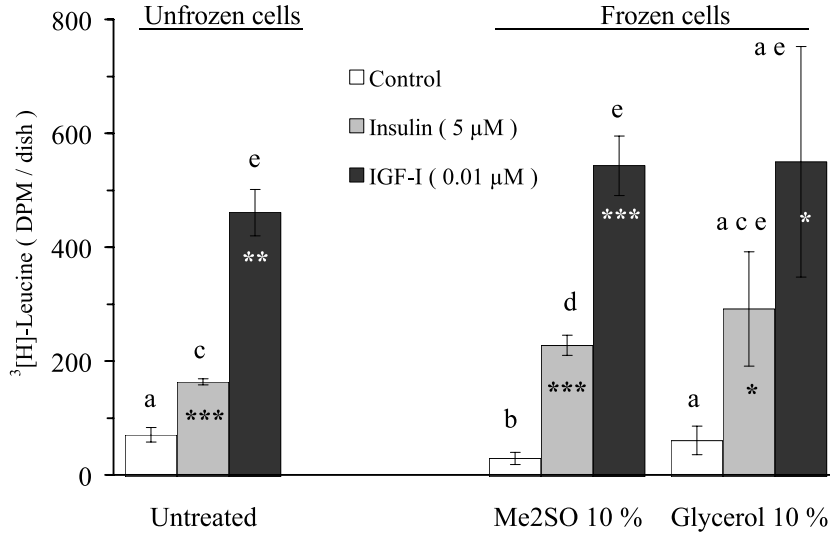

Fig. 3. Effects of insulin and IGF-I on the incorporation of [^{3}H]leucine in fresh cells or after freezing–thawing (10% Me_2SO or 10% or Gly) procedure in 2-day-old culture cells. Cells were seeded at 1.4×10^6 cells per flask and grown in the absence or the presence of insulin (5 µM) or IGF-I (0.01 µM) for 24 h at 15°C in modified Hanks'–99 medium. Each data point represents the mean ± standard deviation of triplicate cultures. Significant differences from respective control cells at $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***). Letters represent the statistical comparison between control and frozen cells (10% Me_2SO or 10% Gly) for an identical treatment (insulin or IGF-I). Histograms with the same letter (a or e) are statistically nondifferent; (b) represents a significant difference from (a) at $P < 0.01$.

stimulation induced by IGF-I is statistically equivalent (see e in Fig. 3) in control (unfrozen cells) and in 10% Me_2SO or 10% Gly protected freeze–thawed cells. For insulin treatment, the stimulation rate remains optimal after the process of freeze–thawing (see a in Fig. 3) and indeed better (b) in the case of the 10% Me_2SO protection.

Discussion

Cryopreservation has a significant role in providing material for fundamental science and biotechnology. Cryopreservation of marine invertebrate cells may lead to applications in studies of aquatic biodiversity and marine ecotoxicology. Despite recent progress in cryopreservation procedures of vertebrate cells, very little information has been published on the viability and more particularly on the maintenance of functional activities after the freeze–thawing of cryopreserved marine invertebrate cells. In this study, experiments were conducted to evaluate the viability, the metabolic and enzymatic activities, as well as physiological stimulation in response to treatment with growth factors before and after cryopreservation of mantle cells from *H. tuberculata*.

First, the use of cryoprotective agents (CPAs) is indispensable for preventing cryoinjury to cells during the cryopreservation process. Aqueous solutions of Me_2SO and Gly are widely used as cryoprotectant agents in protocols for long-term low-temperature preservation of viability, including cells, tissues, and embryos from marine molluscs [6,8,10,13]. Investigations conducted with dissociated molluscan cells, such as mantle or heart cells, have shown that the addition of cryoprotectors at concentrations close to 10% (Me_2SO or glycerol) was the most efficient [8,13].

Addition of CPAs to cells before cooling and removal of CPAs after thawing can result in severe cell injury [7]. The possible negative effects of CPAs on the metabolic capacity of cryopreserved molluscan cells have not been tested in previous studies. Data presented in this report demonstrate that the presence of Me_2SO (10%) or glycerol (10%) has no significant effect on protein content (respectively, 5.11 ± 0.56 and 4.78 ± 0.16 µg/dish versus 4.67 ± 0.14 µg/dish for control cells), on cellular metabolic activity (MTT assay, respectively, 0.122 ± 0.003 and 0.131 ± 0.002 versus 0.133 ± 0.004 for control cells), or on alkaline phosphatase activity (20.8 ± 1.3 and 22.9 ± 1.1 nmol/µg pro-

tein·hour versus 24.6 for control cells) of 2-day-old mantle cell cultures from *H. tuberculata*.

Investigations conducted by Odintsova and Tsal [13] with dissociated mantle cells from mussels have shown viability to be near to 80% after a freeze–thawing procedure using 10% Me_2SO ($77 \pm 1.45\%$ viable cells) or 10% glycerol ($80.5 \pm 2.95\%$ viable cells) as CPA. Cell viability was tested by the trypan blue exclusion test. Based on DNA and protein contents our results agree with these values. Cell viability was ±76% for 10% Me_2SO and 67–68% for 10% glycerol. Similarly, Le Marrec-Croq et al. [8] have found that the viability of *P. maximus* heart cells ranged between 70 and 90% after cryopreservation with 12% Me_2SO. Viability levels of dissociated molluscan cells after cryopreservation appear, therefore, to be roughly similar. However, the analysis of functional activities in freeze–thawed cultured molluscan cells seems more controversial.

RNA synthesis in primary cell cultures of mussel mantle after the freeze–thawing procedure was significantly decreased [13]. In fact, the incorporation levels of [^{3}H]uridine by unfrozen cells were, respectively, 1.45-fold higher than that in cryopreserved cells protected by 10% Me_2SO (11.712×10^3 cpm versus 8.005×10^3 cpm for $1–3 \times 10^6$ cells) and 2.3-fold higher than that in cryopreserved cells protected by 10% glycerol (5.181×10^3 cpm). On the other hand, Le Marrec-Croq et al. [8] reported similar metabolic activities in both unfrozen and frozen cultured heart cells. These metabolic activities were evaluated by DNA and protein syntheses via the incorporation of [^{3}H]thymidine and [^{14}C]leucine. In our study, the metabolic activity observed in cultured frozen cells was 85% with 10% Me_2SO and 80% with 10% Gly in comparison with control unfrozen cells. This slight decrease is in accordance with the decreasing viability quantified with DNA and protein contents. The result was that surviving cells had recovered the same basal MTT activity as that of unfrozen cells. The recovery of intact functional activity of surviving cells is confirmed by the estimation of the alkaline phosphatase activity. In a 2-day-old culture significant increases in ALP activity (9 and 48% for Me_2SO and Gly, respectively) were measured in frozen cells in comparison with unfrozen control cells. The mantle is composed of several cell types, including epithelial cells, different glandular cell types which may or may not be ciliated, nonglandular cells, and hemocytes. No data are available concerning either the ALP activity of these different cell types or their evolution in culture. The observed increase in ALP activity may be due to a differential rate of survival of these cell types.

The response of primary molluscan cells to vertebrate growth factors, such as insulin and IGF-I, has been established [5,9,17]. Vertebrate insulin (10^{-6} M) stimulated [^{3}H]leucine incorporation in cultured hemocytes. The maximal stimulation induced an increase of 196% with respect to 100% for nontreated cells [9]. In the present report insulin (5×10^{-6} M) induced a similar increase in [^{3}H]leucine incorporation in cultured mantle cells. The effect of IGF-I on *Haliotis* mantle cells appeared more potent than that of insulin. This finding is in line with results registered for scallop digestive gland or scallop mantle edge cells [5]. The freeze–thawing procedure had no significant effect on the response of *Haliotis* cultured mantle cells to vertebrate growth factors.

To date, these results taken together represent one of the most convincing demonstrations of the recovery of intact functional activities of cultured molluscan cells after a cryopreservation procedure. Our results indicate that cryopreserved mantle cells will be able to be used in fundamental research or in biotechnology. Recently, we have described a useful in vitro model from primary cultures of mantle cells from *H. tuberculata* and shown the ability of these cultured cells to synthesize proteoglycans and collagen as components of the extracellular matrix [14]. Yoneda et al. [22] have provided evidence that both the abalone collagen mRNA level and collagen content vary seasonally and decrease from December–January to summer. This observation confirms the advantage of the availability of cryopreserved cell banks to provide a source of optimal or standardized biological material. In addition, mantle cell ALP activity has been proposed as a marker of biomineralization, as in vertebrates [18,19]. The maintenance of ALP activity in cryopreserved mantle cells may thus prove useful for further studies concerning the process of biomineralization.

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References

[1] S. Auzoux, I. Domart-Coulon, D. Doumenc, Gill cell cultures of the butterfish clam *Ruditapes decussatus*, J. Mar. Biotechnol. 1 (1993) 79–81.

[2] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[3] K. Burton, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid, Biochem. J. 62 (1956) 315–323.

[4] I. Domart-Coulon, D. Doumenc, S. Auzoux-Bordenave, Y. Le Fichant, Identification of media supplements that improve the viability of primarily cell cultures of *Crassostrea gigas* oysters, Cytotechnology 16 (1994) 109–120.

[5] W. Giard, J.-M. Lebel, E. Boucaud-Camou, P. Favrel, Effects of vertebrate growth factors on digestive gland cells from the mollusc *Pecten maximus* L.: An in vitro study, J. Comp. Physiol. B 168 (1998) 81–86.

[6] C. Horita, H. Mega, H. Kurokura, Grafting of cryopreserved mantle tissues onto cultured pearl oyster, Cryo-Lett. 20 (1999) 311–314.

[7] J.O.M. Karlsson, M. Toner, Long-term storage of tissues by cryopreservation: Critical issues, Biomaterials 17 (1996) 243–256.

[8] F. Le Marrec-Croq, P. Fritayre, C. Chesné, A. Guillouzo, G. Dorange, Cryopreservation of *Pecten maximus* heart cells, Cryobiology 37 (1998) 200–206.

[9] J.-M. Lebel, W. Giard, P. Favrel, E. Boucaud-Camou, Effects of different vertebrate growth factors on primary cultures of hemocytes from the gastropod mollusc, *Haliotis tuberculata*, Biol. Cell 86 (1996) 67–72.

[10] T.T. Lin, N.H. Chao, H.T. Tung, Factors affecting survival of cryopreserved oyster (*Crassostrea gigas*) embryos, Cryobiology 39 (1999) 192–196.

[11] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.

[12] T. Naganuma, B.M. Degnan, K. Horikoshi, D.E. Morse, Myogenesis in primary cell cultures from larvae of the abalone *Haliotis rufescens*, Mol. Mar. Biol. Biotechnol. 3 (1994) 131–140.

[13] N. Odintsova, L. Tsal, Cryopreservation of primary cell cultures of Bivalvia, Cryo-Lett. 16 (1995) 13–20.

[14] J.-M. Poncet, A. Serpentini, B. Thiébot, V. Villers, J. Bocquet, E. Boucaud-Camou, J.-M. Lebel, In vitro synthesis of proteoglycans and collagen in primary cultures of mantle cells from the nacreous mollusks, *Haliotis tuberculata*: A new model for study of molluscan extracellular matrix, Mar. Biotechnol. 2 (2000) 387–398.

[15] B. Rinkevitch, Cell cultures from marine invertebrates: Obstacles, new approaches and recent improvements, J. Biotechnol. 70 (1999) 13–153.

[16] Y. Robledo, M.P. Cajaraville, Isolation and morphofunctional characterization of mussel digestive gland cells in vitro, Eur. J. Cell Biol. 72 (1997) 362–369.

[17] A. Serpentini, C. Ghayor, J.-M. Poncet, V. Hebert, P. Galera, J.-P. Pujol, E. Boucaud-Camou, J.-M. Lebel, Collagen study and regulation of the *de novo* synthesis by IGF-I in hemocytes from the gastropod mollusc, *Haliotis tuberculata*, J. Exp. Zool. 287 (2000) 275–284.

[18] D. Sud, S. Auzoux-Bordenave, M. Martin, D. Doumenc, Cell cultures from the abalone *Haliotis tuberculata*. A new tool for in vitro study of biomineralization, in: Le Gal, Halvorson (Eds.), New Developments in Marine Biotechnology, Plenum, New York, 1998, pp. 165–170.

[19] D. Sud, D. Doumenc, E. Lopez, C. Milet, Role of water-soluble matrix fraction, extracted from the nacre of *Pinctada maxima*, in the regulation of cell activity in abalone mantle cell culture (*Haliotis tuberculata*), Tissue Cell 33 (2001) 1–7.

[20] T. Suzuki, R. Yoshinaka, S. Mizuta, S. Funakoshi, K. Wada, Extracellular matrix formation by amebocytes during epithelial regeneration in the pearl oyster *Pinctada fucata*, Cell Tissue Res. 266 (1991) 75–82.

[21] Y. Takeuchi, K. Inoue, D. Miki, S. Odo, S. Harayama, Cultured mussel foot cells expressing byssal protein genes, J. Exp. Zool. 283 (1999) 131–136.

[22] C. Yoneda, M.N. Ahsan, M. Nakaya, Y. Matsubara, T. Ebihara, I. Shinkichi, Y. Uno, K. Hatae, S. Watabe, Abalone collagens: Immunological properties and seasonal changes of their mRNA levels, Comp. Biochem. Physiol. B 126 (2000) 59–68.