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Morphological Observations on Haemo-Lymphocytes in *Oncomelania hupensis* (Gastropoda: Pomatiopsidae)

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Abstract.- Information concerning morphological and functional basis of defense cells of *Oncomelania hupensis* (Gastropoda: Pomatiopsidae), intermediate host of schistosomiasis parasite, *Schistosoma japonicum*, observed through optical microscopy (light and fluorescence); scanning electron microscopy (SEM); and transmission electron microscopy (TEM) is presented. The results showed that nucleus of *O. hupensis* haemo-lymphocytes were round or oval in shape with rough surface, and contained very high density of granules; the nucleus was encircled by cytoplasm possessing pseudopodia. The haemo-lymphocytes of *O. hupensis* were divided into three sizes groups: large, medium, and small based on the diameter of their nucleus. Large haemo-lymphocytes had a diameter range of 8.1 - 8.6 µm, medium 5.5 - 6.0 µm, and small 4.2 - 4.7 µm. The haemo-lymphocytes could possess pseudopodia for adhering to, or embracing Sheep Red Blood Cells (SRBC), and after incubation, haemo-lymphocytes were surrounded by numerous SRBC. The present study provides precise information concerning nuclear size of haemo-lymphocytes of *O. hupensis* and confirms some previous reports on haemocytes of *O. hupensis*, thereby opening the door for further research into the structure and function of haemo-lymphocytes in *O. hupensis.*

Keywords: *Oncomelania hupensis*, haemo-lymphocytes, morphology, adherence.

INTRODUCTION

Oncomelania hupensis (Gastropoda: Pomatiopsidae) is a unique intermediate host of the schistosomal parasite, *Schistosoma japonicum*, in eastern and southeastern Asian countries, particularly in China, where it still remains an important public health problem (Zhou *et al.*, 2010). For example, *O. hupensis* is widely distributed in many schistosomiasis epidemic areas of China, such as lake/marshland and all water channel areas of Yangtze River. These snails can react through many pathways in terms of penetration of miracadia of *S. japonicum.* The haemocytes of mollusks play an important role in their internal defense system (Monteil and Matricon-Gondran, 1991).

The nomenclature of haemocytes of Mollusca has not been standardized as yet. Most of the morphological studies on haemocytes of *Biomphalaria* sp. (Sminia and Barendsen, 1980; Cavalcati *et al.*, 2012; Noda and Loker, 1989) have reported haemocyte cells of at least two categories: granulocytes and hyalinocytes (agranulocytes). In *Oncomelania* sp., two types of haemocytes have been reported in *O. nosophora* snail, with type I (macrophage-like) and type II (lymphocytes-like) cells (Sasaki *et al.*, 2003). However, Zhang *et al.* (2007) described four types of *O. hupensis* haemocytes: round cells with filiformfilopodia, acidophilic round cells, basophillic round cells without filiformfilopodia, and spindle cells.

More recently, Tang *et al*. (2012) described haemo-lymphocytes (lymphocytes) as a mollusk haemocytes category and illustrated the reaction of haemo-lymphocytes to *S. japonicum* larvae in *O. hupensis* pre-infected with *Exorchis* trematode; these authors also reported three sizes, small, medium, and large, of haemo-lymphocytes of *O. hupensis*, with diameter range of 4.2-4.7 µm, 5.5-6.0 µm, and 8.1-8.6 µm, respectively. Tang *et al.* (2012) also showed that haemo-lymphocytes play an important role in the defense mechanism (immune

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system) of *O. hupensis* from parasites. Nevertheless, haemo-lymphocytes in *O. hupensis* need more investigation because at present, even proper systematics, morphology, and structural information concerning haemo-lymphocytes in *O. hupensis* is generally lacking.

In the present study, morphological and structural characterization of haemo-lymphocytes in haemolymph of *O. hupensis* were investigated. In particular, morphological and functional bases of defense cells mechanism of *O. hupensis* by using optical, fluorescence and electron microscopy were explored.

MATERIALS AND METHODS

Snail collection and rearing

Oncomelania hupensis were bred in the laboratory at the Schistosomiasis Control Station, Xingzi, Jiujiang City, Jiangxi Province, China, during May 2012. The stock snails were field-collected from Poyang Lake, Jiangxi Province, China. The field-collected snails were tested twice for any natural infections using the shedding method, and no parasites or pathogens were found. The snails were maintained on a wet rough straw paper containing antibiotics (200 U/ml penicillin and 200 µg/ml streptomycin) (HyClone Laboratory, Inc., Utah, USA) at 24±1°C for a few days.

Haemo-lymphocytes collection for morphological investigations by light and fluorescent microscopy

Haemolymph of *O. hupensis* was collected by gently crushing the snail shell after cleaning any water on snail surface and foot with absorbent tissue paper. Haemolymph from randomly selected 12 snails was pooled. A 10 µl suspension; haemolymph/saline-antibiotic (SA) solution pH 7.2 (1.16M NaCl, 0.15M Na_2HPO_4, 0.15M KH_2PO_4, 100 U/ml penicillin and 100 µg/ml streptomycin) (Sasaki *et al.*, 2003) was smeared on a glass slide, and then stained by using Wright-Giemsa stain (Jain, 1986). Morphology of haemo-lymphocytes was observed under a light microscope (Olympus BX51, Japan). Nucleus diameter of randomly selected 120 haemo-lymphocyte cells was measured. Another 10 µl of haemolymph suspension was placed on a glass slide and stained with DAPI stain (4, 6-diamidino-2-phenylindole) (Kapuscinski, 1995) to observe morphology of the nucleus and to confirm appearance (shape and structure) of nucleus of haemo-lymphocytes by using fluorescent microscopy.

Scanning electron microscopy (SEM) investigations

For SEM study, *O. hupensis* were killed and haemolymph was collected as described previously.The haemolymph was then processed through a series of SEM procedures on a glass slide, including fixing for 2 hours in 2.5% glutaraldehyde (phosphate buffered preparation), and rinsing three times (each time for 15 minutes) with 0.1M phosphate buffer. Glass slides were post-fixed with 1% Osmium tetroxide (OsO_4) fixative for 2-3 hours, and then washed with 0.1M phosphate buffer three times, each time for 15 minutes. Samples were dehydrated through an ethanol series, then processed to critical point drying and coating with gold (Au). *Oncomelania hupensis* haemo-lymphocytes were examined using an SEM (JSM6390LV, JEOL Ltd, Tokyo, Japan).

Transmission electron microscopy (TEM) investigations

For TEM investigation, *O. hupensis* were killed by removing tail portion of the snail at first, and then the snail's shell was gently crushed. The snail body was carefully removed and immediately fixed (for one hour at 4°C) using 2.5% glutaraldehyde (phosphate buffered preparation). After fixing, the tissue was rinsed three times with 0.1M phosphate buffer, each time for 15 minutes, and then post-fixed with 1% OsO_4 fixative for 2 - 3 hours and then dehydrated through an ethanol series, and embedded via propylene oxide in Taab epoxy resin (Taab Ltd., Aldermaston, UK). Thereafter, using a suitable microtome for ultra-thin sectioning, 60 - 80 nm thick sections of the fixed tissue were cut and double-stained with 3% uranyl acetate, followed by lead citrate, and then examined using the TEM (JEM2100HC, JEOL Ltd, Tokyo, Japan).

Oncomelania hupensis *haemo-lymphocytes SRBC adherence and non-self-recognition by* O. hupensis *haemo-lymphocytes*

To study response of haemo-lymphocytes to a

foreign substance, such as Sheep Red Blood Cells (SRBC), a hole was created on snail's shell with a small wimble (0.5 mm in diameter) and then a suspension of 1% SRBC was injected into the haemocoel of the snail through the hole by using a micropipette. All inoculated snails were maintained at 24±1°C. Twenty two hours after SRBC inoculation, snails were killed to collect haemolymph. The collected haemolymph was taken on a glass slide containing equal volume of SA solution. The glass slides were kept horizontally at 24±1°C for 60 minutes to allow the cells to adhere to the slide. After incubation, the slides were quickly rinsed with SA solution, after which haemo-lymphocytes adhering to the glass slide were fixed by immersing the slide(s) in methanol for at least 5 minutes and thereafter stained with Wright-Giemsa stain. The haemo-lymphocytes were observed by employing a light microscope.

RESULTS AND DISCUSSION

Wright-Giemsa stain yielded useful results concerning morphology of *O. hupensis* haemo-lymphocytes; this stain clearly differentiated nucleus from the cytoplasm. With this stain, the nuclei and granules were stained dark blue to purple, while the cytoplasm was stained sky blue. Their cells were like lymphocyte, and therefore they were termed as haemo-lymphocyte cells. The nucleus was encircled by sky blue stained cytoplasm with 0.3-0.9 x 0.6-0.9 N/C ratio (average = 0.6 x 0.8 N/C ratio). Observation of 120 haemo-lymphocytes revealed nucleus diameter range of 3.1-9.4 µm (average = 5.3 µm) and the entire cell size range of 4.7-15.0 x 4.4- 12.5 µm (average = 8.6 x 7.1 µm). Based on the haemo-lymphocytes measurements, the nucleus of cells were divided into three size groups: large, medium, and small with diameter range of 8.1-8.6, 5.5-6.0 and 4.2-4.7 µm, respectively (Table I, Fig. 1).

Fluorescent microscopy revealed nucleus of cells having round shape (Fig. 2); the nucleus contained very high density of granules with blue fluorescence color, but DAPI stain could not stain cytoplasm. Through this method, the haemo-lymphocytes nucleus also could be separated into three size groups: large, medium, and small, with diameter ranges of 8.1-8.6 µm, 5.5-6.0 µm, and 4.2-4.7 µm, respectively.

Table I.- Three size groups of nucleus of haemo-lymphocytes of *Oncomelania hupensis*.

Haemo-lymphocytes nucleus size	Size range (µm)
Large	8.1 - 8.6
Medium	5.5 - 6.0
Small	4.2 - 4.7

The SEM micrographs showed round shaped nucleus of haemo-lymphocytes with rough surface but the cytoplasm was not visible. The SEM of haemo-lymphocytes also showed the three sizes of nucleus (Fig. 3), with their size ranges similar to the ones observed using fluorescent microscopy (Fig.4).

Through TEM examination, haemo-lymphocytes appeared round and containing a round section shaped nucleus surrounded by cytoplasm (Fig. 5).

Oncomelania hupensis haemo-lymphocytes appeared to adhere quickly to surface of glass slide. Adherence is an important role of haemo-lymphocytes against invading microorganisms in haemolymph circulation. Sheep red blood cells (SRCB) served as foreign particles in this study to simulate attack of microorganisms. Light microscopy observations after Wright-Giemsa stain showed that SRBC were present as red or light purple cells and were round or oval, with diameter approximately around 1-2 µm and had smooth surface. The haemo-lymphocytes possessed pseudopodia for adhering or embracing SRBC as is evident from Figure 6A. After incubation for 60 minutes at 24±1°C, light microscopy revealed that haemo-lymphocytes were surrounded by numerous SRBC (Fig. 6B).

The difficulty of sampling haemolymph from *O. hupensis* due to their rather small body size necessitated employment of a special technique for collecting haemolymph samples from the snail. Morphology of haemo-lymphocytes of *O. hupensis* was unique and different from that of other snails already reported in the relevant scientific literature. However, because of the relatively small size of *O. hupensis* snail and associated difficulty of sampling haemolymph of this snail, there are almost no previous detailed reports on the morphology of

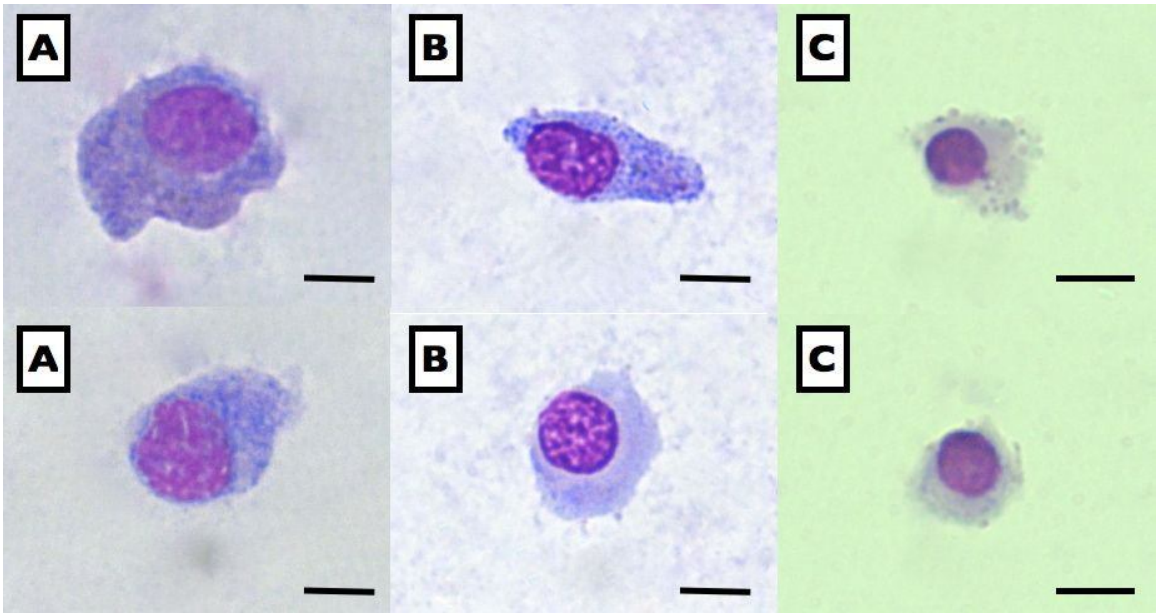

Fig.1. Micrograph of large (A), medium (B), and small (C) nucleus size of haemo-lymphocytes in *Oncomelania hupensis* snail (light microscopy). Bars = 5 µm.

haemo-lymphocytes of *O. hupensis* in the relevant scientific literature.

Three categories of haemo-lymphocytes: large, medium, and small nucleus size, according to their shape, size, surface structure, internal structure, functions, the characterization of cytoplasm, and the processes of pseudopodia, were distinguished. These results are in agreement with those of Tang *et al.* (2012) who had previously separated haemo-lymphocytes (lymphocytes) of *O. hupensis*. In addition, Noda and Loker (1989) described heamocytes in *Biomphalaria glabrata*; their findings were similar to the findings in the present study. The general morphology of the three classes of haemo-lymphocytes is quite similar, except for the difference in cell size. Travers *et al.* (2008) studying haemocytes of abalone (*Haliotis* sp.) had reported smaller haemocytes as "blast cells". In the present investigation, the small nucleus size of haemo-lymphocytes in *O. hupensis* snail could also be blast cells. Several investigations support presence of blast cells in mollusk haemolymph circulation. For example, the heamocytes population studies in the pond snail *Lymnaea staginalis* (Dikkeboom *et al.*, 1984) and *L. truncatula* (Monteil and Matricon-Gondran, 1993) described young/juvenile haemocytes. The ultrastructure of haemo-lymphocytes of *O. hupensis* snail revealed that the cell contained nucleus, which was surround by cytoplasm having no other visible structures. The shape of haemo-lymphocytes observed under light microscopy and SEM did not fully conform to the shape of haemo-lymphocytes observed under TEM. Through TEM, amoeboid shaped cells were noted, while light microscopy and SEM presented cells round in shape. It is likely that haemo-lymphocytes were able to transform into the various shapes depending upon the surrounding environmental conditions to which they may react. However, in agreement with the amoeboid-shaped haemo-lymphocytes observed in the present study, previously Sminia and Barendsen (1980) had named haemo-lymphocytes as amoebocytes in three snail species, *L. stagnalis*, *B. glabrata*, and *B. truncatus*.

The adherence is a major internal defense ability of haemocytes of gastropods. Haemocytes of mollusks are known to efficiently adhere to foreign materials, such as bacteria or parasites. Matozzo *et al.* (2007) suggested that haemocytes have opsonising properties when observed in cockle (*Cerastoderma glaucum*). In the present study, SRBC used as foreign particles to test adherence of

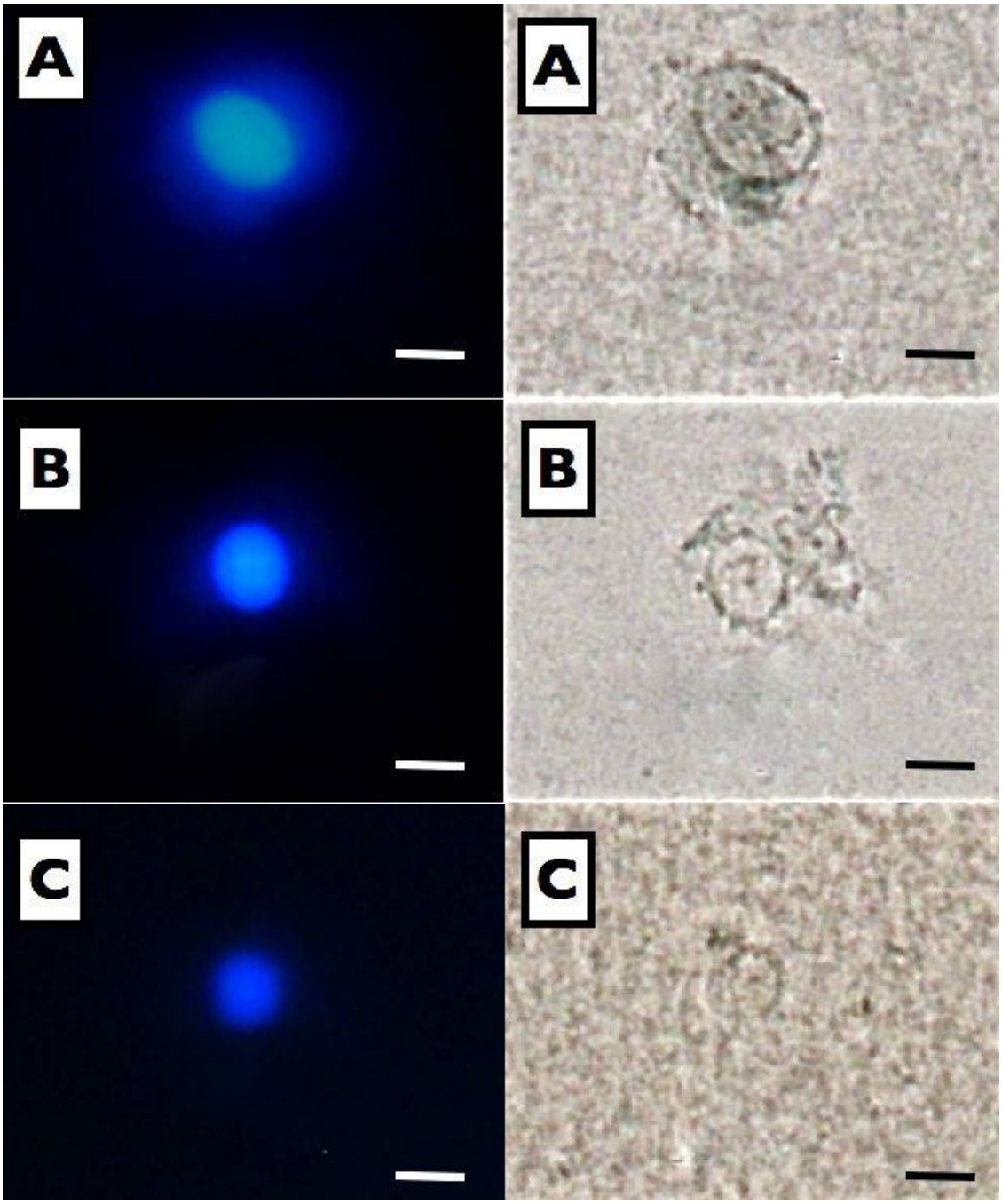

Fig. 2. Large (A), medium (B), and small (C) nucleus of haemo-lymphocytes of *Oncomelania hupensis* snail; Left: blue fluorescence color of nucleus of haemo-lymphocytes, Right: unstained haemo-lymphocytes (light microscopy). Bars = 5 µm.

haemo-lymphocytes, clearly showed adherence activity of haemo-lymphocytes of *O. hupensis* snail: thus, confirming the mechanism of haemo-lymphocytes action against foreign particles or parasites. After 60 minutes of incubation, some cells did not adhere to SRBC meaning not all of haemo-lymphocytes could adhere to SRBC. Although haemocytes nomenclature has not yet been

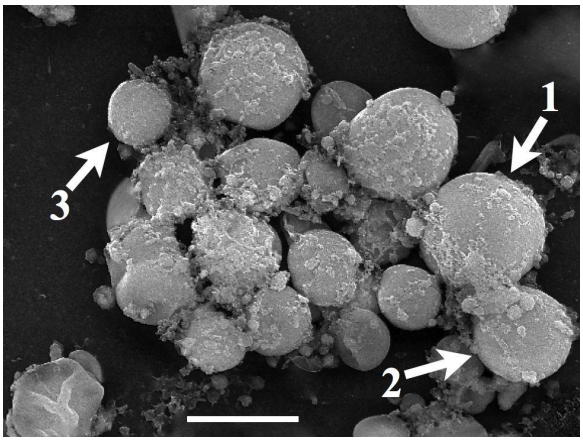

Fig. 3. *Oncomelania hupensis* haemo-lymphoctes of three different nucleus sizes; large (arrow 1), medium (arrow 2) and small (arrow 3) (Scanning Electron Microscopy micrograph) Bar = 10 µm.

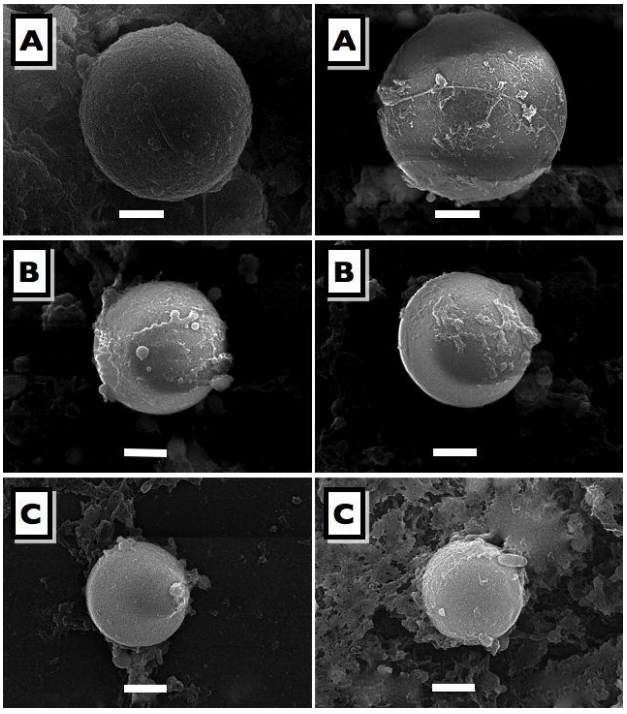

Fig. 4. Three nucleus sizes of *Oncomelania hupensis* haemo-lymphocytes (A = large size, B = medium size, and C = small size) (Scanning Electron Microscopy micrograph) Bars = 2 µm.

standardized, the present study utilized the physical appearance of *O. hupensis* heamo-lymphocytes to unveil their morphology. It has been previously shown that the *S. japonicum* larval development could be blocked in *O. hupensis* already infected with the fish trematode, *Exorchis* sp., suggesting that the innate immunity response and haemo-lymphocytes of *O. hupensis* may play an important role in this process (Tang *et al*., 2009).

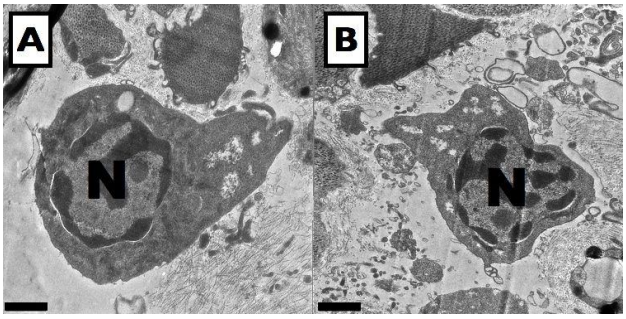

Fig. 5. Ultra structure of *Oncomelania hupensis* haemo-lymphocytes (Transmission Electron Microscopy micrograph); (A, B) with large round shaped nucleus (N) surrounded by cytoplasm. Bars = 1 µm.

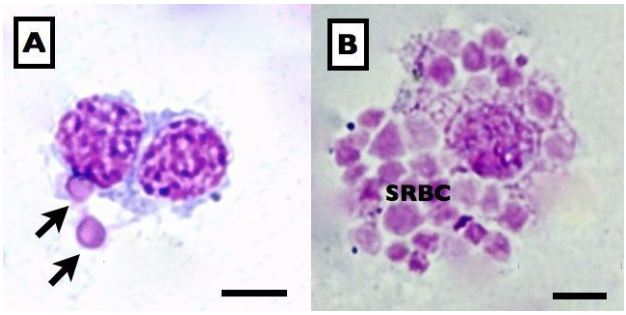

Fig. 6. Haemo-lymphocytes of *Oncomelania hupensis* adhering to Sheep Red Blood Cells (SRBC, arrows) (light microscopy micrograph). (A, B) Bars = 5 µm.

In conclusion, haemo-lymphocytes are vital in defense functions of Mollusca. The results of the present study add to the basic knowledge of haemo-lymphocytes of *O. hupensis*, providing a springboard to researchers to continue investigations concerning haemo-lymphocytes of *O. hupensis*, the intermediate host of schistosomiasis in People's Republic of China and elsewhere.

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