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Primary culture of Zhikong scallop *Chlamys farreri* hemocytes as an *in vitro* model for studying host–pathogen interactions

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ABSTRACT: Primary cultured cells can be a useful tool in studies on physiology, virology, and toxicology. Hemocytes play an important role in animal rapid response to pathogen invasion. In this study, an appropriate medium for primary culture of hemocytes of the bivalve *Chlamys farreri* was developed by adding 5% fetal bovine serum and 1% *C. farreri* serum to Leibovitz L-15 medium. These primary cultured hemocytes were maintained for more than 40 d *in vitro* and were classified into 3 types: (1) granulocytes containing numerous granules in the cytoplasm, (2) hyalinocytes with no or few granules, (3) a small percentage of macrophage-like cells. Furthermore, the primary cultured hemocytes were observed to be sensitive to bacterial and viral challenges. These hemocytes could phagocytose the bacterium *Vibrio anguillarum*, and presented cytopathic effects on the extracellular products (ECPs) of *V. anguillarum*; the mRNA level of *QM*, which plays an important role in immune response, also significantly increased 12 h after infection. When these hemocytes were challenged with ostreid herpesvirus 1 (OsHV-1), virus particles and empty capsids in the cells infected for 48 h were observed by transmission electron microscopy, and the *QM* mRNA level increased significantly at 12 h and 24 h following OsHV-1 challenge. This primary culture system is available for *C. farreri* hemocytes which can be used in the future to study host–pathogen interactions.

KEY WORDS: *Chlamys farreri* · Cell culture · Hemocyte · *Vibrio anguillarum* · Ostreid herpesvirus 1

INTRODUCTION

Hemocytes are considered to be the main immune effector cells participating in innate immune responses such as phagocytosis, inflammation, and wound repair, and modulating organism homeostasis in mollusks (Sparks & Morado 1988, Chu 2000). Based on their morphological and cytochemical features, mollusk hemocytes are mainly categorized into 2 types: granulocytes, with numerous granules in the cytoplasm, and hyalinocytes, with no or few granules (Hine 1999, Cima et al. 2000, Aladaileh et al. 2007, Donaghy et al. 2009, Astuya et al. 2015). Moreover, subpopulations of hyalinocytes and granulocytes are also observed in several mollusk species, such as the clam *Ruditapes philippinarum* (López et al. 1997, Cima et al. 2000, Lambert et al. 2003, 2007) and abalone *Haliotis tuberculata* (Hine 1999, Travers et al. 2008). In addition to the common granulocytes and hyalinocytes, a small quantity of

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large hyalinocytes possessing a phagocytic function are deemed to be macrophages in the Zhikong scallop *Chlamys farreri* (Sun & Li 2001).

In vitro culture cell is an important tool for investigating physiology, virology, toxicology, and carcinogenesis (Zhang et al. 2011). In marine organisms, many cell lines of fish have been established and are commonly used in diagnosing and characterizing viral and bacterial pathogens as well as exploring pathogenic mechanisms (Fryer et al. 1992, Chen et al. 2004, Gong et al. 2011, Hasoon et al. 2011). However, no immortalized cell line in marine invertebrates has been established to date, although great efforts have been made over the last several decades (Rinkevich 2005, Yoshino et al. 2013, Cai & Zhang 2014). Therefore, research has been devoted to establishing stable primary culture systems for related studies, including investigations of the propagation of white spot syndrome virus (WSSV) in primary cultured ovarian cells of the kuruma shrimp *Marsupenaeus japonicus* (Maeda et al. 2004); susceptibility of WSSV *in vitro* in primary cultured lymphoid cells from *Penaeus monodon*, *Metapenaeus ensis*, and *Litopenaeus vannamei* (Han et al. 2013, Li et al. 2014, Puthumana et al. 2015); electrophysiological analysis in primary cultured neurons of the crab *Carcinus maenas* and lobster *Homarus americanus* (Saver et al. 1999, Stepanyan et al. 2004); and genotoxicity and cytotoxicity of the antibacterial agent Triclosan in primary cultured hemocytes of the zebra mussel *Dreissena polymorpha* (Binelli et al. 2009a,b).

The Zhikong scallop *C. farreri* is an important commercial bivalve that is cultured widely along the coast of northern China. In this study, we established a primary culture system for hemocytes of *C. farreri* and investigated the sensitivity of these hemocytes to infection by *Vibrio anguillarum*, a common pathogen in scallop aquaculture (Zhou et al. 2011, Wang et al. 2012a,b), and by ostreid herpesvirus 1 (OsHV-1), which can cause mass mortality of cultivated bivalves around the world (Renault et al. 1995, Le Deuff & Renault 1999, Davison et al. 2005, 2009, EFSA 2010, Segarra et al. 2010, Bai et al. 2015, Barbosa Solomieu et al. 2015). Our data will be helpful in developing hemocyte cultures *in vitro* and understanding pathogenic mechanisms in marine mollusks.

MATERIALS AND METHODS

Animals and sampling

Adult scallops *Chlamys farreri* with a mean shell height of 6.28 ± 0.43 (SD) cm were collected from Shazikou bay (Qingdao, China) and maintained in filtered seawater (FSW, 14 ± 2 psu) for 2 d. Hemolymph was extracted using a sterile syringe from *C. farreri* adductor muscle after the scallop shell and adductor muscle were decontaminated with a common swab soaked in 75% ethanol cotton. Part of the hemolymph was seeded onto plastic Petri dishes or 24-well plates (Nunc) for *in vitro* culture. Others were transferred to a sterile container overnight at 4°C, and then centrifuged at 2000 × *g* (20 min at 4°C). The supernatant was then filter-sterilized and labeled *C. farreri* serum (CFS), which was stored at −20°C until use.

Optimization of the medium

To increase survival of the cultured *C. farreri* hemocytes, the medium was first optimized by supplementing fetal bovine serum (FBS, Hyclone) and CFS to the basic L15 medium (BM-L15, pH 7.2–7.4). BM-L15 medium consists of 13.7 g l^{-1} Leibovitz's 15 medium (Gibco), 20.2 g l^{-1} NaCl, 0.54 g l^{-1} KCl, 0.60 g l^{-1} $CaCl_2$, 1 g l^{-1} $MgSO_4$, 3.9 g l^{-1} $MgCl_2$, 2 mmol l^{-1} glutamine (Sigma), 100 U ml^{-1} penicillin (Sigma), and 100 µg ml^{-1} streptomycin (Sigma). The different media used are summarized in Table 1. Hemolymph was seeded (0.2 ml; 5×10^4 cells) onto each of the 24-well plates with 0.5 ml BM-L15 and supplements

Table 1. Characteristics of different media for culturing primary hemocytes of *Chlamys farreri.* The composition of BM-L15 medium is given in 'Materials and methods'; FBS: fetal bovine serum, CFS: *C. farreri* serum. Cell confluence time is the elapsed time between seeding of primary hemocytes and formation of a monolayer. Living cells (% of total cells) were measured 24 h after seeding. Cell longevity is the elapsed time until ~50% of cells were still alive. '–' indicates primary hemocytes did not form cell confluence. Data are given as means ± SD

Group	Medium (v/v)	Cell confluence time (h)	Living cells (%)	Cell longevity (d)
1	BM-L15	–	60 ± 5	3 ± 2
2	BM-L15 + 2% FBS	72–96	73 ± 4	7 ± 3
3	BM-L15 + 5% FBS	48–60	86 ± 5	12 ± 3
4	BM-L15 + 10% FBS	60–72	85 ± 3	10 ± 3
5	BM-L15 + 5% FBS + 1% CFS	8–12	98 ± 1	48 ± 4
6	BM-L15 + 5% FBS + 2% CFS	8–12	97 ± 2	45 ± 3
7	BM-L15 + 5% FBS + 5% CFS	12–24	96 ± 1	40 ± 3

(Table 1), and 3 wells were employed for each group. The primary culture was conducted in an SHP-080 biochemistry incubator at 23°C. The cultured cells were observed and photographed daily using a TS100 inverted microscope (Nikon). The cultured cells were digested with 0.25% trypsase at primary culture for 24 h and counted using a hemocytometer after staining with Trypan blue (Cao et al. 2003) to determine the percentage of living cells (Table 1).

Primary culture of hemocytes

Hemolymph was collected from adductor muscle of *C. farreri* with a 1 ml syringe, and seeded (0.5 ml; 1.25×10^5 cells) onto a 60 mm diameter plastic Petri dish containing 0.5 ml CF-L15 medium which consisted of BM-L15 medium supplemented with 5% FBS and 1% CFS. The culture was conducted according to the method described above. The CF-L15 medium (pH 7.2–7.4) was refreshed at intervals of 2 to 3 d during the primary culture, and the cells were monitored and photographed daily using the TS100 inverted microscope.

Giemsa staining

Giemsa staining was employed to identify types of the cells according to their morphological characteristics. Hemocytes cultured for 3 d were fixed with 4% paraformaldehyde (Sigma) for 15 min and washed twice in phosphate-buffered saline (PBS, pH 7.4), and then stained with Giemsa stain solution (Sigma) for 20 min and rinsed with distilled water. After air-drying, the samples were examined and photographed with a Nikon E80i microscope. Six 400 × 400 μm^2 sized squares of the samples were randomly selected and observed at 800-fold magnification under the inverted microscope. Cells of different types were counted and their proportions were calculated.

Challenge of primary cultured hemocytes with *Vibrio anguillarum*

The Gram-negative bacterium *V. anguillarum* was grown in marine broth (2216E; tryptone 5 g l^{-1}, yeast extract 1 g l^{-1}, $C_6H_5Fe{\cdot}5H_2O$ 0.1 g l^{-1}, pH 7.6) at 28°C, and then centrifuged at 2000 × *g* (5 min) to harvest the bacteria when the OD_{600} was 0.2. The pellet was suspended in PBS and adjusted to 1×10^5 CFU ml^{-1}. The monolayer hemocytes were cultured in 60 mm diameter plastic Petri dishes with CF-L15 medium for 12 h. The CF-L15 medium (pH 7.2–7.4, without penicillin and streptomycin) was then refreshed after washing the monolayer hemocytes twice with PBS. Based on pre-experimental data, the hemocytes were challenged using *V. anguillarum* at a multiplicity of infection (MOI) of 0.16 (16 bacteria per 100 cells). Phagocytosis by hemocytes was monitored and photographed daily using the TS100 inverted microscope.

Challenge of primary cultured hemocytes with extracellular products (ECPs) of *V. anguillarum*

ECPs of *V. anguillarum* were produced using the cellophane overlay method as described by Liu et al. (1957). The supernatant containing the ECPs was filtered with a 0.22 µm membrane and stored at −80°C until use. The protein concentration of the ECPs was measured using Coomassie brilliant blue staining (Bradford 1976). Three ECP concentrations (2, 20, and 200 µg μl^{-1}) were used as treatment groups, and the negative control used the same volume of PBS without ECPs. Monolayer hemocytes cultured in 24-well plates with CF-L15 medium for 12 h were challenged using either the ECPs at different concentrations or the PBS. Three wells were used for each group. These cells were collected using 0.25% trypsin (Sigma) and centrifugation at 800 × *g* after being challenged for 3, 6, 12, 24, or 48 h, and then stored at −80°C for total RNA extraction. Cytopathic effects (CPEs) of ECPs on the hemocytes were observed and photographed using the TS100 inverted microscope during the experiment.

Challenge of primary cultured hemocytes with OsHV-1

OsHV-1 supernatant was prepared from OsHV-1 positive tissues of *Scapharca broughtonii* according to the method described by Schikorski et al. (2011). The number of viral DNA copies in the supernatant was examined by quantitative real-time PCR (qRT-PCR) according to the method described below. Based on pre-experimental data, monolayer hemocytes cultured in CF-L15 medium for 12 h were challenged with OsHV-1 at an MOI of 864 (864 viruses $cell^{-1}$). PBS and tissue extract of healthy *S. broughtoni* were used as negative controls. After being infected for 0.5 h, the hemocytes were washed twice in PBS and then cultured in 1 ml CF-L15

medium. CPEs were observed under the TS100 inverted microscope. To detect virus amplification, some of the hemocytes were collected at 0.5 and 24 h (when obvious CPE had occurred) after OsHV-1 infection, then stored at −80°C as per the method described above for DNA extraction. Other primary cultured hemocytes were collected and stored at −80°C for 3, 6, 12, 24, and 48 h after OsHV-1 infection for total RNA extraction. Others were fixed in 2.5% glutaraldehyde for transmission electron microscopy.

Transmission electron microscopy (TEM)

The samples fixed in 2.5% glutaraldehyde were transferred into 1% OsO_4 for 30 min, then dehydrated in a graded series of acetone (30, 50, 70, 80, 100%) and embedded in Epon-812. Ultrathin-sections were made with an LKB-8000 ultramicrotome and stained with lead acetate and post-stained with uranyl acetate before examination under TEM (H-7000, Hitachi).

DNA extraction and OsHV-1 quantification by qRT-PCR

Total DNA was extracted from the stored hemocytes described above using the phenol chloroform–sodium dodecyl sulfate method according to Molecular Cloning III (Sambrook 2001). qRT-PCR was carried out with the primers BF/B4 (BF, 5′-GTC GCA TCT TTG GAT TTA ACA A-3′; B4, 5′-ACT GGG ATC CGA CTG ACA AC-3′) and the TaqMan probe BP (BP, 6FAM-ATC GGG GGG GGG GGT TTT TTT TTT ATC G-BHQ1). The reaction mixture contained 1 µl of each primer (20 µM), 1 µl of TaqMan probe (10 µM), 7 µl of water, and 1 µl of DNA. The PCR reaction was performed using the method described by Martenot et al. (2010). Triplicate assays were conducted for each DNA sample. The number of OsHV-1 DNA copies was quantified according to the method of Le Deuff & Renault (1999) by comparing C_t (cycle threshold) values of each sample using the standard curve. The results are presented as the virus DNA copy number per total DNA of the control or treatment group.

RNA isolation and cDNA synthesis

Total RNA was extracted from the samples stored at −80°C using the thiocyanate–phenol–chloroform method according to Molecular Cloning III (Sambrook 2001), and treated with DNase I to remove genomic DNA. Quality and quantity of the RNA were measured by agarose gel electrophoresis and spectrophotometry at 260 nm. The RNA was reverse-transcribed to cDNA for qRT-PCR analysis according to the protocol of the Prime script cDNA Amplification Kit (Takara).

QM expression levels analyzed by qRT-PCR

QM (GenBank accession no. AAQ09228) is an immune-related gene which encodes a 60S ribosomal subunit protein. Expression levels of *QM* in the hemocytes after challenge by bacteria or viruses were analyzed through qRT-PCR, which was performed using SYBR Green Real-Time PCR Master Mix (Takara) and an ABI 7500 Real-Time PCR System (Applied Biosystems). Two *QM*-specific primers (q-QmF: 5′-GAG GAG ATG AGG CAG GAT GG-3′ and q-QmR: 5′-ACA GTC GCA AGG CAG TAA GA-3′) were designed to amplify a 109 bp fragment of *C. farreri QM* (*Cf-QM*) transcript (Chen et al. 2015). Two elongation factor 1a (*EF-1α*)-specific primers (*EF-1α*F: 5′-ATC CTT CCT CCA TCT CGT CCT-3′, *EF-1α*R: 5′-GGC ACA GTT CCA ATA CCT CCA-3′) were used for an 86 bp fragment of *C. farreri EF-1α* transcript (GenBank accession no. AEX08674.1) as a reference gene (Ma et al. 2017). Triplicate assays for each cDNA sample were conducted. Data were analyzed by the ABI 7500 system SDS software version 1.4 (Applied Biosystems) with an automatically set baseline and C_t values. Relative *Cf-QM* mRNA levels were calculated based on the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Unless noted otherwise, all data are presented as means ± SEM of 3 samples with 3 parallel repetitions. Differences between means were tested using 1-way analysis of variance followed by the least significant difference test with a significance level set at $p < 0.05$ by SPSS software version 17.0.

RESULTS

Optimal medium for culturing primary hemocytes

When seeded onto different media, *Chlamys farreri* hemocytes first clustered into cell masses of various sizes on the plate surface, and then formed

pseudopods and gradually migrated out of the cell masses. Finally, they spread to form cell monolayers (Fig. 1). However, confluence capability and survival time of the cultured hemocytes differed among various media (Table 1). BM-L15 medium could only maintain the survival of *C. farreri* hemocytes ephemerally, and these cells could not adhere well and form a monolayer. FBS improved the confluence and longevity of the hemocytes to some extent, and 5% FBS was more effective than the other 2 FBS groups. When CFS at different concentrations was added to the medium (BM-L15 with 5% FBS), cell survival rate was >96% when in primary culture for 24 h, and longevity of the primary cells was extended to more than 40 d. Based on these results, the best medium was identified as BM-L15 supplemented with 5% FBS and 1% CFS (Table 1). This medium was named CF-L15.

Types and morphological characteristics of primary cultured hemocytes

When *C. farreri* hemocytes were primary cultured in CF-L15 medium for 12 h, approximately 90 ± 2% (mean ± SD) of primary hemocytes were fibroblast-like (Fig. 1D) and nearly 10 ± 2% (mean ± SD) of cells were macrophage-like (Fig. 1D) under inverted microscopy. Giemsa staining showed that fibroblast-like cells contained 25 ± 3% (mean ± SD) granulocytes (Fig. 2B) and 75 ± 4% (mean ± SD) hyalinocytes (Fig. 2A).

Phagocytosis of *Vibrio anguillarum* by primary cultured hemocytes

Phagocytosis by the primary hemocytes could be observed using the inverted microscope (Fig. 3). *V. anguillarum* was first observed in the margin of primary cultured hemocytes after infection for 6 h (Fig. 3A,B). With extension of the challenge time, the number of bacteria phagocytosed by each hemocyte cell increased from 7 ± 2 (means ± SD) to 26 ± 5 (means ± SD) (n = 6), and these bacteria were located in the margin or center of hemocytes infected for 12 h (Fig. 3C,D).

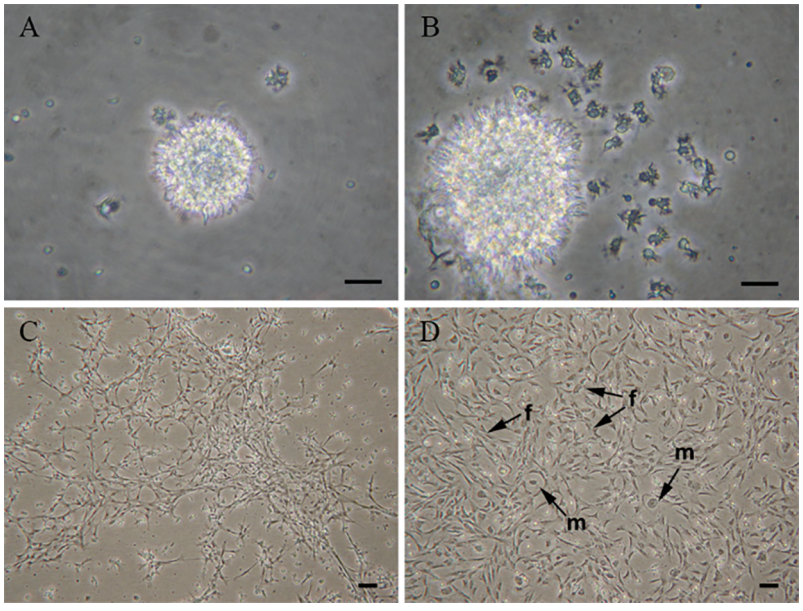

Fig. 1. Photomicrograph of *Chlamys farreri* primary hemocytes in CF-L15 medium at (A) 0 h, (B) 4 h, (C) 8 h, and (D) 12 h. f: fibroblast-like hemocytes; m: macrophage-like hemocytes. Scale bars = 20 µm

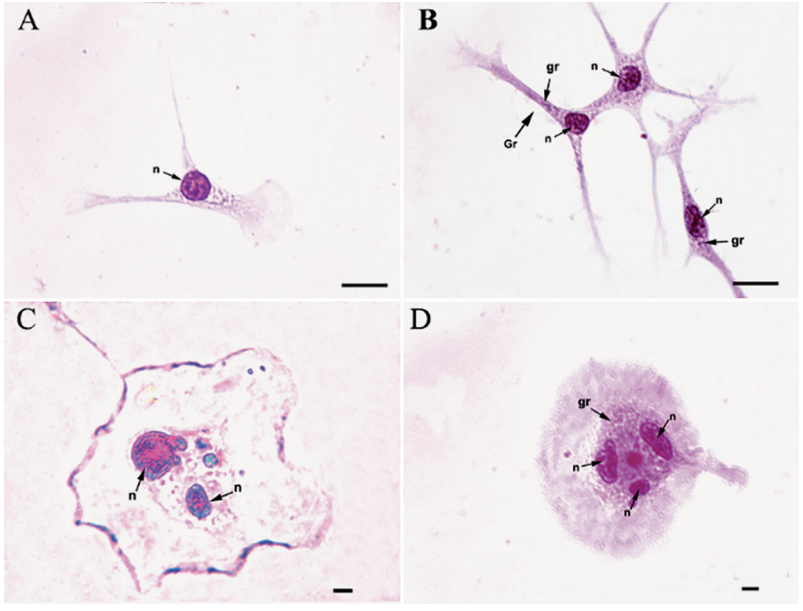

Fig. 2. Photomicrograph of *Chlamys farreri* hemocytes in CF-L15 medium (Giemsa stain). (A) Hyalinocyte; (B) granulocytes; (C,D) macrophage-like cells. gr: granules; n: nucleus. Scale bars = 10 µm

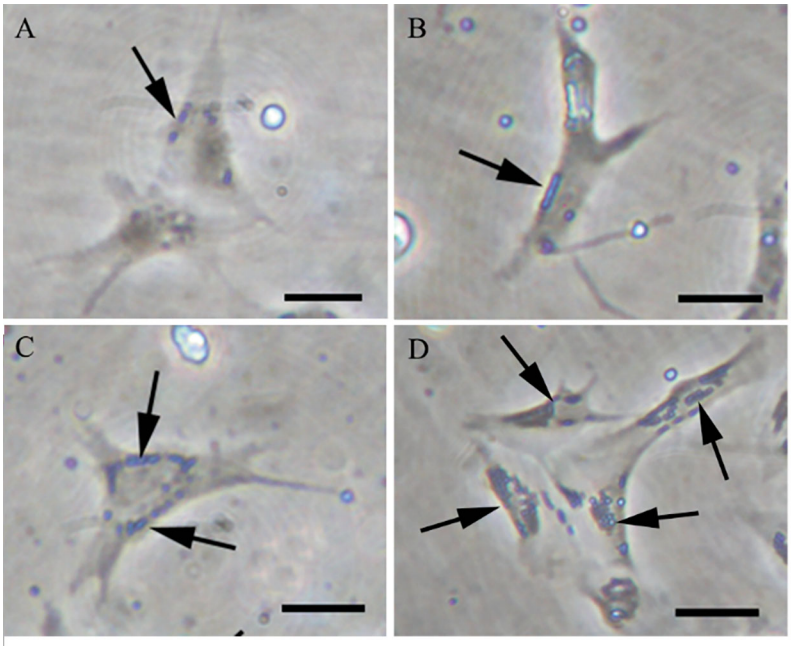

Fig. 3. Primary cultured *Chlamys farreri* hemocyte phagocytosing *Vibrio anguillarum*. Primary hemocytes infected for (A,B) 6 h and (C,D) 12 h. Black arrows show *V. anguillarum*. Scale bars = 20 µm

CPEs of *V. anguillarum* ECPs on primary cultured hemocytes

When *C. farreri* primary hemocytes were challenged using ECPs of *V. anguillarum* at concentrations of 2 and 20 µg µl^{-1}, no obvious cytotoxicity was presented (Fig. 4B,C). However, hemocyte cytotoxicity was observed at 200 µg µl^{-1} ECPs after the hemocytes were challenged for 12 h. Under inverted microscopy, the morphology of the challenged hemocytes changed from fibroblast-like into rounded and shrunken cells (Fig. 4D). Moreover, these cells gradually lost their adhesion properties, and ultimately the cell monolayer was destroyed (Fig. 4D).

Infection and replication of OsHV-1 in primary hemocytes

The number of viral DNA copies was estimated to be approximately 5.4 × 10^5 in 1 µl of OsHV-1 supernatant by qRT-PCR. After the primary cultured hemocytes were infected with OsHV-1 for 24 h, apparent CPE was observed in the cells (Fig. 5E). These infected cells initially exhibited shrinkage or aggregated, and then most of the infected cells became more rounded at 48 h post incubation (Fig. 5F). Finally, the cell monolayer completely detached from the culture dishes or broke up at 5 d post incubation. In contrast, no obvious CPE was observed in cells that were inoculated with the same volume of PBS and tissue extract of healthy *Scapharca broughtonii* (Fig. 5A–D).

qRT-PCR revealed that the number of OsHV-1 DNA copies in the primary hemocytes infected for 24 h was significantly higher (by ca. 7-fold) than that at 0.5 h post infection (Fig. 6). Meanwhile, no OsHV-1 DNA was detected in the hemocytes of the PBS group or in tissue extract of the healthy *S. broughtonii* group. Furthermore, TEM revealed OsHV-1 particles in the nucleus of primary cultured hemocytes infected for 48 h (Fig. 7A). In the infected primary cells, the karyotheca of some nuclei were incomplete (Fig. 7A) or were in karyolysis. Intra-nuclear empty capsids and nucleocapsids were observed in primary cultured hemocytes (Fig. 7B). The nucleocapsids which contained an electron-

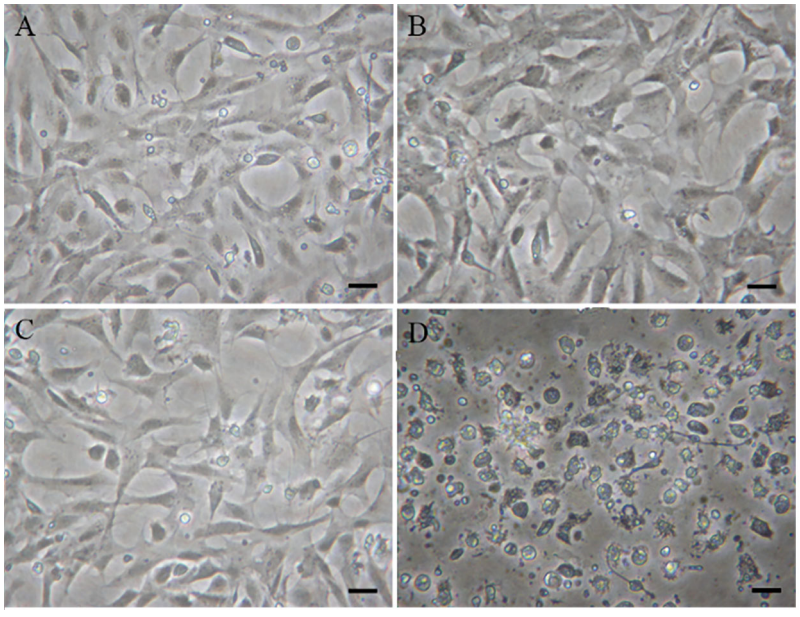

Fig. 4. Morphologic features of primary cultured *Chlamys farreri* hemocytes after challenge with extracellular products (ECPs) of *Vibrio anguillarum* for 12 h at different concentrations. (A) Phosphate-buffered saline (control), and ECPs at (B) 2 µg µl^{-1}, (C) 20 µg µl^{-1}, and (D) 200 µg µl^{-1}. Scale bars = 20 µm

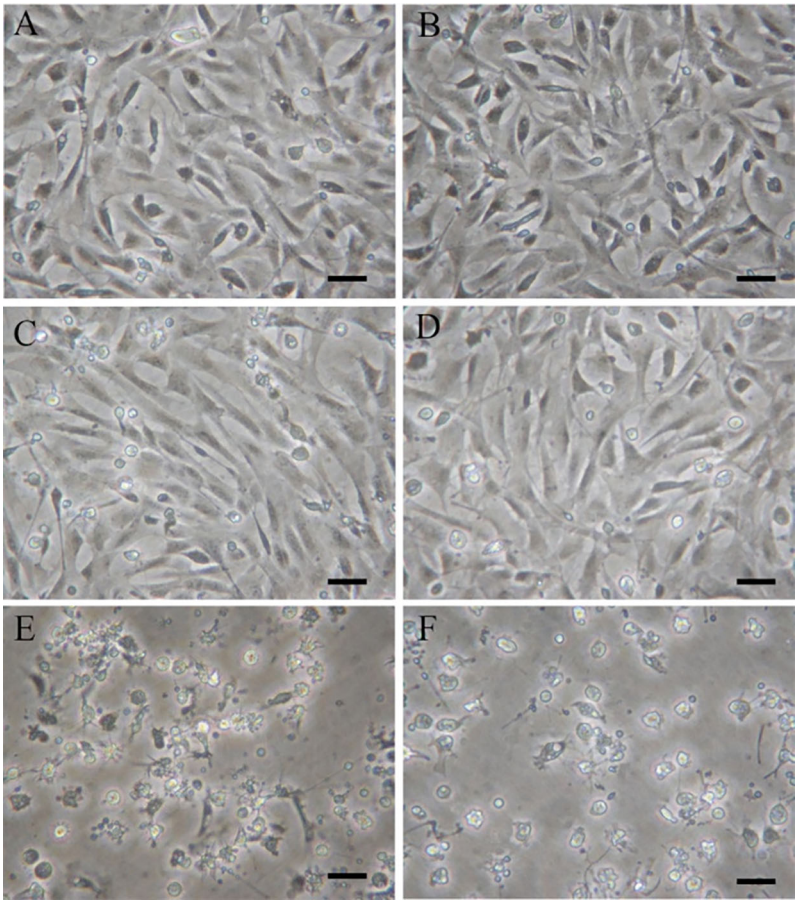

Fig. 5. Morphologic features of *Chlamys farreri* hemocytes challenged with OsHV-1 virus. (A) Phosphate-buffered saline (PBS), 24 h; (B) PBS, 48 h; (C) tissue extract of healthy *Scapharca broughtonii*, 24 h; (D) tissue extract of healthy *S. broughtonii*, 48 h; (E) OsHV-1, 24 h; (F) OsHV-1, 48 h. Scale bars = 20 µm

dense core were polygonal in shape and 130–150 nm in diameter (Fig. 7B).

Expression of *Cf-QM* in primary hemocytes challenged by bacteria and viruses

The level of *Cf-QM* mRNA showed a slight increase in primary hemocytes before the challenge with ECPs of *V. anguillarum* for 6 h, while a significant increase of *Cf-QM* mRNA level occurred at 12 h, which was about 2.5-fold higher than that of the control. After that, it decreased significantly to the level of the control (Fig. 8).

The *Cf-QM* expression level also increased significantly in primary hemocytes challenged with OsHV-1 viruses for 12 h, to about 2-fold higher than that of the control at the same time. It then reached a peak at

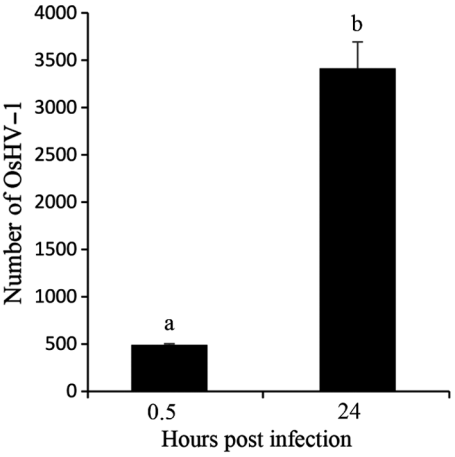

Fig. 6. Number of virus particles in primary *Chlamys farreri* hemocytes after infection with OsHV-1 virus detected by qRT-PCR. Data are means ± SEM. different letters indicate significant differences ($p < 0.05$)

24 h, at 4-fold higher than that of the control, and then decreased significantly to the level of the control at 48 h (Fig. 9).

DISCUSSION

Stable primary hemocytes of the scallop *Chlamys farreri*

In this study, we established a stable primary culture system for *C. farreri* hemocytes which adhered rapidly and formed cell monolayers on the plastic Petri dishes, and were maintained for more than 40 d. In this system,

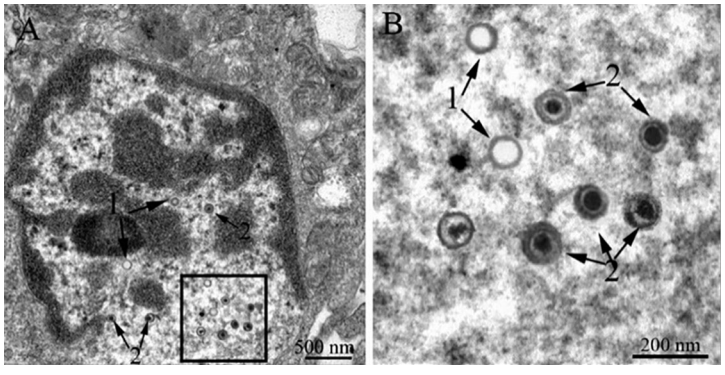

Fig. 7. Transmission electron micrographs of primary *Chlamys farreri* hemocytes 48 h after infection with OsHV-1 virus. (A) Primary hemocyte infected with OsHV-1 in its nucleus (n). (B) Higher magnification of the box in Panel A; 1: empty capsids of the OsHV-1 virus; 2: OsHV-1 virus particles

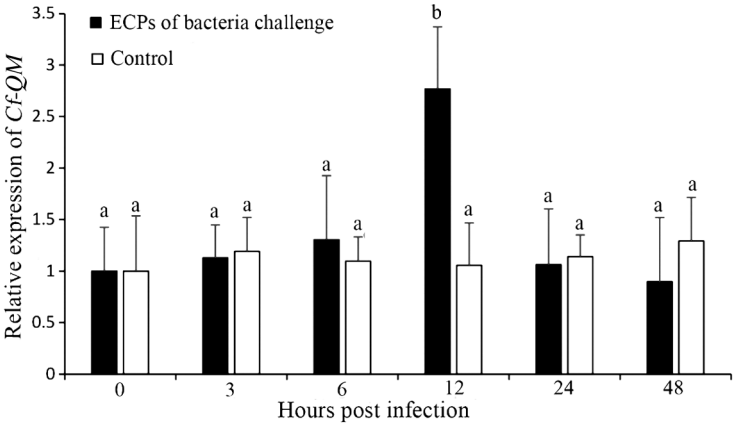

Fig. 8. Relative expression of the *Cf-QM* gene in *Chlamys farreri* hemocytes challenged by extracellular products (ECPs) of *Vibrio anguillarum*. The level of *Cf-QM* mRNA in primary hemocytes of the control group at 0 h was set as 1.00 to calibrate the relative levels. Data are means ± SEM; different letters above bars indicate significant differences ($p < 0.05$)

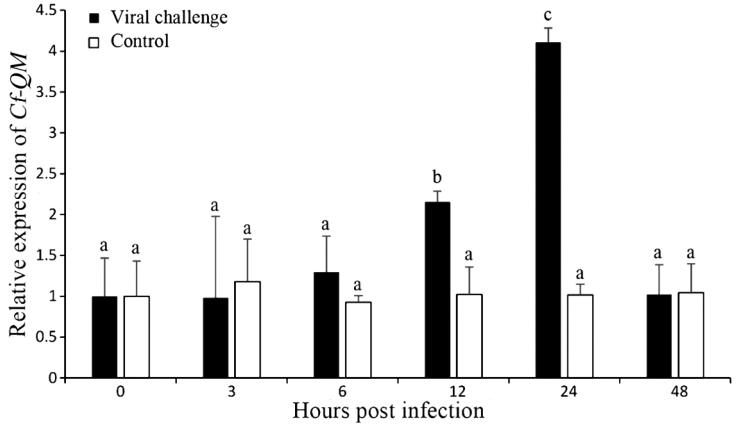

Fig. 9. Relative expression of the *Cf-QM* gene in *Chlamys farreri* primary hemocytes challenged with OsHV-1 virus. The level of *Cf-QM* mRNA in primary hemocytes of the control group at 0 h was set as 1.00 to calibrate the relative levels. Data are means ± SEM; different letters above bars indicate significant differences ($p < 0.05$)

FBS and CFS were important factors in improving the confluence ability and longevity of the primary hemocytes. The optimal concentrations were 5% FBS and 1% CFS in BM-L15 medium. In previous studies, hemocytes of the abalone *Haliotis tuberculata* were primary cultured for 7–10 d in Hanks' 199 sterile medium (Lebel et al. 1996, Serpentini et al. 2000, Farcy et al. 2007, Latire et al. 2012), oyster *Crassostrea gigas* hemocytes were maintained for 7 d in L-15 medium supplemented with 10% FBS, and mussel *Mytilus galloprovincialis* hemocytes were maintained for 20 d in L-15 medium supplemented with inactivated hemolymph (Domart-Coulon et al. 1994, Cao et al. 2003). Therefore, the concentration of FBS and the activity of hemolymph seem to have an obvious impact on the survival of primary hemocytes. Furthermore, 3 types of *C. farreri* hemocytes, i.e. hyalinocytes, granulocytes, and macrophage-like cells, were identified in this primary culture, which is similar to that reported by Sun & Li (2001), indicating that characteristics of the hemocytes are retained.

Primary cultured hemocytes to study host–pathogen interactions and immune-related genes

Over the past several decades, pathogen infections have occurred frequently, causing mass mortalities of *C. farreri* and seriously affecting the development of the scallop aquaculture industry in China. Therefore, an in-depth understanding of the pathogenic mechanisms involving in host–pathogen interactions and immune responses is crucial for sustainable aquaculture (Wang et al. 2012a). To date, many researchers have focused on studying the pathogenicity of bacteria and viruses to *C. farreri in viv*o (Wang et al. 2004, Zhang et al. 2006, Sun et al. 2014, Chen et al. 2015). However, *in vivo* experiments retain some deficiencies, including a long experimental period and high cost. In this study, an *in vitro* approach was shown to be useful for studying host–pathogen interactions in *C. farreri*. We challenged the primary cultured hemocytes with *Vibrio anguillarum* and observed the process of the bacteria being phagocytosed by hemocytes. Moreover, cytoxic effects of *V. anguillarum* ECPs appeared on the primary hemocytes of *C. farreri* (Figs. 4 & 8), which is similar to that of *V. aestuarianus* in the hemocytes of the oyster *C. gigas* on cellular and molecular levels *in vivo* (Labreuche et al. 2006). Furthermore, we revealed that the primary cultured hemocytes of *C. farreri* were highly susceptible to OsHV-1 viruses from *Scapharca broughtonii*, in which the typical CPE appeared and virus replication took place (Figs. 5–7 & 9). These data indicated that the primary hemocytes of *C. farreri* can be used to study pathogenic mechanisms, such as the processes of bacterial infection, isolation and propagation of viruses, and identification and function of immune-related genes in shellfish.

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