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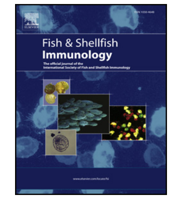

Full length article

The hematopoietic organ of *Macrobrachium rosenbergii*: Structure, organization and immune status

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ABSTRACT

The hematopoietic organ (HO) of the giant freshwater prawn *Macrobrachium rosenbergii* is a discrete, whitish mass located in the epigastric region of the cephalothorax, posterior to the brain. It is composed of hematopoietic cells arranged in a thick layer of numerous lobules that surround a central hemal sinus from which they are separated by a thin sheath. At the center of the sinus is the muscular *cor frontale*. The lobules extend radially outward from the sinus in three developmental zones. Basal Zone 1 nearest the sinus contains large hematopoietic stem cells with euchromatic nuclei that stain positive for proliferation cell nuclear antigen (PCNA). Zone 2 contains smaller, actively dividing cells as indicated by positive 5-bromo-20-deoxyuridine (BrdU) staining. Distal Zone 3 contains small, loosely packed cells with heterochromatic nuclei, many cytoplasmic granules and vesicles indicating that they will eventually differentiate into hemocytes and enter circulation. Three main arteries, namely the ophthalmic and the 2 branches of the antennary, connect the heart to the HO. Use of India ink and 0.1 µm fluorescent micro-beads injected into the heart revealed that the *cor frontale* could immediately remove foreign particles from hemolymph by filtration. Fluorescent beads were also detected in the hematopoietic tissue at 30 min after injection, indicating that it could be penetrated by foreign particles. However, the fluorescent signal completely disappeared from the whole HO after 4 h, indicating its role in removal of foreign particles. In conclusion, the present study demonstrated for the first time the detailed histological structures of the HO of *M. rosenbergii* and its relationship to hematopoiesis and removal of foreign particles from hemolymph.

1. Introduction

The immune system plays an important role in fighting against infection and can be classified as innate or adaptive [1]. Innate immunity is the first line of defense characterized by relatively non-specific host responses via immune cells and molecules, whereas adaptive immunity is characterized by specifically induced immune responses with a memory capability based on recognition of foreign proteins called antigens and production of host response proteins called antibodies. However, there is no evidence that invertebrates have adaptive immunity based on antibodies. Thus, it is widely believed that they rely solely on innate immunity and are incapable of a specific, adaptive immune response. However, recent results suggest that they may be capable of an adaptive immune response based on recognition and specific response to foreign viral RNA [2]. In crustaceans, hemocytes are the most important cells for the innate immune response, they act as a reservoir for humoral defense molecules and also participate in nodule formation, encapsulation and phagocytosis of pathogens [3].

Abbreviations: Br, brain; Cnt, connective tissue; Co, cor frontale; Cs, cor frontale sinus; Cu, cuticle; G, Golgi apparatus; Hl, hematopoietic lobule; HO, hematopoietic organ; Hpt, hematopoietic tissue; Hr, heart; Hs, hemal sinus; Mi, mitochondria; Mu, muscle; N, nucleus; Op, ophthalmic artery; RER, rough endoplasmic reticulum; Ro, rostrum; St, stomach; V, vacuole

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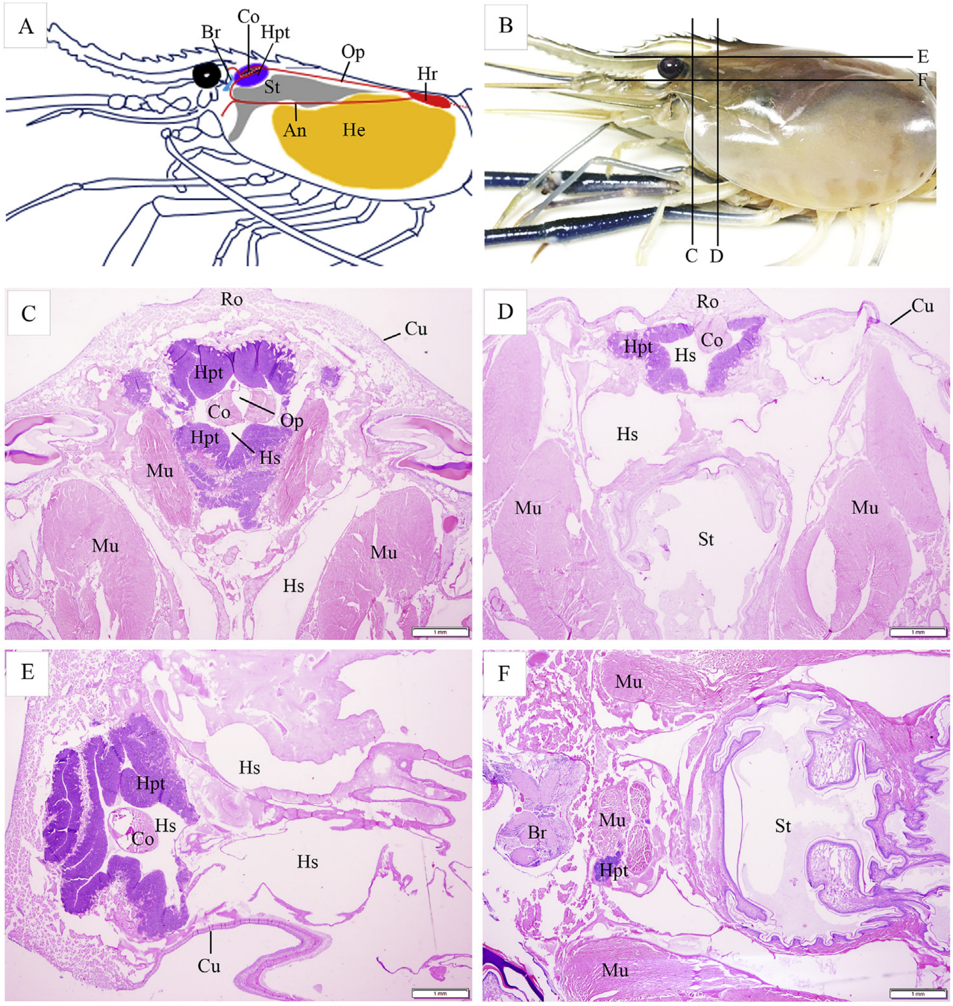

Fig. 1. Location of the HO and related structures in *M. rosenbergii.* (A) Diagram of the cephalothorax of *M. rosenbergii.* (B) Cephalothorax diagram showing lines of sectioning in C to F. (C–D) Coronal sections showing hematopoietic tissue (Hpt) surrounding the *cor frontale* (Co) and separated from it by a hemal sinus (Hs). (E–F) Horizontal sections showing Hpt located in the epigastric region under the base of rostrum. An, antennary artery; Br, brain; Cu, cuticle; Hr, heart; Mu, muscle; Ro, rostrum; Op, ophthalmic artery; St, stomach.

Although the life span of hemocytes as 129–156 h and decrease in number in response to infection, their population is homeostatically maintained by a compensatory, continuous renewal process via stem cells in hematopoietic tissue that is sometimes contained in a specifically identifiable organ [4–10]. The hematopoietic tissues of several crustacean species have previously been described: for the ridgeback prawn *Sicyonia ingentis* [10], the lobster *Homarus americanus* [11], the penaeid shrimp *Penaeus (Litopenaeus) vannamei* [12], *P. (Penaeus) monodon* [7] and *P. (Feneropenaeus) chinensis* [14], the crayfish *Pacifastacus leniusculus* [13] and the Chinese mitten crab *Eriocheir sinensis* [3]. Early studies on hematopoietic tissue focused on histological analysis for the presence of dividing cells and maturing hemocytes, while more recent molecular techniques such as gene expression have been applied for a more detailed study of its functions and hemocyte lineages [11,15]. The architecture and location of hematopoietic tissue in various crustacean species is variable. For example, in penaeid shrimp it is scattered in nodule-like areas in the cephalothorax while in the crayfish *P. leniusculus* it is localized in a discrete lobular hematopoietic organ (HO) that is highly vascularized by extensive branches of the ophthalmic arteries [10]. Lobular HO have been described in lobsters, crabs and crayfish. They are enveloped in a thin connective-tissue sheet covering the stomach in the epigastric region. Hematopoietic stem cells are situated in the apical border of each lobule, while young and maturing hemocytes are released into adjacent hemal spaces before entering into the circulation [11,13,16].

The classification of the hematopoietic cells has been performed through the comparison between the morphological characteristics of the cells to those of circulating hemocytes. Moreover, classification has also depended on cytochemical and functional features used to describe circulating hemocytes and applied when determining hemocyte lineages in some crustaceans. Additionally, proteins associated with cellular development have been applied for lineal determination such as proliferation cell nuclear antigen (PCNA). Accordingly, PCNA was used to study the proliferative property of hematopoietic stem cells of *P. leniusculus* [17]. Furthermore, the cellular progeny from the hematopoietic tissue can be tracked using bromodeoxyuridine (BrdU) labeling [9,18]. As such, the hyaline and granular lineages have been described in *H. americanus* with the large and small granular lineages as described in *P. monodon* and *P. leniusculus* [11,13,14,19]. Nevertheless, the identification of hematopoietic stem cells is yet to be definitively determined [14].

The giant freshwater prawn, *M. rosenbergii*, is an economically important species in Thailand and other Southeast Asian countries. Research on its immune system is primary concerned with disease control, which is reflected by the majority of studies focused on circulating hemocytes [20,21]. Therefore, information on the HO and its functions on hematopoiesis and immunity remain to be elucidated. In the present study, the organization and histological features of the HO

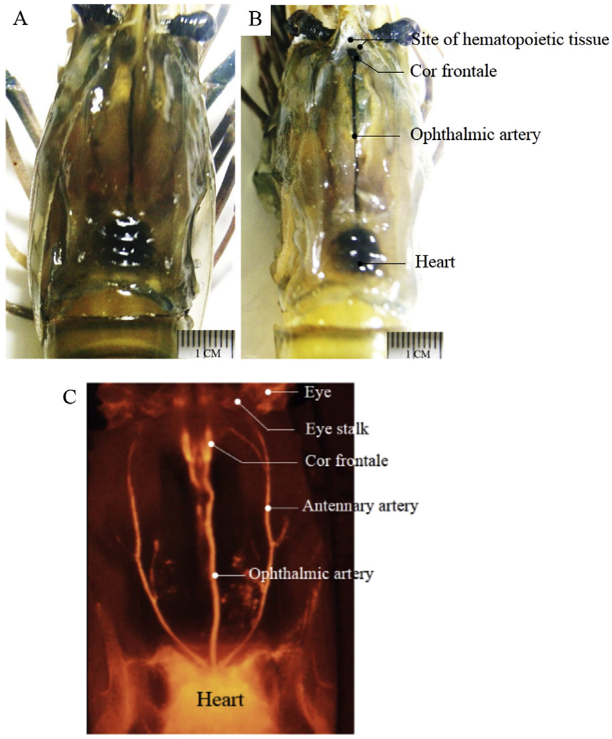

Fig. 2. Distribution of blood vessels to the HO. (A–B) Cephalothorax of shrimp after injection with India ink into the heart. Panel A shows the India ink in the heart and ophthalmic artery. Panel B shows the accumulation of India ink in the *cor frontale* area close to the hematopoietic tissue. (C) Cephalothorax of shrimp after injection with micro-fluorescent beads (0.1 μm) into the heart. The image shows the ophthalmic artery and branches of the antennal artery supplying the HO. The accumulation of fluorescent beads was also observed in the *cor frontale* area.

of *M. rosenbergii*, as well as morphology of hematopoietic cells, were investigated for the first time. Additionally, the ability of the HO to remove foreign particles from circulation was also examined.

2. Materials and methods

2.1. Experimental animals

Fully mature *M. rosenbergii* were obtained from a local farm in Suphanburi province, Thailand. They were maintained in 500 L tanks at ambient temperature (22–26 °C) with aeration and natural light and fed daily with the commercial food pellets at rate of 5% body weight per day. They weighed 32.76 ± 2.70 g with lengths ranging from 11.99 ± 0.24 cm (from eyestalk bases to telson) and were used for the investigation on the HO, (including histology, morphology and cell types) and for their response to injection of foreign particles. Smaller prawns weighing 0.84 ± 0.11 g and with lengths of 3.25 ± 0.20 cm were utilized for visualizing vascularization from the heart to HO using fluorescent micro-beads.

2.2. Histology by light microscopy (LM)

Prawns were anesthetized on ice and the cephalothorax region was excised and immediately fixed with Davidson's fixative for 24 h followed by processing and staining using hematoxylin and eosin (H&E) and Picro-Sirius Red staining for conventional LM [22]. Serial tissue sections were cut through horizontal and coronal planes. The microscope was a Leica DM750 model with a Leica ICC 50W digital camera. Additional samples for light microscopy were prepared from epoxy resin blocks as semi-thin sections prior to use of the electron microscope.

2.3. India ink injection studies

Since the HO was suggested to have a high vascular supply, India ink (70 μl) was injected into the heart of small, live animals (32.76 ± 2.70 g) using a 1-ml tuberculin syringe fitted with a 21G needle to aid in the localization of hematopoietic tissue. To visualize hemolymph vessels and the organ, the cephalothorax of the prawns were fixed and dissected 5 min after injection [10]. They were then processed for normal examination by LM.

2.4. Fluorescent micro-bead injection studies

For video capture, approximately 5–10 μl of a solution containing 0.1 μm red fluorescent micro-beads (FluoSpheres™ Carboxylate-Modified Microspheres 580/605, Invitrogen™) at concentration 1×10^6 beads/ml was injected into the heart of live prawns (0.84 ± 0.11 g) with a 1-ml tuberculin syringe fitted with a 26G needle. They were then observed live using an MVX10 Research Macro Zoom Microscope (Olympus) equipped with a video camera to record VDO files.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.fsi.2019.03.011.

In addition to samples processed for video analysis, other prawns (23.10 ± 0.97 g) were injected with 50 μl of fluorescence beads at a concentration of 1×10^7 beads/ml and sacrificed at 10 min, 30 min, 1 h, and 4 h after injection to be processed for examination of frozen tissue sections. Briefly, the dissected hematopoietic tissue was placed in embedding medium (O·C.T. Embedding Compound, Tissue Tek) and frozen followed by cutting and fixing in 4% paraformaldehyde for 20 min in dark chamber before washing 2 times with PBS. Then, the tissue sections were stained with TOPRO-III (Invitrogen™) to visualize the nucleus before viewing and photographing using an FV10i-DOC confocal laser scanning microscope (Olympus).

2.5. Electron microscopy

Dissected HOs were immersed in 2.5% glutaraldehyde with 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4 °C. After being washed with 0.1 M PB, the tissues were post-fixed in 1.0% osmium tetroxide in 0.1 M PB at 4 °C. Then the tissues were washed with the buffer, dehydrated in ethanol series, and subsequently processed for embedding in Epon 812. Semithin sections were prepared at 500 nm thickness, stained with Toluidine blue, and observed under a light microscope. Ultrathin sections were cut at 60–90 nm thickness and collected on copper grids for staining with uranyl acetate and lead citrate. The stained sections were viewed at 100 kV using a Hitachi H-800 transmission electron microscope.

For scanning electron microscopy (SEM), the tissues were dried in a Hitachi HCP-2 critical point drying machine under liquid CO_2 and coated with platinum and palladium in a Hitachi E – 120 ion sputter device. Then, the specimens were observed using a Hitachi scanning electron microscope S-2500.

2.6. PCNA and BrdU staining

To identify hematopoietic tissue stem cells, paraffin embedded tissue sections were stained with proliferating cell nuclear antigen (PCNA). Briefly, the sections were rehydrated through decreasing concentrations of ethanol before endogenous peroxidase and free aldehydes were blocked by incubation in 1% H_2O_2 in 70% ethanol for 30 min followed by 1% glycine in PBS for 5 min. Antigen retrieval was then performed by boiling the slides in sodium citrate buffer (pH 6.0) for 15 min. The tissue was subsequently permeabilized with 0.5% Triton X-100 in PBS for 8 min. After washing with PBS containing 0.1% Tween 20 (PBST), the tissues were incubated with blocking buffer

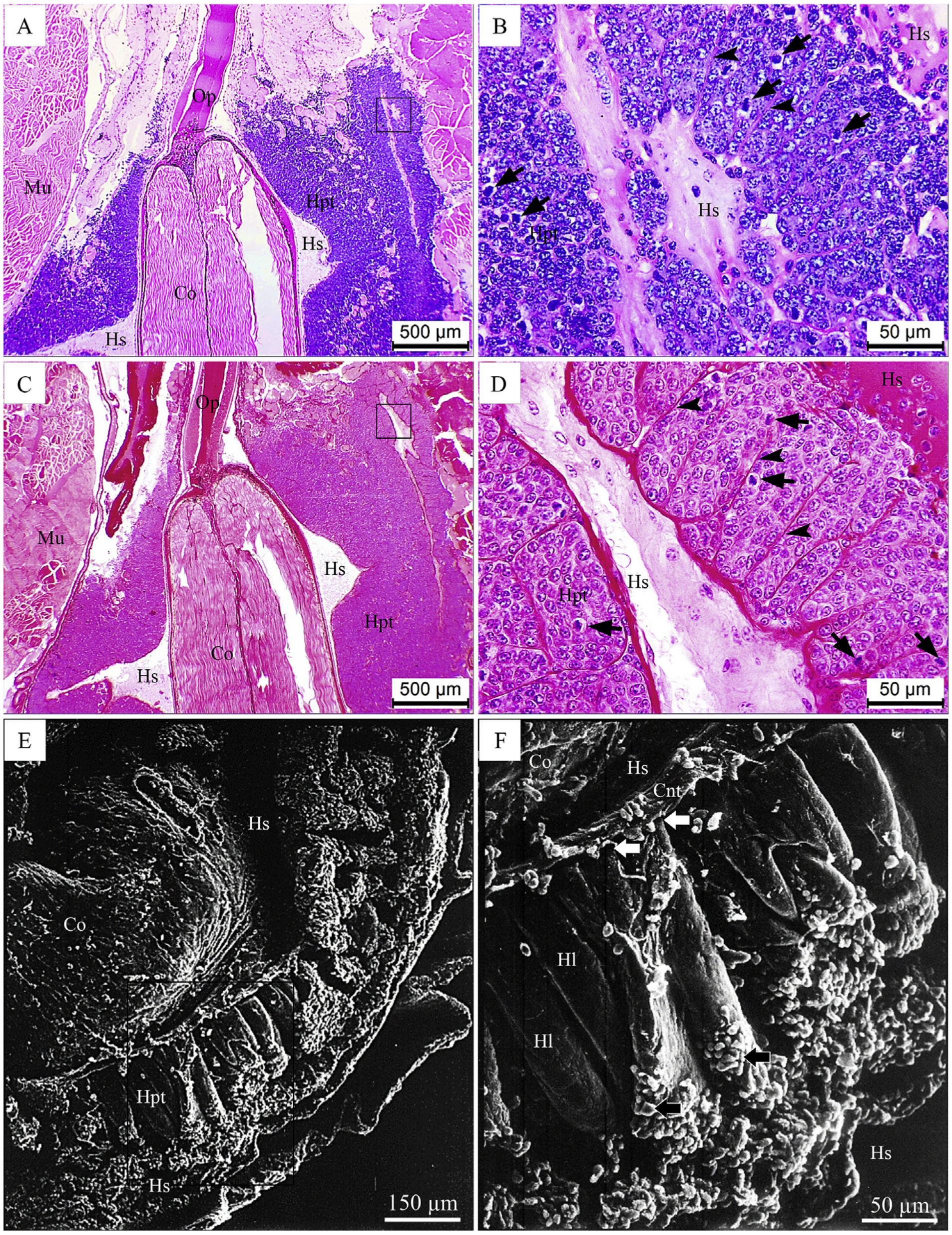

Fig. 3. Histology of the HO. (A–B) H&E stained sections showing hematopoietic tissue (Hpt), the hemal sinus (Hs) and *cor frontale* (Co). Hpt cells were arranged in lobules (Hl) surrounded with a connective tissue sheath on the proximal sinus side. The Co comprised two bundles of skeletal muscular tissue surrounded by Co sinus. The ophthalmic artery (Op) was also observed at the anterior end of Co. Hpt lobules bounded by a connective tissue sheath adjacent to the Hs but diffuse on the distal Hs side. Hpt cells with mitotic figures (black arrows) were usually found in the mid region of the Hl. Arrow heads show the connective tissue wall of the Hpt adjacent to the sinus. (C–D) Sirius Red stained sections showing the connective tissue sheath (arrow head) of the Hpt next to the sinus. (E–F) Scanning electron micrographs showing the relationship of the Hpt, Co and Hs. White arrows indicate the connective tissue sheath of the Hpt. The diffuse distal side of the Hpt lobules showing hematopoietic cells that have been released into the Hs (black arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(0.3% Triton X-100, 5% normal donkey serum in PBS) and incubated with goat anti-PCNA (Santa Cruz Biotechnology. Inc; 1:300) overnight at 4 °C. After washing two times with PBST, the sections were incubated with biotin-SP-affinity pure conjugated donkey anti-goat antibody (Jackson Immuno Research Laboratory, Inc; 1:500) for 1 h at room temperature. The sections were washed and incubated with horseradish peroxidase for 1 h at room temperature. After washing two times with PBST, the signal of PCNA was detected with peroxidase substrate kit (VECTOR NovaRED, USA) and observed by LM.

Similarly, 5-bromo-20-deoxyuridine (BrdU) was used to detect cell proliferation indicated by DNA synthesis by injecting 5 mg of BrdU per 100 g of body weight into small shrimp followed by sacrifice 4 h later. The HO was then removed and processed for immunohistochemistry as described above. The sections were incubated with mouse anti-BrdU monoclonal antibody (1:50) overnight at room temperature and incubated with HRP conjugated goat anti-mouse antibody (1:300) for 2 h at 37 °C. BrdU expression was detected using a peroxidase substrate kit (VECTOR NovaRED, USA) and observed by LM.

3. Results

3.1. Location of the HO and its relation to the cor frontale and major vessels

The HO of *M. rosenbergii* was situated in the anterior part of cephalothorax under the base of the rostrum, adjacent to the anterior end of the cardiac stomach and posterior to the brain (Fig. 1). The tissue was removed and measured by using a ruler. It is a whitish, ellipsoid body of tissue approximately 6.20 ± 1.21 mm in length, 5.0 ± 1.29 mm in width, and 4.2 ± 0.30 mm in thickness. Hematopoietic tissue was not found in any other location. Frontal and horizontal sections of the H&E-stained cephalothoraxes showed a purple-stained layer of hematopoietic tissue lobules covered by a thin sheath and surrounding a hemal sinus that separated it from a muscular structure called the *cor frontale* at the center of the sinus (Fig. 1A–F). When India ink or fluorescent micro-beads were injected into the heart of living animals to trace the hemolymph vessels, two main vessels were revealed, including a single ophthalmic artery and a pair of antennary arteries projecting from the heart into the HO. Moreover, accumulations of injected India ink particles and micro-fluorescent beads were

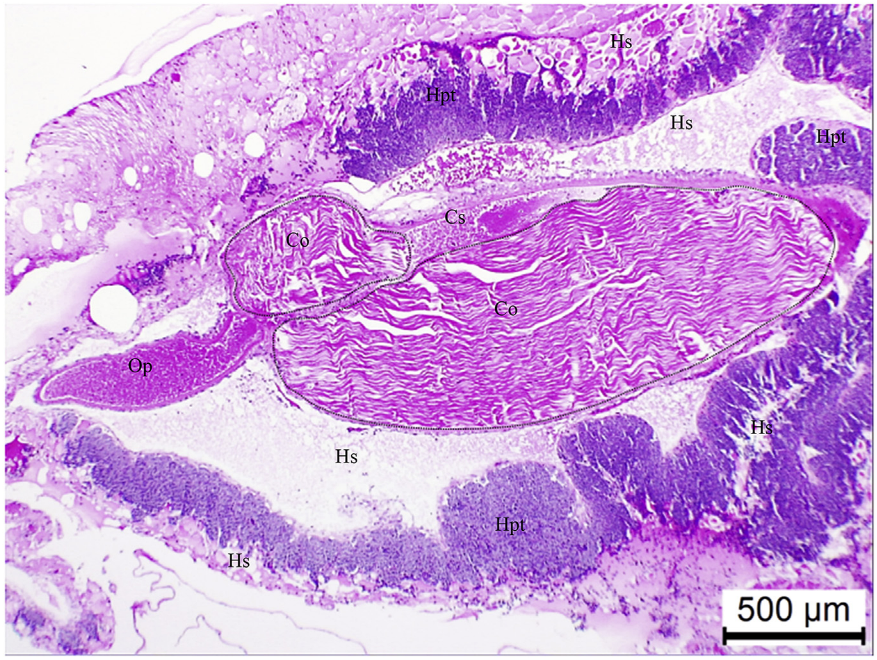

Fig. 4. H&E stained sections showing the *cor frontale* (Co) structure. It was composed of two strips of skeletal muscle wrapped by the dilation of the ophthalmic artery (Op) which formed the *cor frontale* sinus (Cs). A hemal sinus (Hs) separates the sheathed hematopoietic tissue (Hpt) lobules from the *cor frontale* wall.

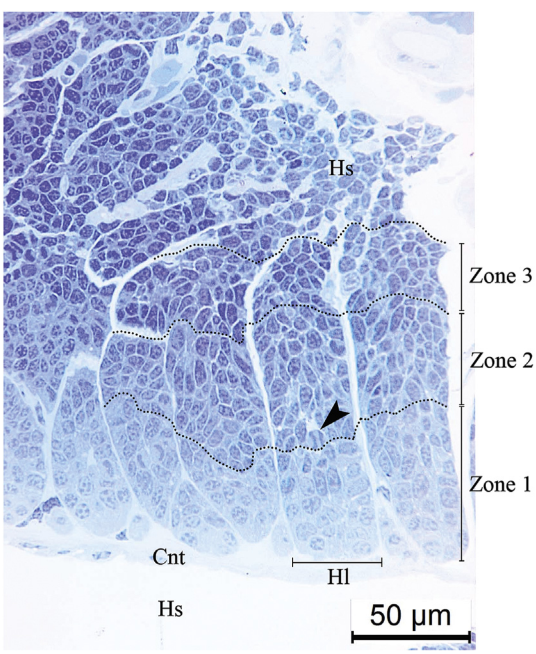

Fig. 5. Semithin sections stained with Toluidine blue showing different degrees of cell differentiation in hematopoietic lobules (Hl). Based on the characteristics of the cells and color shade after staining, Hl could be divided into three zones. Zone 1 is the connective-tissue-sheathed region next to the proximal sinus and composed of large cells with light staining. Zone 2 is the middle region of the lobule occupied by smaller cells with moderate staining. Zone 3 is the distal, diffuse end of the lobule adjacent to the hemal sinus (Hs). Cells in Zone 3 are reduced in size with dark staining. Cnt, connective tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

detected within the *cor frontale* structure. (Fig. 2A–C and Supplementary VDO clip).

3.2. HO histology

Although the *cor frontale* was found to be surrounded by hematopoietic tissue, the two tissues were separated from one another by a hemal sinus that contained some hemocytes (Fig. 3A and B). The hematopoietic tissue was arranged in somewhat columnar lobules of densely packed hematopoietic cells that were separated from the central sinus and its narrow branches by a thin sheath (Fig. 3C and D). The connective tissue sheath separating the lobules from the sinus was clearly revealed by Sirius Red staining. By contrast, the distal side of the hematopoietic tissue layer appeared diffuse, open and directly linked to the surrounding connective tissue and hemal sinus (Fig. 3B, D). The central, sinus and its inter-lobular extensions usually contained mature hemocytes. Scanning electron micrographs of the HO clearly showed the connective tissue sheath covering the hematopoietic lobules on the proximal sinus side and its open-endedness on the distal side (Fig. 3E and F).

The *cor frontale* was comprised of two strips of striated muscle flanked by the ophthalmic artery at their anterior and posterior ends. However, intercalated discs characteristic of cardiac muscle were also observed in one section of the *cor frontale* examined in this study (data not shown). The wall of *cor frontale* was formed by a dilation of the ophthalmic artery that enclosed the muscular structure within its wall. Double layers of lining tissue could be observed in the *cor frontale* such that a sinus filled with hemolymph was formed between the two layers (Fig. 4). This *cor frontale* sinus was connected to the ophthalmic artery at its anterior and posterior ends.

3.3. HO cell types

Within the hematopoietic tissue lobules, there was a gradation of developing hematopoietic tissue cells. Although there was no precise line of demarcation between the developing types, it was possible to divide the lobules roughly into three parts based upon the locations of cells showing differences in cellular morphology and degrees of

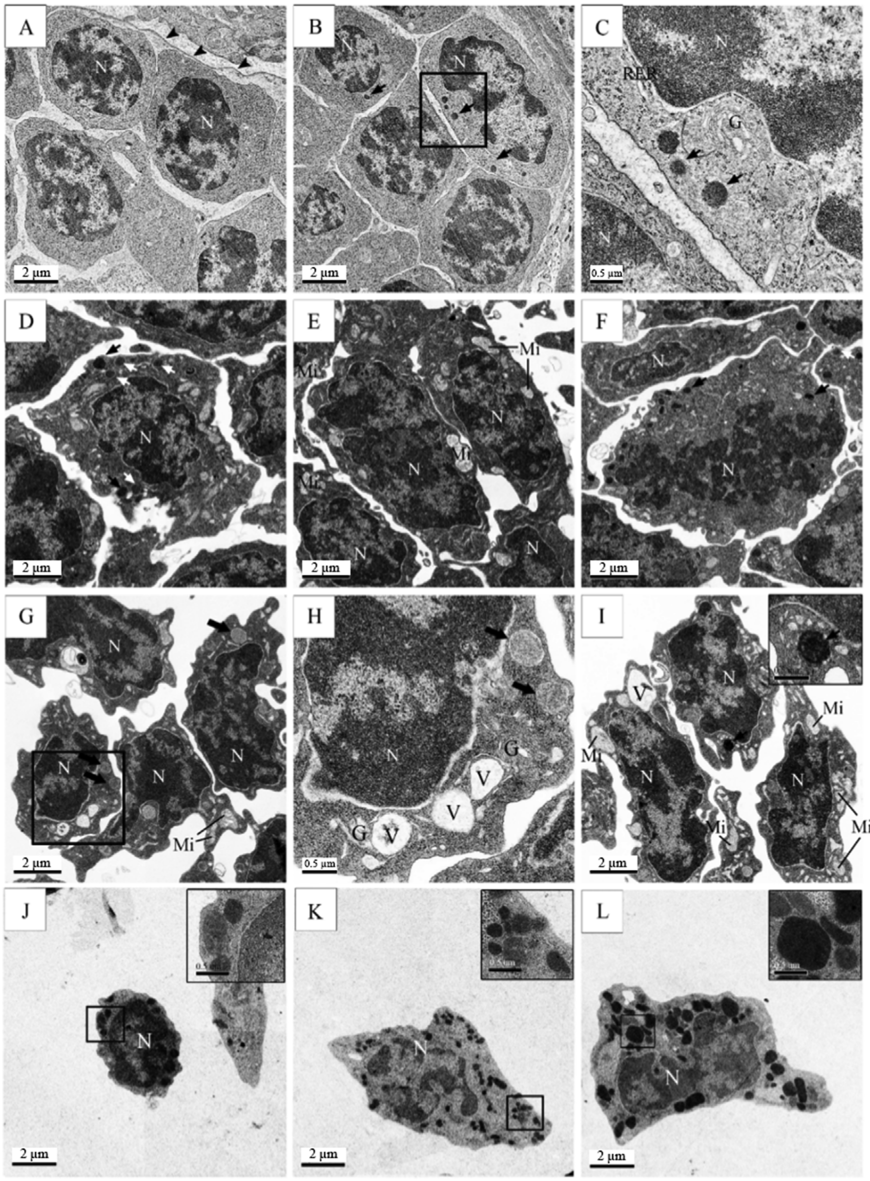

Fig. 6. TEM micrographs showing morphological characteristics of hematopoietic cells and mature hemocytes. (A–C) Hematopoietic cells in Zone 1 covered with a sheath that separates them from the central hemal sinus. Most of the cells are closely attached to the sheath (arrow heads) and lack electron-dense granules (A). Some cells in this zone also show electron-dense granules in their cytoplasm (B, arrows). Panel C shows higher magnifications of the area in the box in panel B, revealing granules (arrows), rough endoplasmic reticulum (RER) and a Golgi apparatus. (D–F) Hematopoietic cells in Zone 2, located the middle region of the lobule. The hematopoietic cells show both electron-dense granules (black arrow) and intermediate electron-dense granules (white arrow) (D). However, cytoplasmic granules are absent in some cells (E) and many cells show characteristics of mitosis (F). (G–I) Hematopoietic cells in Zone 3, located at the distal, diffuse end of the lobule. The cells contain granules with less electron density (thick arrow); vacuoles (V) can be observed in the cytoplasm (G, H). Panel H shows a higher magnification of the box in panel G. Some cells contain numerous mitochondria (Mi) and electron-dense granules (I, inset and black arrow). (J–L) Circulating hemocytes in hemolymph. Oval-shaped hyaline cells showing a high nucleo-cytoplasmic ratio (J). Cytoplasm containing intermediate and electron-dense cytoplasmic granules (J, inset). Small granular hemocytes containing numerous intermediate and electron-dense small granules composed (K, inset); some vacuoles can be observed. Large granular hemocytes showing numerous intermediate and electron-dense cytoplasmic granules of variable size (L, inset). N, nucleus.

Table 1
Hematopoietic cells size in different parts of the hematopoietic lobules.

Hematopoietic cell	Size (µm)	
	Length (Average ± SD)	Width (Average ± SD)
Basal part (Zone 1)	10.41 ± 2.50	8.89 ± 1.87
Middle part (Zone 2)	12.71 ± 2.41	4.59 ± 0.88
Distal part (Zone 3)	7.17 ± 1.84	5.07 ± 1.10
Hemal sinus	6.48 ± 1.59	5.18 ± 0.88

differentiation (Figs. 5 and 6). The proximal end of the lobules adjacent to the inner hemal sinus (Zone 1) contained large, round to oval-shaped, tightly packed cells (size = 10.41 ± 2.50 µm length and 8.89 ± 1.87 µm width) (Table 1) with euchromatic nuclei (Fig. 5). Some of these cells contained small, rounded, and electron-dense granules within the cytoplasm while some showed no granules (Fig. 6A and B). Cytoplasmic organelles included rough endoplasmic reticulum, free ribosomes and Golgi apparatuses (Fig. 6C). This group of cells was stained lightly with Toluidine blue in LM images and also in TEM images. The middle part of the lobules (Zone 2) was occupied by smaller cells when compared to those in the Zone 1 (Fig. 5). The cells contained round to more elongated, oval-shaped nuclei and a small amount of cytoplasm. The cytoplasm contained numerous mitochondria. Cytoplasmic granules were observed in some cells and had two different characteristics, those that were electron-dense and those of intermediate electron density (Fig. 6D–F). Many mitotic cells could be observed in this area (showing in Fig. 3B, D and Fig. 6F). In contrast, Zone 3 (the distal lobule end) contained more loosely packed cells (Fig. 5) that were comparatively smaller than that in the Zone 2. Most of the cells contained oval to round nuclei showing higher degrees of heterochromatin condensation than in Zones 1 and 2 (Fig. 6G–I). In addition, mitochondria were prominently observed and some intracytoplasmic vesicles were also evident (Fig. 6H). It was likely that these were nacent hemocytes that would be released into the adjacent hemal space and enter circulation. In the region of the hemal sinus near the end of Zone 3, the majority of hemocytes had small heterochromatic nuclei like the cells in the distal part of the lobule.

To compare hematopoietic cells with mature hemocytes, circulating hemocytes were also observed by TEM. There were 3 types of hemocytes including hyaline, semi-granular, and large granular cells (Fig. 6J-L). Hyaline cells were the smallest cells showing oval (Fig. 6J) or spindle 2D shapes (data not show). They contained oval or elongated

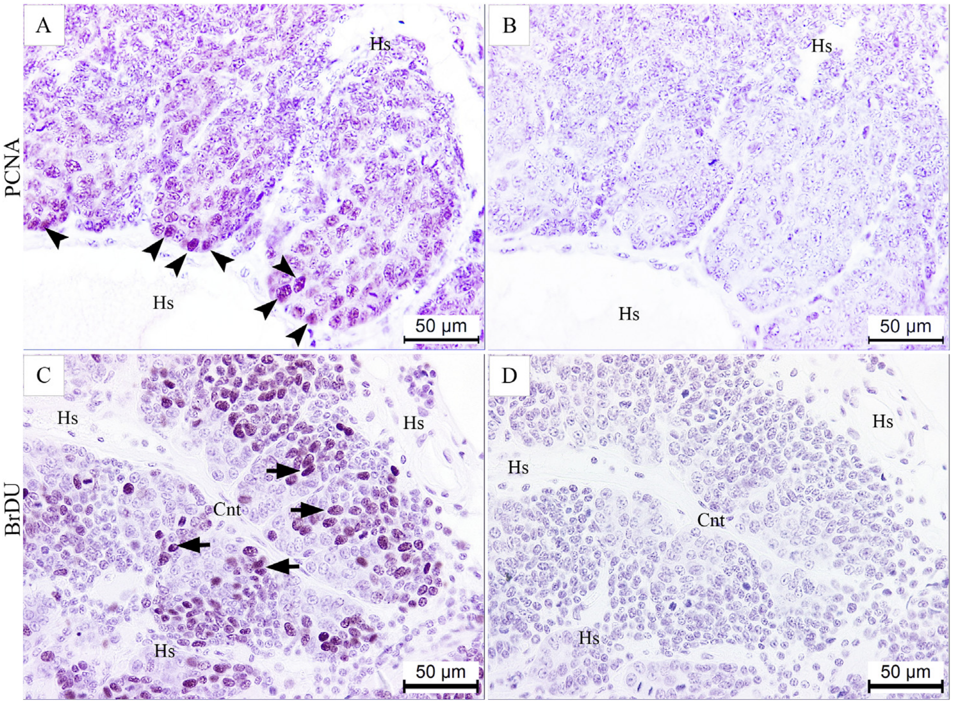

Fig. 7. Micrographs showing cell proliferation markers in the HO. (A–B) HO sections immunostained with PCNA. PCNA-positive cells can be seen in the basal part of the hematopoietic lobules (A, arrow heads), while the control section shows only background staining (B). (C–D) HO sections immunostained with BrdU. Most BrdU-positive cells can be seen in the middle region of the lobules (C, arrows), while the control section shows negative staining (D). Cnt, connective tissue; Hs, hemal sinus.

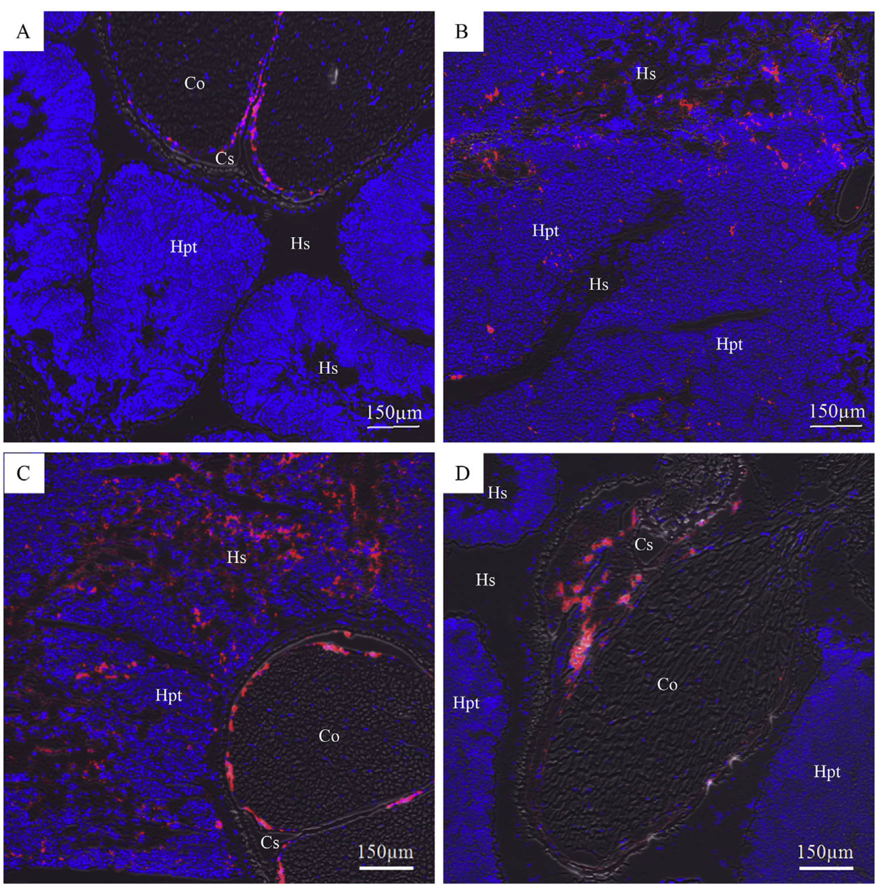

Fig. 8. Confocal laser scanning micrographs show the distribution of micro-fluorescent beads in the hematopoietic tissue (Hpt) after injection into the heart. (A) Micro-fluorescent beads seen only in the *cor frontale* sinus (Cs) immediately after injection into the heart. (B) Micro-fluorescent beads observed in the hematopoietic lobules (Hl) within 30 min after injection, particularly in the hemal sinus (Hs). (C) Numerous micro-fluorescent beads seen within the hematopoietic tissue (Hpt) 1 h after injection. (D) At 4 h after injection, beads are absent in the Hpt but they still remain in Cs surrounding the *cor frontale* (CO).

nuclei and showed a high nucleo-cytoplasmic ratio. The cytoplasm contained few granules with differing degrees of electron density (Fig. 6J, inset). Semi-granular cells were variable in shape and size, but mostly large oval-shaped cells were observed. They contained variable numbers of prominent, small electron-dense granules in the cytoplasm (Fig. 6K). Large granular cells were the largest cells among three hemocyte types. Their cytoplasm contained numerous, large sized, electron-dense and less electron-dense granules (Fig. 6L). Additionally, both semi-granular and large granular cells exhibited a few electron translucent vesicles in their cytoplasm (Fig. 6K-L). From the ultrastructural observation of hematopoietic cells and circulating hemocytes, it was indicated that the HO contained various stages of developing hematopoietic cells but not mature hemocytes. Therefore, the immature hematopoietic cells appeared to continuously develop into mature hemocytes after release into hemal circulation.

3.4. Cell proliferation in the HO

The proliferation of hematopoietic cells was determined using PCNA and BrdU staining. PCNA positive cells were prominently found in Zone 1 of the HO lobules (Fig. 7A and B) characterized by cells showing large euchromatic nuclei and a large amount of cytoplasm. These cells can be classified as hematopoietic progenitor cells (i.e., stem cells). By contrast, BrdU incorporation was localized mostly in Zone 2 and Zone 3 (i.e., the mid and distal regions of the lobules (Fig. 7C and D).

3.5. Foreign particle accumulation within the cor frontale sinus

When fluorescent micro-particles (0.1 μm size) were injected into the heart to evaluate the function of the *cor frontale*, cryosections of the hematopoietic organ collected at various intervals after injection, revealed by confocal microscopy that the fluorescent micro-particles collected within the *cor frontale* sinus immediately after injection and remained there for up to 4 h (Fig. 8A). This suggested a hemolymph filtration role for the *cor frontale*. Infiltration of micro-beads was also detected in the hematopoietic tissue at 30 min after injection (Fig. 8B). This may suggest that the route of micro-particle entry into the hematopoietic tissue was probably via the hemal sinus, rather than via the *cor frontale* sinus and cavity. The highest signal intensity of the fluorescence beads inside the hematopoietic tissue was at 1 h post injection (Fig. 8C). However, it disappeared completely by 4 h post-injection at which time it could still be clearly detected in the *cor frontale* sinus (Fig. 8D). These results suggested that the hematopoietic tissue was susceptible to infiltration by foreign particles but could eliminate them within 4 h.

4. Discussion

In invertebrates, hemocytes provide a rapid cellular immune response to foreign particles [6]. Thus, hematopoiesis is crucial for replacement of circulating hemocytes lost during infection or as a result of the normal process of homeostasis [8]. Recently, hematopoietic tissue and hematopoiesis in crustaceans has become the focus of more studies to providing new insights into the cellular immunity with the aim of improving disease control in the aquaculture industry. A critical, initial step in the process is to determine the location and structure of the hematopoietic tissue for each cultivated species [14]. This work showed by histological analysis that the hematopoietic tissue of *M. rosenbergii* is a discrete lobular organ situated on the dorsal side of stomach beneath the base of the rostrum and posterior to the brain. In contrast, in some species such as *P. monodon, S. ingentis*, and *P. chinensis* [7,10,14] the hematopoietic tissue is scattered in the dorsal region of the cephaloghorax and at the base of the maxillipeds extending towards the antennal gland. In *M. rosenbergii* the hematopoietic tissue was instead organized into lobules surrounding an inner hemal sinus and a central *cor frontale* that comprised a distinct HO. The hematopoietic lobules were surrounded by connective tissue that formed a sort of base for the hematopoietic lobule layer and the collagen fibers that containing fibroblast-like cells to separate the lobule layer from the proximal sinus [11]. In *M. rosenbergii*, the thick basement membrane was found to be associated with undifferentiated hematopoietic progenitors, whereas the thin basement membrane was associated with the hemocyte differentiation zone. These characteristics have also been shown in the HO of *Drosophila melanogaster*. The extracellular matrix protein Trol has been suggested to regulate HO organization and hemocyte differentiation [23].

Although it is difficult to classify cell lineages from ultrastructural observations, there were striking patterns in the organization of the hematopoietic tissue cells. The hematopoietic cells in *M. rosenbergii* could be classified into three groups based on the morphology and degrees of cellular differentiation. The characteristics of these cells were similar to those described in the blue crab *Callinectes sapidus* [24]. The first group was the cells in the basal part of the lobule (Zone 1) associated with thick connective tissue. These cells contained large euchromatic nuclei with or without cytoplasmic granules. Moreover, they were positive to PCNA staining that is a specific biomarker for hematopoietic progenitor cells showing active mitotic activity [17,25]. These PCNA positive cells were considered as the undifferentiated hematopoietic stem cells that give rise to all mature hemocytes similar to those described in *C. maenus* and *P. leniusculus* [13,16]. The second group consisted of cells that were smaller in size and located in Zone 2. Most of the cells contained condensed chromatin and numerous mitochondria. Expectedly, mitotic cells were often observed in this group. The actively dividing property of the cells was also supported by positive BrdU staining. BrdU is known to incorporate into DNA during the S-phase of dividing cells and its passage to their daughter cells [26]. Positive BrdU-stained cells were observed intensively in the middle region of the lobules (Zone 2) but were also seen scattered in distal Zone 3. The cells in Zone 3 were more dispersed and closely related to those seen in the hemal sinus. Altogether, these observations suggested that stem cells were located in Zone 1 located close to Co and were progressively developed further in the distal Zones 2 and 3 before finally being released into the hemal sinus as immature hemocytes. Therefore, immature hemocytes can be found in the hemolymph of *M. rosenbergii* [27] and maturation proceeds further in the hemal circulation [11,12].

In general, the hematopoietic tissue in the HO is composed of both stem cells and cells undergoing differentiation, hence requiring a higher supply of nutrients. Accordingly, there were extensive vessels entering and supplying the HO. In *S. ingentis*, the ophthalmic artery is branched and forms contortions within its hematopoietic tissue [10].

By injection of India ink and micro-fluorescent beads we showed the antennary arteries supplying the HO of *M. rosenbergii*. At the anterior end of ophthalmic artery before its branching, an associated structure called the *cor frontale* was found, similar to that described in the HO of other decapod crustaceans [28]. The *cor frontale* was made up with two strips of skeletal muscles that originated from tendons outside the ophthalmic artery. The connective tissue covering the *cor frontale* was continuous with part of the ophthalmic arterial wall. It has been reported to act as an accessory heart [29]. Hemolymph was observed to enter the space between the *cor frontale* halves and to have originated from the ophthalmic artery to form the *cor frontale* sinus before leaving the sinus and joining the distal part of the ophthalmic artery to supply the head region [29]. Interestingly, India ink dye and fluorescent micro-beads (0.1 μm in size) were trapped within the *cor frontale* sinus immediately after they were injected into the heart. This result indicated that the *cor frontale* sinus could act as a small reservoir of the hemolymph during its transport from the heart and that it could help in the pumping of hemolymph to the head region where the brain, eyestalks, and antennal glands are located. Moreover, the accumulation of fluorescent beads in the *cor frontale* sinus could still be observed more than 4 h after injection.

In Panaeid shrimp, for example *P. monodon,* entrapment of foreign particles was observed in the lymphoid organ before they go from the arterial system into the open circulatory system [30]. However, lymphoid organ, which is an important site for immune activation against invading pathogens, has been found exclusively in penaeid prawns and may not exist in the freshwater prawn [31]. In this way, the *cor frontale* could act as a filter or create an effective trap of micro-fluorescent beads or other foreign particles by rapidly removing them from circulation. In addition, micro-fluorescent beads could also be detected in the hematopoietic tissue at 30 min suggesting that the hematopoietic tissue might be accessible to foreign pathogens. The clearance of micro-fluorescent beads from the hematopoietic tissue within 4 h also suggested that it may play a role in the clearance of foreign antigens. It has been previously reported in the shrimp *S. ingentis* that once bacteria are within the hematopoietic tissues, they adhere to and are phagocytosed by small-granular hemocytes [32]. Recently, the hematopoietic tissue of *M. rosenbergii* has been shown to respond to *Mr*NV infection by up-regulation of many immune-related genes [33].

5. Conclusions

These constitute for the first time a general view of the histological structures of the HO of *M. rosenbergii* and its roles in hemocytes production and its possible role in clearance of foreign particles. This evidence suggested that HO might play a crucial role during infection and immune response. However, this possibility requires further investigation for providing valuable information for effective strategies to improve shrimp health status or prevent microorganism infection.

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