Ontogenic Maturation of Hemocyte Production and Gene Expression in the Hematopoietic System of *Procambarus clarkii*

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Abstract: The red swamp crayfish, *Procambarus clarkii* is an important aquaculture decapod species as well as invasive species in some countries. The present study investigates the association between the moult stages-related pattern and the abundance of different cell types with the lymphoid organs of *P. clarkii*. Studies on the effect of moult cycle on the lymphoid organ spheroid (LOS) cells of *P. clarkii* were also studied. The spheroid to total tissue (STT) ratio of the lymphoid organ varied during the life span of the crayfish. Kazal-type serine proteinase inhibitor hcPcSP12 with complete cDNA sequence, were identified from cDNA library of the red swamp crayfish, *P. clarkii*. RT-PCR shows that hcPcSP12 exists mainly in hemocytes. Western both demonstrates that hcPcSP12 only exists in semi-granular cells.

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Keywords: *Procambarus clarkii*; Moulting; Hemocytes; Spheriod cells; Kazal-type serine proteinase inhibitor.

1. Introduction

Crustaceans have a cyclic phenomenon known as moulting for development and growth in which all biochemical and physiological changes in various tissues of crustaceans occur during moulting cycle (Chang, 1995; Phlippen *et al*., 2000; Cheng *et al.*, 2002). Changes to the immune components of prawns in association with moulting have also been studied to a lesser extent. Hematological variation in both total hemocyte count (THC) and differentiated hemocyte count (DHC) was recorded in individuals and between species during the moult cycle (Le Moullac *et al*., 1997: Cheng and Chen, 2001; Liu *et al*., 2004). Furthermore, the resistance of prawns to pathogens was suggested to relate to this physiological mechanism (Le Moullac *et al*., 1997; Liu *et al*., 2004; Ho *et al*., 2009).

In penaeid species, the lymphoid organ (LO) has an important role in immuno-defence against invading pathogens (Anggraeni and Owens, 2000; van de Braak *et al*., 2002). The LO has a phagocytic function (Kondo *et al*., 1994), bacteriostatic effect (Burgents *et al*., 2005) and trapping ability to immobilise foreign material from the hemolymph before this material enters the open circulatory system (van de Braak *et al*., 2002). Furthermore, the lymphoid organ is believed to be a major site for viral degradation by forming lymphoid organ spheroid (LOS) cells (Anggraeni and Owens, 2000). This supposition was supported by the work of Rajendran *et al.* (2006) in that viral load in the lymphoid organ was higher compared to the other organs in infected prawns. The spheroid formation might be related to the tolerance of penaeid prawns to viral infection (Rodriguez *et al*., 2003; Anantasomboon *et al*., 2008).

Kazal-type SPIS exist widely in crustaceans (Visetnan *et al*., 2009; Kong *et al*., 2009; Wang *et al*., 2009; Cerenius *et al*., 2010), some of them were found to have important roles in the innate immunity of their respective organisms (Donpudsos *et al*., 2009). To date, four kazal-type inhibitors with full length in hemocytes was up regulated after WSSV infection, whereas previous study demonstrated hcPcSP11 was down regulated (Li *et al*., 2009) though both originated from hemocytes, they showed low identity. Taking into account that inhibitors from hemocytes evolve rapidly, we speculate that hcPcSP11 and hcPcSP12 are two different kinds of Kazal-type inhibitors and may participate in anti-pathogenic immunity in two different ways in *P. clarkia* (Cheng and Chen, 2001; Ho *et al*., 2009; Li *et al*., 2009; 2010; Cerenius *et al*., 2010).

Western blots show that hcPcSP12 exists in semi-granular cells only. This result is similar with a marker protein residing in semi-granular hemocytes from crayfish *P. Leniusculus* (Wu *et al*., 2008) since hcPcSP12 have comparatively high identity with this marker protein, we believe that hcPcSP12 is also a marker protein of semi-granular cells in crayfish, *P. clarkii*. In addition, the hcPcSP12, was detected in heart and gills mainly because of these tissues always full of hemocytes, while the fact that it was found in stomach and intestine might be due to the contamination of hemocytes since the hemolymph in crayfish sequences have been identified from WSSV infected *P. clarkii* (Rodriguez *et al*., 2003; Anantasomboon *et al*., 2008; Li *et al*., 2009; 2010;

Cerenius *et al*., 2010). So, the present study indicated a moult effect on the abundance of the spheroid cells in the lymphoid organs.

2-Materials and Methods

Experimental animals

Fresh water red swamp crayfish, *Procambarus clarkii* were collected from Mansouria Canal, Giza Governorate, Egypt; during spring 2011, and transported to the Zoology Department, Faculty of Science, Cairo University. Crayfish were immediately acclimated and kept in two 1000 L plastic bins with a recirculating system. They were screened for white spot syndrome virus (WSSV) infection by RT-PCR Only crayfish that tested positive for WSSV were selected at maintained in 300L aquarium tanks filled with filtered sea water with continuous aeration at room temperature for at least 21 days. Each surviving crayfishes were considered as chronically infected with WSSV and used in this study (Fig 1A).

Moult staging

Moult was staged by microscopical examination of setal development (setogenesis) and epidermal withdrawal (apolysis) in the inner uropod near the telson tip. Prior to staging the moult, crayfish were anaesthetized by placing them in ice water for a few minutes. Five animals were sampled at every time point for each experiment. Five crayfish were kept alive as controls to observe the cycles of the moulting during the experiment.

Histological studies

Light microscopical study

Crayfish were fixed in Davidson's fixative by injecting 0.5 ml of the fixative into the hepatopancrease and adjacent areas of the cephalothorax. The organs were cut in half longitudinally, stored in 70% ethanol and then processed for routine histological examination using standard paraffin embedded procedure. Section were cut at 5 µm and stained with H & E.

Electron microscopy

Dissected lymphoid organs were fixed in 3% glutaraldehyde and 0.2 m cacodylate buffer, post-fixed in 1% OsO_4, dehydrated through ascending concentrations of ethanol, and then embedded in Araldite-502 resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate before being observed under Zeiss 902A transmission electron microscope at 60 KV.

Nested RT-PCR detection of WSSV using 1Q2000 Kit RNA extraction

Crayfish pleopods were excised and homogenized in 1 mL of TRI ™ reagent (Pacific Science, USA). Chloroform (200 mL) was then added to the homogenate and the mixture was centrifuged to separate the insoluble materials at 12.000 xg for 10 min. The aqueous phase was collected and then 200 ml of chilled isopropanol was added to precipitate RNA. The RNA pellet was collected by centrifuged at 12.000 xg for 10 min, washed with cold 75% (v/v) ethanol, dried and re-suspended in 20 mL diethyl pyrocarbonate treated water.

Nested RT-PCR

Two microliters (50 ng) of extracted crayfish RNA were added to RT-PCR reaction consisting of 7 mL of RT-PCR premix, 0.5 mL of 1Qzyme DNA polymerase, and 0.5 mL RT Enzyme Mix. The RT-PCR reaction profile consisted of reverse transcription at 42 ^{0}C for 30 min, denaturation at 94 ^{0}C for 2 min, 15 cycles of denaturation at 94 ^{0}C for 30 s, annealing at 62 ^{0}C for 20 s, and extension at 72 ^{0}C for 30s. The nested–PCR was carried out by adding 15 mL of nested PCR mixture consisting of 14 mL Nested PCR premix and 1 mL 1Qzyme DNA polymerase to THE total RT-PCR reaction mixture, then the nested–PCR was performed by 30 cycles of denaturation at 94 ^{0}C for 20 s, annealing at 62 ^{0}C for 20 s and extension at 72 ^{0}C for 30s. Ten 10 mL for the mixture was fractionated by using 1.5% agarose gel electrophoresis. The bands at 277 and/or 777 base pairs indicated WSSV infection.

Nucleotide sequence translation and deduced protein forecast were performed using EXPASy (http://www.exposy.ch). Prediction of signal peptide and motife forecasting was carried out using signal P and SMART (http://www.smart.de), respectively (Nielsen *et al*., 1997).

3. Results

Moult stage determination

Setogenesis is the most used criteria to distinguish moult stages and sub-stages. Three moult stages were defined: postmoult, intermoult and premoult (Fig. 1A). Each moult stage is subdivided in several sub-stages according to the retraction of the epithelium within setae of the antennal scale. There are five moult stage classification were identified by the degree of hardness of the exoskeleton (A, B, C, D0/D1, D2/D3) (Fig. 1B).

Tissue distribution and expression pattern analysis

The tissue distribution of hcPcSP12 kazal–type inhibitor was detected using semi-quantitative RT-PCR. The results showed that hcPcSP12 was highly expressed in hemocytes, but also could be detected in the heart, gills, stomach and intestine of normal crayfish (Fig. 2A). Quantitative RT-PCR was used to analyze the temporal expression profiles in pathogen–infected crayfish. In WSSV infected animals, hcPcSP12 levels was significantly increased reaching 10 fold increase. It is mainly distributed in the hepatopancreas and heart (Fig. 2B).

Tissue, hemocytes distribution and expression profile of hcPcSP12 at protein level

Western blot analysis was performed to investigate the tissue and hemocytes distribution, and expression pattern of hcPcSP12 during different moult stage. The results indicate that, consistent with the semi–quantitative RT-PCR results, hcPcSP12 was found in hemocytes, heart, gills, intestine and stomach during postmoult, but not in cell free hemolymph or in the hepatopancreas in normal crayfish during intermoult (Fig. 3A). Furthermore, it was found that hcPcSP12 existed during premoult only in semi-granular hemocytes, not in hyaline or granular hemocytes (Fig. 3B). However, in WSSV Infected animals, the transcripts of hcPcSP12 appeared during premoult and postmoult in hemocytes and the cell–free hemolymph (Fig. 3C).

Variations of hemocytes during the moult cycle

Table (1) shows that the THC observed in intermoult (stage C) was significantly lower ($P< 0.1\%$) than the THC observed in postmoult (stage B) and premoult (stages Do/D1 and D2/D3). The relative percentage for the different hemocyte types was evaluated for the different moult stages. While no significant differences were seen with HC and SGC at the different moult stages, the LGC relative percentage was significantly higher during the stage C (Table 1). Variations of THC during the moult cycle are mainly related to the variations of HC representing about 80% of THC. SGC relative percentage does not vary significantly during the moult cycle; while in contrast, the relative variations of LGC show a maximum in intermoult and a minimum in premoult.

Structure of the lymphoid organs (LO)

The LO of postmoult stage consists of the distinct lobes ventro-anterior to the hepatopancreas. Each of the lobes consists of folded tubules with a central hemal lumen, which branch several times. The tubules are afferent hemolymph vessels that originate from the LO duct, which arises from the anterior aorta near the heart (Figs. 4A and B). Although the LO duct lumen of intermoult stage has generally a larger diameter, the cells in the LO duct and tubules have an identical morphology. The LO tubules become smaller in diameter and central hemal lumen may be nearly occluded at the end. The larger central hemal lumen of the tubules, the more evident is the lining by a layer of flattened endothelial cells. Encircling the endothelium, the cells in the inner layer of the tubule walls are tightly packed, whereas lesser cells, often observed with poorly or unstained cytoplasm (Fig. 4C). The LO tubules of premoult stage are surrounded by fibrous connective tissue. Hemocytes seemed to penetrate into the tubule wall, where they might settle or leave at the outer layer of the tubule wall. The hemocytes may enter the hemolymph sinuses of the open circulatory system or might settle in the surrounding clusters of spheroids cells. Mitotic cells were occasionally observed in the LO tubules (Figs. 4D and E).

UIltrastructure of the lymphoid organ (LO)

The central hemal space of the LO tubules of postmoult stage contained young circulating hemocytes of the large and to a much lesser extent, the small-granular cell type (Fig. 5A). Cells apparently migrating into the tubule wall were often observed (Fig. 5B). The central lumen of intermoult stage was frequently lined with a flattened layer of elongated endothelial cells, followed by a densely packed layer of cells (Figs. 5C and D). These cells often contained many vacuoles and up to 30 granules, varying in size and electron density, per cell section, and strongly resembled the small granular hemocytes. Those small-granular cells of premoult stage contained many pseudopods. The pseudopods apparently surrounded foreign material and led into the outer layer of the tubule wall.

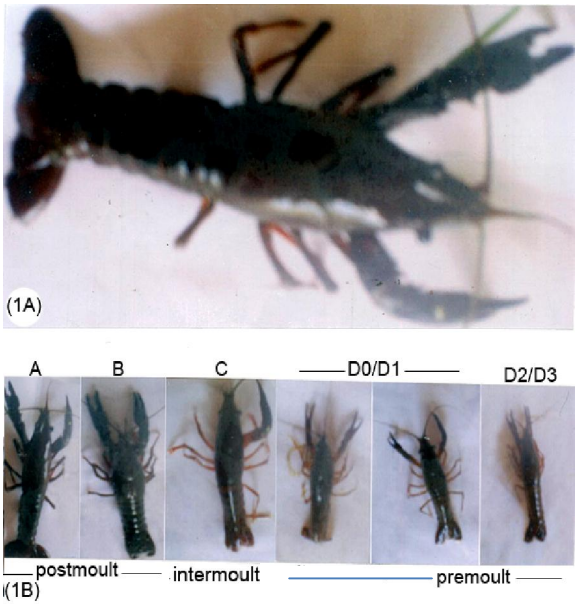

Fig. 1: (A) The red swamp crayfish, *Procambrus clarkii*. (B) Different moult stages of *P. clarkii*.

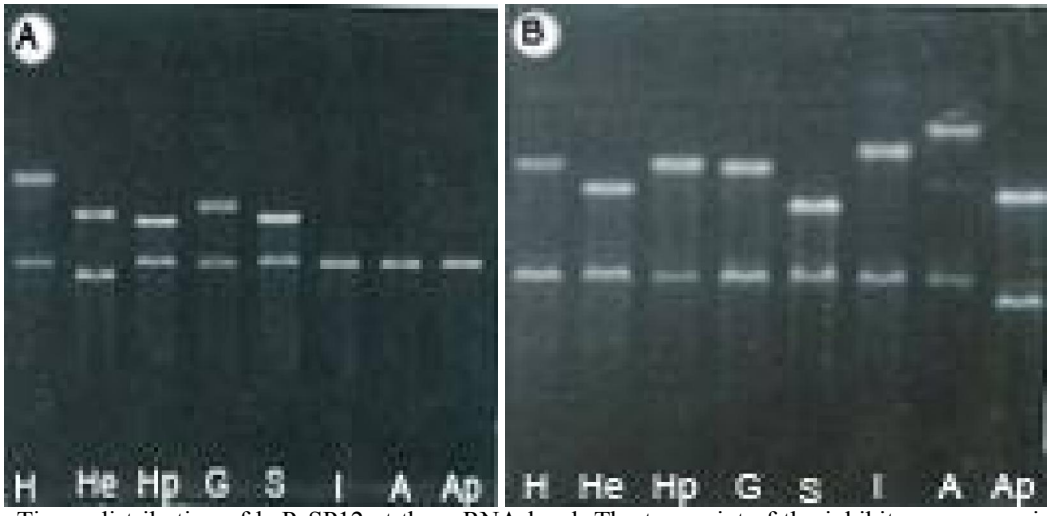

Fig. 2: Tissue distribution of hcPcSP12 at the mRNA level. The transcript of the inhibitor was examined by semi-quantitative RT-PCR. The top panel show the results of the inhibitor detected with 29 cycles. The bottom panel indicates 18sRNA amplified as an internal control. (A) Non-infected animal. (B) WSSV-infected animal. C: cell-free hemolymph, H: hemolymph, He: heart, Hp: hepatopancreas, G: gills, S: stomach, I: intestine, A: antennal gland, Ap: appendage.

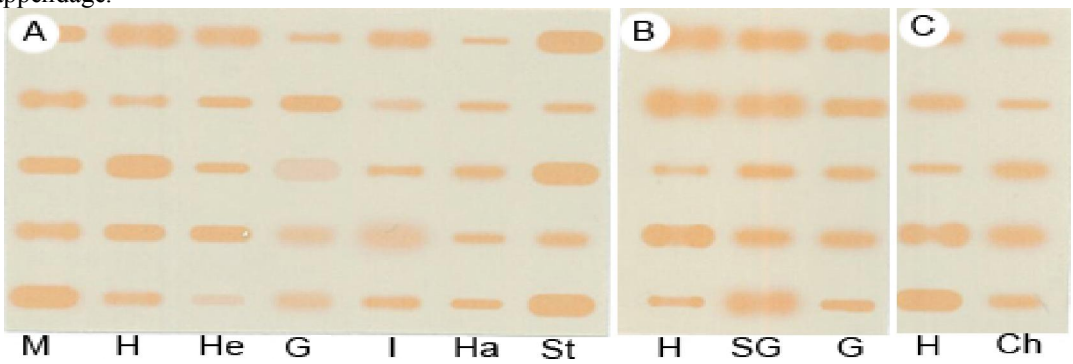

Fig. 3: Western blot analysis of tissue and hemocyte type distribution, and expression pattern of hcPcSP12 at protein level. Tissue distribution (A) and hemocyte type distribution (B and C) of hcPcSP12 in normal crayfish. M: protein marker; H: hyaline hemocytes; SG: semi-granular hemocytes; G: granular hemocytes; Hi: hemocytes; He: heart; G: gill; I: intestine; Ha: hepatopancreas; St: stomach; Ch: cell-free hemocytes.

Table 1: Total hemocyte count (THC mm^{-3}) of hemolymph in *Procambanus clarkii* and the relative percentage of each hemocyte type during the moult cycle. Means are presented with standard deviation (1) sub-stage A,B in postmoult, sub-stage C in intermoult, sub-stages D0/D1 and D2/D3 in premoult. HC: hyaline cells, SGC: semi-granular cells, LGC: large granular cells

Moult Substages (1)	No. of crayfish	THC mm^{-3} (200)	HC (%)	SGC (%)	LGC (%)
A	16	218±85	78±4	17±3	5±
B	12	196±67	79±10	13±7	7±3
C	15	131±81	80±5	10±3	10±2
D0	6	201±42	83±4	13±3	4±3
D1	12	223±51	82±5	11±4	7±3
D2	20	311±67	80±5	13±7	7±3
D3	16	212±39	81±3	13±	6±3

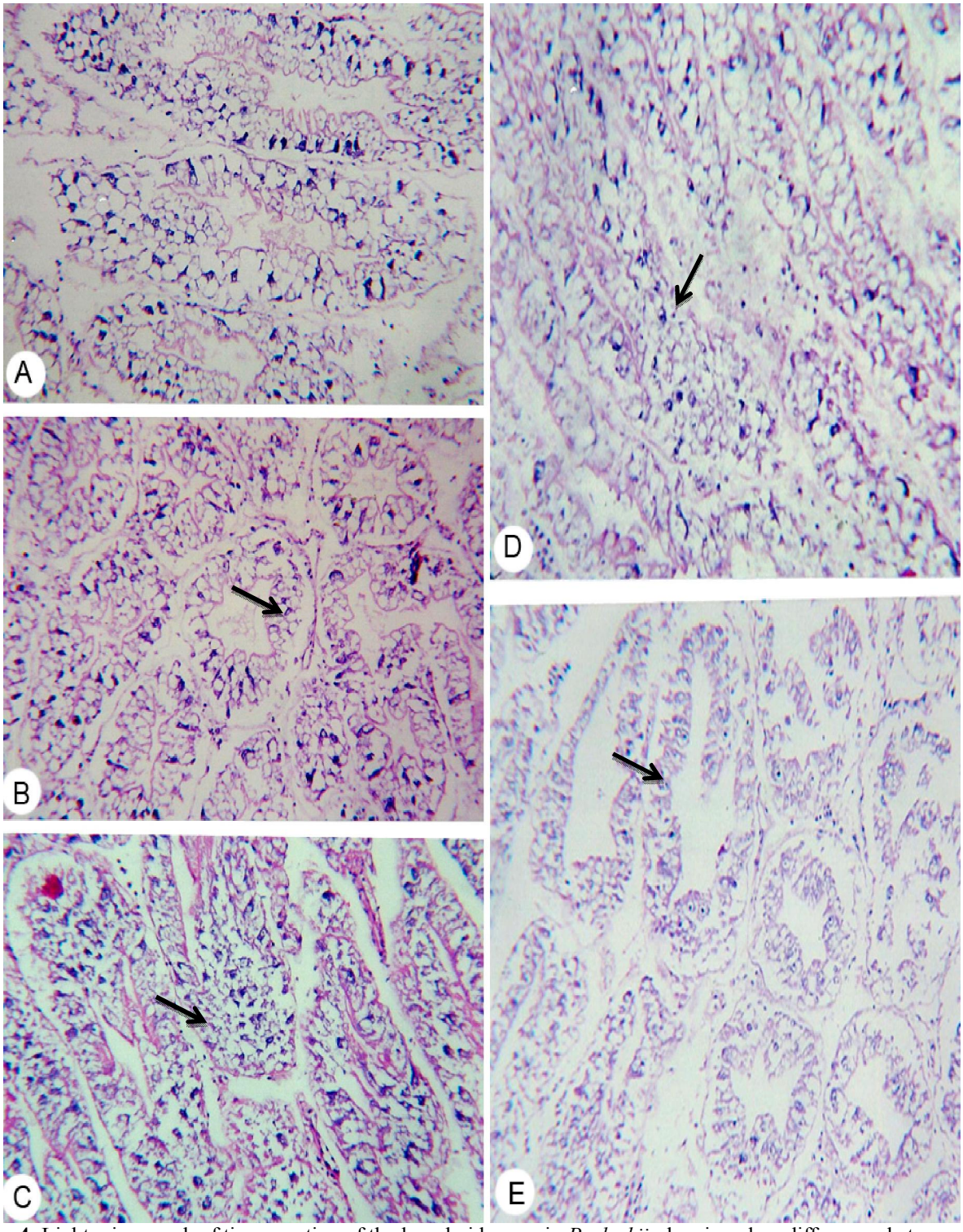

Fig. 4: Light micrograph of tissue section of the lymphoid organ in *P. clarkii*, showing clear differences between the lymphoid organ tubules and spheroids. A tubule with a central haemal lumen is observed next to a tubule with occulted haemal centre (A and B). Arrow indicates the beginning of the lymphoid organ duct (C). The LO cells are found in the haemal sinuses and appear to have a more basophilic cytoplasm and each of a central lumen compared to normal lymphoid tubule (D and E). (A) (B) postmoult; (C) intermoult; (D) (E) premoult. X 400

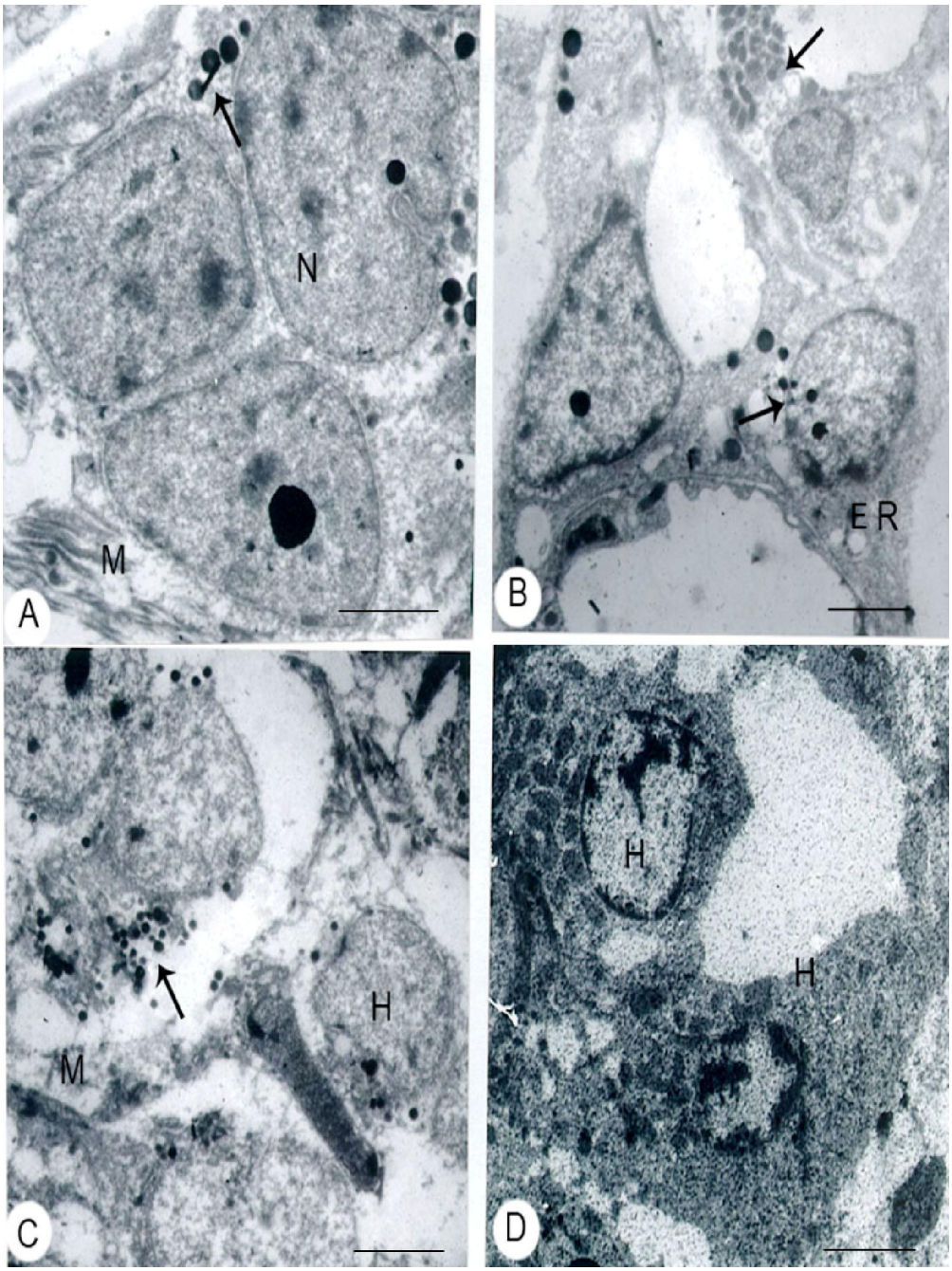

Fig. 5: Electron micrographs of the lymphoid organ in *P. clarkii*. (A) (B) A LO tubule is composed of endothelial cells and stromal matrix with small granules and vacuoles (arrow). The necrotic cells can be observed in the tubule wall. Arrow points to the basal lamina covering the endothelia. (C) (D) Besides the stromal matrix cells, there are hemocytes (H) fiber, granules (arrows) and other inclusions in the outer layer of a LO tubule. X1000

4. Discussion

The circulating hemocytes of crustaceans play a central role in innate immunity. They are involved in nodule formation, encapsulation and in phagocytosis. Three hemocytes types are commonly described in crustaceans: hyaline hemocytes, small granules hemocytes, and large granules hemocytes (Johansson *et al*., 2000; Battison *et al*., 2003; Giulianin *et al*., 2007). The morphological classifications are essentially based on granule number, size and nucleus to cytoplasm ratio.

Le Moullac *et al.* (1997) reported that the total hemocytes count (THC) measured in intermoult stage were significantly lower than the THC observed in

premoult stages and B stage. Moreover, *L. vannamei* displayed a pattern of granular cells and THC higher at C stage and lower at A stage. It was also found that the phagocytic activity of *L. vannamei* against *Vibrio alginolyticus* was higher during the intermoult stages. These facts support the idea that *L. vannamei* is more resistant to *V. alginolyticus* in this stage. Cheng *et al*. (2003) also observed that both phagocytic activity and clearance efficiency of *M. rosenbergii* to *L. garvieda* were higher during the intermoult stage. This may be because the cuticle is sclerotinized and not permeable during the intermoult, whereas during the premoult stages, the detachment of old cuticle could allow bacteria to penetrate in the body though the new cuticle (Le Moullac *et al*., 1997).

Kazal-type SPIs exist widely in crustaceans. For instance, five Kazal-type inhibitors in the Chinese white shrimp, *F. chinesis*, six transcripts in the black tiger shrimp, *P. monodon* and twelve transcripts in the crayfish *P. leniusculus* were reported recently (Cerenius *et al*., 2010). To date, four Kazal-type inhibitors with full-length sequences have been identified from crayfish (Li *et al*., 2010). Kazal-type inhibitors from the hepatopancreas are believed to be secreted in the plasma or gastrointestinal system to exert different physiological functions. This means that hcPcSP12 may be released into the hemolymph and has a role in antiviral immunity. In addition, at protein level there was no obvious change for the hcPcSP12 in hemocytes except for a decrease after WSSV-infection. Interestingly, hcPcSP12 was also detected in cell-free hemocytes after WSSV-infection during postmoult and premoult stages (Wu *et al*., 2008).

Western blot shows that hcPcSP12 exists in semi-granular cells only during premoult stage. This result is similar with a marker protein residing in semi-granular hemocytes from crayfish *P. leniusculus* (Wu *et al.,* 2008). Since hcPcSP12 have comparatively high identity with this marker protein, we believe that hcPcSP12 is also a marker protein of semi-granular cells during moult staging in *P. clarkii*. In addition, the hcPcSP12 was detected in heart and gills during postmoult mainly because of these tissues always full of hemocytes, while the fact that it was found in stomach and intestine might be due to contamination of hemocytes since the hemolymph in crayfish was ubiquitous (Li *et al*., 2010).

In penaeid species, the lymphoid organ (LO) has an important role in immuno-deficient against invading pathogens (Kondo *et al*., 1994; Martin *et al*., 1996; Van de Braak *et al*., 2004). The LO has a phagocytic function (Kondo *et al*., 1994), bacteriostatic effect (Burgens *et al*., 2005) and trapping ability to immobilize foreign material from the hemolymph before this material enters the open circulatory system. The organ also appears as a primary site for accumulation of foreign substances (Burgents *et al*., 2005). Furthermore, the lymphoid organ is believed to be a major site for viral degradation by forming lymphoid organ spheroid (LOS) cells (Anggraeni and Owens, 2000). This supposition was supported by the work of Rajendran *et al*. (2000) in that viral load in the lymphoid organ was higher compared to the other organs in infected prawns. The spheroid formation might be related to the tolerance of penaeid prawns to viral infection. In the case of survivors of a viral episode or in a chronic infection, the spheroids were formed when animals controlled the infectious agents or at least reacted to them (Rodriguez *et al*., 2003; Anontasomboon *et al*., 2008).

There is no study examining the relationship between the LOS cells and moulting cycle in penaeid prawns. Anggraeni and Owens (2000) suggested that if the spheroid cells accumulated in the hemal sinus of the LO, then the area of the spheroids would increase with increased animal size. However, these authors found that the area of spheroid varied during the life of the animals and there was no effect of prawn size on spheroid mass. Therefore, there must be a mechanism driving the removal of the spheroid cells. Since some immuno-defence factors of prawns are related to moulting cycle, it was hypothesized by Anggraeni and Owens (2000) that the spheroid cells were possibility disposed of during moulting (ecdysis). The ratio of the LOS cells generally decreased during intermoult stage. This implication could be made that there was elimination mechanism of LOS during the life span of the animals, as the animals for the three cycle stages came from the same farm. It seemed more likely that the LOS cells were eliminated gradually during the life span of the animals.

Anggraeni and Oweans (2000) suggested that LOS cells were possibly disposed during the moulting. Unfortunately, there is no evidence on the changes of spheroid cell to total cells during the life of the animals (Cheng *et al*., 2003; Liu *et al*., 2004; Rusaini and Owens, 2010). However, the present study indicated a moult effect on the abundance of the spheroid cells in the lymphoid organs. Thus, we suggest that the spheroid cells are gradually disposed of and it is more likely stimulated by moulting rhythmicity. What factors or processes are driving the variability of the spheroid cells within the moulting rhythms is unknown and needs further investigation. Despite this absence, the presence of moulting related patterns on the abundance of LOS cells in laboratory held animals suggest that this effect is endogenous and hereditary, without environmental cues being necessary.

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