Selected morpho-chemical features of hemocytes in farmed shrimp, *Fenneropenaeus indicus* in Iran

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Abstract

The aim of the present study was to determine various types of hemocytes, total and differential hemocyte count and to show some indices of phagocytosis, including percent phagocytosis and phagocytic index in Indian white shrimp, *Fenneropenaeus indicus* in Iranian shrimp farms. The hemolymph was extracted from the shrimps and mixed with anticoagulant. It then stained and Yeast was added as foreign particles to samples. Based on the cell size and presence of the granules and nucleocytoplasmic ratio, three major groups of hemocytes, including hyaline cells, small granular cells (SGC) and large granular cells (LGC) were identified. Hyaline cell (HC) was the smallest hemocyte. HC (hyaline cell) about was 10-15%, lower quantity in comparison to some species and those of LGC and SGC were 20-25% and 60-65%, respectively, suggesting that relative percentage of differential hemocyte count in *Fenneropenaeus indicus* is different from other decapods. Also, in this study, it was shown that SGH and LGH could engulf the yeast particles. In Contrast to some previous studies, no engulfment was observed by hyaline cells in vitro. In this study it was showed that there are some variations in total and differential hemocyte count compare to other species in the family of Penaeidae.

Keywords: Hemocytes,morpho chemical Feature, Phagocytosis, *Fenneropenaeus indicus*

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Introduction

Shrimp culture is the most important beneficial trade sector in south-east Asia, started in Iran in 1994, and has rapidly expanded during last years. However, the intensive culture systems which adopted by shrimp farmers frequently causing stress to the animals and consequently, predispose them to infection. Infectious diseases have affected the profitability of the shrimp farming industry in Iran, too. During 2002, a rapid and high mortality occurred in cultured *F. indicus* farms in Iran (Tokhmafshan *et al*., 2004). In involved farms the mortality reached up to 90% within 3-10 days (Afshanasab *et al*., 2007). Disease prevention has been a priority and shrimp immunology has become a prime area of research. As reported by Lightner (1992), the need to reduce the lethal and weakening effects of pathogens is stimulating a renewed interest in the defense mechanisms and the immune system of crustaceans. In shrimp, the most important role of the circulation hemocytes is the protection of animals against invading microorganisms by participating in recognition, phagocytosis and melanization (Tzou *et al*., 2002;Cerenius & Soderhall, 2004; ; Hsieh *et al*., 2008). Despite the variety of shrimp responses, many of them originate from hemocyte. Shrimp hemocytes are involved in defense mechanisms such as phagocytosis, encapsulation, clot formation and melanization (Johansson *et al*., 2000). Thus hemocytes number sometimes used as an indicator of shrimp health status (Perazzolo *et al*., 2002). The aim of this study was to classify the hemocytes of the Indian white shrimp, *F. indicus*, based on morphology, cell size, granules and the nucleocytoplasmic ratio and to illustrate phagocytosis of the foreign particles (Yeast), by hemocytes using a light microscope and some other techniques according to Hose *et al*. (1990a) and Kondo (2003).

Materials and methods

10 adult shrimps, *F. indicus* (weight ranging from 9 - 11 g) obtained from a semi-intensive farm in Bushehr Province, in south of Iran and acclimatized in a tank containing clean and well aerated seawater with 34 ppt salinity at 24-26^{o}C in Persian Gulf Higher Education Center for Fisheries Science of Bushehr Province for 2 days before collecting the hemolymph. Hemolymph (0.5ml) was withdrawn from the ventral sinus located at the base of the first abdominal segment of each shrimp by 10 ml-syringe along with 26 gauge needle containing 9.5 ml fixative, 2-4% formaldehyde in 0.2 M sodium cacodylate buffer plus 10% sucrose in pH 7.4 and finally modified Alsever solution as an anticoagulant. The former was used to classify hemocytes (and/or total and differential hemocyte count) and phagocytic activities, respectively (Kondo, 2003; O.I.E, 2005; Art *et al*., 2007).The Alsever solution prepared by mixing Sodium citrate (27mM)Nacl (336m M), Glucose (115mM) and EDTA. (9mM) and then Adjusted to pH 7.0 with 1 N NaOH and stored at 5$^{o}_{C}$ (Kondo, 2003). Hemolymph was transferred to tubes, mixed gently and finally fixed for 10 to 15 minutes at 5^{o}c. The smear of the fixed hemocyte was

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prepared on slide glass. The films were airing dried and used for treating with Periodic Acid Schiff (PAS) and May Grundwald_Giemsa. PAS reagent was used to show carbohydrates in the cytoplasm of hematocytes to realize variation between granulocytes and others, particularly semi-granulocyte. MGG was used to identify and classify the hemocytes. Staining with Wright's solution that illustrated the phagocytosis of Yeast as foreign particles was the following process (Kondo, 2003).

10 samples were undergone differential hemocyte count (DHC) and total hemocyte count by Thoma's type hemocytometer (0.1 mm dimensions; with 16 small squares, 1/400 mm^2 each), counting the different hemocyte types and calculating their relative percentages in blood smears. To calculate the cell numbers, the total volume of 5 large squares was calculated as: $1/400\times16\times5\times0.1=1/50mm^3$ (Stolen *et al*., 1995; Kondo, 2003;).

The blood smears, were incubated with 1% periodic acid for 15 min., rinsed with distilled water and treated with Schiff´s reagent for 1 hour. The blood smears were then rinsed for 3 minutes in each of three consecutive 1% sulfurous acid bath (90 ml distilled water, 5 ml of 1N Hydrochloric acid in 5 ml of 10% NaHSO3), left in running water for 5 min., placed in distilled water briefly and then staining with mayer´s hematoxylin (commercial). PAS reaction is a good method to recognize and differentiate granulocytes, agranulocytes and semi-granulocytes, generally (Kondo, 2003). The blood smears, were placed into the May Grunwald solution (1.5 ml/slide for 5 minutes) then added Giemsa stain solution for 20 min., and rinsed with distilled water, finally. This is a proper method to differentiate hyaline cell (agranulocyte), small granular cell and large granular cell in the blood smear (Stolen *et al*., 1995 &Kondo 2003).

Suspension of *Saccharomyces cerevisiae* was prepared by adding 0.9% NaCl solution and then was boiled for 1 hour, followed by washing in prawn saline (g/l). The solution prepared by mixing NaCl (28.4), $MgCl_2 . 6H_2O$ (1.0), $MgSO_4 . 7H_2O$ (2.0), $CaCl_2 . 2H_2O$ (2.25), KCl (o.7), Glucose (1.0) and Hepes (2.38) then Adjusted to pH 7.6 with 1 N NaOH.

The yeast was suspended in the saline and the hemocyte concentration adjusted at 1×10^6 cells/ml., then stored at 5^oC and spread the hemocyte suspension (200µl) on cover slip in the plastic dish. 2 ml of heated killed Yeast was incubated 1-2 hours. We washed the yeast by saline. It fixed by the fixative for 10 min. and washed by distilled water whenever 1 ml of Wright solution added and left it for 5 minutes and finally 5 ml of phosphate buffer added(1/15 M, PH 6.6) (Sritunyalucksana *et al.,* 2005; Kondo, 2003).

Ingestion of microorganisms by hemocytes of the shrimp was studied by preparing blood cell monolayer on glass coverslips, according to the method of Johansson and Soderhall (1985) and Kondo (2003). The blood cells were collected, kept in the same anticoagulant described above, and washed in a saline solution that consisted of 0.4 M NaCl, pH7.6, for the penaeid shrimps (adapted from Smith and Ratcliffe, 1980). Monolayers were prepared

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by allowing the cells (200 µl.) to attach in the presence of 10 mM $CaCl_2$ (final concentration) for 15 min at room temperature (20°C), on pyrogen-free glass cover slips (baked at 180°C for 4hrs).The monolayer was rinsed with the saline solution and incubated with 120 µl of a *Saccharomyces cerevisiae* (baker's yeast) suspension (see below) for 1hr at 20°C. The monolayer was then carefully rinsed three times with the saline solution, fixed 10 min with methanol, and stained with the Wright solution (7 diluted with distilled water) for 30 min. The cover slips were then placed on mounted samples and observed under a light microscope to detect phagocytosis of microorganisms by the hemocytes. In this study we calculated:

Percent Phagocytosis =Number of active phagocytes/100 phagocytes (Stolen *et al.*, 1995). PI (Phagocytic Index) =(No. of hemocytes ingesting Yeasts/no. of hemocytes observed)× (no. of Yeasts ingested/no. of hemocytes observed) ×100 (Itami *et al.*, 1994). ABPC (Average number of the beads ingested per cell) =No. of Yeasts ingested/no. of hemocytes ingesting Yeasts (Itami *et al.*, 1994).

Results

Three types of hemocytes, including agranular (hyaline), small-granule, and large-granule cells were found. Hyaline cells (HC) represented about 10-15% of the circulating hemocytes in *F. indicus* (Table 1). It was round, ovoid, or fusiform hemocyte (7.07 ×5.14 µm) had a relatively large nucleus (5.84 × 4.316 µm). They were cytoplasmic dense and red in color with no granule in the cytoplasm (Figs. 1a and b, & 2). Small granule cells (SGC) comprised about 60_65% of the total circulating hemocytes in *F. indicus* (Table 1).These ovoid or fusiform (Fig. 3) cells (11.24×4.92 µm) had low nucleucytoplasmic ratio (with average size of nuclei, 6.29×4.26 µm) compared to that of the HC.

Table 1: **Total and differential hemocyte counts**

	THC(cc)		DHC%		
Fenneropenaeus indicus	mean	SD	HC	SGC	LGC
	5.3×10^6	2×10^6	10-15	60-65	20-25

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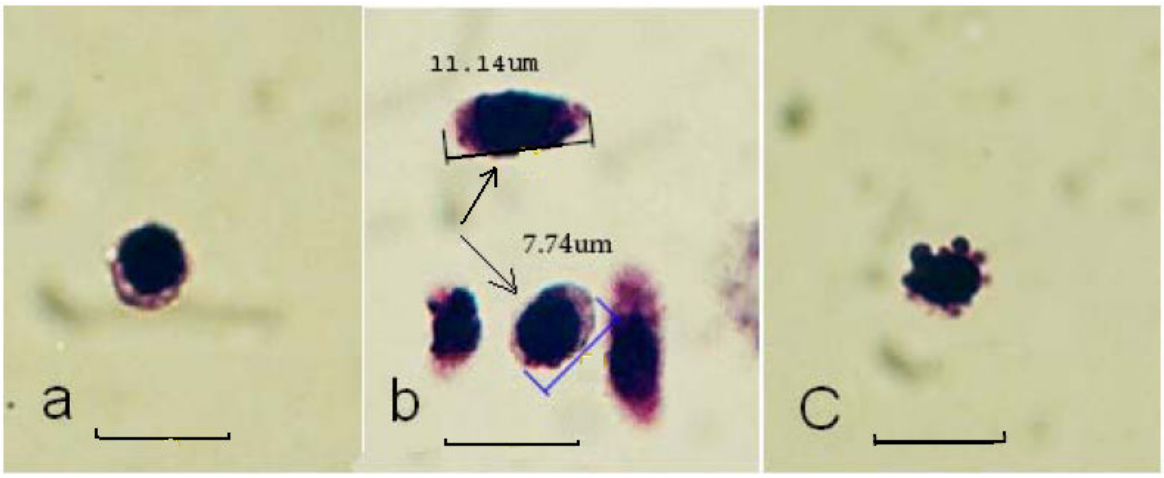

Figure 1: shows variety in size and shape of HC. Note the large nucleocytoplasmic ratio in a and b and podocytes in c; MGG stained. Scale bar= 10 μm,×1000

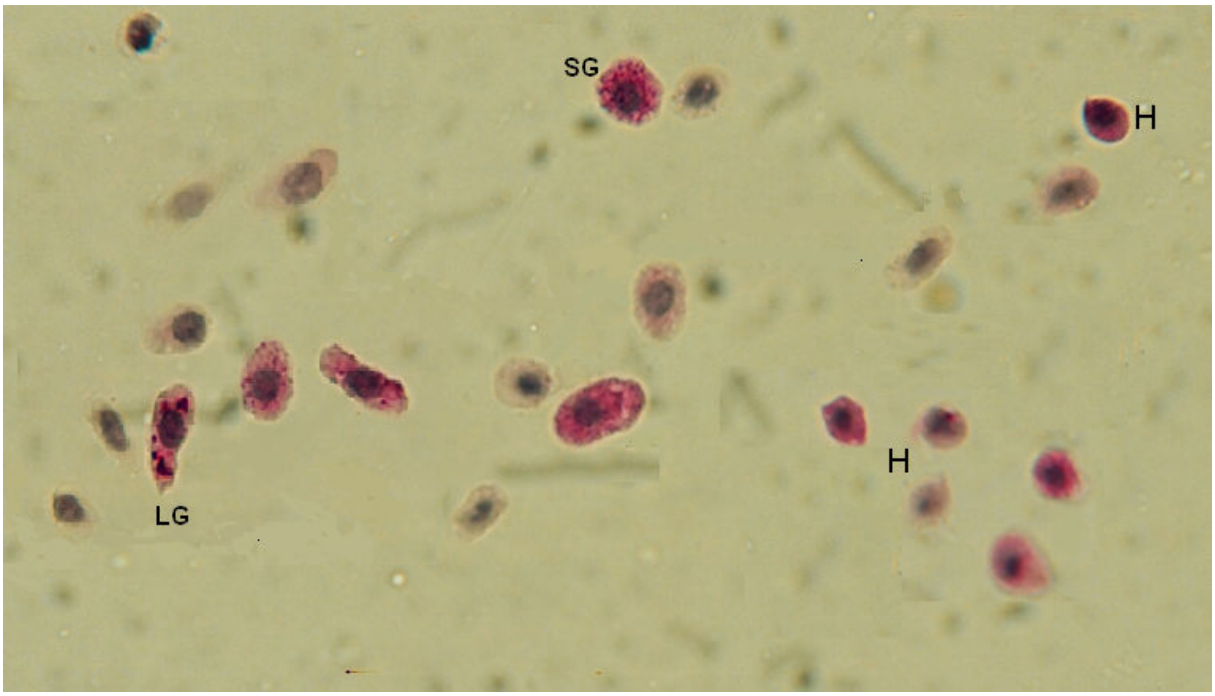

Figure 2: Light microscopy of recirculated hemocytes in reaction with PAS. SG: small granolucyte; LG: large granulocytes; H: hyaline cell (H). Mayer´s Hematoxylin.×1000

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SGC had numerous granules (Figs. 3a, c, d, and f) that usually were smaller than those of the LGC (large granular cells) (Fig. 4). Its cytoplasm sometimes was not clearly observed and represented cloudy appearance and some of the granules appeared weakly dense (Figs. 3b, e). The cytosol of the SGH (small granular hemocetes) and LGH (large granular hemocytes) were rich in carbohydrates [(PAS positive) Fig. 2. Large granule cells (LGC) represented about 20-25% of the total circulating hemocytes in *F. indicus* (Table 1). These cells were round (Fig.4a) or ovoid (Figs. 4b, c) and stable cells (10.97×7.84 µm) which have low nucleucytoplasmic ratio (with average size of nuclei, 6.8×6.26 µm) compare to that of the HC. The cytoplasm was filled with numerous large refractile granules. The granules were stained dark blue or red with MGG (Fig. 4). Under light microscopy, the nucleus of LGC's exhibited condensed chromatin masses. Some deposits (Fig. 2) were apparently polysaccharides, as they were strongly positive with PAS, were frequently observed in the LGC. In PAS reactions, some SGC-like cells with any variation in the color of their cytoplasms observed too. The monolayer hemocytes of all cell types were mounted on glass coverslip. The phagocytosis of yeast particles was investigated. It was possible to determine that SGH and LGH could engulf and involve in some immune response, such as clot formation in exposing to the yeast particles (Fig. 5a). After exposing to yeast particles, granular hemocytes undergo profound morphological changes characterized by pseudopodia and philipodia in LGC and SGC respectively Discoloration in nucleus and cytoplasm and releasing the granules after exposing to foreign particles during the phagocytosis showed in fig.5. The feature of nucleus and cell size of SGC become more colorless and bigger respectively; compare to the condition that Yeast particles did not exist. The whitish cytoplasm became more visible. Pseudopodia and philipodia performed. Few granular hemocytes were observed undergoing mitosis (Fig.5b) that has rarely described in crustaceans. However, the number of dividing hemocytes was very low (less than 1%).The yeast ingested by hemocytes was light blue and non-ingested one was dark blue. The clear space around the yeast in hemocyte was phagosome (Fig.5).Percent phagocytosis, phagocytic index (PI) and A.B.P.C. were calculated as 48% ±2.2, 38.4±2.7 and 1.66±0.2, respectively, in vitro. Few clotting formations were observed and showed a central hemocyte (usually HC) surrounding by numerous granulocytes. Clot formation was started by hyaline cell cytolysis (center of the clot) which surrounded by a few of granulocytes, SGC and LGC.

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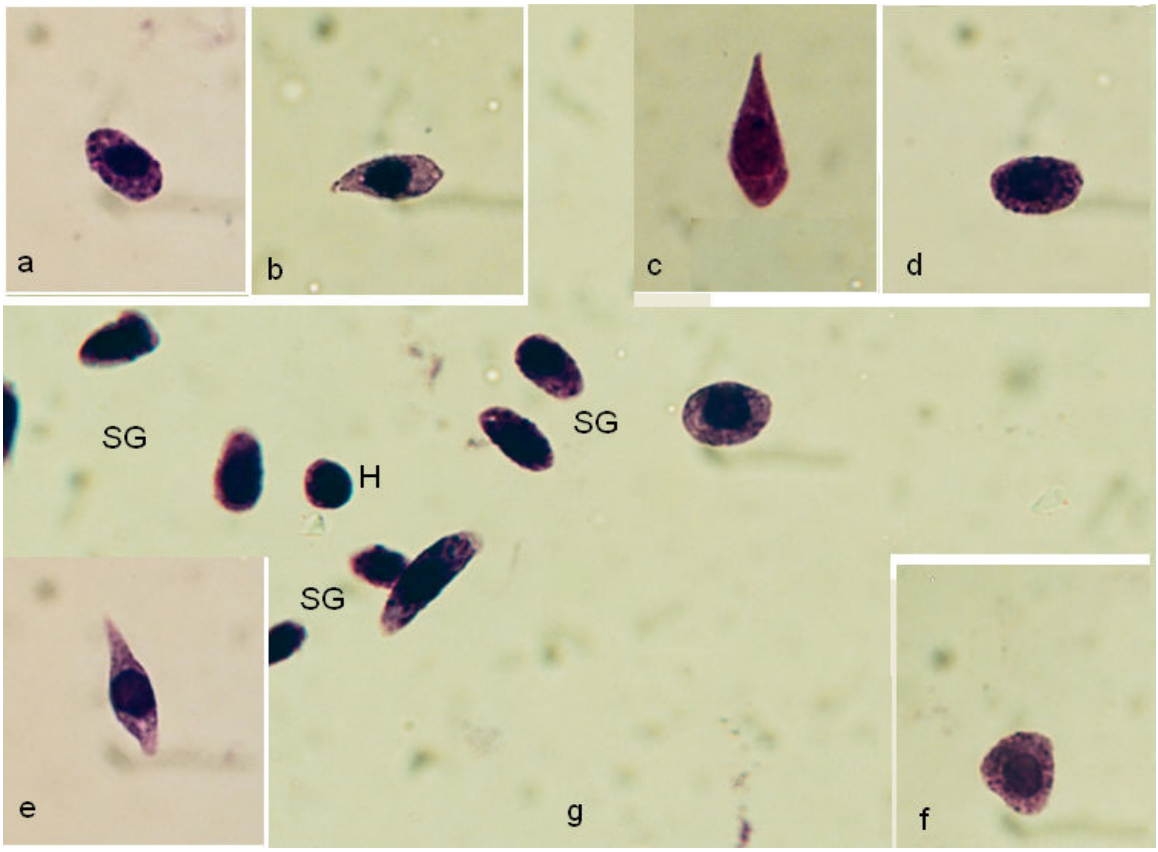

Figure 3: Light microscopy of a monolayer smear of hemolymph (g) which contains hyaline cell (H) and a maximum percentage of small granular cell (SG). Different features of SGC were shown (a, b, c, d, e, and f). MGG stained. ×1000

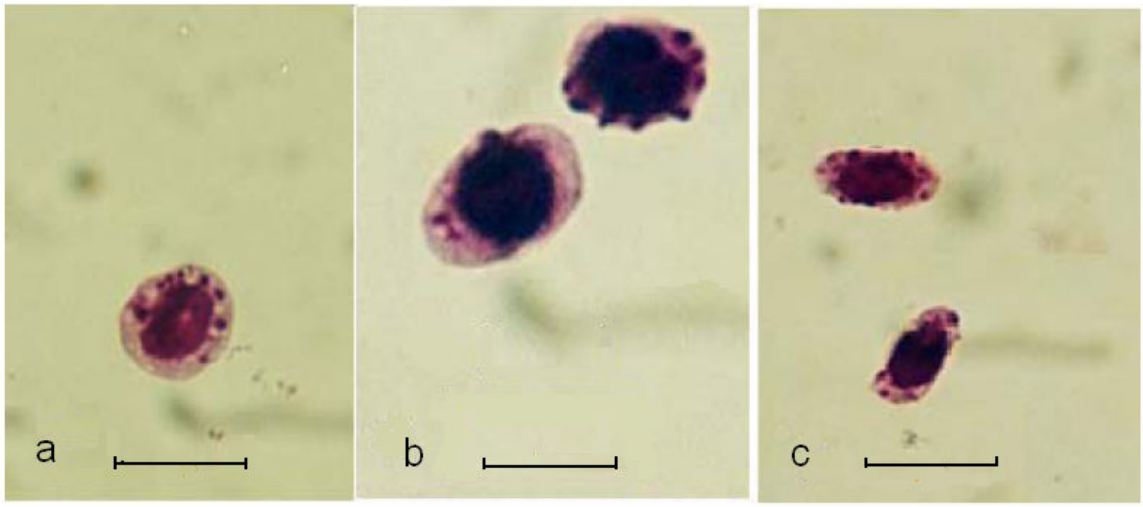

Figure 4: LM of large granule cell (LGC) showing either larger granules in the cytoplasm or larger cell size than those of SGC. MGG stained. Scale bar=10μm, × 1000

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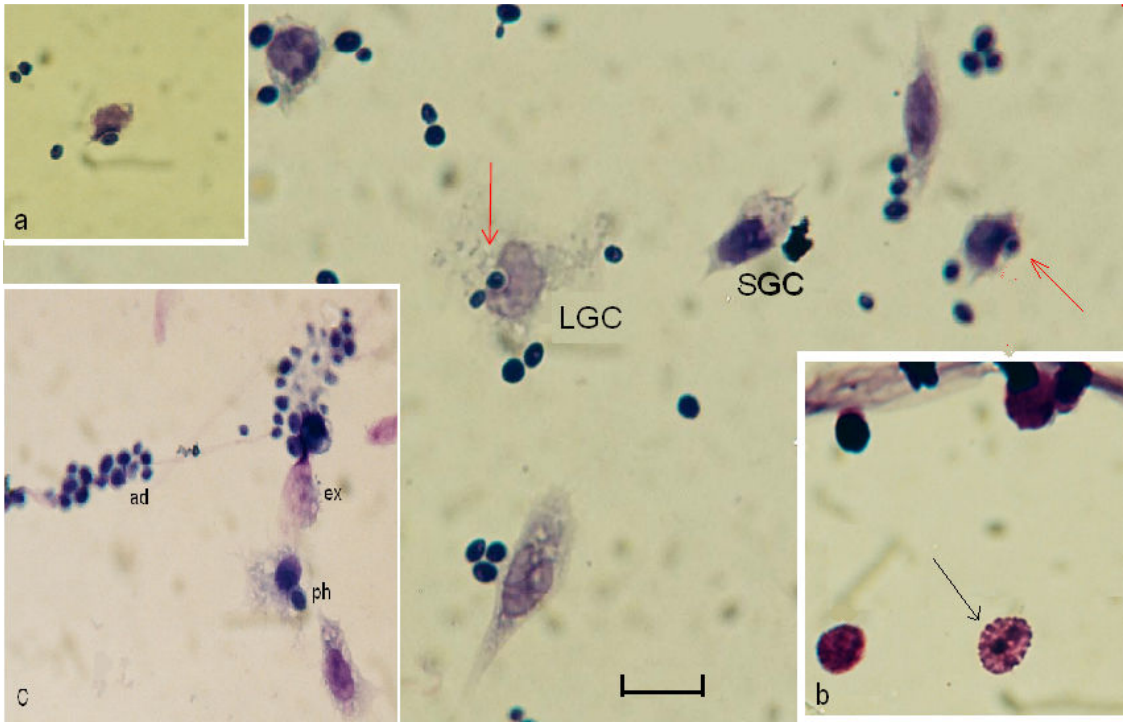

Figure 5: Arrows of the background shows the phagosome which contains a clear space around itself. a) the yeast particle phagocyted .b) a hematocyte represents mitosis. c) Shows adhesive factor (ad) excretes from the lysed hemocyte (ex) to enable foreign particles adhere together. A granulocyte shows a yeast particle in phagocytosis process (ph). Wright stained, Scale bar=10μm, ×1000

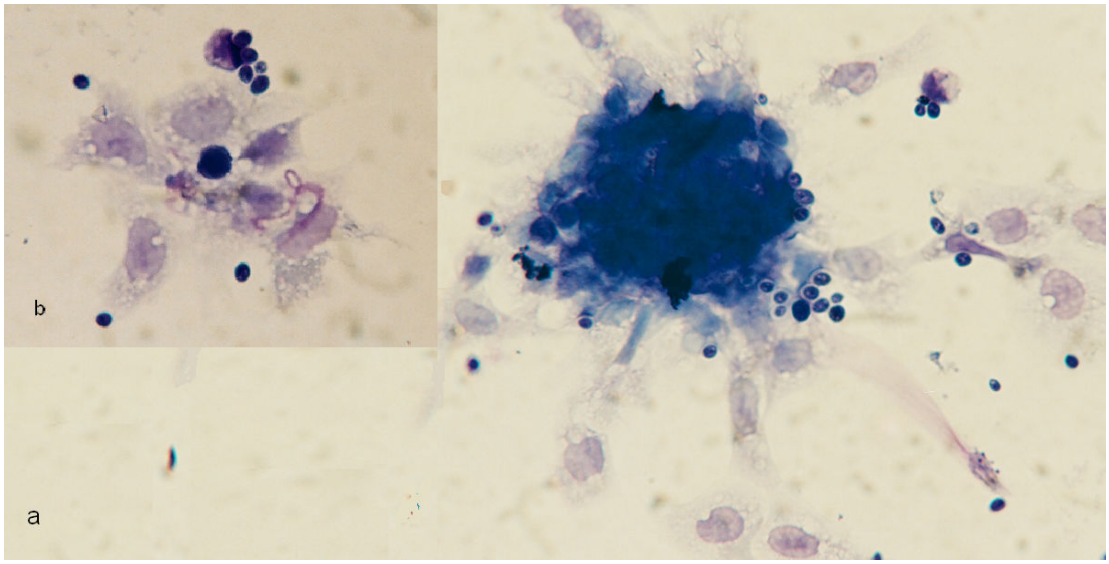

Figure 6: A clotting formation from beginning (b) to complete process (a) to show a central point surrounding with numerous granulocytes (SGC & LGC). Wright stained ×1000

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Discussion

Fisheries researchers have adopted different criteria for hemocytes classification although the classification of crustacean hemocytes remains controversial (Hose *et al*. 1987, 1990a). Hose and Martin (1989) and Tsing *et al*., (1989) represented common and unified terms for decapods blood cells. The results of the present study suggest that hemocytes in *F. indicus* comprise three major groups, including hyaline cells, small granule cells (SGC) and large granule cells (LGC) depend on cell and granules size and the nucleocytoplasmic ratio. The high nucleocytoplasmic ratio of the HC is useful for its identification. It is agranule and the smallest SGC-like hemocytes were observed on PAS reaction slides with distinct differences in color of cytoplasm to granulocytes. It seems these cells can be semi-granulocytes which observed by other researchers. These cells had less color compared to SGC in PAS reaction (Fig. 2). Kondo (2003) believed that these cells are similar to SGC but has large granules and PPO (prophenoloxidase). Therefore, he believed that in *Penaeus japonicus*, semi-granulocytes are the immature LGC. Granulocytes involved the immune system as well as hyaline cells. However it seems hyaline cells start some reaction such as clot formation. Lavine and Strand (2002), Cerenius and Söderhäll (2004) and Iwanaga and Lee (2005) proved that these reactions are often observed to become melanized, through the action of phenoloxidase. This discoloration was observed in our study, as well. A few mitotic figures (less than 1%) were observed in the granulocytes of *F. paulensis*, but not in the palaemonids (Gargioni and Barracco, 1998). In the present study, it was found that mitosis occurs in granular cells. Few granular hemocytes were found to undergo mitosis. This has rarely been reported by other researchers. Gargioni and Barracco (1998) stated that the SGH and LGH were actively phagocytic when examined on blood cell monolayers incubated with the yeast *Saccharomyces cerevisiae*. According to Rengpipat *et al*., (2000) the phagocytic activities such as the percent of phagocytosis , phagocytic index and A.B.P.C. after 2 months of feeding and then challenging with WSSV as in vivo study were 16.3 ± 0.8, 12.8±4.3 and 4.9± 1.6, respectively. Compare to our study, it seems that the phagocytosis rate and phagocytic index were higher. Therefore, it is likely that the yeast particles could be more stimulant than probiotic in defense system, particularly in vitro study. According to Hose *et al*. ($1990_{a\ \&\ b}$) and Kondo *et al*. (1998), the hemocytes of the shrimp (Penaeidae) are classified into three types, hyaline cells (HC), small granular cells (SGC) and large granular cells (LG) associated with the presence of cytoplasmic deposit and size of granules. We observed that Hyaline cells, which are the smallest cells in *F. indicus* are easily lysed. They looked like round or oval cells, with a high nucleocytoplasmic ratio. They probably had a very few granules (Bauchau, 1981; Tsing *et al*., 1989; Martin & Graves, 2005) but no granules within, were observed in our study. This finding suggests that the HC undergoes a rapid lysis in vitro. Hyalines

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cytolysis initiated coagulation of hemolymph and followed by rupture of the plasma membrane. Cells then looked to leach and aggregated deposits appeared to form long filamentous strands within the cytoplasm. Sphere coagulation was formed around individual hyaline cell while granulocytes were pushed to the periphery(Omori *et al*., 1989). This system may be triggered by endotoxin and β-1 ,3-glucans (Levin 1967; Söderhäll, 1981; Durliat, 1985). On the other hand, the HC were nonphagocytic and primarily involved with coagulation of hemolymph (Omori *et al*., 1989). Small granulocytes were responsible for the phagocytosis of foreign particles (Hose & Martin, 1989). The granulocytes of *Penaeus paulensis* were easily identified as SGH and LGH according to the descriptions of other decapods (Bauchau 1981; Martin & Graves, 2005; Hose *et al*., 1990_a; Barracco & Amirante, 1992). They proved these can be actively phagocytic cells and could ingest microorganisms in vitro, such as yeast particles. Our results suggested that the HC in *F. indicus* is an unstable cell that rapidly lost its viability and can help to initiate coagulation and small granulocyte and large granulocyte involved in phagocytosis in vitro. Differential hemocyte count values were recorded in the relative percentage (differential hemocyte count) of the hemocytes in the different species. In *Sicyonia ingentis*, Hose *et al.* (1992) recorded that the HC comprises 50–60% of the circulating hemocytes, whereas the SGH and LGH represented 30% and 10%, respectively. Our calculation about the relative percentage of HC in *F. indicus* is varied and approximately was 10-15%, lower value compared to former species and LGC and SGC were 20-25% and 60-65%, respectively. Hemocyte types, particularly the hyaline cells, are not analogous between different crustacean species, both morphologically and functionally (Bachere *et al.,* 1995). In the shrimp, *P. japonicus,* in accordance with Tsing *et al.* (1989) in the sense that the hyaline cells are relatively less numerous than in other decapods. The hyaline cells of *Macrobrachium rosenbergii*, posses 70% of hemocytes (Vazquez *et al*., 1997), in contrast to Penaeidae shrimp. In the lobster, *Homarus americanus,* and the crab, *Loxorhynchus grandis*, the SGH were highest (similar to *F. indicus)* reaching more than 60% of the total cell number (Hose *et al*., 1990_a). High variation of total hemocyte count (THC) in different crustacean species has been usually reported by researchers. Martin and Graves (2005) reported a relatively low THC (about 11×10^3 cells/mm^3) in the *Penaeus californiensis* while Tsing *et al*. (1989) reported about $5\text{-}14\times10^6$ cells/mm^3 in the hemolymph of the *Penaeus japonicus* as our record in *F. indicus*. In shrimp, *Penaeus setiferus* the THC was approximately recorded 8.9×10^6 (Yeager & Tauber, 1935). There were some variations in result of hemolymph contents of serum in *Pontastacus leptodactylus* after exposing to varied pH (Soltani *et al*., 2008). The present study showed morphologic features of hemocytes and occurrence of phagocytosis in *F. Indicus* which can be led to further research on shrimp immunity and to look for new or modified approaches to

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decrease the risk of pathogens in shrimp culture farms.

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