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Full Length Article

Flow Cytometric Analysis of the Defense Functions of Hemocytes from Oyster (*Crassostrea ariakensis*)

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Abstract

Morphology and defense function of the hemocytes from oyster (*Crassostrea ariakensis*) were analyzed using flow cytometry (FCM) method. Two sub-populations of hemocytes (granulocytes and hyalinocytes) were identified by FCM and subsequently confirmed by light and electron microscopy observation. FCM analysis of cell viability for *C. ariakensis* hemocytes based on propidium iodide staining was carried out. The mortalities of the hemocyte collected in pH 7.0 acidic Alsever solution (AS), pH 3.5 AS or 4% formaldehyde were 5.5780 ± 1.0117, 22.5250 ± 6.4263, 91.6020 ± 1.3754%, respectively. FCM was also used to evaluate active phagocytosis of the oyster hemocytes according to fluorescence level of engulfed yellow-green fluorescent latex beads. The average percentage of phagocytic hemocytes was 24.6290 ± 2.0608%, significantly higher than that treated with sodium azide which was 8.5020 ± 1.4078%. Respiratory burst was measured using FCM at 530 nm with 2', 7'-dichlorofluorescein diacetate staining. The dichlorofluorescein mean fluorescence level in the oyster hemocytes stimulated with phorbol-12-myristate-13-acetate (PMA) was higher than non-stimulated cells. Respiratory burst activity of the hemocyte samples pre-incubated with calmodulin antagonist W-13 was significantly lower than the cells only stimulated with PMA. Respiratory burst activity did not decrease significantly when the hemocyte samples were pre-incubated with N_G-methyl-L-arginine. In this study, more research methods and data associated with the oyster hemocytes base on FCM analysis were provided, and they contributed to the further study on their defense functions and mechanism. © 2018 Friends Science Publishers

Keywords: Flow cytometry; *Crassostrea ariakensis*; Hemocyte; Cell viability; Phagocytosis; Respiratory burst

Introduction

Bivalve mollusks have no specific immune response based on the antibody. They mainly rely on the hemocytes responsible for the innate immune response, such as phagocytosis, respiratory burst (RB) activity subsequent generation of reactive oxygen intermediates, and production of antimicrobial peptides. Since the bivalve hemocytes play the important role in the internal defense, many studies on their morphology, classification, and function have ever been carried out. Conventional cytological and biochemical techniques were used for most of these previous studies. Since they were usually combined with the light microscopy as an analytical method, the subjective and biased data for the studies were sometimes obtained.

Flow cytometry (FCM) is a powerful tool for measuring certain physical and chemical characteristics of cells or particles. These measurements are made as cells are interrogated individually. FCM allows for multiparametric readouts at the single cell level within heterogeneous cell populations in suspension. And this method avoids the subjective opinions and bias that occur when researchers use the light microscopy as an analytical method.

The oyster *Crassostrea ariakensis* Gould, 1861 is one of the most economically important cultured bivalve species in China (Wu and Pan, 2000). In recent years, mass mortality in *C. ariakensis* has been emerging and caused great economic losses due to the infection of a rickettsia-like organism (RLO) (Sun and Wu, 2004; Xu *et al*., 2012). A better and further understanding of the innate immune response based on the oyster hemocytes may contribute to controlling RLOs and avoiding mass mortality. However, only few studies were conducted associated with the morphology and classification of the hemocytes from *C. ariakensis* (Sun *et al*., 2006), and there was still deficient information about the defense functions of the oyster hemocytes. Here we reported classification and further investigated the defense functions including viability, phagocytosis, and RB acitivity of the hemocytes from *C. ariakensis* using FCM method.

Materials and Methods

Oyster Hemolymph Collection

Healthy oysters, *C. ariakensis*, with the length of 6.5–9.9

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cm, width of 5.3–6.8 cm and height of 8.0–13.0 cm, were obtained from Leizhou bay (Guangdong, China). Five hundred microliters of hemolymph were collected from each oyster and directly transferred into 0.5 mL Alsever solution (AS, 27 m*M* Na citrate, 336 m*M* NaCl, 115 m*M* glucose, 9 m*M* EDTA, pH 7.0). The suspensions were centrifuged at 800 g for 10 min at 4℃, and then the sediments were suspended in the sterile phosphate-buffered saline (PBS).

Hemocyte Types

The hemocyte suspensions were analyzed with a BD FACSCalibur flow cytometer and at least 10000 events were counted for each hemocyte sample. Two parameters, forward scatter height (FSC) and size scatter height (SSC), were used to analyze hemocyte types. FSC value is a measure of relative cell size. SSC value reflects the extent of the inner complexity of the cell. FCM dot plot and density curve cytograms of FSC against SSC were displayed for the results. For the confirmation of the hemocyte subpopulation identified using FCM, the hemocyte smears were observed with light microscopy after stained with Hemacolor kit (Merck, Darmstadt, Germany). For the transmission electron microscopy study, ultrathin sections of the oyster hemocytes were done according to the method of Sun *et al.* (2006). The utrastructure of the oyster hemocytes was observed under a JEM-100CXII transmission electron microscope (TEM, Jeol, Japan).

Cell viability estimation

Thirty oysters were used to study the viability of hemocytes using FCM method. Hemocyte samples from each of ten oysters were collected in physiologically neutral AS of pH 7.0, those of another ten oysters in acidic AS of pH 3.5. Hemocyte samples from each of the rest ten oysters collected in 4% formaldehyde solution were used as the control. Propidium iodide (PI, Sigma, St. Louis, MO, USA) was added to the hemocyte samples at a final concentration of 10 μg/mL, and the hemocytes suspensions were immediately analyzed using FCM. PI red fluorescence of each of the hemocyte samples was detected at FL-2 channel. Dead cells presented stronger red fluorescence than alive cells.

Phagocytosis

Five hundreds of microlitre of the hemolymph sample from each of fifteen oysters was collected. The hemocytes were suspended in PBS at a concentration of 10^6 cells/mL. The yellow-green fluorescent latex beads (Molecular Probes, Eugene, OR, USA) with the diameter of 1.0 μm were used for phagocytosis assay. The hemocyte suspensions were incubated with beads for 14 h at 16℃ (Xue *et al*., 2001) and the bead/hemocyte ratio was about 30:1. The fluorescent intensity associated with the cells was analyzed with FCM. The hemocyte sub-samples as control were pre-incubated with 0.1% sodium azide for 30 min prior to incubation with the fluorescent beads. Data were presented as histograms of cell numbers versus fluorescence level recorded with 10,000 cells per sample. For phagocytosis experiments, the fluorescence of hemocytes containing beads was detected at FL-1 channel. Phagocytic activity was measured and analyzed according to the fluorescence level using the Cell Quest software (BD, San Jose, CA, USA). Cells with fluorescence level higher than one bead were included for the phagocytosis evaluation.

RB activity

The fluorescence dye, 2', 7'- dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) was used as indicator to evaluate the RB activity of the oyster hemocytes. DCFH-DA infiltrates into the cells and is rapidly oxidized to highly florescent dichlorofluorescein (DCF) in cell cytoplasms. The mean fluorescence intensity of DCF represented the RB activity of the oyster hemocytes. The hemocytes were suspended in ice cold PBS at a concentration of 10^6 cells/mL and were incubated with DCFH-DA (5 m*M*) at 0℃ for 30 min (Goedken and Guise, 2004; Agrawal *et al*., 2011). The hemocytes were washed, and then incubated with phorbol-12-myristate-13-acetate (PMA, Sigma) at final concentration of 33.2 n*M* for 60 min at 0℃. The fluorescence level of the hemocyte samples indicated the RB activity and was recorded with the flow cytometer at 530 nm.

In order to investigate which kind of reactive oxygen species mediated the oxidation of DCFH to DCF, NG-methyl-L-arginine (NMMA, Sigma) or W-13 (Sigma) with a final concentration of 100 m*M* were added to the hemocyte sub-samples, and then incubated for 30 min at 0℃ prior to incubation with PMA. NMMA inhibits the H_2O_2 production, and W-13 inhibits NO production (Rao *et al*., 1992). For all the RB activity assays, the hemocyte sub-samples without PMA were used as control.

Statistical Analyses

The SPSS 16 system for Windows was used for statistical analysis. One-way ANOVA with Student-Newman-Keuls test was used to analyze the differences for the data in the cell viability assay and differences for the RB activities of the hemocyes stimulated with PMA, pre-incubated with NMMA or W-13, and controls. A paired *t*-test was used to analyze the differences for the phagocytosis between the oyster hemocyte sub-samples with and without sodium azide. Values were showed as mean ± S.E. and differences were considered statistically significant at $p < 0.05$ level.

Results

Hemocyte Types

Two distinct hemocyte sub-populations were identified by FCM. One sub-population with the higher SSC and FSC values was entitled granulocytes, and another with the lower SSC and FSC values hyalinocytes (Fig. 1). The percentages (n=11) of hyalinocytes and granulocytes were evaluated using Cell Quest software as 38.3491± 2.0001 and 59.1318 ±1.9456%, respectively.

Light microscopy observation confirmed the two types of hemocytes. Granulocytes had plenty of cytoplasm and larger than hyalinocytes. Hyalinocytes had thin cytoplasm and were spherical in shape and usually smaller than granulocytes (Fig. 2). Ultratructure examination of hemocyte types was carried out by electron microscopy (Fig. 3). The granulocytes (Fig. 3A) showed abundant cytoplasm which contained a variable number of mitochondria, Golgi complex, endoplasmic reticulum, abundant electron-dense particles and electron-lucent granules. The pseudopodia sprouting off the granulocyte surface was usually observed. Their nuclei often were in one end of the cells. The hyalinocytes (Fig. 3B) had thin cytoplasms and presented a total absence of cytoplasmic granules or a few small electron-lucid vesicles. Their nuclei were in a central position of the cell and round or oval in shape. The hyalinocytes sometimes presented fewer pseudopodia on their surfaces.

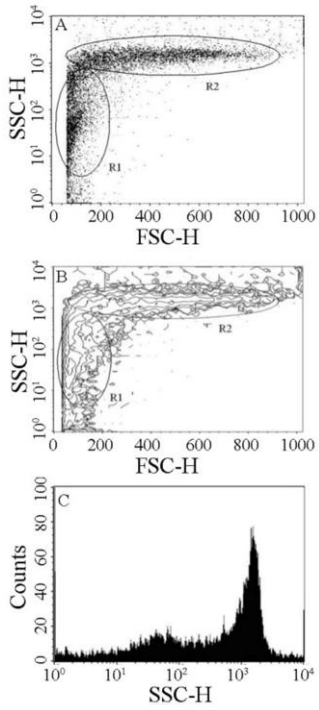

Fig. 1: Two distinct hemocyte sub-populations (Regions R1 and R2) from *Crassostrea ariakensis* were identified on the flow cytometric diagrams. A. dot plot graph; B. density contour plot graph; C. sides scatter parameter graph

Hemocyte Viability

The hemocytes with high (10^2 to 10^4, FL-2) and low fluorescence (10^0 to 10^2, FL-2) were respectively used to determine those of dead and live cells (Fig. 4). The mortalities of the hemocyte in pH 7.0 AS, pH 3.5 AS or 4% formaldehyde were 5.5780 ± 1.0117, 22.5250 ± 6.4263, 91.6020 ± 1.3754%, respectively.

Phagocytic Activity

Phagocytic activity was analyzed using FCM for the oyster hemocytes. Only cells that had phagocytized beads were contained in the fluorescence distribution and were easily distinguished from non-phagocytic hemocytes with basal fluorescence (Fig. 5). Peaks 1 through 4 observed from the flow cytogram showed the number of oyster hemocytes containing one, two, three, or four beads, respectively. The percentage of cells with beads measured on 15 normal oysters was 24.6290 ± 2.0608%. And the percentages of the phagocytic hemocytes containing one , two, three, four, and more than four beads were respectively 10.08 ± 1.55, 6.53 ± 0.83, 4.07 ± 0.46, 3.04 ± 0.33, 3.71 ± 0.80%. The fluorescence level of the phagocytic cells decreased after the hemocyte samples were treated with sodium azide (Fig. 6). FCM data showed 8.5020 ± 1.4078% of the oyster hemocytes contained beads when exposed to sodium azide.

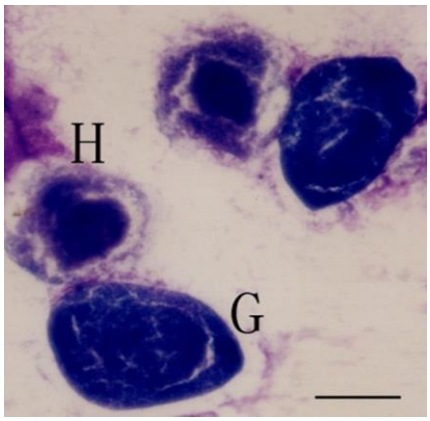

Fig. 2: Hemocytes of *Crassostrea ariakensis* stained with the Hemacolor kit. G, granulocyte; H, hyalinocyte. Bar, 6.5 μm

And the phagocytosis percentages were significantly different between the oyster hemocytes exposed to and not to sodium azide.

RB Activity

The stimulation of the hemocytes with PMA resulted in

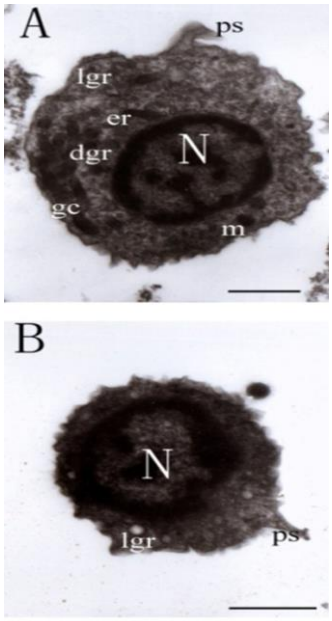

Fig. 3: Electron microscopy observation of the hemocytes from *Crassostrea ariakensis*. The granulocytes (A) showed abundant cytoplasm. The pseudopodia sprouting off cell surface was usually observed. The hyalinocytes (B) had thin cytoplasms and a few small electron-lucid vesicles. Sometimes the pseudopodia can be observed on their surfaces. N, nucleus; dgr, electron-dense granules; lgr, electron-lucent granules; er, endoplasmic reticulum; ps, pseudopodia; gc, Golgi complex; m, mitochondrion. Bar, 1.5 μm

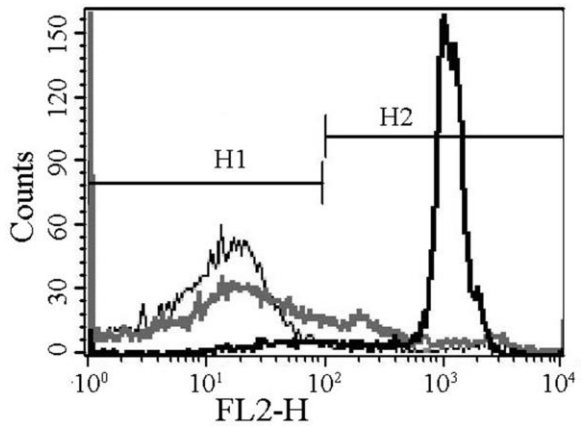

Fig. 4: Flow cytometric cytoplasm representing the oyster hemocyte viability. Black thin line, hemocytes in pH 7.0 Alsever solution; grey thick line, hemocytes in pH 3.5 Alsever solution; black thick line, hemocytes in 4% formaldehyde. H1, hemocytes with low fluorescence; H2, hemocyte with high fluorescence

the occurrence of RB. The increase of the DCF fluorescence represented the RB activity. DCF mean fluorescence level in the oyster hemocytes stimulated

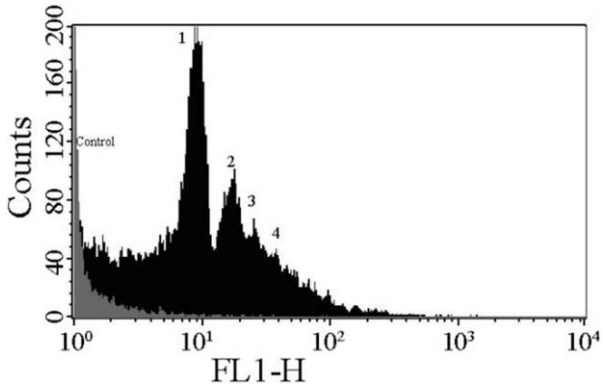

Fig. 5: Fluorescence histograms representative of phagocytosis activity of the oyster hemocytes. The basal fluorescence of hemocytes without beads (grey) was easily distinguished from the hemocytes containing beads (black). Peaks 1, 2, 3 and 4 were respectively corresponding to the number of hemocytes containing one, two, three, or four fluorescent beads

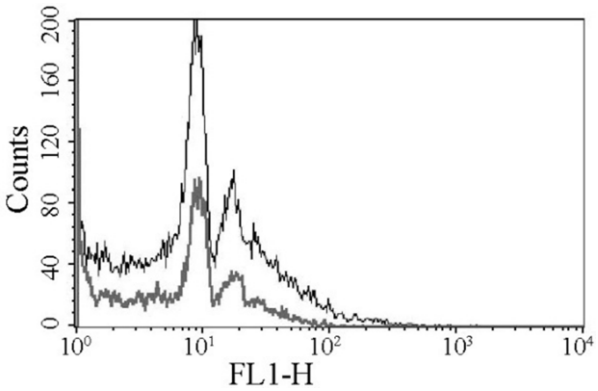

Fig. 6: The fluorescence level of the phagocytic cells decreased after the hemocytes were treated with sodium azide. Grey black curves represented the hemocytes pre-incubation with sodium azide; Black curves represented the hemocytes without sodium azide

with PMA was higher than non-stimulated cells (Fig. 7). RB activity of the hemocyte samples pre-incubated with W-13 was significantly lower than the cells only stimulated with PMA. RB activity did not decrease significantly when the hemocyte samples were pre-incubated with NMMA (Fig. 8).

Discussion

The classification schedules for the bivalve hemocytes are inconsistent, dependent on the observer and the technique used (Cima *et al*., 2000; Chang *et al*., 2005). In this study, two main hemocyte types in *C. ariakensis*, granulocytes and hyalinocytes, were identified with FCM method and then confirmed with light and electronic microscopy observation. These results were in agreement with the point of Cheng

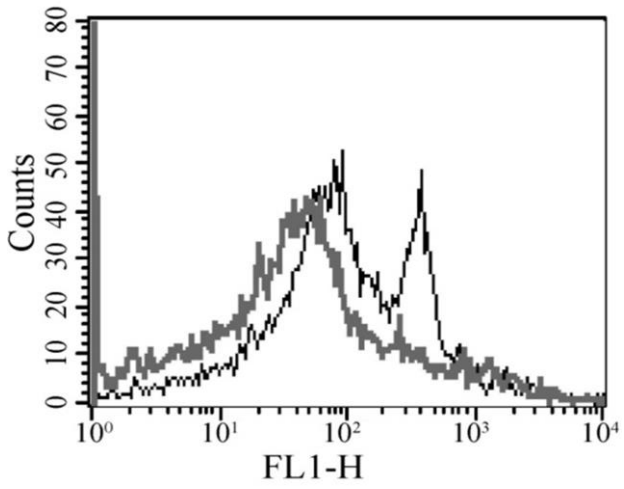

Fig. 7: Mean fluorescence level of dichlorofluorescein in the oyster hemocytes stimulated with phorbol-12-myristate-13-acetate (black curve) was higher than non-stimulated cells (grey curve)

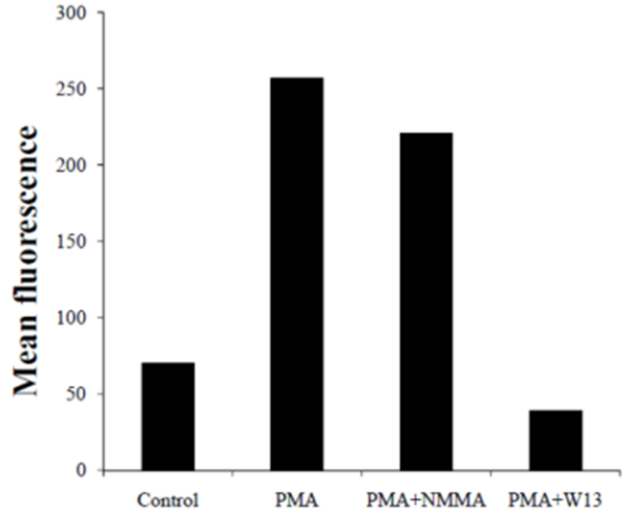

Fig. 8: Respiratory burst activity of the hemocyte samples pre-incubated with W-13, was significantly lower than the cells only stimulated with phorbol-12-myristate-13-acetate (PMA). While, NG-methyl-L-arginine (NMMA) was not

(1981) and Hine (1999) with respect to the classification scheme for the hemocytes of the whole Mollusca phylum.

The abundance of the two hemocyte sub-populations of *C. ariakensis* obtained from FCM analysis in the present study showed that granulocytes were more than the hyalinocytes, and this finding was agreement with the research in *C. ariakensis* using light microscopy (Sun *et al*., 2006). Similar results were obtained for other mollusks, such as *Scrobicularia plana* (Wootton and Pipe, 2003), *Pinctada imbricate* (Kuchel *et al*., 2010). However, some reported the hyalinocytes were the predominant cell type in *C. virginica* (Hégaret *et al*., 2003) and *Patinopecten yessoensis* (Xu *et al*., 2006). This inconsistency for the abundance of the hemocyte sub-populations probably resulted from discrepant classification standard used and the different life stages of mollusks.

PI label was used to evaluate the hemocyte viability in the present study. The high mortality rates of the hemocyte in non-physiological buffer confirmed the reliability in determining cell viability using FCM for *C. ariakensis* hemocytes.

Previous phagocytic assays in the invertebrate hemocytes have applied multiple research methods such as radiolabelling (Chu, 1988), light and electron microscopy (Sun *et al*., 2006; Kuchel *et al*., 2010) and FCM (Tu *et al*., 2007). Radiolabelling achieves information at the population level and has the inconvenience of radioactivity. Microscopic techniques just deal with examination of small numbers of cells. Compared with the conventional techniques, FCM has many advantages for the measurement of phagocytosis. And it has been used to accurately measure the phagocytosis in the invertebrate hemocytes such as *C. virginica* (Goedken and De Guise, 2004), *Penaeus vannamei* (Sun *et al*., 2010) and *Pomacea canaliculata* (Cueto *et al*., 2015). In the present study, FCM was used to evaluate phagocytosis of *C. ariakensis* hemocytes. The cells that had fluorescence greater than that of one bead were considered involved in phagocytosis. Since sodium azide inhibits cell ATP synthesis, therefore, inhibiting its phagocytosis (Fang *et al*., 2012), the finding of the decrease of the fluorescence level of cells pre-incubated with sodium azide was indicative that the flow cytometric assay for phagocytosis was proved to be valid and specific.

In the process of RB, the invertebrate hemocytes generated microbicidal reactive oxygen intermediates (ROIs), such as superoxide anion (O_2^-), which is spontaneously dismutated to microbicidally active H_2O_2. Some methods of measurements for RB activity were previously used, including chemiluminescence assay, nitroblue tetrazolium (NBT) reduction assay.

In this study, FCM was used to detect H_2O_2, which presented RB activity. NMMA inhibits production of NO, while W-13 inhibits O_2^-, and subsequent H_2O_2 generation (Goedken and De Guise, 2004). The findings that W-13 significantly suppressed the RB and NMMA failed to inhibit RB implied that the oyster hemocyte RB was associated with H_2O_2. This was in agreement with the results in the *C. virginica* hemocyte (Goedken and De Guise, 2004). Herein, PMA was chosen to elicit the RB activity due to its ability of binding directly to protein Kinase C (Zhou *et al*., 2016). And the low incubation temperature (0^oC) avoided the spontaneous activation of the cells.

The FCM provided more research methods and data associated with *C. ariakensis* hemocytes, and proved to be an effective tool for studying some characteristics and defense functions of the oyster hemocytes.

Acknowledgments

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References

Agrawal, M., V. Kumar, M.P. Kashyap, V.K. Khanna, G.S. Randhawa and A.B. Pantbc, 2011. Ischemic insult induced apoptotic changes in pc12 cells: protection by trans resveratrol. *Eur. J. Pharmacol.*, 666: 5–11

Chang, S.J., S.M. Tseng and H.Y. Chou, 2005. Morphological characterization via light and electron microscopy of the hemocytes of two cultured bivalves: a comparison study between the hard clam (*Mretrix lusoria*) and pacific oyster (*Cassostrea gigas*). *Zool. Stud.*, 44: 144–152

Cheng, T.C., 1981. Bivalves. *In: Invertebrate Blood Cells*, pp: 233–300. N.A. Ratcliffe and A.F. Rowley (eds.), Academic Press, New York, USA

Ratcliffe, N.A. and A.F. Rowley (eds.). Academic Press, London

Chu, F.L., 1988. Development and evaluation of techniques to study acquired immunity to *Perkinsus marinus* in the oyster *Crassostrea virginica*. *J. Shellf. Res.*, 7: 51–55

Cima, F., V. Matozzo, M.G. Marin and L. Ballarin, 2000. Haemocytes of the clam *Tapes philippinarum* (Adams and Reeve, 1850): morphofunctional characterisation. *Fish Shellf. Immun.*, 10: 677–693

Cueto, J.A., C. Rodriguez, I.A. Vega and A. Castrovazquez, 2015. Immune defenses of the invasive apple snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae): phagocytic hemocytes in the circulation and the kidney. *PLoS One*, 10: e0123964

Fang, C., D. Bourdette and G. Banker, 2012. Oxidative stress inhibits axonal transport: implications for neurodegenerative diseases. *Mol. Neurodegener.*, 7: 1–13

Goedken, M. and S.D. Guise, 2004. Flow cytometry as a tool to quantify oyster defence mechanisms. *Fish Shellf. Immun.*, 16: 539–552

Hégaret, H., G.H. Wikfors and P. Soudant, 2003. Flow-cytometric analysis of haemocytes from eastern oysters, C*rassostrea virginica*, subjected to a sudden temperature elevation. I. Haemocyte types and morphology. *J. Exp. Mar. Biol. Ecol.*, 293: 237–248

Hine, P.M., 1999. The inter-relationships of bivalve haemocytes. *Fish Shellf. Immun.*, 9: 367–385

Kuchel, R.P., D.A. Raftos, D. Birch and N. Vella, 2010. Haemocyte morphology and function in the Akoya pearl oyster, *Pinctada imbricata*. *J. Invertebr. Pathol.*, 105: 36–48

Rao, K.M., J. Padmanabhan, D.L. Kilby, H.J. Cohen, M.S. Currie and J.B. Weinberg, 1992. Flow cytometric analysis of nitric oxide production in human neutrophils using dichlorofluorescein diacetate in the presence of a calmodulin inhibitor. *J. Leukocyte Biol.*, 51: 496–500

Sun, J.F. and X.Z. Wu, 2004. Histology, ultrastructure, and morphogenesis of a rickettsia-like organism causing disease in the oyster, *Crassostrea ariakensis* Gould. *J. Invertebr. Pathol.*, 86: 77–86

Sun, J.F., A.L. Wang and T.J. Zhang, 2010. Flow cytometric analysis of defense functions of hemocytes from the penaeid shrimp, *Penaeus vannamei*. *J. World Aquac. Soc.*, 41: 92–105

Sun, J.F., X.Z. Wu and W.Z. Zhang, 2006. Morphological, structural and functional characteristics of the hemocytes of the oyster, *Crassostrea ariakensis*. *J. Shellf. Res.*, 25: 55–64

Tu, C.Y., S.W. Hung, L.T. Tsou, Y.C. Chang and W.S. Wang, 2007. Simultaneous flow cytometric assessment for cellular types and phagocytic abilities of the haemocytes of the hard clam, *Meretrix lusoria*. *Fish Shellf. Immunol.*, 23: 16–23

Wootton, E.C. and R.K. Pipe, 2003. Structural and functional characterisation of the blood cells of the bivalve mollusc, *Scrobicularia plana*. *Fish Shellf. Immunol.*, 15: 249–262

Wu, X.Z. and J.P. Pan, 2000. An intracellular prokaryotic microorganism associated with lesions in the oyster, *Crassostrea ariakensis* Gould. *J. Fish Dis.*, 23: 409–414

Xu, T., J. Xie, J. Li, M. Luo, S. Ye and X. Wu, 2012. Identification of expressed genes in cDNA library of hemocytes from the RLO-challenged oyster, *Crassostrea ariakensis* Gould with special functional implication of three complement-related fragments (CaC1q1, CaC1q2 and CaC3). *Fish Shellf. Immunol.*, 32: 1106–1116

Xu, X.Q., Y.Y. Wang and H.S. Sun, 2006. Flow cytometric comparison of haemocyte classification in four species of bivalve mollusks. *Trans. Oceanol. Limnol. (in Chinese)*, 26: 46–50

Xue, Q.G., T. Renault and S. Chilmonczyk, 2001. Flow cytometric assessment of haemocyte sub-populations in the European flat oyster, *Ostrea edulis*, haemolymph. *Fish Shellf. Immunol.*, 11: 557–567

Zhou, H., Y. Wang, Q. Zhou, B. Wu, A. Wang, W. Jiang and L. Wang, 2016. Down-regulation of protein kinase C-ε by prolonged incubation with PMA inhibits the proliferation of vascular smooth muscle cells. *Cell. Physiol. Biochem.*, 40: 379–390

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