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METHOD IN CELL SCIENCE

A novel medium for the development of in vitro cell culture system from *Penaeus monodon*

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Abstract Lack of a valid shrimp cell line has been hampering the progress of research on shrimp viruses. One of the reasons identified was the absence of an appropriate medium which would satisfy the requirements of the cells in vitro. We report the first attempt to formulate an exclusive shrimp cell culture medium (SCCM) based on the haemolymph components of *Penaeus monodon* prepared in isosmotic seawater having 27 ‰ salinity. The SCCM is composed of 22 amino acids, 4 sugars, 6 vitamins, cholesterol, FBS, phenol red, three antibiotics, potassium dihydrogen phosphate and di-sodium hydrogen phosphate at pH 6.8–7.2. Osmolality was adjusted to 720 ± 10 mOsm kg^{-1} and temperature of incubation was 25 °C. The most appropriate composition was finally selected based on the extent of attachment of cells and their proliferation by visual observation. Metabolic activity of cultured cells was measured by MTT assay and compared with that in L-15 (2×), modified L-15 and Grace's insect medium, and found better performance in SCCM especially for lymphoid cells with 107 % increase in activity and 85 ± 9 days of longevity. The cells from ovary and lymphoid organs were passaged twice using the newly designed shrimp cell dissociation "cocktail".

Keywords Shrimp cell line · *Penaeus monodon* · Shrimp cell culture medium (SCCM) · MTT assay · Lymphoid cell culture

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Introduction

Attempts on the development of continuous cell lines from shrimps have a long and arduous history amidst the absolute requirement of certified cell lines to address the viral diseases which have been hampering the shrimp industry. Lack of a cell line has been hampering the progress of research in shrimp viruses especially in the study of viral morphogenesis and in the development of prophylactic and therapeutic measures. The most prominent reason for the non attainment of cell lines from shrimps and for that matter from crustaceans altogether might be the lack of an appropriate medium like Grace's insect cell culture medium which paved the way for the development of over 500 insect's cell lines (Lynn 2001). Considering this necessity, several commercially available media have been modified over decades to suit the requirements of cell cultures (Jose 2009).

Among them Leibovitz's—15 (L-15) has been the most popular one subjected to modifications (Chen et al. 1986, 1988; Chen and Kou 1989; Nadala et al. 1993; Lu et al. 1995; Tapay et al. 1995; Tong and Miao 1996; Toullec et al. 1996; Mulford and Austin 1998; Chen and Wang 1999; Shike et al. 2000; Wang et al. 2000; Kumar et al. 2001; Shimizu et al. 2001; Chun-Lei et al. 2003; Maeda et al. 2003, 2004; Jiang et al. 2005; Assavalapsakul et al. 2006; Jose et al. 2010, 2011, 2012) They have been modified by supplementing with growth factors in isolation as well as in multiples (Chen et al. 1986, 1988; Machii et al. 1988; Chen et al. 1989; Luedeman and Lightner 1992; Nadala et al. 1993; Ghosh et al. 1995; Lu et al. 1995; Tapay et al. 1995; Toullec et al. 1996; Mulford and Austin 1998; Chen and Wang 1999; Itami et al. 1999; Shike et al. 2000; Wang et al. 2000; Kumar et al. 2001; Mulford et al. 2001; Fan and Wang 2002; Lang et al. 2002; Maeda et al. 2003, 2004; Jiang et al. 2005; George and Dhar 2010; Jose et al. 2010, 2011, 2012). Meanwhile, Shimizu et al. (2001) reported a modified L-15 medium based on the haemolymph composition with some success in prolonging the longevity of primary cell culture from shrimp. More recently, Jose et al. (2010) modified L-15 (2x) by supplementing it with 2 % glucose, MEM vitamins (1×), tryptose phosphate broth (2.95 mg ml^{-1}), 20 % FBS, and 0.2 mM N-phenylthiourea. Mulford et al. (2001) revealed that 2 × Leibovitz's medium supplemented with 10 % (v/v) heat inactivated FBS, 5 % (v/v) muscle extract, 0.06 g l^{-1} of L-proline, 1 g l^{-1} glucose prepared in 25 ‰ seawater was effective for cell migration, survival and longevity. Though the hemolymph composition of *P. aztecus* and *P. stylirostris* had been reported earlier (Najafabadi et al. 1992; Shimizu et al. 2001), a medium exclusively for shrimp cell culture based on it could not be attained so far, other than the modification of the existing media. This might be cited as one of the reasons for the non attainment of immortal cell line from shrimp. In this context we made an attempt to develop seawater based cell culture medium exclusively for shrimp cell culture and named it as shrimp cell culture medium (SCCM). Experiments were carried out using various tissues from *P. monodon* for determining its suitability to develop cell cultures. Primary cell cultures developed by employing this medium from lymphoid and ovarian tissues could be sub-cultured twice using shrimp cell dissociation cocktail developed in this study.

Materials and methods

Design of the experiment

The whole experiment was designed to formulate a medium exclusively for shrimp cell culture. The haemolymph components of *P. monodon,* the free amino acids, fatty acids and metal ions were used as background information about the physiological conditions required for in vitro growth of cells. Seawater and artificial seawater were screened for suitable base for the medium. Physical observation was carried out to screen the most suitable combinations initially and further confirmations were done based on MTT assay.

Experimental animals

Shrimps required for the experiments were maintained in Recirculating Aquaculture System (RAS) integrated with nitrifying bioreactor (Kumar et al. 2009) maintained at 27 ‰ salinity. Post larvae, negative for white spot syndrome virus (WSSV) by nested PCR, were stocked in the system and reared for 3 months, maintaining the water quality parameters within a narrow range (pH 6.8–7.8; total ammonia–nitrogen <0.1 mg l^{-1}; nitrite–nitrogen < 1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75–125 mg l^{-1}; total hardness 5,000–6,000 mg l^{-1}) and fed pelleted feed (Higashimaru). Shrimps weighing 15–20 g were used as the donor animals for various tissues besides nauplii directly collected from a seed production centre.

Analysis of haemolymph

Collection of haemolymph

For free amino acid and fatty acid analysis, haemolymph was withdrawn aseptically from rostral sinus using capillary tubes containing 100 µl of 10 % sodium citrate (Jose et al. 2011) and the total volume of each sample was measured to calculate the dilution factor (Shimizu et al. 2001). Pooled haemolymph from 20 animals weighing 20–30 g was centrifuged at 1,000 g for 10 min to remove haemocytes, lyophilized and stored at −20 °C. Haemolymph was collected without anticoagulant also for metal ion analysis. Osmolality of the haemolymph was measured immediately after collection using a Fiske 1–10 Osmometer (Fiske Associates, Norwood, MA, USA).

Analysis of free amino acids

Aliquot of 160 μg pooled lyophilized haemolymph was collected in a test tube and 10 ml of 6 N HCl were added. The test tube was filled with nitrogen, sealed and kept at 121 °C for 24 h. The hydrolysed sample was filtered and flash evaporated repeatedly adding distilled water until the traces of chlorine were removed. The residue obtained was made up to 10 ml with 0.05 M HCl.

Samples were filtered through a polyvinylidene fluoride membrane filter (PVDF, Millipore) of 0.45 μm pore size and 20 μl were injected to an amino acid analyzer (HPLC-LC 10 AS) equipped with cation exchange column packed with a strong acidic cation exchange resin (styrene divinyl benzene copolymer, with sulphonic groups). The column used was Na type, ISC-O7/S 1504 Na, having a length of 19 cm and diameter 5 mm. The instrument was equipped with Shimadzu FL 6A fluorescent detector and Shimadzu CR 6A Chrompac recorder. A gradient mobile phase was applied with buffer A and buffer B for the effective separation of amino acids. The oven temperature was maintained at 60 °C. The total run was programmed for 62 min. The amino acid analysis was done with non-switching flow method and fluorescence detection after post-column derivatization with o-phthaldehyde. In the case of proline and hydroxyproline, imino group was converted to amino group with hypochlorite. Amino acid standard (Sigma Chemical Co., St. Louis, MO, USA) was also run to calculate the concentration of the amino acids in the sample. The amount of each amino acid was expressed as μmol ml^{-1} haemolymph (Antoine et al. 1999).

Analysis of fatty acids

For fatty acid analysis, gas chromatograph with flame ionization detector (GC-FID) was employed (Agilent Technologies, model 6890). The pooled and lyophilized haemolymph was converted into fatty acid methyl esters (FAMEs) by saponification, methylation and extraction (Carvalho and Malcata 2005) with hexane: methyl *tert*-butyl ether. A 25 m (length) × 0.2 mm ID × 0.33 μm film thickness, cross linked 5 % phenylmethyl silicone fused silica capillary column was used to separate the fatty acids. While operation the initial temperature of 170 °C was increased to 310 °C at the rate of 40 °C min^{-1} and held for 1.5 min. Hydrogen was used as the carrier gas at a constant flow rate of 1.3 l min^{-1}. The peaks were analyzed using the software Sherlock (MIDI, Inc., Newark, DE, USA) to identify the relative amounts of fatty acids in the sample and were expressed as percentage of the total fatty acids. Identification of the peaks was accomplished by comparison of retention times to those of authentic standards.

Analysis of metal ions

As the analysis of major ions in the hemolymph is crucial to formulate cell culture medium (Najafabadi et al. 1992), metal ion strength of haemolymph and seawater at 27 ‰ salinity were analyzed using Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES Thermo Electron IRIS INTREPID II XSP DUO). Before analysis, seawater was sterilized by autoclaving at 15 lbs for 15 min, filtered through Whatman No. 1 filter paper to remove precipitates. Lyophilized haemolymph (100 μg) from different age group of *P. monodon* was diluted with Milli-Q water to the volume required for analysis (Shimizu et al. 2001; Huang et al. 1999). Identification of the elements was accomplished by comparison with authentic standards (Merck, Darmstadt, Germany).

Formulation of the basal composition of shrimp cell culture medium (SCCM)

All chemicals used for the preparation, unless specifically stated otherwise, were purchased from Sigma-Aldrich, USA. The Shrimp Cell Culture Medium contained (mg l^{-1} in artificial/natural seawater) L-alanine 70, L-arginine 45, L-asparagine 15, L-aspartic acid 10, L-cystine 1, L-cysteine 1, L-histidine 15, L-leucine 20, L-lysine 60, L-isoleucine 10, L-methionine 5, L-phenyl alanine 10, L-proline 100, L-serine 15, L-taurine 100, L-threonine 15, L-tryptophan 15, L-glutamine 150, L-glutamic acid 10, glycine 20, L-tyrosine 80, L-valine 20, choline bitartarate 1.8, D-pantothenic acid (hemicalcium) 1, folic acid 1, myo-Inositol 2, pyridoxal-HCl 1, riboflavin 0.1, thiamine 1, niacinamide 1, glucose 1,000, ribose 10, trehalose 10, sodium pyruvate 500, potassium dihydrogen phosphate 2, di-sodium hydrogen phosphate 11.5, cholesterol 0.2, and phenol red 0.01. This composition was considered as the base for SCCM to which additional ingredients such as antibiotic mixture and fetal bovine serum (FBS) were added as

described elsewhere, and the efficacy was evaluated through development of cell cultures and their subsequent visual observation under inverted phase contrast microscope (Leica DMIL) connected with a CCD camera (Leica DFC 420C).

Artificial seawater and natural seawater as liquid base

Based on the report of Dall (1981), the isosmotic point of *P. monodon* was identified equivalent to 27 ‰ salinity. Natural seawater at 27 ‰ having 810 ± 20 mOsm kg^{-1} osmolality was used for the whole experiment. Seawater was sterilized by autoclaving at 15 lbs for 15 min, filtered through Whatman No. 1 filter paper to remove precipitates and stored in a 20 l container at room temperature. Artificial seawater was prepared by dissolving the following ingredients one after the other: NaCl, 2.3926 g; Na_2SO_4, 0.4 g; KCl, 0.0677 g; $NaHCO_3$, 0.0196 g; KBr, 0.0098 mg; H_3BO_3, 0.0026 g and NaF, 0.0003 g in 75 ml MilliQ water with constant stirring. To this solution, 5.327 ml of 1.0 M $MgCl_2{\cdot}6H_2O$, 1.033 ml of 1.0 M $CaCl_2{\cdot}2H_2O$ and 0.09 ml of 0.1 M $SrCl_2{\cdot}6H_2O$ were added and made up to 100 ml with MilliQ water (Kester et al. 1967). The differential effects of artificial and natural seawater were evaluated by visual observation of the extent of attachment and monolayer formation of primary cell cultures from various tissues using an inverted phase contrast microscope (Leica DMIL).

Effect of inorganic salts and trace elements

To equalize the inorganic salt and trace element concentration of SCCM to those of haemolymph, additional inorganic salts and trace elements were added to the tune of 0.518 mg l^{-1} barium chloride, 124.2 mg l^{-1} copper chloride, 37.38 mg l^{-1} zinc sulphate, 8.28 mg l^{-1} ferric citrate and 0.26 mg l^{-1} manganese chloride. The effect of this modification was evaluated visually by observing the extent of attachment and monolayer formation of the cells using an inverted phase contrast microscope (Leica DMIL).

Effect of organic supplements

Five different mixtures of organic supplements were added to the SCCM and the effect of these supplements were evaluated by the extent of cell attachment and monolayer formation and compared with that of the basal SCCM as control. The following supplements were added to the SCCM (final concentration).

Supplement A: consisted of a mixture of arachidonic acid 0.02 μg l^{-1}, linoleic acid 0.1 μg l^{-1}, linolenic acid 0.1 μg l^{-1}, myristic acid 0.1 μg l^{-1}, oleic acid 0.1 μg l^{-1}, palmitic acid 0.1 μg l^{-1} stearic acid 0.1 μg l^{-1}, cholesterol 2.2 μg l^{-1}, Tween-80 20.2 μg l^{-1}, and tocopherol acetate 0.7 μg l^{-1}.

Supplement B: consisted of precursors of signal molecules at a concentration, 0.83 mg l^{-1} flavin adenine dinucleotide (FAD-Na_2), 0.55 mg l^{-1} adenosine 5′-triphosphate magnesium salt (ATP), 1.14 mg l^{-1} nicotinamide adenine dinucleotide phosphate (NADP), 0.35 mg l^{-1} adenosine 5′-monophosphate (AMP-Na_2), 0.77 mg l^{-1} Coenzyme A (CoA-Na_2).

Supplement C: consisted of precursors of nucleic acid synthesis such as, adenosine 0. 27 mg l^{-1}, guanosine 0.28 mg l^{-1}, cytosine 0.11 mg l^{-1}, thymine 0.13 mg l^{-1}, deoxy ribose 0.13 mg l^{-1}, uracil 0.11 mg l^{-1}, uridine 5′-triphosphate (UTP 0.55 mg l^{-1}).

Supplement D: consisted of Kreb's cycle intermediates which included ketoglutaric acid 0.15 mg l^{-1}, malic acid 0.13 mg l^{-1} and succinic acid 0.12 mg l^{-1}.

Supplement E: consisted of vitamins viz., ascorbic acid 0.01 mg l^{-1}, biotin 0.05 mg l^{-1} nicotinamide 0.01 mg l^{-1}, nicotinic acid 0.01 mg l^{-1}, pyridoxin, 0.01 mg l^{-1} calciferol 0.01 mg l^{-1}, tocopherol 0.01 mg l^{-1}, p-aminobenzoic acid 0.01 mg l^{-1} .

Preparation of shrimp cell culture medium (SCCM)

Different constituents of SCCM were prepared separately and mixed as follows. A 100 × concentrated amino acid mixture ("amino mix-I") containing L-alanine, L-arginine, L-asparagine, L-cysteine, L-histidine, L-lysine, L-methionine, L-proline, L-serine, L-taurine, L-threonine, glycine, L-valine was prepared in MilliQ water. The "amino mix-II" was prepared by dissolving 100 × concentration of L-aspartic acid, L-cystine, L-leucine, L-isoleucine, L-phenyl alanine, L-tryptophan, L-glutamic acid, L-tyrosine in 1 N HCl. An aliquot of 100 × concentration of "sugar mix" was prepared by dissolving glucose, ribose, trehalose, sodium pyruvate, potassium dihydrogen phosphate and di-sodium hydrogen phosphate in MilliQ water. A "Vitamin mix" was prepared by mixing 100 × concentrations of choline bitartarate, D-pantothenic acid (hemicalcium), myo-Inositol, pyridoxal-HCl,

thiamine and niacinamide in MilliQ water with 100 × concentration of riboflavin and folic acid in 1 M NaOH. Cholesterol was prepared (100x) separately in MilliQ water. Phenol red solution was prepared by dissolving 1 mg phenol red (100x) in 1 ml NaOH (1 M). All above mixtures were separately filtered through PVDF (Millipore) of 0.22 µm pore size and stored in amber colored bottle at −20 °C till use.

The final SCCM (1,000 ml) was prepared by mixing 10 ml each of amino mix I, amino mix II, sugar mix, vitamin mix, cholesterol and phenol red solution in 100 ml double distilled water, mixed with 685 ml sterile seawater of 810 ± 20 mOsm kg^{-1} osmolality (27 ‰). An aliquot of 100 ml fetal bovine serum was added, the osmolality of the medium was adjusted to 720 ± 10 mOsm kg^{-1} by adding NaCl and measuring by means of osmometer (Fiske One-Ten Osmometer, Fiske Associates, Norwood, MA, USA). pH of the medium was adjusted to 6.8 using 1 N NaOH or 1 N HCl, the medium was made up to 1,000 ml with distilled water. Further, the medium was supplemented with antibiotic mixture containing penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and chloramphinicol (0.0 6 µg ml^{-1}) and added filter sterilized 150 mg l^{-1} glutamine was added just before use.

Development of primary cell cultures

Prior to dissection, the juvenile shrimp and nauplii were chilled in ice and surface sterilized by immersion in 800 ppm sodium hypochlorate in ice cold seawater for 10 min followed by thorough washing in sterile seawater. Lymphoid organ, heart, hepatopancreas, nerve code, eye stalk, muscle, testis and ovary were removed aseptically and collected in holding medium (SCCM without FBS) of 720 mOsm kg^{-1} osmolality. The tissues and nauplii were washed three times with PBS and minced into very small pieces using sterile surgical knife. The clumps of tissue were separated using cell dissociation sieves (CD-1, Sigma) with a 60 mesh screen (Mulford et al. 2001); the suspension was mixed thoroughly with the medium and seeded on 25 mm^2 culture flask/wells (Greiner Bio-One) and incubated at 25 °C. For haemocyte culture, haemolymph was withdrawn aseptically from rostral sinus using capillary tubes containing 100 µl anticoagulant (Tris HCl 0.01 M, sucrose 0.25 M, tri sodium citrate 0.1 M) and diluted to obtain 5 × 10^5 cells ml^{-1} using SCCM supplemented with N-phenylthiourea (0.2 mM), 0.06 mg ml^{-1} chloramphenicol, 100 mg l^{-1} streptomycin and 100 IU ml^{-1} penicillin (Jose et al. 2011) and aliquots of 200 µl were dispensed into the wells of 96 well plates (Greiner Bio-One) and incubated at 25 °C.

MTT reduction assay for measuring cellular metabolism

In spite of the visual observation, mitochondrial dehydrogenase activity was measured as the cell viability and metabolic activity which depended on an intact mitochondrial membrane and the respiratory chain. MTT assay measures the mitochondrial dehydrogenase which reflects the metabolic activity of the cells. Succinate dehydrogenase system which belongs to the mitochondrial respiratory chain reduces MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma-Aldrich Co.) to insoluble formazan crystals, solubilized in dimethyl sulphoxide (DMSO) yielding a purple-coloured solution (Mosmann 1983). Accordingly, aliquots of 200 µl cell suspension of ovary, lymphoid organ, heart, hepatopancreas and the hemocytes were seeded on to 96 well plates and after desired incubation period (2, 4, 6 and 8 days), the medium was pipetted out and replaced with 50 µl MTT solution (5 mg ml^{-1}) prepared in PBS (720 mOsm kg^{-1}), and the plate was kept for incubation (in dark) at 25 °C for 5 h (Jose et al. 2012). The control consisted of the medium alone with MTT added. The MTT solution was removed and 200 µl of DMSO (HiMedia Laboratories, Mumbai) were added and well mixed to confirm the dissolution of formazan crystals, the absorbance was measured at 570 nm in a microplate reader (InfiniteM-200 Tecan, Grödig, Austria).

Comparison of SCCM with other selected media

The efficacy of SCCM with respect to in vitro growth and viability of cells from ovary, lymphoid organ, heart, hepatopancreas and the hemocytes was determined by comparison with 2 × L-15 with 10 % FBS (v/v), modified L-15 (Jose et al. 2010) and Grace's insect medium with 10 % FBS (v/v). Metabolic activity (viability) of the cultures in vitro was analyzed on the 4th day of culture using MTT assay, and the percentage difference was compared.

Cell dislodgement and passaging

Cell dislodgement was performed for passaging the primary cell cultures developed from explants of ovary, lymphoid organ, heart, hepatopancreas and the hemocytes. All enzymes used for this experiment were purchased from Sigma Aldrich, USA. Trypsin (0.25 %, w/v), collagenase type V (1 %), accutase (1 %), non enzymatic cell dissociation solution-I (prepared in PBS), non enzymatic cell dissociation solution-II (prepared in HBSS without calcium and magnesium) and shrimp cell dissociation 'cocktail' were tested. The 'cocktail' contained 0.25 g trypsin, 0.02 g EDTA, 0.02 g EGTA, 0.04 g polyvinyl pyroline and 0.05 g glucose dissolved in 100 ml PBS (720 mOsm kg^{-1}). pH was adjusted to 6.8 within a range 6.8-7.2 using 1 N NaOH or 1 N HCl and supplemented with antibiotic mixture containing penicillin (100 U ml^{-1}) streptomycin (100 µg ml^{-1}) and chloramphenicol (0.06 µg ml^{-1}), filtered through 0.22 µm pore size polyethersulfone (PES) (Millex GP, Millipore) membrane using syringe filter.

Statistical analyses

The results in the figures are average values of 3–6 replicates ± standard deviation. The effects of treatments were statistically analyzed by single factor and two factor analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

Results

Analysis of haemolymph

Amino acid composition from haemolymph was recorded to be aspartic acid (47.02 ± 28.68 mg l^{-1}) threonine (11.51 ± 0.98 mg l^{-1}), serine (13.89 ± 7.72 mg l^{-1}), glutamic acid (92.17 ± 54.19 mg l^{-1}), proline (26.45 ± 13.51 mg l^{-1}), glycine (14.77 ± 6 mg l^{-1}), alanine (26.55 ± 8.56 mg l^{-1}), cystine (1.64 ± 0.27 mg l^{-1}), valine (18.58 ± 10.64 mg l^{-1}), isoleucine (11.16 ± 3.24 mg l^{-1}), leucine (27.3 ± 7.64 mg l^{-1}), tyrosine (11.08 ± 3.42 mg l^{-1}), phenyl alanine (25.11 ± 10.10 mg l^{-1}), histidine (15.63 ± 10.56 mg l^{-1}), lysine (13.33 ± 11.36 mg l^{-1}), and arginine (20.3 ± 17.96 mg l^{-1}).

Fatty acid profile exhibited long chain fatty acids (up to 20-carbon atoms) along with polyunsaturated fatty acids (PUFA) (Table 1). Among the fatty acid components of the haemolymph lipids, 81.63 % were contributed by palmitic acid (16:0), linoleic acid (18.2 ω-6), oleic acid (18.1 ω-9) and stearic acid (18:0). Other fatty acids recorded were capric acid (0.07 %) lauric acid (0.17 %), myristic acid (1.28 %), pentadecyclic acid (0.48 %), margaric acid (1.95 %), linolenic acid (0.16 %), nonadecyclic acid (0.53 %), arachidic acid (0.46 %), eicosenoic acid (0.34 %), eicosadienoic acid (0.34 %) and arachidonic acid (2.58 %).

Of the ten elements analyzed, concentration of copper, zinc, barium, iron and manganese were lower in the seawater (27 ‰) than in the haemolymph of *Penaeus monodon*. Copper and manganese levels in the seawater were very low (< 0.01 mg l^{-1}) compared to those of the haemolymph, 148.6 ± 34.50 mg l^{-1} and 0.33 ± 0.10 mg l^{-1}, respectively. However, the concentration of sodium, potassium, calcium, boron and strontium were more or less the same (Table 2). Concentration of zinc in seawater was 0.035 ± 0.02 mg l^{-1} and iron 0.053 ± 0.01 mg l^{-1}, whilst in haemolymph the concentrations were 39.415 ± 2.88 mg l^{-1} and 12.98 ± 6.63 mg l^{-1}, respectively. Moreover, the barium level in seawater (0.015 ± 0.01 mg l^{-1}) was hundred times lower than that of the haemolymph (15.29 ± 14.27 mg l^{-1}). Inorganic salts and trace elements were added to the SCCM as supplements to adjust these differences. Osmolality of haemolymph and 27 ‰ seawater were found to be 730.5 ± 51.2 and 810 ± 20 mOsm kg^{-1} respectively.

Artificial seawater and natural seawater as liquid base

Based on the visual observation of growth and monolayer formation of various primary cell cultures from different tissues and organs (Table 3), it was clear that the artificial seawater was not promising as the liquid base for SCCM in terms of limited proliferation and less attachment of cells compared to natural seawater inspite of the fact that there were risks of variations in the composition of natural seawater from place to place.

Effect of inorganic salts, trace elements and organic supplements

The inorganic salts and trace elements added to equalize the haemolymph metal ion concentration

Table 1 Fatty acid profile of *P.monodon* haemolymph

S. No.	Retention time (RT)	Carbon atom	Fatty acid	Percentage
1	2.808	10:0	Capric acid	0.07
2	4.302	12:0	Lauric acid	0.17
3	6.682	14:0	Myristic acid	1.28
4	8.166	15:0	Pendadecylic acid	0.48
5	9.782	16:0	Palmitic acid	29.30
6	11.476	17:0	Margaric acid	1.95
7	13.208	18:0	Stearic acid	13.84
8	12.807	18.1 ω-9	Oleic acid	17.96
9	12.719	18.2 ω-6	Linoleic acid	20.53
10	12.485	18.3 ω-6	Linolenic acid	0.16
11	14.939	19:0	Nonadecyclic acid	0.53
12	16.651	20:0	Arachidic acid	0.46
13	16.250	20:1 ω-9	Eicosenoic acid	0.34
14	16.184	20.2 ω-6	Eicosadienoic acid	1.31
15	15.605	20.4 ω-6	Arachidonic acid	2.58

Table 2 Comparison of the metal ion composition of *P. monodon* haemolymph with hemolymph from *P. stylirostris*, and from *P. aztecus* and seawater (27 ‰)

S. No	Parameter analyzed	Seawater 27 ‰ (mg l^{-1})	Concentration in haemolymph (mg l^{-1})		
			P. monodon	*P. stylirostris* (Shimizu et al. 2001)	*P. aztecus* (Najafabadi et al. 1992
1	Sodium	8,075.5 ± 260.92	6,784.34 ± 785.83	8,411.1 ± 549.7	6,188.00 ± 795.6
2	Potassium	512.935 ± 73.16	524.535 ± 157.88	328.44 ± 50.8	281.52 ± 62.56
3	Calcium	443.72 ± 63.10	488.84 ± 107.93	439.2 ± 44.3	410 ± 70
4	Copper	BDL (<0.01)	148.595 ± 34.50	81.6 ± 13.9	
5	Zinc	0.035 ± 0.02	39.415 ± 2.88	11.4 ± 1.9	
6	Barium	0.015 ± 0.01	15.29 ± 14.27	NA	
7	Iron	0.053 ± 0.01	12.98 ± 6.63	0.1 ± 0.0	
8	Strontium	7.05 ± 0.44	7.27 ± 1.90	6.3 ± 0.6	
9	Manganese	BDL (<0.01)	0.325 ± 0.10	NA	
10	Boron	4.87 ± 0.65	8.42 ± 7.68	0.4 ± 0.3	
11	Osmolality (mOsm kg^{-1})	810 ± 20	730.5 ± 51.2	784 ± 36	625 ± 46

were found to have negative effect on growth and monolayer formation of various cell types (Table 3). Moreover, the experiments by addition of organic supplements like vitamin mixture, lipid mixture, citric acid cycle intermediates, nitrogenous base and energy precursors in the basal medium did not bring forth any enhancement in the attachment of cells, their proliferation and confluence.

Preparation of shrimp cell culture medium (SCCM)

The basal composition of SCCM was finalized by conducting a series of experiments by trial and error method (data not shown) by incorporating different permutation combination of amino acid mix I and II, sugar mix, vitamin mix, cholesterol, FBS and pH.

Table 3 Effect of different combinations of shrimp cell culture medium (SCCM) on primary cell culture of ovary (OV), lymphoid organ (LY), heart (HT), hepatopancreas (HP), hemocytes (HC), nerve cord (NC), testis (TS), nauplii (N), eyestalk (ES) and muscle (MS)

Exp. No	Media combinations pH 6.8, osmolality 720 ± 10 mOsm kg^{-1}	Primary culture's growth and monolayer formation									
		LY	HT	HC	OV	HP	NC	TS	N	ES	MS
I	BASE + ASW + 0 % FBS	−	−	−	−	−	−	−	−	−	−
	BASE + ASW + 1 % FBS	−	−	−	−	−	−	−	−	−	−
	BASE + ASW + 2.5 % FBS	−	−	−	−	−	−	−	−	−	−
	BASE + ASW + 5 % FBS	−	−	−	−	−	−	−	−	−	−
	BASE + ASW + 7 % FBS	+	+	+	−	−	−	−	−	−	−
	BASE + ASW + 10 % FBS	++	+	+	+	+	−	−	−	−	−
	BASE + ASW + 15 % FBS	++	+	+	+	+	−	−	−	−	−
	BASE + ASW + 20 % FBS	++	+	+	−	−	−	−	−	−	−
II	BASE + SW + 0 % FBS	−	−	−	−	−	−	−	−	−	−
	BASE + SW + 1 % FBS	−	−	−	−	−	−	−	−	−	−
	BASE + SW + 2.5 % FBS	+	+	−	−	−	−	−	−	−	−
	BASE + SW + 5 % FBS	+	+	−	−	−	−	−	−	−	−
	BASE + SW + 7 % FBS	++	++	−	−	−	−	−	−	−	−
	BASE + SW + 10 % FBS	+++++	++++	+++	++++	+++	+	+	+	+	+
	BASE + SW + 15 % FBS	++++	++++	++	++++	+++	+	+	+	+	+
	BASE + SW + 20 % FBS	++++	++++	++	+++	++	+	+	+	+	+
III	BASE + SW 5 ‰ + 10 % FBS	+++	+++	++	+	+	−	−	−	−	−
	BASE + SW 10 ‰ + 10 % FBS	+++	+++	++	+	+	−	−	−	−	−
	BASE + SW 15 ‰ + 10 % FBS	+++	+++	++	+	+	−	−	−	−	−
	BASE + SW 20 ‰ +10 % FBS	+++	+++	++	+	+	+	+	+	+	+
	BASE + SW 27 ‰ +10 % FBS (SCCM)	+++++	++++	+++	++++	+++	+	+	+	+	+
	BASE + SW 30 ‰ +10 % FBS	+++	+++	++	+	+	+	+	+	+	+
	BASE + SW 35 ‰ + 10 % FBS	+++	+++	+	+	+	−	−	−	−	−
IV	SCCM	+++++	++++	+++	+++	+++	+	+	+	+	+
	SCCM + inorganic salts & trace elements	+++	++	+	++	+	+	+	+	+	+
	SCCM + supplement A	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement B	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement C	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement D	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement E	+++++	++++	+++	++++	++	+	+	+	+	+

BASE: All ingredients except FBS (Fetal bovine serum) and seawater. SCCM: BASE with 27 ‰ SW and 10 % FBS

ASW artificial seawater, *SW* seawater (natural)

The confluence of primary cell cultures was categorized as: +tissues/cells attached; ++ attached tissues/cells started multiplying; +++ and ++++ started monolayer formation, +++++ confluent monolayer formed, − no attachment

Phenol red, antibiotic mix and glutamine were used at fixed quantity. Potassium dihydrogen phosphate and disodium hydrogen phosphate were added as the buffering agents incorporated in the sugar mix. Cholesterol, 0.2 mg l^{-1}, was added as the lipid component and phenol red as the pH indicator. The most appropriate composition was finally selected based on the extent of attachment of the explants and proliferation of cells by visual observation (Table 3). The pH, temperature of incubation and osmolarity of the medium were 6.8–7.2, 25 °C and 720 ± 10 mOsm kg^{-1}, respectively. Natural seawater as the liquid base at 27 ‰ gave better performance. Concentration of 10–15 % FBS (v/v) was found to be most effective.

Development of primary cell cultures

Among the combinations studied, SCCM supplemented with 10 % FBS prepared in 27 ‰ seawater was the most effective one for attachment of cells, their proliferation and confluence for all cell types tested (Table 3). Among the cell types tested, lymphoid and ovarian cells were the most promising ones having the cell longevity exceeding 50 days, 85 ± 9 days for lymphoid cells and 63 ± 6 days for ovarian cells followed by heart cells (29 ± 1 days) and hepatopancreas (25 ± 5 days). Meanwhile, the longevity of other cell types were, testis 21 ± 3 days, haemocytes 10 ± 3 days, eyestalk 9 ± 2 days, muscle 7 ± 1 days, nerve cord and cells from nauplii 6 ± 1 days (Fig. 1). Moreover, metabolic activity of lymphoid cells in terms of MTT assay showed a significant increase ($p < 0.05$) from day 2 to day 8 compared to the other cell types which were to a large extent static (Figs 2, 3).

Morphology of lymphoid cells in culture was spherical or elliptical initially, and the cells were found getting attached within 3 h and after 24 h, 90 % of the cells were anchored, judged by visual observation. They were epitheloid with large nucleus and granulated cytoplasm exhibiting mitotic division (Fig. 4). Subsequently, 42 % increase in cell metabolic activity was observed by MTT assay within 48 h (Fig. 2). Moreover, additional increase of 27 and 16 % were observed within 72 and 96 h, respectively. A rapid monolayer formation and cell proliferation were characteristic of the lymphoid cells compared to all other cell types. Hemocytes in culture appeared to have similar shape as lymphoid cells, however within 6 h they transformed to anchor dependent spindle shaped cells (Fig. 5). Even though, haemocytes were viable for 10 days (judged through MTT assay) direct mitotic division could not be observed under microscope. In ovarian culture, fibroblastic as well as round cells were found proliferating (Fig. 7). Meanwhile, cells from hepatopancreas (Fig. 8) required longer duration to attach to and proliferate (>24 h) than the heart cells which showed better multiplication within 24 h of seeding (Figs. 6, 7). Cell culture developed from testis was very small compared to all other cell types (Fig. 8, 9). Despite the attachment to the substratum, viability and longevity, cell migration and multiplication were not satisfactory with respect to the cells originated from muscle, eyestalk, nerve cord and nauplii.

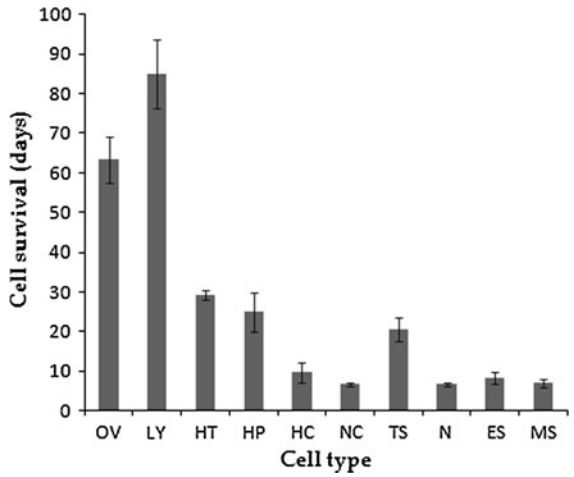

Fig. 1 Survival of primary cell cultures of ovary (*OV*), lymphoid organ (*LY*), heart (*HT*), hepatopancreas (*HP*), hemocytes (*HC*), nerve cord (*NC*), testis (*TS*), nauplii (*N*), eyestalk (*ES*) and muscle (*MS*) of *P. monodon* cultured in SCCM supplemented with 10 % FBS

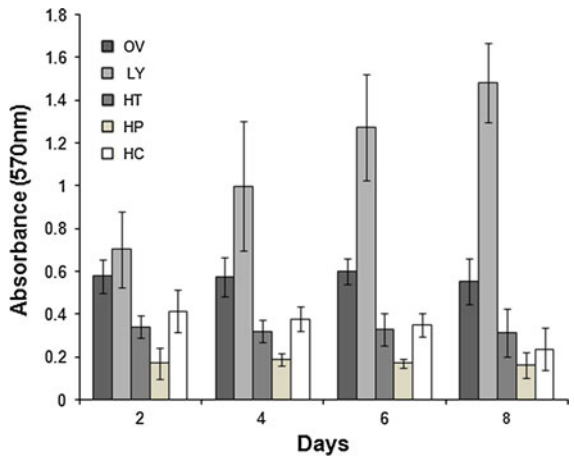

Fig. 2 Viability (in terms of MTT assay) of primary cultures of ovary (*OV*), lymphoid organ (*LY*), heart (*HT*), hepatopancreas (*HP*) and hemocytes (*HC*), of *P. monodon* cultured in SCCM supplemented with 10 % FBS

Comparison of SCCM with other selected media

Lymphoid and ovarian cells ameliorated in SCCM compared to other selected media. Relative increase of 107 % growth in terms of MTT assay ($p < 0.05$) was observed in lymphoid cells in SCCM in comparison to 2 × L-15, and 59 and 82 % with modified L-15 (Jose et al. 2010) and Grace's insect medium, respectively (Fig. 3b). Ovarian cells showed an increase of 45, 37 and 36 % ($p < 0.05$) in SCCM, 2 × L-15, modified

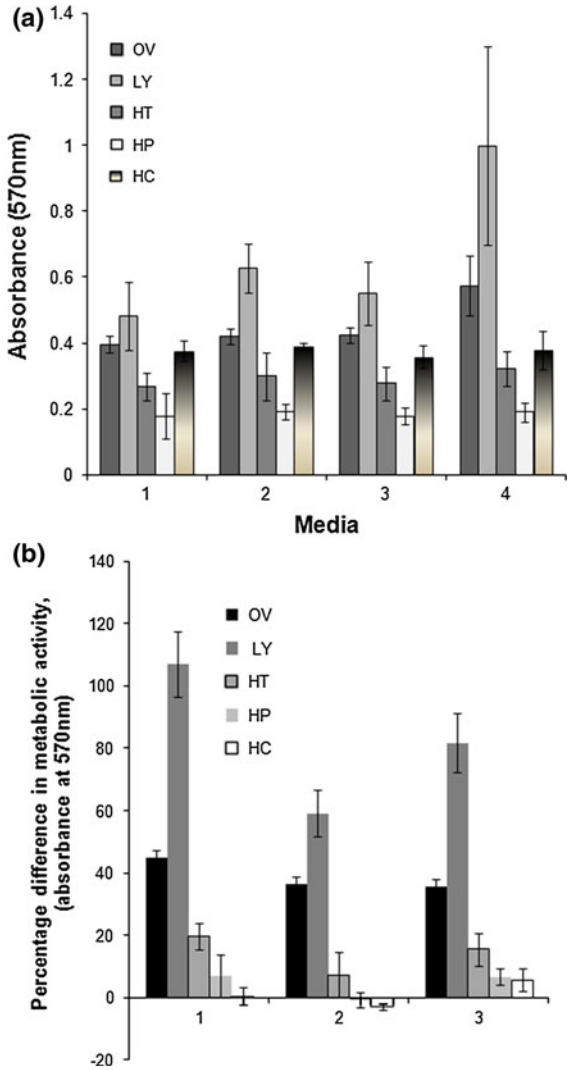

Fig. 3 **a** Effect of different media on in vitro growth (4th day results by MTT assay) of different cell types from *P. monodon*. 1. L-15 + 10 % FBS, 2. Modified L-15, 3. Grace's insect medium +10 % FBS, 4. SCCM + 10 % FBS. OV- ovary, LY-lymphoid organ, HT-heart, HP-hepatopancreas and HC- hemocytes. **b** Percentage difference in the metabolic activity (4th day results by MTT assay) of various cell types grown in SCCM in comparison with selected media. 1. L-15 + 10 % FBS, 2. Modified L-15, 3. Grace's insect medium +10 % FBS. OV-ovary, LY-lymphoid organ, HT-heart, HP-hepatopancreas and HC- hemocytes

L-15 (Jose et al. 2010) and Grace's medium respectively (Fig. 3a, b). However, increase in growth and multiplication of cells from heart, hepatopancreas and hemolymph in SCCM was less than 20 % compared to the other media.

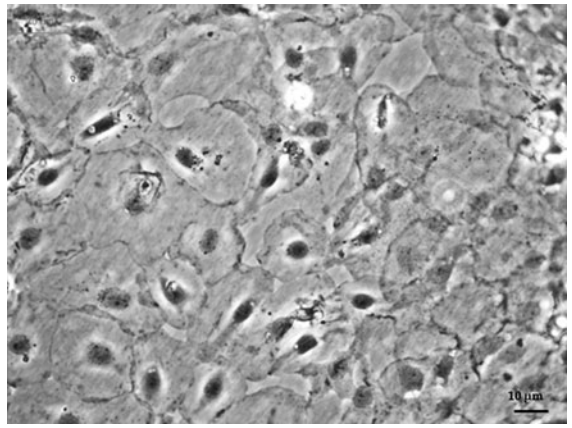

Fig. 4 Primary cell culture developed from *P. monodon* lymphoid cells in SCCM

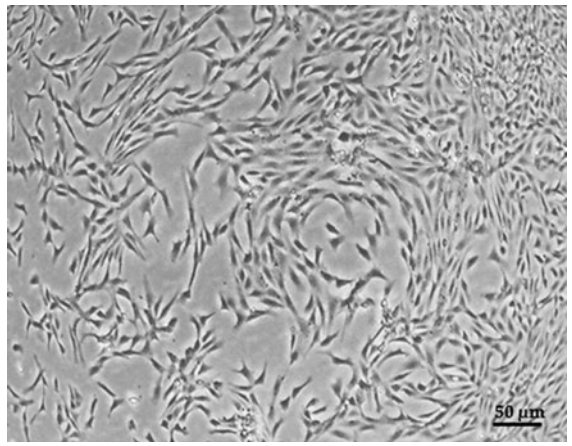

Fig. 5 Primary cell culture from *P. monodon* haemocytes grown in SCCM

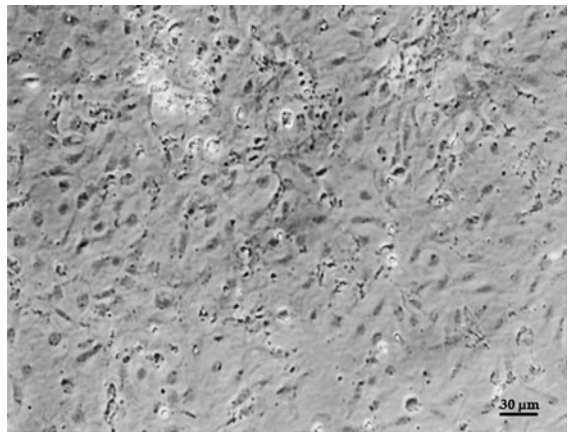

Fig. 6 Primary cell culture developed from *P. monodon* heart cells in SCCM

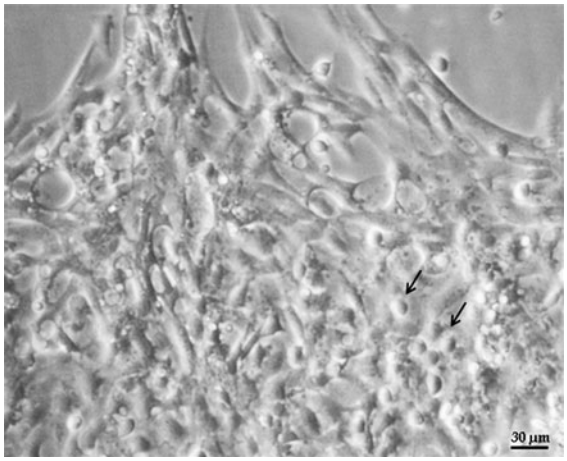

Fig. 7 Primary cell culture in SCCM developed from ovarian cells of *P. monodon*. Proliferating fibroblastic and round cells (*black arrow*) can be seen

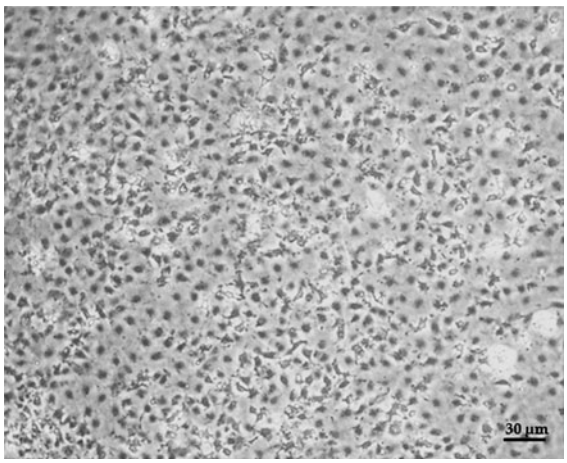

Fig. 8 Primary cell culture developed from hepatopancreatic cells of *P. monodon* in SCCM

Cell dislodgement and passaging

The results of cell dislodgement and passaging of primary cell cultures from ovary, lymphoid organ, heart, hepatopancreas and haemocytes using different dissociation methods and their efficacy in cell reattachment and growth are summarized in Table 4. Of the six dissociation agents tested shrimp cell dissociation 'cocktail' showed better survival (40 %) of lymphoid cells after two passages. Heart cells and ovarian cells showed a survival rate of up to 30 % whereas for hepatopancreas and haemocytes it was toxic with a survival rate of 20 % and less than 10 %,

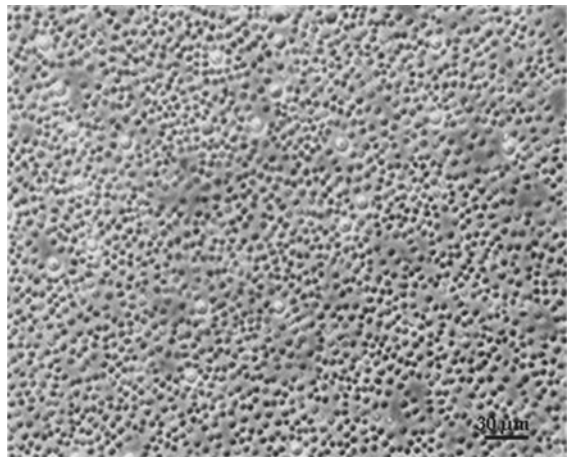

Fig. 9 Primary cell culture developed from *P. monodon* testicular cells in SCCM

respectively. Trypsin (0.25 %) and accutase (1 %) were found toxic to cells with a survival rate of less than 10 %. The non-enzymatic solutions I and II were proven to be not suitable for shrimp cells altogether. However, in 1 % collagenase V, all cell types showed comparatively better survival (>10 %) except haemocytes, for which it was less than 10 %.

Discussion

On surveying the literature it has been observed that shrimp cell line development has not yet attained considerable success mainly because of the absence of an appropriate growth medium (Jayesh et al. 2012). Even though the medium composition is very important among the factors which affect the proliferation of cells in primary cultures (Mitsuhashi 2001), what done so far has been to modify and use the available media designed for mammalian cell culture systems. Among the commercially available cell culture media used Leibovitz's -15 (L-15) has been the most popular one (Chen et al. 1986, 1988; Chen and Kou 1989; Nadala et al. 1993; Lu et al. 1995; Tapay et al. 1995; Tong and Miao 1996; Toullec et al. 1996; Mulford and Austin 1998; Chen and Wang 1999; Shike et al. 2000; Wang et al. 2000; Kumar et al. 2001; Shimizu et al. 2001; Chun-Lei et al. 2003; Maeda et al. 2003, 2004; Jiang et al. 2005; Assavalapsakul et al. 2006; Jose et al. 2010, 2011; 2012). Other media such as M199 (Ghosh

Table 4 Effect of different cell dissociation agents on passaging primary cell cultures

z	Cell dissociation agent	Cell type	Cells survived	Passage no
I	Collagenase type V	LY	++	1
		HT	++	1
		HC	+	1
		OV	++	1
		HP	++	1
II	Trypsin	LY	+	1
		HT	+	1
		HC	–	–
		OV	+	1
		HP	+	1
III	Accutase	LY	–	–
		HT	–	–
		HC	–	–
		OV	+	1
		HP	–	–
IV	Non enzymatic solution-I	LY	–	–
		HT	–	–
		HC	–	–
		OV	–	–
		HP	–	–
V	Non enzymatic solution-II	LY	–	–
		HT	–	–
		HC	–	–
		OV	–	–
		HP	–	
VI	"Cocktail"	LY	+++++	2
		HT	++++	1
		HC	+	1
		OV	++++	2
		HP	+++	1

OV ovary, *LY* lymphoid organ, *HT* heart, *HP* hepatopancreas, *HC* hemocytes

The confluence of primary cell cultures was categorized as: +<10 % of cells passaged, ++10 % of cells passaged, +++20 % of cells passaged, ++++30 % of cells passaged, +++++>30 % of cells passaged, − no survival of cells

et al. 1995; Toullec et al. 1996; Itami et al. 1999; Shimizu et al. 2001; Lang et al. 2002), MPS (Tong and Miao 1996; Fan and Wang 2002; Hu et al. 2010;), and Grace's insect medium (Luedeman and Lightner 1992; Nadala et al. 1993; Toullec et al. 1996; Wang et al. 2000; George et al. 2011; George et al. 2011; Jose et al. 2012) were also tested alone as well as with additives for growth of shrimp cells in vitro.

Seawater based shrimp cell culture medium (SCCM) was formulated in 27 ‰ natural seawater as the base since the isosmotic point of salinity for *P. monodon* had indirectly been calculated (Dall 1981) to be 27 ‰ by comparing with the coexisting *Penaeus merguiensis*. Earlier to this Mulford et al. (2001) had prepared L-15 medium in 25 ‰ seawater and found it effective for cell migration, survival, and cell longevity and claimed rapid migration of cells from explants of hematopoietic tissue of the lobster *Nephrops norvegicus*. Meanwhile, in our study the experiment with artificial seawater was not found to be as effective

for growth and proliferation of cells as those in natural seawater.

The great success achieved in the development of insect cell line by Grace (1962) was due to the contributions by Wyatt et al. (1956) in insect biochemistry. May be motivated by them, Najafabadi et al. (1992) and Shimizu et al. (2001) attempted to investigate the biochemistry of haemolymph from *P. aztecus* and *P. stylirostris* and modified commercially available L-15 to suit the requirement of shrimp cell culture in vitro. Following these lines we undertook determination of the composition of haemolymph of *P. monodon* and quantified 15 fatty acids, 16 amino acids and 10 metal ions, which were hitherto not recorded in this species. Taking queues from this information we formulated a novel shrimp cell culture medium having natural seawater as the base. This was due to the fact that the haemolymph metal ions such as sodium (6,784.3 ± 785.8 mg l^{-1}), potassium (524.5 ± 157.9 mg l^{-1}) and calcium (488.8 ± 107.9 mg l^{-1}) were within the range of 27 ‰ seawater where they were 8,075.5 ± 260.9 mg l^{-1}, 512.935 ± 73.2 mg l^{-1} and 443.7 ± 63.1 mg l^{-1}, respectively. These values supported the results of Najafabadi et al. 1992 who observed 6,188.00 ± 795.6 mg l^{-1} sodium, 281.5 ± 62.6 mg l^{-1} potassium and 410 ± 7 mg l^{-1} calcium in the haemolymph of *P. aztecus*. In *P. stylirostris* (Shimizu et al. 2001) the values were 8,411.1 ± 549.7 mg l^{-1}, 328.44 ± 50 mg l^{-1}, and 8,439.2 ± 44.3 mg l^{-1}, respectively (Table 2). Concentration of iron, zinc and strontium in the haemolymph of *P. monodon* were 12.9 ± 6.6 mg l^{-1}, 39.42 ± 2.9 mg l^{-1} and 7.3 ± 2 mg l^{-1}, respectively, whilst in seawater it was 0.053 ± 0.01 mg l^{-1}, 0.035 ± 0.02 mg l^{-1} and 7.05 ± 0.44 mg l^{-1}. However, Shimizu et al. (2001) estimated the concentrations of these elements in *P. stylirostris* haemolymph as 0.1 ± 0.0 mg l^{-1}, 11.4 ± 1.9 mg l^{-1} and 6.3 ± 0.6 mg l^{-1}, respectively.

In our study, the addition of salts such as barium chloride, copper chloride, zinc sulphate, ferric citrate and manganese chloride and the organic supplements such as vitamin mixture, citric acid cycle intermediates, nitrogenous base and energy precursors to 27 ‰ natural seawater did not make any observable changes in the growth and multiplication of primary cell cultures over and above what has been observed in SCCM. This may be due to the availability of sufficient organic and inorganic elements from the supplemented fetal bovine serum (Freshny 2000) and the elemental complexity of the natural seawater (Bruland et al. 1991).

Amino acid and fatty acid constituents in the haemolymph provided a basic knowledge about the concentration to be used in the new medium. Accordingly, various concentrations of the amino acid mixture were tried to find out the most appropriate one which provided maximum cell attachment, multiplicity and survival. Despite the requirement of 0.02 % cholesterol for better performance of the shrimp cell culture in SCCM, the addition of lipid mixture (in addition to cholesterol) did not contribute to any observable changes. In similar lines Kasornchandra et al. (1999) and George and Dhar (2010) had recommended 0.01 % cholesterol for better performance of shrimp cell culture in media.

Even though the maintenance of proper pH is essential for the successful growth of cells (Nadala et al. 1993) no direct measurements of haemolymph pH of *P. monodon* or any penaeid has been published. However, Huang et al. (1999) calculated in culture flasks, the optimum pH for hepatopancreatocytes of *Penaeus chinesis* as 6.5 with a suggested range of 6.0–7.2. Meanwhile, most researchers selected a pH within the range of 7–7.2 (Tong and Miao 1996; Fan and Wang 2002; Jiang et al. 2005) and 6.8–7.2 (Chen and Wang 1999: Kumar et al. 2001). In the present study pH of SCCM was selected as 6.8 within a range of 6.8–7.2 where the medium remained clear without any precipitation supporting better growth of the cell cultures.

Osmolality of the medium was fixed at 720 ± 10 mOsmol kg l^{-1} in accordance with the haemolymph osmolality, which was found to be 730.5 ± 51.2 mOsmol kg l^{-1} and the cells in vitro were in isosmotic state at this osmolality as judged by visual observation. This was supported by the findings of Kasornchandra et al. (1999) and Fraser and Hall (1999), who used 730 ± 10 and 720 ± 20 mOsmol kg l^{-1}, respectively, for maintaining cells from *P. monodon* in vitro.

In SCCM, we could maintain ovarian cell culture for 63 ± 6 days, lymphoid cells for 85 ± 9 days during which they showed better proliferation among all the cell types tested, and also as evidenced through MTT assay with significant increase in cell metabolic activity ($p < 0.05$). They exhibited better survival (40 %) after two passages. Further, cell cultures could

be maintained from hepatopancreas for 25 ± 5 days, heart for 29 ± 1 days, haemocytes for 10 ± 3 days, testis for 21 ± 3 days, eyestalk for 9 ± 2 days, nerve cord, and cells from nauplii for 6 ± 1 days and muscle for 7 ± 1 days in SCCM. Several authors claimed to have maintained ovarian cell culture for 45–66 days (George and Dhar 2010 Maeda et al. 2003), to several months (Toullec et al. 1996; Tong and Miao. 1996). Lymphoid cell culture was reported to be maintained for 54 days (Itami et al. 1999) to 3 months (Tong and Miao. 1996), hepatopancreas for 30 days (George and Dhar. 2010), heart tissue for 4 days (Chen and Wang. 1999) and haemocytes for 48 days (George and Dhar. 2010). Even though, Toullec et al. (1996) maintained embryonic culture for several months, Chun-Lei et al. (2003) maintained nerve cells for 15 days and Nadala et al. (1993) for 3 months, such results were not found to have been reproduced by other workers for reasons not known.

Growth and multiplication of cells in SCCM were compared with those in other media based on MTT assay. The lymphoid cells grown in SCCM exhibited a relative increase of 107 % growth in comparison with 2 × L-15, and 59 and 82 % with modified L-15 (Jose et al. 2010) and Grace's insect medium, respectively ($p < 0.05$). Meanwhile, ovarian cells showed an increased growth of 45, 37 and 36 % ($p < 0.05$) in SCCM in comparison with 2 × L-15, modified L-15 (Jose et al. 2010) and Grace's insect medium respectively. However, increase in growth and multiplication of cells from heart, hepatopancreas and hemolymph in SCCM was less than 20 % compared to the same in the other media.

The passaging of primary shrimp cell cultures and their survival have been found to be the most difficult tasks (Fraser and Hall. 1999; Chun-Lei et al. 2003). However, we could passage lymphoid cell culture twice using shrimp dissociation cocktail. But only about 40 % cells were found surviving after the passage as evidenced by visual observation. Even though, lymphoid cell culture were reported to be passaged 3 times (Chen and Wang. 1999) to 44 times (Tapay et al. 1995), neither confirmation of their results nor adoption of methods by other shrimp cell culture research labs could be found reported elsewhere.

From the difficulties experienced by researchers over the last few decades, it is obvious that several untold hindrances stand in the way of the development of shrimp cell lines. The major unsettling fact was that the media used for the study were mostly by the modification of commercially available ones which were designed mainly for supporting cells from mammalian origin or mostly from terrestrial animals. Virtually, application of none of the media could end up with a lead in cell line development specific for shrimp cell culture. Availability of growth medium and optimal conditions for supporting cell cultures could be one of the major possible ways to tackle the issue of immortalization. In this context, the medium developed here gives the lead to work towards attainment of immortality of shrimp cell culture especially from lymphoid tissue. However, it may require further improvement.

In conclusion, through this study, a seawater based novel cell culture medium for the development of shrimp cell culture has been formulated and validated with various tissues of *P. monodon*. The success in extending longevity, metabolic activity and the passaging efficiency of the cells grown in SCCM in vitro suggests that this medium should help researchers in the development and establishment of shrimp cell lines for various applications.

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