Investigating the establishment of primary cell culture from different abalone (*Haliotis midae*) tissues

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Received: 24 May 2010 / Accepted: 24 July 2010 / Published online: 3 August 2010
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Abstract The abalone, *Haliotis midae*, is the most valuable commodity in South African aquaculture. The increasing demand for marine shellfish has stimulated research on the biology and physiology of target species in order to improve knowledge on growth, nutritional requirements and pathogen identification. The slow growth rate and long generation time of abalone restrict efficient design of in vivo experiments. Therefore, in vitro systems present an attractive alternative for short term experimentation. The use of marine invertebrate cell cultures as a standardised and controlled system to study growth, endocrinology and disease contributes to the understanding of the biology of economically important

molluscs. This paper investigates the suitability of two different *H. midae* tissues, larval and haemocyte, for establishing primary cell cultures. Cell cultures are assessed in terms of culture initiation, cell yield, longevity and susceptibility to contamination. *Haliotis midae* haemocytes are shown to be a more feasible tissue for primary cell culture as it could be maintained without contamination more readily than larval cell cultures. The usefulness of short term primary haemocyte cultures is demonstrated here with a growth factor trial. Haemocyte cultures can furthermore be used to relate phenotypic changes at the cellular level to changes in gene expression at the molecular level.

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Keywords *Haliotis midae* · Cell culture · Larval ·
Haemocyte · Viability

Introduction

The practice of cell culture in marine invertebrate species, specifically molluscs, is not as well developed as in vertebrate species. Despite the need for immortalised cell lines from marine invertebrates in aquaculture, biotechnology and the pharmaceutical industry, attempts made to establish such cell lines have been ineffective. The lack of knowledge pertaining to invertebrate cell physiology and biochemistry, the slow rate of cell proliferation in vitro and the formulation of appropriate culture media

remain unsolved challenges in the quest for marine invertebrate cell lines (Lebel et al. 1996; Rinkevich 2005; Rosenfield 1993).

Primary cell cultures provide suitable models for short term in vitro investigations, in spite of the disadvantages of increased risk of contamination, limited number of cells and short lifetime associated with them. The isolation and maintenance of primary cell cultures for limited periods, ranging from a few days to several weeks, has been accomplished in a number of aquatic species. Endeavours to improve aquaculture have particularly stimulated the development of primary cell cultures from economically important marine mollusc species. Mantle explant cultures of the mussel, *Lamellidens marginalis*, have been maintained for 70 days in an attempt to utilise cell culture as a way of producing tissue-cultured pearls (Barik et al. 2004). Primary cell cultures from oyster heart and clam gills have also been used as experimental in vitro models to assess cytotoxicity of aquatic pollutants (Domart-Coulon et al. 2000). Furthermore, mussel and abalone larval cell cultures have been maintained for three and 12 weeks, respectively, to investigate myogenesis (Naganuma et al. 1994; Odintsova et al. 2001). Other abalone primary cell cultures, including mantle explants and haemocyte cell culture of *Haliotis tuberculata*, have been maintained for up to 2 weeks to investigate the control of biomineralization (Auzoux-Bordenave et al. 2007) and the effect of vertebrate growth factors on haemocyte physiology (Lebel et al. 1996). Abalone haemocyte primary cell cultures were also used to investigate transcription expression patterns under conditions of thermal stress (Farcy et al. 2007) and to investigate the effect of insulin-like growth factor 1 (IGF-1) on haemocyte collagen synthesis (Serpentini et al. 2000). It is clear from these studies that relevant knowledge of the biology and physiology of molluscs can be gained from the use of primary cell culture.

Primary cell cultures are initiated by tissue fragmentation (explants) or by dissociating tissues to isolate individual cells. Cell isolation can be accomplished by mechanical, chemical and enzymatic dissociation methods. When a cell population consisting of individual cells is required, the method of choice will be one which delays re-aggregation of cells; thus chemical or enzymatic dissociation (Mulcahy 2000; Pomponi and Willoughby 2000). To isolate

single cells, the extracellular matrix is disrupted by proteolytic enzymes such as collagenase or trypsin, that digest extracellular matrix proteins or by chemical agents such as EDTA which bind the Ca^{2+} on which cell–cell adhesion depends (Alberts et al. 2002; Mulcahy 2000). During all types of dissociation, cells are vulnerable to mechanical and toxic damage and therefore care should be taken to limit the duration of the dissociation procedure (Pomponi and Willoughby 2000).

Culture medium significantly affects the success of primary cell culture initiation and because different cell types have different growth requirements, the most appropriate medium should be formulated. When experimenting with new cell cultures, such as marine invertebrate cell culture, it is often challenging to formulate an appropriate medium as most commercial media have been developed for use in mammalian cell culture. These commercial media may be used as a foundation and adapted in terms of osmolarity, pH and nutritional components to simulate the marine environment.

The tissues of origin for the present study are veliger larvae and haemolymph from *Haliotis midae*. The larval development stages of *Haliotis midae* are similar to that described by Hahn (1989), for *Haliotis discus hannai*, and Courtois De Viçose et al. (2007) for *Haliotis tuberculata*. Larvae hatch as trochophores at 10–12 h post-fertilization and develop through the veliger stages from fifteen to 48 h post-fertilization. The length of free swimming larvae of $166.6 \pm 7.6 \mu\text{m}$ and width, $124.7 \pm 4.75 \mu\text{m}$ as reported for *Haliotis tuberculata* (Courtois de Viçose et al. 2007; Vacquier et al. 1990) corresponds to the size in *Haliotis midae* (personal observation). At the veliger larval stage, differentiation into various cell types, including those comprising the larval retractor muscle, larval shell, foot mass, eye-spot and velum with cilia has occurred (Courtois de Viçose et al. 2007).

Abalone haemocytes represent a heterogeneous population both in morphological and functional terms. Three types of abalone (*Haliotis tuberculata*) haemocytes exist: large hyalinocytes, basophilic granulocytes and small blast-like cells (with a high nuclear-to-cytoplasm ratio). Haemocytes have been suggested to play roles in defense mechanisms (like cell-mediated cytotoxicity, encapsulation and phagocytosis), production of antimicrobial peptides, digestion,

metabolite transport, wound and shell repair and secretion of extracellular matrix components (Cima et al. 2000; Humphries and Yoshino 2003; Lebel et al. 1996; Travers et al. 2008). Haemocytes provide a suitable model to investigate the effects of factors involved in proliferation and differentiation and various reports on haemocyte primary cell culture are available (Auzoux-Bordenave et al. 2007; Gagnaire et al. 2006; Lebel et al. 1996; Serpentine et al. 2000; Terahara et al. 2005).

Haliotis midae cell cultures can provide a method for investigating a wide range of cell-mediated responses. This paper investigates the suitability of larval and haemocyte tissues for establishing primary cell cultures. Culture initiation, maintenance in terms of media suitability and cell viability, and susceptibility to contamination are described. The utility of primary haemocyte cell cultures is demonstrated at the hand of a growth supplement trial.

Materials and methods

Tissue collection

All materials originated from a commercial abalone hatchery situated on the Southwestern coast of South Africa. Care was taken throughout tissue collection to sterilise all equipment and surfaces with 70% ethanol and 1% Virkon disinfectant. All procedures were carried out in a laminar flow cabinet. For larval cell cultures, normal embryo division was verified after fertilisation by light microscopic observation and healthy developing embryos at the four to eight cell stage were collected at a temperature of 18 °C in 0.2 µm-filtered seawater supplemented with 1% Penicillin/Streptomycin, 2.5 µg/mL Amphotericin B and 50 µg/mL Gentamycin (AB wash solution). Embryos were transported within 2 h from the abalone farm to the laboratory in this solution on ice. The solution was replaced by filtered seawater and embryos were transferred to sterile glass bottles and incubated at 18 °C overnight. Hatched larvae were collected by pouring the seawater in which they hatched through 100 µm mesh and then rinsing the larvae off with a small amount of FSW into sterile 50 mL conical tubes (Greiner Bio-One). Larvae were collected by centrifugation (300 g, 17 °C, 5 min) and washed several times with AB wash solution.

Following the final centrifugation, the supernatant was aspirated carefully and the larval pellet was used for subsequent dissociation steps.

Before haemolymph collection, adult abalone of size 70–80 mm were starved and kept in clean ultra-violet sterilised seawater for 3 days. Dissection, haemolymph collection and culture initiation were performed in a laminar flow cabinet. The foot of each animal was wiped with 70% ethanol before dissection to clean the surface of mucous and debris. The foot was dissected away from the shell and organs and placed, cut side down, in a 90 mm Petri dish where it was allowed to bleed for 10 min. Care was taken not to sever the gut or gonad, as this would release contaminating material. The volume of haemolymph collected ranged from six to 12 mL per abalone.

Cell dissociation

Mechanical, enzymatic and a combination of chemical and mechanical dissociation methods were tested for larval dissociation. Mechanical dissociation of larvae consisted of maceration of larvae with a pestle in an eppendorf tube. Enzymatic methods included treatment with trypsin at concentrations of 0.15 or 0.25% at room temperature for 1 h or at the same concentrations at 17 °C for 3 h. Another enzymatic dissociation method consisted of treatment with 0.125% collagenase in Calcium- and Magnesium-free artificial seawater solution (CMFSS: 25.5 g/L NaCl, 0.8 g/L KCl, 3.0 g/L Na₂HPO₄, 3.0 g/L Glucose, 2.86 g/L HEPES, personal communication N.A. Odintsova 2007) for 1 h at room temperature, with gentle agitation. Chemical–mechanical dissociation was accomplished by treating larvae with AB wash solution for 45 min followed by [CMFSS + 15 mM EDTA + 1% Penicillin/Streptomycin] for 10 min at room temperature. After collecting the tissue pellet by centrifuging at 300 g for 3 min, it was resuspended in 3 mL [CMFSS + 15 mM EDTA + 1% Penicillin/Streptomycin] and gently pipetted 10 times. Following dissociation and quantification (by haemocytometer counting) cells were seeded in six-well and 12-well plastic tissue culture plates (CELLSTAR, Greiner Bio-one), and culture medium was added.

For haemocyte culture initiation, the initiation method reported by Lebel et al. (1996) and Auzoux-Bordenave et al. (2007) was followed. Haemolymph

Table 1 Culture media formulations

Culture medium A (Domart-Coulon et al. 1994)		Culture medium D (Artificial Seawater Medium; ASM) (Sea Urchin Embryology: Artificial Seawater 1997)	
NaCl	10.1 g	NaCl	6.15 g
KCL	0.27 g	KCL	0.1675 g
CaCl ₂	0.3 g	CaCl ₂ ·2H ₂ O	0.34 g
MgSO ₄ ·7H ₂ O	0.5 g	MgSO ₄ ·7H ₂ O	1.5725 g
MgCl ₂ ·6H ₂ O	1.95 g	MgCl ₂ ·6H ₂ O	1.165 g
		NaHCO ₃	0.045 g
Dissolve in 500 mL Leibovitz-L15		Dissolve in 250 mL dH ₂ O	
		Adjust pH to 7.6. Filter through 0.2 µm into sterilized bottle	
Add		Add	
Penicillin/Streptomycin	1% (v/v)	Penicillin/Streptomycin	1% (v/v)
Gentamycin	0.5% (v/v)	Gentamycin	0.5% (v/v)
Amphotericin B	0.4% (v/v)		
Adjust pH to 7.6. Filter through 0.2 µm into sterilized bottle			
Add glutamax (or L-glutamine)	2% (v/v)		
osmolarity ~ 1,000 mmol/kg			

was collected into a sterile 50 mL conical tube (Greiner Bio-One) and an equal volume of AB wash solution was added. The mixture was passed through a 70 µm cell strainer into a clean 50 mL tube, to eliminate debris, before two volumes (to one volume of haemolymph) of anticoagulant Alsever Solution (Lebel et al. 1996) was added. A sample from the haemolymph mixture was counted using a haemocytometer and cells were seeded in the required densities in 96-well tissue culture plates. The cells were incubated at 18 °C and allowed to adhere to the surface of the tissue culture dish for 1–2 h. Following this incubation, the solution was removed and replaced with a mixture of 50% culture medium and 50% AB wash solution. After another 1–2 h in the incubator, the medium was changed to 100% culture medium (100 µL per well).

Conditions for cell maintenance and media

Cells were incubated at 18 °C in a Hotpack low temperature incubator in specified culture medium. Tissue culture plates were sealed with parafilm and no artificial humidity- or CO₂ environment was simulated in the incubator. The pH of seawater from the abalone hatchery was verified as 7.6–7.8 and the

osmolarity as 1,030–1,070 mmol/kg. This was taken into consideration when formulating culture media. Various formulations of culture media were tested for larval cell culture maintenance before optimal cell culture media (media A and D, Table 1) were adopted from the formulations of Domart-Coulon et al. (1994) and the Stanford University Sea Urchin Embryology group (Sea Urchin Embryology: Artificial Seawater 1997). Domart-Coulon et al. (1994) reported the use of media similar to medium A, as basal media. When supplemented, it supported oyster heart cell culture for up to 3 months (Domart-Coulon et al. 1994). Artificial seawater (the foundation of medium D) is routinely used to maintain sea urchin gametes, embryos and larvae (Alvarez et al. 2008; Qiao et al. 2003) and supplemented media similar to medium D have been used to maintain cardiac cells of the surf clam for up to 4 months (Cecil 1969). Haemocyte cultures were also maintained in culture media A and D (Table 1). After preparation of cell culture medium, it was filtered at 0.2 µm and stored at 4 °C for a maximum of 3 weeks. Glutamine was only added prior to use. Cultures were observed under light and phase contrast on an Olympus CKX31 inverted microscope. Cultures were inspected regularly for contamination. A rapid change in pH

Table 2 Medium D supplementation to study the effect on viability of cultured haemocytes

Medium name	Supplement/supplement combination	Concentration
D-1	Bovine insulin	50 µg/mL
D-2	Epidermal growth factor (EGF)	25 ng/mL
D-3	Catalase	20 µg/mL
D-4	Collagenase	100 µg/mL
D-5	50% Insulin + 50% EGF	25 µg/mL + 12.5 ng/mL
D-6	50% Insulin + 50% Catalase	25 µg/mL + 10 µg/mL
D-7	50% Insulin + 50% Collagenase	25 µg/mL + 50 µg/mL
D-8	50% EGF + 50% Catalase	12.5 ng/mL + 10 µg/mL
D-9	50% EGF + 50% Collagenase	12.5 ng/mL + 50 µg/mL
D-10	50% Catalase + 50% Collagenase	10 µg/mL + 50 µg/mL

(visible by a colour change from red to orange/yellow in the medium), cloudiness of the medium, extracellular granularity and visible floating or moving material were all regarded as indicators of contamination (Freshney 1992).

Medium supplementation

For haemocyte cell culture, medium D was supplemented with the different factors summarised in Table 2 to investigate the effect of these known growth stimulants on haemocyte viability. These factors used alone or in combination are reported to result in an increase in metabolic activity in cultured mollusc cells (Chen and Wen 1999; Domart-Coulon et al. 1994; Lebel et al. 1996). Insulin and EGF have been reported to stimulate cell viability in *Haliotis tuberculata* haemocytes (Lebel et al. 1996) and *Crassostrea gigas* heart cells (Domart-Coulon et al. 1994). Also, catalase was shown to have a positive effect on *Crassostrea gigas* heart cell growth (Domart-Coulon et al. 1994). Collagenase has been reported to enhance growth in *Crassostrea gigas* and *Meretrix lusoria* heart cells (Chen and Wen 1999).

Viability assessment

The Trypan Blue (TB) exclusion test was employed to verify larval cell viability after culture initiation. TB (0.4 %) was added to the cells and incubated for 5 min before microscopic examination. Cell metabolic activity of larval cells and haemocytes was also monitored over time by interpreting the results of XTT assays on specified days of culture. The XTT assay relies on the cleavage of a yellow tetrazolium

salt into an orange formazan product by metabolically active cells (Roche Applied Science 2003). Measurement of such metabolic activity over time can be regarded as indicative of cell viability or cell density variations. Five milliliter XTT (sodium 3'-[1- (phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) and 100 µL PMS (*N*-methyl dibenzopyrazine methyl sulfate) were thawed, combined and mixed thoroughly according to the manufacturers' instructions. Fifty microliters of XTT/PMS mix was added to each well of the 96-well plate containing the cells in 100 µL of culture medium. The plate was wrapped in foil and returned to the incubator (18 °C) for 6 hours. After incubation, an absorbance reading was performed on the 96-well plate with a Bio-Rad model 680 Microplate Reader using a measurement filter at 490 nm and a reference filter at 655 nm.

Statistical analysis

Results of the viability assays were analysed by using Students' *t*-tests and Analyses of Variance (ANOVA). ANOVAs that confirmed a significant difference between treatment groups ($p < 0.05$) were followed by pairwise *t*-tests to evaluate the difference between individual treatment groups and the untreated control. Prior to performing a *t*-test, an *F*-test for equal variances was performed. According to the results of this *F*-test, the appropriate *t*-test (assuming equal or unequal variances) was performed. *t*-Tests that returned *p*-values of <0.05 were regarded as confirmation of significant differences between treated and control groups.

Results

Dissociation

For larval dissociation, method 1 (mechanical) resulted in a low yield of cell number and resulted in cell cultures that remained viable for 19 days. Dissociation method 2 (enzymatic, trypsin) resulted in higher cell numbers forming cell cultures that remained viable for 12–21 days. Larval cells dissociated with method 3 (enzymatic, collagenase) remained viable for 8 days and those dissociated with method 4 (chemical and mechanical) remained viable for 10 days. Methods 3 and 4 had similarly high yields of cell number as dissociation method 2 (enzymatic, trypsin). Viability of resultant cell cultures decreased over time, regardless of the dissociation method used. Because of the similar success of dissociation methods 2, 3 and 4, all three are suitable for use in future experiments. These methods delivered material where the larvae were dissociated into a mixture of cell clumps and single cells. Cell viability in the form of adhering cells or ciliated movement was apparent after initiation of culture.

Larval cell morphology and viability

Larval cells in culture tended to separate into two groups: cells adhering to the tissue culture dish within the first 24 h of culture, and cells that clustered together and remained in suspension. Both populations were characterised by movement; the adherent cells by contractile movement and the suspension cells by ciliated movement. Adherent larval cells have fibroblast-like, epithelial-like and large round morphologies. Groups of adherent epithelial-like cells were observed to communicate with neighbouring clusters through thin tube-like protrusions (Fig. 1). Such communication is evident during contraction, when two neighbouring cell clusters will contract in succession. Suspension cells are characterized as clusters of small cells, some of which are ciliated.

The relationship between absorbance and cell number was determined as linear in a XTT assay over 7 days of culture (Fig. 2). Primary larval cells resulting from enzymatic dissociation with 0.125% collagenase in CMFSS were cultured in 96-well tissue culture plates (Greiner Bio-one) in culture

Fig. 1 Cultured abalone larval cells dissociated with method 3 (0.125% collagenase in CMFSS) at day 4 of culture in medium A. Epithelial-like (E), fibroblast-like (F) and large round (R) morphologies can be distinguished. Clusters are connected by thin tube-like structures (T)

Fig. 2 Linear relationship of increasing absorbance with increase in larval cell number determined by XTT assay over 7 days of culture. Cells were cultured in 96-well plates in medium A and each absorbance value is calculated as the mean absorbance over six wells

medium A. At the time of culture initiation, larval cell viability was determined as 91.7% by the Trypan Blue (TB) exclusion test.

A consistent increase in absorbance with increased cell number per well was observed with no detrimental effects evident at the highest density of cells (25,000 cells per well). From the same assay, it was evident that cell viability remained stable for 7 days. A similar pattern was observed in earlier trypan blue exclusion tests where larval cell viability decreased from 92% on day 3 to 85% on day 6 and 52% on day 10.

The effect of media A and D on larval cell viability were compared over a period of 10 days, taking absorbance measurements following XTT assays on days 3, 5, 7 and 10. The result from this experiment is represented in Fig. 3.

Although medium A seemed to increase cell viability initially, when compared to medium D, this difference disappears over the last 3 days of cell culture. This may be ascribed to initial abundance and later depletion of the vitamin and mineral supplements included in Leibovitz-L15 medium.

Haemocyte cell morphology and viability

The majority of *Haliotis midae* haemocytes appeared as small round brightly coloured amoeboid-like cells that readily clumped together (Fig. 4). The cells

Fig. 3 Effect of medium composition on larval cell viability determined by XTT assay. Cells were cultured in 96-well plates at 15,365 cells per well, in medium A (solid line) and medium D (dashed line)

Fig. 4 Abalone haemocytes in medium D (Table 1) attached to the surface of a six-well plate at day 5 of culture: A amoeboid-like, F fibroblast-like

attached individually and in clusters to the surface of the culture dish or even to the surface of the glass haemocytometer within 30 min and started spreading out by the next day of culture, adopting a fibroblast-like morphology upon adhering. Thin pseudopodial connections between cells became visible after 1 day of culture. Cells were observed to cover an increasing portion of the well area over the first 3 days of culture after which it did not change until diminishing at day 9–10 of culture when medium was not changed. Amoeboid- and fibroblast-like cells remained present throughout in vitro culture, with fibroblast-like cells decreasing and amoeboid-like cells increasing in number towards the second week of culture.

The relationship between absorbance and cell number was determined as linear in a XTT assay over 9 days of culture (Figs. 5, 6). Cells were cultured in 96-well plates in culture medium A (Table 1). A consistent increase in absorbance with increased cell number per well is observed with no

Fig. 5 Linear relationship of increasing absorbance with increase in haemocyte number determined by XTT assay at day 2 of culture. Cells were cultured in 96-well plates in medium A

Fig. 6 XTT assay of increased haemocyte densities over 9 days of culture. Cells were cultured in 96-well plates in medium A

detrimental effects evident at the highest density of cells (109,500 cells per well). From the same viability assay, it was evident that cell viability remained stable for 7 days before drastically decreasing by day 9. An estimated haemocyte cell number of 75,000 cells per well returned XTT absorbance values between 0.76 and 0.86 for the first 7 days. At day nine the absorbance value decreased to 0.07 (Fig. 6). This may be due to medium depletion and cell deterioration. Subsequent primary haemocyte cultures were performed in 96-well tissue culture plates at a density of 50,000 cells per well. This was regarded as an adequate cell number for optimal cell viability.

When viewing the progression in viability associated with different cell densities over the course of 9 days, it is clear that cell viability at all densities rapidly decrease after day seven of culture (Fig. 6). At all cell densities a pattern of initial increase in viability at day 2 of culture is followed by a period of relative stability in viability between days 2 and 7.

Although media A and D were similar in their ability to maintain *H. midae* haemocytes with constant viability for 7 days, medium D was used for later haemocyte cultures described below, due to the simple nature of the medium (which would facilitate observation of the effect of added factors) and the close resemblance of it to natural seawater in terms of pH and osmolarity.

Effect of media supplements on cultured haemocytes

To investigate the effect of media supplements on haemocyte viability, different supplements, known to stimulate cell metabolism, were added to culture medium D (see Table 2). Cells were incubated in the respective media in 96-well plates at 50,000 cells per well for 60 h before an XTT assay was initiated. Media that lead to a significant decrease in haemocyte viability and that was supplemented with collagenase (D-4, D-9 and D-10) are contrasted with control medium and media D-1 and D-7 in Fig. 7. Significant reduction ($p < 0.01$) of haemocyte viability is apparent for media D-4 (100 µg/mL collagenase), D-9 (12.5 ng/mL EGF + 50 µg/mL collagenase) and D-10 (10 µg/mL catalase + 50 µg/mL collagenase) when compared to the control. No significant difference in haemocyte viability was observed between medium

Fig. 7 Change in cell viability of cultured *H. midae* haemocytes (60 h): control = culture medium D; medium D with different supplements (Table 2): D-1 = 50 µg/mL insulin, D-2 = 25 ng/mL EGF, D-3 = 20 µg/mL catalase, D-4 = 100 µg/mL collagenase, D-5 = 25 µg/mL insulin + 12.5 ng/mL EGF, D-6 = 25 µg/mL insulin + 10 µg/mL catalase, D-7 = 25 µg/mL insulin + 50 µg/mL collagenase, D-8 = 12.5 ng/mL EGF + 10 µg/mL catalase, D-9 = 12.5 ng/mL EGF + 50 µg/mL collagenase, D-10 = 10 µg/mL catalase + 50 µg/mL collagenase. Error bars indicate the standard error. Significant difference from control and D-1 and D-7 at $p < 0.01$ (**). D-4, D-9 and D-10 also significantly differ from D-2, D-3, D-5, D-6 and D-8 at $p < 0.05$

D and media D-1 (50 µg/mL bovine insulin) and D-7 (25 µg/mL bovine insulin + 50 µg/mL collagenase). Although medium D-7 also contains collagenase as a supplement, viability was not significantly decreased as for other collagenase-containing media.

Contamination

Overall, contamination by protozoa and bacteria was widespread throughout cultures despite the addition of antibiotics. Protozoa presented as oval-shaped bodies exhibiting rapid, directional movement. Bacteria presented as small round or rod-shaped bodies that had a very fast proliferation rate. After first appearing, the surface of the entire tissue culture plate would be covered in bacteria within 24 h. Addition of amphotericin B kept fungal contamination at bay, but concurrently had a detrimental effect on cultured cells, causing them become shriveled and to loosen from the culture plate surface. This confirmed previous reports that antifungals may be toxic to molluscan cells (Mulcahy 2000). When amphotericin B was omitted, fungal contamination presented as cylindrical, thread-like hyphae, which branched and proliferated to a network of hyphae that would fill the

culture well if left to grow. When contamination was apparent, cultures were discarded. Contamination was less of a problem in haemocyte cultures when compared to larval cell cultures.

Discussion

This is the first time that cell culture trials using *Haliotis midae* larval and haemocyte tissues are reported. There are however reports of similar investigations for other abalone species (Auzoux-Bordenave et al. 2007; Lebel et al. 1996; Serpentine et al. 2000). As these trials were pilot studies, they were met with various challenges. Attaining culture conditions, including optimal pH, osmolarity and temperature, that support cell viability while keeping contamination at bay with the addition of antibiotics, summarize the main challenges.

Although it is generally not recommended, antibiotics were added to wash solutions and culture media throughout trials. The trade-off when doing this is that cell integrity is challenged and that resistant bacterial strains, which are difficult to eradicate, may develop (Buchanan et al. 1999; Domart-Coulon et al. 1994; European Collection of Cell Cultures (ECACC) and Sigma-Aldrich 2001). The use of antifungal agents was discontinued after it was observed that such treatment was detrimental to cultured cells. Other studies with molluscan larval cell culture also reported the use of antibiotics. Chen and Wen (1999) used Streptomycin and Penicillin; Odintsova et al. (2001) used gentamycin and Naganuma et al. (1994) used amphotericin B, Ampicillin, Penicillin G and Streptomycin Sulphate.

In an attempt to isolate larval cells, various dissociation protocols were assayed on larval tissues. Enzymatic dissociation with trypsin and collagenase and chemical-mechanical dissociation with EDTA and gentle pipetting demonstrated to be the most effective methods for dissociating abalone larvae into various cell types, while retaining cell viability. Viability of ~90% after dissociation with these methods compares well with viability of 90–95% reported for *Mytilus trossulus* larvae after dissociation with 0.25% collagenase (Odintsova et al. 2001). Larval dissociation with 0.25% trypsin, reported for abalone (*Haliotis rufescens*), also resulted in

successful short-term primary cell cultures (Naganuma et al. 1994).

Morphology and cellular behaviour similar to that described in other molluscan larval cell cultures were confirmed during this study. Although Naganuma et al. (1994) reported viable larval cell cultures for up to 12 weeks, the same pattern of decreased cell number after approximately day 7 of culture was observed. The same authors also reported contracting myocytes after one to 2 days of culture, which was confirmed as parallel development to the *in vivo* situation. They did however not report synchronicity of contraction in neighboring contractile cells, as was the case for *H. midae* larval cell cultures.

Culture media A (Leibovitz-based) and D (artificial seawater-based, results not shown) both sustained short-term larval cell cultures for up to 10 days. The successful maintenance of primary larval cell cultures from *Haliotis midae* in Leibovitz-based medium is supported by reports of primary cell culture from other molluscan larvae. Examples include oyster (*Crassostrea gigas*) larval cell cultures that were maintained for 50 days in 2 × Leibovitz's L-15 medium (Chen and Wen 1999) and mussel (*Mytilus trossulus*) larval cell cultures that were maintained for at least 3 weeks in modified Leibovitz-L15 medium. A slight decrease in the proportion of viable cells was observed at day 10 of culture (from 90 to 95% at culture initiation to 87–89% at day 10) (Odintsova et al. 2001). Larval cell cultures from red abalone (*Haliotis rufescens*) were also maintained in modified Leibovitz L-15 medium for up to 12 weeks (Naganuma et al. 1994). The use of artificial seawater for maintenance of larval cell cultures cannot be supported by literature, but from the present study it seems a feasible alternative to Leibovitz-based media. Cardiac cells of the surf clam (*Spisula solidissima*) was successfully cultured for up to 4 months using artificial seawater, supplemented with amino acids, vitamins, fetal calf serum and whole egg ultrafiltrate (Cecil 1969). Sterilised (0.22 µm filtered) natural seawater, supplemented with antibiotics, is also frequently used for sponge cell cultures (Cao et al. 2007; De Rosa et al. 2001; Klautau et al. 1993).

From initiation of larval cell cultures, contamination threatened culture quality and stability. Regardless of the utmost care that was taken in assuring sterile working conditions, the nature of the tissue of

origin guaranteed the presence of contamination from initiation of culture. Since abalone are broadcast spawners and their gametes are released through their respiratory pores into the environment, the sterility of materials originating from gametes are jeopardized. It is recommended that future studies on material originating from gametes rather be conducted on mature excised gametes from adult animals. This is however not a feasible practice when working with farmed animals as the mature, spawning adults constitute the valuable broodstock of the farm and excising gametes cannot be performed without sacrificing the animal. Another modification that may improve *H. midae* larval cell culture is the coating of tissue culture surfaces with extracellular matrix (ECM) factors, like collagens, fibronectins and laminins. Such coatings are used in cell culture frequently and have been shown to have profound effects on cell attachment, survival and maintenance (Zhang et al. 2009). The effect of adhesion polypeptide Poly-D-Lysine coating on adherence behaviour of larval cells has also been reported to enhance adherence and spreading in oyster (*Crassostrea virginica*) heart cells (Buchanan et al. 1999).

Abalone haemocytes adapted well to in vitro conditions, adhering rapidly and firmly to the surface of the tissue culture plate and forming morphologically distinct populations that remained well defined for the duration of the experiments. Haemocyte cell cultures were maintained in both Leibovitz-based (medium A) and artificial seawater-based (medium D) media for up to 7 days. When cultured for longer periods, the condition of the cells deteriorated and increasing numbers of cells started lifting away from the surface of the tissue culture dish. Cultured *H. tuberculata* haemocytes, however, can be maintained successfully in medium similar to medium A for at least 9 days without any marked decrease in viability (Auzoux-Bordenave, unpublished data). The amoeboid-like cells observed in the present study likely correspond to the blast-like cells in *Haliotis tuberculata*, which are described as exhibiting characteristics of undifferentiated cells and poor adhesion behaviour. The fibroblast-like cells observed here correspond to the large hyalinocytes in *Haliotis tuberculata*, which are proposed to play roles in glycogen storage and phagocytosis (Travers et al. 2008).

From XTT viability assays, it can be concluded that haemocytes have a higher metabolic activity than

larval cells. Approximately 20,000 haemocytes per well returned absorbance values between 0.5 and 0.66, while for the same number of larval cells the absorbance values varied between 0.27 and 0.37. The high metabolic activity of haemocytes has been previously ascribed to their high respiratory burst effect; an early defense mechanism to infection or contamination of haemocytes in culture (Auzoux-Bordenave et al. 2007; Boulo et al. 1991). This immune response, together with the phagocytic characteristics of the fibroblast-like cells may contribute to lower susceptibility to contamination and improved health of haemocyte cultures when compared to larval cell cultures.

The effects of specific medium supplementations on cell viability reported for other molluscan cell cultures were not confirmed in 60-h-old *Haliotis midae* primary haemocyte cultures. Catalase and EGF did not have any significant effect on metabolic activity of the haemocytes. Likewise, bovine insulin (at a suggested concentration of 50 µg/ml) did not have a significant stimulatory effect such as reported for haemocytes of *Haliotis tuberculata* and cultured oyster heart cells (Domart-Coulon et al. 1994; Lebel et al. 1996). Differences in haemolymph composition and culture conditions could contribute to failure in reproducing the results published by other researchers. Further studies with *Haliotis midae* haemocytes, where the time of incubation and concentration of supplements are varied, are required to elucidate possible growth stimulatory effects.

The supplementation experiment suggests that collagenase has a detrimental effect on haemocyte cell viability. This is in contrast with the report of Chen and Wen (1999) which indicated that 100 µg/mL collagenase might enhance the growth of oyster and hard clam heart cell cultures. Various reports on the detrimental effect of collagenase on cultured cells are however available. When human fibroblast cell cultures were used to study the wound healing process, collagenase was used to reduce the number of viable fibroblast cells, in a dose dependant manner. A concentration of 0.5 mg/mL decreased fibroblast cell number by 60% in 72 h and lower concentrations (0.01, 0.05 and 0.25 mg/mL) also confirmed a trend toward decreasing cell viability (Zamboni et al. 2004). Lo and Kim (2004) showed that collagenase caused apoptosis in primary human chondrocyte cell culture in a time and dose dependent

manner (0.02–0.08% collagenase from 12 to 48 h). While outlining dissociation procedures for initiating nerve and muscle cell cultures from *Xenopus laevis*, Gómez et al. (2003) also warns against prolonged exposure to collagenase, due to its detrimental effect on cell survival. In cell cultures, collagenase probably causes degradation of the extracellular matrix components, which leads to an impairment in the interaction between cells and matrix components (Zamboni et al. 2004).

The detrimental effect of collagenase in the present study was markedly less severe when bovine insulin was added to haemocyte cell cultures (Fig. 7). Bovine insulin at a concentration of 25 µg/mL may have a protective effect against collagenase-induced cell damage. Insulin-like growth factor I (IGF-1) was previously shown to be an effective inhibitor of collagenase-induced chondrocyte apoptosis, in a dose dependent manner (100 and 200 ng/mL) (Lo and Kim 2004). A study by Han et al. (2005), where insulin increased live cell number of porcine aortic endothelial cells after damaging treatment, also points to the protective function of insulin. Insulin has been reported to protect cultured neuronal cells from neonatal rat retina (R28 cell line) against apoptosis via a PI 3-kinase/Akt-dependent pathway (Barber et al. 2001). Insulin is also present in fetal bovine serum (FBS). Burke and Vuk-Pavlovic (1993) showed that insulin and insulin-like growth factor elicit a similar proliferative response in human neuroblastoma cells as when these cells are treated with 10% FBS. The fact that FBS is also widely used during subculturing of vertebrate cell lines to inactivate proteases after cells have been detached from the flask bottom, indicates that some FBS components counter the action of proteases. Insulin might play a role in protecting abalone haemocyte cultures against degradation by collagenase.

Primary cell cultures from marine molluscs have been successfully applied in various research fields to study the biology, physiology and pathology of economically important species. Examples include studies on cell differentiation, regulation of metabolic processes (biomineralization), enzyme characterisation, cytotoxicity assessment as well as pathogenicity studies (Auzoux-Bordenave et al. 2007; Badariotti et al. 2007; Barik et al. 2004; Domart-Coulon et al. 2000; Faucet et al. 2003; Le Pennec and Marcel Le Pennec 2001; Naganuma et al. 1994; Odintsova et al.

2001; Suja et al. 2007). The successful short term culture of larval and especially haemocyte cells from *Haliotis midae* reported here may be used as a foundation for similar in vitro assays. This will facilitate advances in knowledge pertaining to physiology, biochemistry and behaviour at the cellular level, for the South African abalone. One application of haemocyte cell cultures is the measurement of growth response of cultured cells to exogenous growth stimulants. Haemocyte cultures can be used in this way for analyses at the molecular level. RNA extraction and quantitative analysis of gene expression can contribute information about the genes involved in cell viability, growth and proliferation.

Acknowledgments This work was supported by a research grant from the NRF Innovation Fund and Industry partners (Irvin & Johnson Limited, Abagold (Pty) Ltd, Aquafarm Development Company (Pty) Ltd, HIK Abalone (Pty) Ltd and Roman Bay Sea Farm (Pty) Ltd). Stellenbosch University is thanked for facilities provided and HIK Abalone for providing samples.

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