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Characterization of a primary cell culture from lymphoid organ of *Litopenaeus vannamei* and use for studies on WSSV replication

Wenfeng Li [a,*], Van Thao Nguyen [a,b], Mathias Corteel [a], João José Dantas-Lima [a], Khuong Van Thuong [a], Vo Van Tuan [a], Peter Bossier [b], Patrick Sorgeloos [b], Hans Nauwynck [a]

[a] Laboratory of Virology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium
[b] Laboratory of Aquaculture & Artemia Reference Center, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University, Belgium

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ABSTRACT

Shrimp aquaculture is a booming agro-industry worldwide. Due to intensification of shrimp farming, pathogens emerge. Control of these pathogens especially viral pathogens is essential for a further expansion of this industry. Until now, the lack of shrimp cell lines has limited research on shrimp viral pathogens. In this context, a primary culture from the lymphoid organ of *Litopenaeus vannamei* was developed and standardized as a platform for further research on white spot syndrome virus (WSSV). Explants from the lymphoid organ of *L. vannamei* were cultured in 2× L-15 (Leibovitz-15) medium supplemented with 20% fetal bovine serum (FBS), 10% Chen's salt, penicillin (100 IU/ml) & streptomycin (100 μg/ml), gentamicin (50 μg/ml) and fungizone (0.25 μg/ml) with a pH of 7.5. Gelatin (0.1%)-coated culture plates promoted the migration of cells from the explants and cell survival. 600 μg/ml cholesterol and 1000 μg/ml L-glutathione (GSH) both enhanced cell survival and performance *in vitro*. Susceptibility of lymphoid organ cells for infection with white spot syndrome virus (WSSV) was determined by indirect immunofluorescence (IIF) staining by monoclonal antibodies against VP28 (W29) and goat anti-mouse IgG-FITC. FITC positive signals in the nuclei starting from 9 h post inoculation (hpi) demonstrated the susceptibility of the cells for WSSV infection in these cultures. This cell culture system will be used in the future as a tool for studying host–virus interactions in WSSV pathogenesis.

1. Introduction

Penaeid shrimp, the most important cultured crustacea, are threatened by diseases worldwide. Mainly viral diseases have resulted in disastrous economic losses to the shrimp agro-industry with white spot syndrome virus as the main shrimp killer (Kasornchandra et al., 1995; Wongteerasupaya et al., 1995). PCR, immunohistochemistry, immunofluorescence and electron microscopy have been of great value for diagnosis and research. However, lack of shrimp cell cultures and stable continuous cell lines have limited research on this virus. A better understanding of WSSV-associated problems relies on the development of standardized techniques to maintain and culture host cells. Plenty of publications on primary cell cultures of various organs from different shrimp species and using different media appeared already in the literature in the past 27 years. The most commonly used species were *Penaeus monodon* (Hsu et al., 1995; Jose et al., 2012; Owens and Smith, 1999) and *L. vannamei* (George et al., 2011; Lu et al., 1995; Nadala et al., 1993; Toullec et al., 1996). Explant and dissociation methods have been used to obtain cells from diverse tissues and organs, such as lymphoid organ (Assavalapsakul et al., 2003; Chen and Kou, 1989; Han et al., 2013; Tapay et al., 1995; Wang et al., 2000a), ovary (George and Dhar, 2010; Kasornchandra et al., 1999; Luedeman and Lightner, 1992), heart (Chen and Wang, 1999; Goswami et al., 2010; Tapay et al., 1997), hemocytes (Dantas-Lima et al., 2012; Jiang et al., 2006; Jose et al., 2010; Lang et al., 2002), hepatopancreas (Ke et al., 1990), nerve cord (Lang et al., 2002; Nadala et al., 1993; Owens and Smith, 1999), muscle (Chen et al., 1986; Wang et al., 2000b), hematopoietic tissue (Jiravanichpaisal et al., 2006; West et al., 1999), gills (Chen et al., 1986; Hsu et al.,1995; Nadala et al.,1993), gut (Nadala et al., 1993), eye stalk (George and Dhar, 2010) and embryonic cells (Frerichs, 1996). Primary cells from the lymphoid organ have been used in WSSV research and some could be infected by WSSV (Itami et al., 1999; Kasornchandra and Boonyaratpalin, 1998; Kasornchandra et al., 1999; Tapay et al., 1995, 1997). Until now, there is no report on primary cell cultures from the lymphoid organ of *L. vannamei* used for white spot syndrome virus (WSSV) research. In the present study, a reproducible protocol was developed for a primary cell culture from the lymphoid organ of *L. vannamei* and the susceptibility of these cultured cells for an infection with WSSV was examined.

* Corresponding author.

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2. Materials and methods

2.1. Experimental animals

Penaeus (*Litopenaeus*) *vannamei* post-larvae which were imported from Syaqua Siam Co. Ltd. (Thailand) were certified to be specific pathogen-free for the viruses WSSV, TSV, YHV, IHHNV and IMNV. After arrival, the post-larvae were stocked in a recirculating aquaculture system with 3.5% salinity in the Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium. These shrimp were reared with commercial pelleted feed. After 4 months, shrimp in premolt stage weighing 20 ± 2 g were used for the collection of lymphoid organ.

2.2. Primary cell culture of lymphoid organ

The shrimp were disinfected by immersion in 4.0% hypochlorite solution and 70% ethanol prepared in cold seawater (3.5% salinity) for 2 min, respectively. Finally the shrimp were rinsed several times in sterile cold seawater. The lymphoid organ consists of two white small ovoid-shaped tissues between the lateral side of the stomach and the anterior edge of the hepatopancreas. The organs were rinsed in washing medium (2× L-15 (Sigma Aldrich), supplemented with 20% FBS, 10% Chen's salt (Chen and Kou, 1989), penicillin (1000 IU/ml, Gibco) & streptomycin (1000 μg/ml, Gibco), gentamicin (500 μg/ml, Gibco) and fungizone (2.5 μg/ml, Gibco) (pH: 7.5)) four times and chopped into 0.5 mm^3 cubic explant. All the explants were transferred into wells of 24-well plates with 150 μl of culture medium per well and after 12 h post seeding another 750 μl of culture medium was added into each well. Basic culture medium consisted of 2× L-15 supplemented with 20% fetal bovine serum, 10% Chen's salt, penicillin (100 IU/ml) & streptomycin (100 μg/ml), gentamicin (50 μg/ml) and fungizone (0.25 μg/ml) (pH: 7.5). The cultures were incubated at 27 °C and examined every day with an inverted microscope. One-third of the culture medium was changed every 3 days.

2.3. Effect of coating on primary cell cultures of lymphoid organ

Gelatin (0.1%, w/v) and poly-L-lysine (0.005%, Sigma Aldrich) were tested as attachment factors. In brief, the 24-well plate (with glass insert) was coated with 1000 μl per well gelatin or poly-L-lysine for 2 h at 37 °C. Then, the excess fluid was removed and all coated wells were washed twice by UP water. Explants were introduced into the coated wells and cultured with 1 ml basic culture medium at 27 °C. The control wells were without coating. The number of living cells per explant was counted at 48 h, 96 h and 144 h.

2.4. Effect of cholesterol on primary cell cultures of lymphoid organ

Cholesterol is an essential molecule for living shrimp. In the hemolymph of *L. vannamei*, the concentration of cholesterol is 1480 μg/ml (Najafabadi et al., 1992), which is much higher than in FBS (330 μg/ml). Therefore, the effect of different concentrations of cholesterol was examined. Cells from the lymphoid organ were cultured with different concentrations of cholesterol (66 μg/ml, 200 μg/ml, 600 μg/ml and 1800 μg/ml, Sigma Aldrich) in 24-well plates at 27 °C. The number of living cells per explant was counted at 48 h, 96 h and 144 h.

2.5. Effect of L-glutathione on primary cell cultures of lymphoid organ

L-Glutathione is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species. The cells from the lymphoid organ were cultured with different concentrations of L-glutathione (0 μg/ml, 500 μg/ml, 1000 μg/ml and 2000 μg/ml, Sigma Aldrich) in 24-well plates at 27 °C. The number of living cells per explant was counted at 48 h, 96 h and 144 h.

2.6. Cell counting

Hoechst and EMA stainings were performed to count total and dead cells. The cultures on glass inserts were subsequently (i) incubated with 200 μl EMA solution (20 μg/ml in medium, Invitrogen) for 30 min in dark and on ice, (ii) exposed to candescent light for 10 min on ice, (iii) fixed with 500 μl of 4% paraformaldehyde for 10 min, (iv) incubated with 200 μl Hoechst solution (10 μg/ml in PBS, Sigma Aldrich) for 10 min, (v) washed once with PBS and once with UP and finally mounted upside down on top of 2 μl of glycerin-DABCO on slides. All samples were checked by fluorescence microscopy.

2.7. Identification of hemocytes in primary cell cultures of lymphoid organ

The cells from the lymphoid organ were cultured on glass inserts in 24-well plates at 27 °C and hemocytes were stained with the hemocyte-specific monoclonal antibody WSH8 (Van de Braak et al., 2001) every 24 h post seeding to determine the proportion of hemocytes. The procedure was as follows: the glass inserts with cells were immersed in 200 μl of 4% paraformaldehyde for 10 min and in 200 μl of 0.1% Triton X-100 for 5 min at room temperature. After washing in PBS for 5 min, the glass inserts were incubated in 200 μl of monoclonal antibody WSH8 (1:100) at 37 °C for 1 h. Then after three washings with PBS for 5 min, they were incubated with goat anti-mouse IgG-FITC (1:100, Sigma Aldrich) at 37 °C for 1 h. Afterwards, the cultures were washed again three times with PBS and incubated with Hoechst solution (10 μg/ml in PBS, Sigma Aldrich) in dark for 10 min. After a washing with PBS and UP, they were mounted on a slide with glycerin-DABCO and stored at 4 °C.

2.8. Authentication of cells cultured in vitro

Especially in invertebrate cell culture, contaminations from protozoa and thraustochytrids occur. Authentication is hence needed. After 6-day culture, the cultured cells were collected for DNA extraction. DNA extraction was done according to Bossier et al. (2004). DNA was also extracted from shrimp tissue. The primer combinations Vanna16S (forward)/Vanna12S (reverse) were used in order to amplify the 1800 bp fragment of mitochondrial rDNA by PCR in a Hybaid PCR Express (Labsystemsk, Belgium).

The primer sequences were as follows: Vanna16S: 5′-CCGGTCTGAA CTCAGATC-3′, Vanna12S: 5′-AACCAGGATTAGATACCC-3′. The thermal cycler PCR conditions were as follows: 1 cycle of 94 °C for 2 min, 34 cycles of 1 min 15 s at 94 °C, 45 s at 52 °C, 2 min at 72 °C and a final extension cycle of 72 °C for 10 min. After purification, the PCR fragments were sequenced with primers Vanna16S/Vanna12S and blasted against the EMBL-EBI database.

2.9. Preparation of WSSV inoculum

50 g shrimp body without stomach and hepatopancreas from moribund WSSV (Thailand strain) infected shrimps was minced in 100 ml shrimp PBS on ice. The extract was centrifuged at 5500 *g* for 30 min at 4 °C. Then, the supernatant was collected and passed through a 0.45 μm membrane (Sarstedt). This WSSV stock (10^8 SID_{50}/ml) was diluted 10 times in 2× L-15 containing 10% Chen's salt and 500 μg/ml lactalbumin hydrolysate before use. The negative stock was prepared from healthy shrimp following the same procedure.

2.10. Detection of WSSV infected cells in lymphoid cell cultures by indirect immunofluorescence (IIF)

The lymphoid cell cultures (15 explants/well, 96 h old) were prepared on gelatin (0.1%) coated glass inserts in 24-well plates. 200 μl of

WSSV solution ($10^{6.3}$ SID_{50}) was added to each well with lymphoid cell culture and incubated at 27 °C for 1 h. At the same time, the negative control was inoculated with 200 μl of healthy shrimp stock per well. Then, the inoculum was removed and cells were washed three times with medium. 1 ml of medium was added to each well and the plates were incubated at 27 °C. Cell cultures were fixed at 3 h, 6 h, 9 h, 12 h and 24 h post inoculation and stained. In brief, the samples were fixed in 200 μl of 4% paraformaldehyde for 10 min and then in 200 μl of 0.1% Triton X-100 for 5 min at room temperature. After one washing with PBS for 5 min, the cell cultures were incubated in 200 μl of monoclonal antibody w29 (1:100 in PBS, Chaivisuthangkura et al., 2004) which is directed against WSSV viral protein VP28 at 37 °C for 1 h. Then, after three washings with PBS for 5 min, they were incubated in goat anti-mouse IgG-FITC (1:100 in PBS, Sigma Aldrich) at 37 °C for 1 h. After three washes with PBS for 5 min, the cell cultures were incubated in Texas Red-X Phalloidin (1:40 in PBS, Invitrogen) at 37 °C for 1 h. Then after three washes with PBS for 5 min, the cell cultures were incubated in Hoechst solution (10 μg/ml in PBS, Sigma Aldrich) for 10 min. After washings with PBS and twice with UP water for 5 min, the cultures were mounted on slides with glycerin-DABCO and stored at 4 °C. The inoculated cell cultures were analyzed with a confocal fluorescence microscope. All images were processed and merged by ImageJ software.

2.11. Statistical analysis

All results given in this paper were average values from three independent replicates with standard deviation. The effects of different treatments were statistically analyzed by ANOVA in SPSS 19.0. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Characteristics of a cell culture from lymphoid organ

The lymphoid organ explants of *L. vannamei* stuck well to the polystyrene surface of culture wells and once attached, cells started to migrate out of the explants. The first migrating cells had a round appearance and these cells stayed in the surroundings of the explants in suspension or semi-suspension at 24 h post seeding (Fig. 1A). These cells attached to the surface of the culture well and changed their shape. They became fusiform with branches (Fig. 1B). Afterwards, fibroblast-like cells were migrating from the explants. Many round cells were seen between and on top of the fibroblast-like cells (Fig. 1C). Islands of epitheloid cells were observed in the cultures at 72 h post seeding (Fig. 1D). The final cell monolayer consisted of three cell types: round cells, fibroblast-like cells and epitheloid cells.

3.2. Effect of coating on lymphoid cell culture

The effect of coating materials (gelatin and poly-L-lysine) on growth of primary lymphoid cell cultures was evaluated (Fig. 2). Significant differences in number of living cells/explant between gelatin (0.1%) coated wells and control wells were obtained at 48 h ($p < 0.05$), 96 h ($p < 0.001$) and 144 h ($p < 0.001$). The number of living cells/explant in gelatin (0.1%) coated wells and poly-L-lysine coated wells was also significantly different at 96 h ($p < 0.001$) and 144 h ($p < 0.001$). No significant difference was obtained between the poly-L-lysine group and the control group.

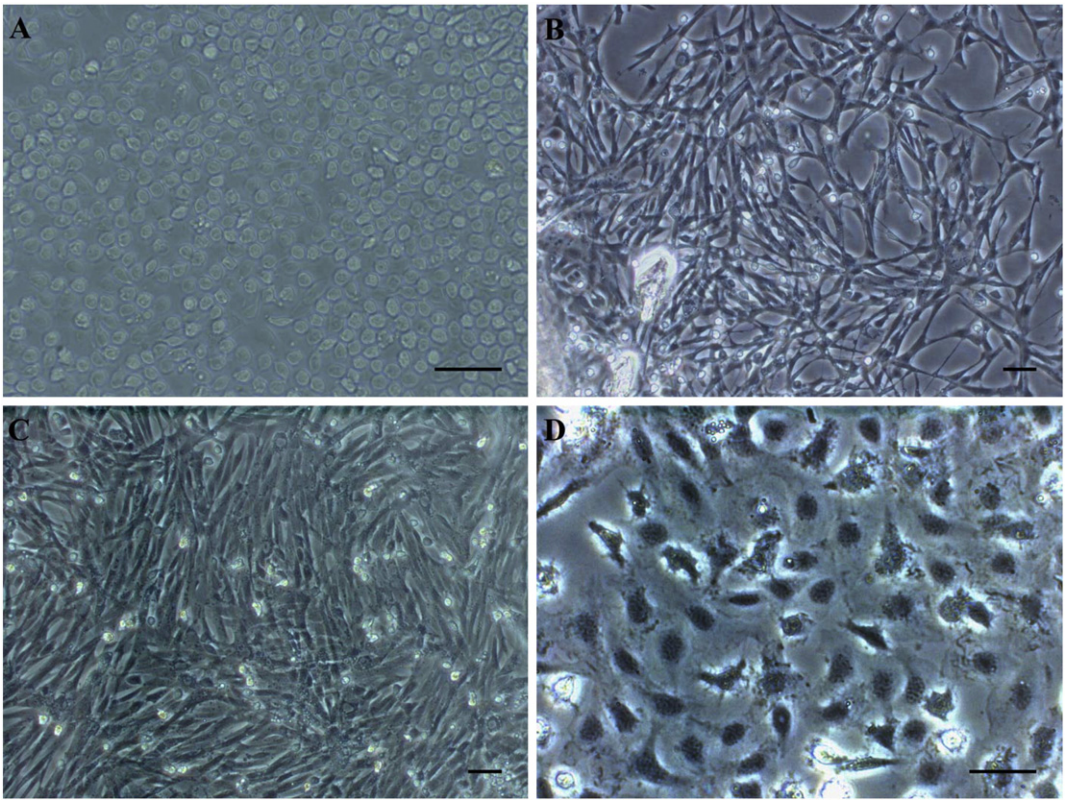

Fig. 1. Monolayer of primary cell culture developed from lymphoid organ of *L. vannamei*. A: round cells migrating from explants at 24 h post seeding; B: fusiform cells with branches transformed from round cells at 48 h post seeding; C: fibroblast-like cells that grew out from explants at 48 h post seeding; D: epitheloid cells at 72 h post seeding (scale bar = 30 μm).

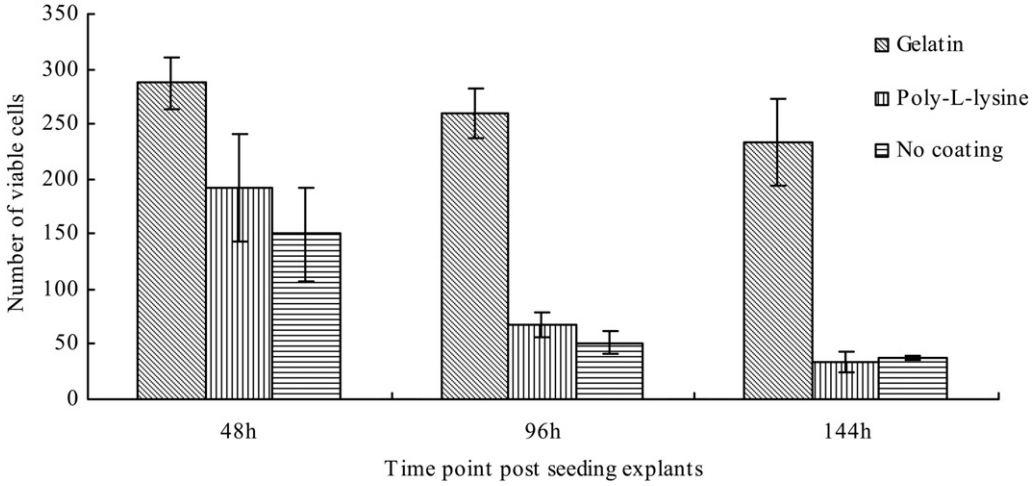

Fig. 2. Effect of coating (gelatin and poly-L-lysine) on the performance of primary cell cultures developed from lymphoid organ of *L. vannamei* at 48 h, 96 h and 144 h post seeding.

3.3. Effect of cholesterol on lymphoid cell culture

The effect of different concentrations of cholesterol (66 μg/ml (control), 200 μg/ml, 600 μg/ml and 1800 μg/ml, Sigma Aldrich) on the growth of primary lymphoid cell cultures was evaluated (Fig. 3). The highest number of living cells/explant was obtained with 600 μg/ml cholesterol, followed by 200 μg/ml cholesterol. Significant differences in number of living cells/explant between 600 μg/ml and 66 μg/ml cholesterol were obtained at 48 h ($p < 0.01$), 96 h ($p < 0.001$) and 144 h ($p < 0.001$). The number of living cells/explant cultured in the presence of 200 μg/ml cholesterol was also significantly different with the controls at 48 h ($p < 0.05$), 96 h ($p < 0.01$) and 144 h ($p < 0.001$) post seeding. Between 200 μg/ml and 600 μg/ml cholesterol there was no significant difference. No significant difference was obtained between 1800 μg/ml and 66 μg/ml (control).

3.4. Effect of L-glutathione on lymphoid cell culture

In this study, different concentrations of L-glutathione (0 μg/ml, 500 μg/ml, 1000 μg/ml and 2000 μg/ml) were added to the culture medium to evaluate their effect on cell performance. Overall, cells treated with 1000 μg/ml and 500 μg/ml of L-glutathione showed better results on the number of living cells/explant (Fig. 4). Significant differences on living cell number/explant between the 1000 μg/ml L-glutathione group and controls were obtained at 48 h ($p < 0.01$), 96 h ($p < 0.05$) and 144 h ($p < 0.01$) after incubation. The living cell number in the 500 μg/ml L-glutathione group was also significantly different from that of the controls at 48 h ($p < 0.05$) and 144 h ($p < 0.01$) post seeding. With 2000 μg/ml L-glutathione, no significant difference with the control was recorded at 144 h post seeding.

3.5. Final culture medium formula

In this experiment, basic culture medium supplemented with either 1000 μg/ml L-glutathione or 600 μg/ml cholesterol or 1000 μg/ml L-glutathione + 600 μg/ml cholesterol were evaluated on their effect on cell performance at 48 h, 96 h and 144 h post seeding explants (Fig. 5). Significant differences on number of living cells/explant between the medium with cholesterol (600 μg/ml) + L-glutathione (1000 μg/ml) and medium with cholesterol (600 μg/ml) were obtained at 48 h ($p < 0.05$), 96 h ($p < 0.05$) post seeding. Significant differences on number of living cells/explant between the medium with cholesterol (600 μg/ml) + L-glutathione (1000 μg/ml) and medium with L-glutathione (1000 μg/ml) were obtained at 48 h ($p < 0.05$), 96 h ($p < 0.001$) and 144 h ($p < 0.05$) post seeding.

Based on these results above, the final culture medium was defined: 2× L-15 (Sigma Aldrich) supplemented with 20% FBS, 10% Chen's salt, 600 μg/ml cholesterol, 1000 μg/ml L-glutathione, penicillin (100 IU/ml) & streptomycin (100 μg/ml), gentamicin (50 μg/ml) and fungizone (0.25 μg/ml) (pH: 7.5). The cell monolayer was formed completely or partly in 4–6 days post seeding explants.

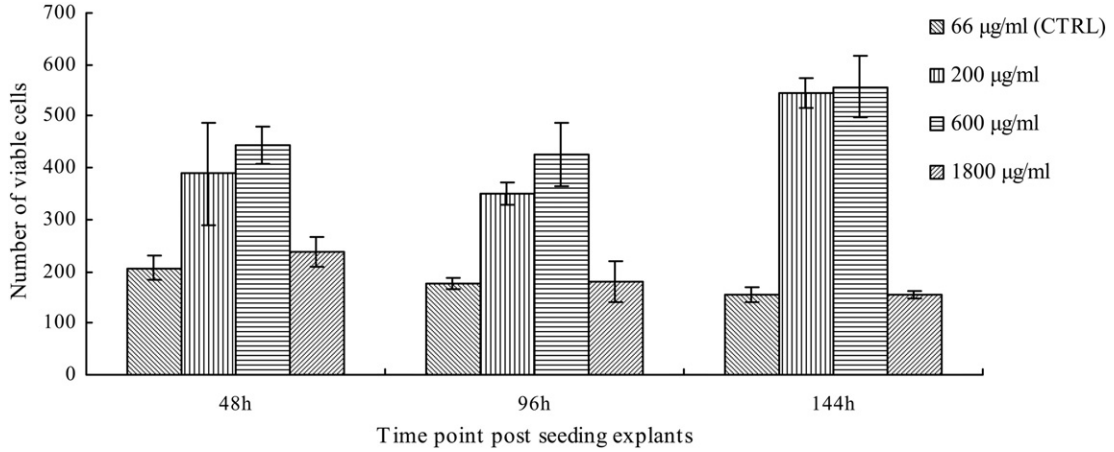

Fig. 3. Effect of cholesterol on the performance of primary cell cultures developed from lymphoid organ of *L. vannamei* at 48 h, 96 h and 144 h post seeding.

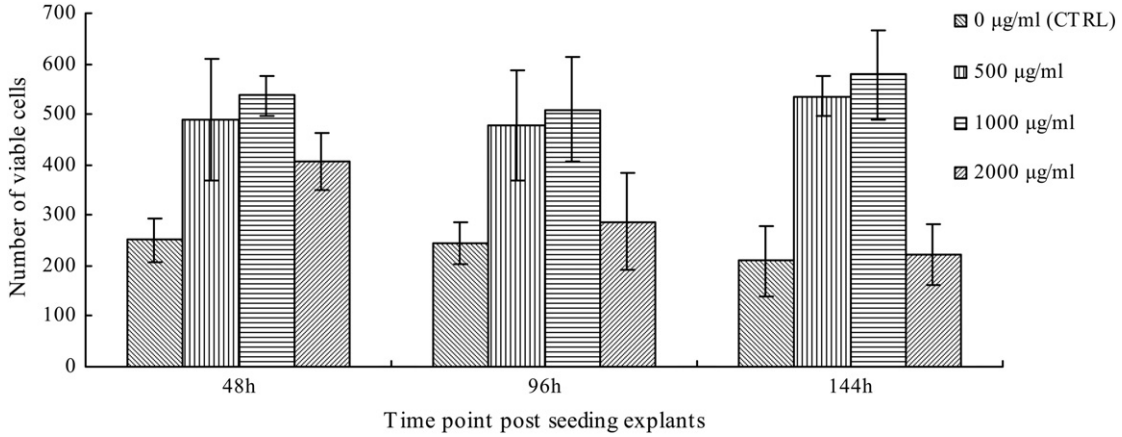

Fig. 4. Effect of L-glutathione on the performance of primary cell cultures developed from lymphoid organ of *L. vannamei* at 48 h, 96 h and 144 h post seeding.

3.6. Proportion of WSH8-positive cells (hemocytes) in primary lymphoid cell culture

WSH8 is a monoclonal antibody directed against a component of granules of hemocytes (Van de Braak et al., 2001). It is a marker for hemocytes in shrimp. In order to determine the proportion of WSH8-positive cells in primary cell cultures from the lymphoid organs of *L. vannamei*, an IIF staining was performed using monoclonal antibody WSH8. The data showed that the proportion of WSH8 positive cells decreased gradually from 81 ± 4% at 24 h of cultivation to 49 ± 5% at 72 h post seeding and 12 ± 1% at 96 h post seeding (Fig. 6).

3.7. Authentication of the cells cultured in vitro

The first 20 hits from the blast of the sequence of the PCR fragment obtained from cultured cells against the EMBL-EBI database were all from *L. vannamei*. The sequence of the PCR fragment obtained from cultured cells showed 99% identity with the sequence of mitochondrial rDNA of *L. vannamei* from EMBL-EBI (DQ534543.1: *L. vannamei* mitochondrion, complete genome), indicating that the cultured cells were from the shrimp *L. vannamei*, and not from contaminations.

3.8. WSSV replication in primary lymphoid cell cultures

Upon WSSV inoculation, WSSV VP28-positive signals were observed in some nuclei of infected cells starting from 9 h post virus inoculation (Fig. 7A) and became more widespread at 24 h post inoculation (Fig. 7B).

4. Discussion

In the present study, the coating and composition of the medium for making primary cell cultures from the lymphoid organ of *L. vannamei* were developed. Compared with other tissues and organs of penaeid shrimp, the lymphoid organ was preferred by most researchers due to its good performance *in vitro* (Assavalapsakul et al., 2003; Chen and Kou, 1989; Chen and Wang, 1999; Hsu et al., 1995; Hu et al., 2008; Itami et al., 1999; Jose et al., 2012; Lang et al., 2002; Nadala et al., 1993; Shike et al., 2000; Tapay et al., 1997; Tong and Miao, 1996). A few of those primary cell cultures from the lymphoid organ of *P. monodon* (Kasornchandra and Boonyaratpalin, 1998; Kasornchandra et al., 1999), *Litopenaeus stylirostris* (Tapay et al., 1995, 1997) and *Marsupenaeus japonicus* (Itami et al., 1999) were reported to be susceptible to white spot syndrome virus. Up till now, there was no report on the susceptibility of primary cell cultures of the lymphoid organ of *L. vannamei in vitro*.

In primary cell cultures of the lymphoid organ, the cells migrating from the explants changed in time post seeding. In the first 24 h, round cells dominated in the proximity of explants, which were reported by Jose et al. (2012), Tsing et al. (1989) and Wang et al. (2000a). In the present study these cells were confirmed to be hemocytes by monoclonal antibody WSH8, a marker for granules of shrimp hemocytes (Van de Braak et al., 2001). After 24 h cultivation, fibroblast-like cells started to grow out from explants and epitheloid cells were usually observed

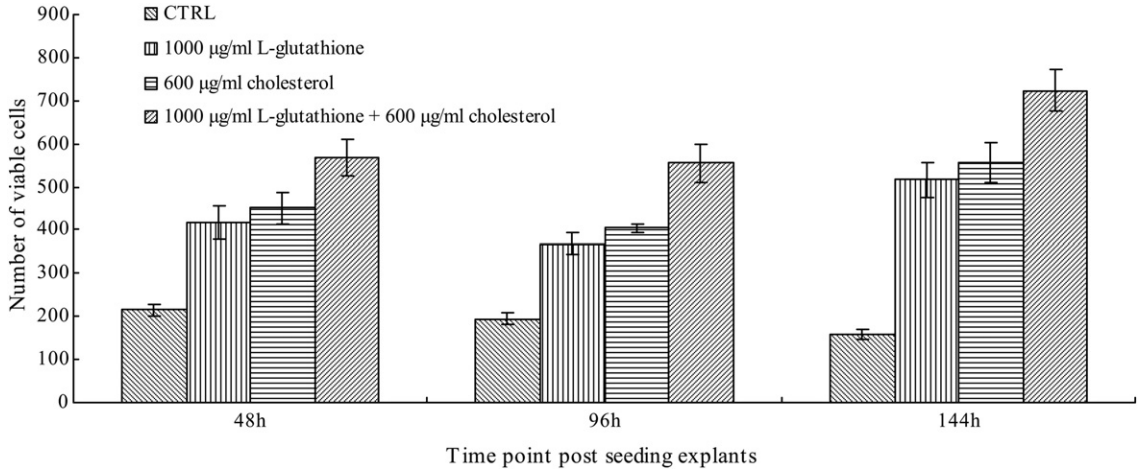

Fig. 5. Effect of basic culture medium with L-glutathione (1000 μg/ml) and/or cholesterol (600 μg/ml) on performance of primary cell cultures developed from lymphoid organ of *L. vannamei* at 48 h, 96 h and 144 h post seeding.

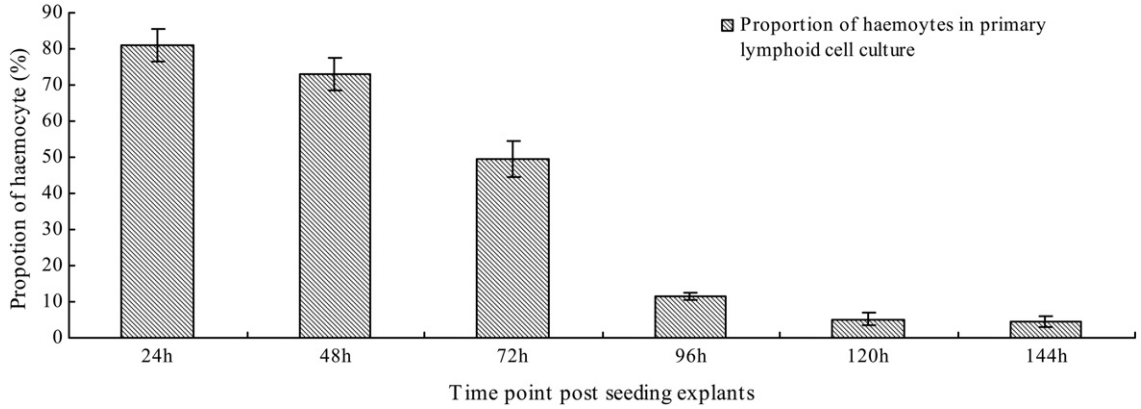

Fig. 6. Proportion of WSH8-positive cells (hemocytes) in primary cell cultures developed from lymphoid organ of *L. vannamei* at different time points post seeding.

after 48 h. These two cell types were also reported before (Itami et al., 1999; Wang et al., 2000a). The percentage of hemocytes in the cultures decreased gradually during the experiment, dropping from 81 ± 4% at 24 h post seeding to 12 ± 1% at 96 h post seeding. This drop may be explained by (i) the increase of non-hemocyte cells by migration and proliferation, (ii) death of hemocytes and (iii) losing the granule marker by differentiation.

Double strength L-15 medium with 20% FBS gave good results on cell survival and performance of primary cell cultures of lymphoid organ in the present study, which supported earlier reports (Fan and Wang, 2002; Itami et al., 1999; Jose et al., 2010; Nadala et al., 1993; Shike et al., 2000; Shimizu et al., 2001; Tapay et al., 1997). The osmolality of growth media varied greatly in between studies, ranging from 470 mOsmol·kg^{-1} (Hsu et al., 1995), up to 770 mOsmol·kg^{-1} (Nadala et al., 1993). The osmolality of adult *P. vannamei* shrimp weighing around 20 g, cultured in 35 ppt seawater, was determined to be 900 mOsmol·kg^{-1}. In culture medium, the osmolality was increased to about 900 ± 20 mOsmol·L^{-1} by 10% Chen's salt mixture (Chen and Kou, 1989). The pH of growth medium is crucial as inappropriate pH may result in poor cell maintenance and growth (Mulford et al., 2000). In the field of shrimp cell cultures, the pH used in growth media is within the range of 7.0–7.5 (Fan and Wang, 2002; Mulford and Austin, 1998; Tapay et al., 1995; Toullec et al., 1996). During earlier studies in our laboratory, pH values were tested for culturing hemocytes and 7.5 was chosen to be the best (Dantas-Lima et al., 2012). In the present study, culture medium with a pH of 7.5 gave good results.

Gelatin and poly-L-lysine are both commonly used coating materials in cell culture research. In our experiment, gelatin (0.1%) and poly-L-lysine (0.005%) both showed good results at the first 48 h post seeding explants. However, gelatin performed significantly better than poly-L-lysine at 96 h and 144 h post seeding explants. Gelatin contained higher concentrations of glycine (27.5%) and proline (16.4%) (Eastoe, 1955), which were also much higher than other amino acids in shrimp body (Vázquez-Ortiz et al., 1995). This might be helpful for the attachment and migration of lymphoid cells from *P. vannamei*.

Cholesterol is an important precursor of steroid hormones and is the major sterol in shrimp (Gong et al., 2000). Like other crustaceans, shrimp are unable to synthesize sterols and thus require cholesterol from dietary source for growth, development and survival (Fox et al., 1994). Kanazawa et al. (1971) reported that cholesterol is required in the shrimp diet for normal growth. Kasornchandra et al. (1999) reported that 0.01% cholesterol (100 μg/ml) enhances growth and performance of cells from the lymphoid organ of *P. monodon*. In the present study, 600 μg/ml cholesterol significantly enhanced survival and performance of lymphoid cells from *L. vannamei in vitro*.

L-Glutathione (GSH) is very important for cell growth and viability. Addition of GSH has been reported to improve mammalian tissue and cell cultures (Ozawa et al., 2006; Wang and Day, 2002). However, in the field of cell cultures of crustaceans, there has been no report on the effect of GSH towards cell growth. GSH has been proven to be helpful for hemocyte survival of *L. vannamei in vitro* (Dantas-Lima et al., 2012), which might be due to the strong anti-oxidant activity of GSH.

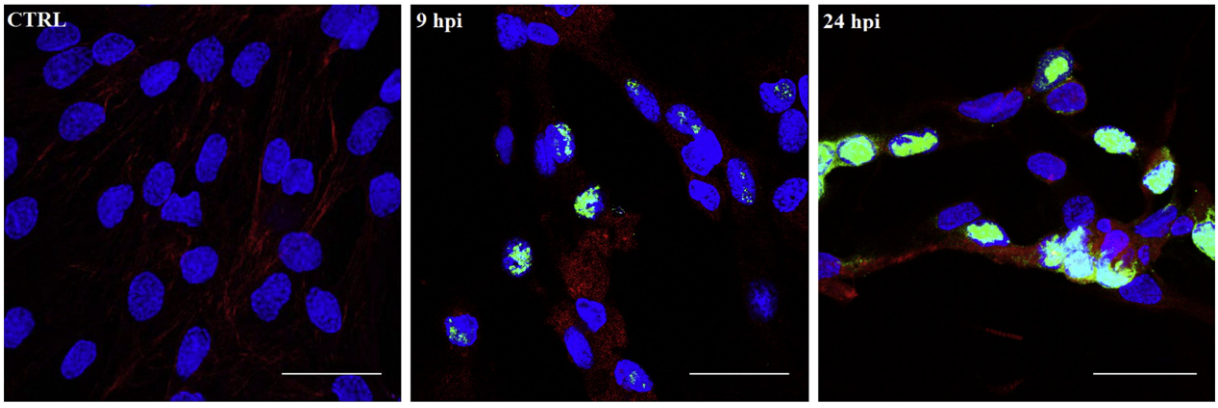

Fig. 7. Indirect immunofluorescence (IIF) staining of WSSV VP28 in primary lymphoid cell cultures (4-day old) from *L. vannamei* at 9 h and 24 h post inoculation (scale bar = 30 μm). Blue: Hoechst staining for nuclei; Green: W29 and F2761-FITC staining for WSSV VP 28; Red: Texas Red-X Phalloidin staining for cytoplasm.

In the present study, a concentration of 1000 μg/ml in culture medium was found to give the highest viable cell number.

In the present study, 96-h-old primary lymphoid cell cultures were inoculated with WSSV. VP28 was detected in the nuclei of lymphoid cells staining for 9 hpi and positive signals became more widespread at 24 hpi. In the near future, more work will be done on the replication cycle of WSSV in the lymphoid cell cultures of *L. vannamei*.

Acknowledgments

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