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On the ultrastructure and classification of hemocytes of freshwater crab *Cylindrothelphusa steniops* (Crustacea: Decapoda)

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ABSTRACT: The present study employs light and electron microscopy methods to elucidate the morphological and ultrastructural details of hemocyte profile of the freshwater crab *Cylindrotelphusa steniops.* Hemocytes were classified based on the cell shape, nucleus characteristics, appearance of cytoplasmic organelles, pseudopodia, presence and nature of granules. We identified four cell types which include agranulocyte (Ag), granulocyte I (GI), granulocyte II (GII), and semigranulocytes (SGC). Agranulocytes were oval or irregular shaped cells, devoid of granules in their cytoplasm. They had prominent pseudopodia and phagosome-like structures in the cytoplasm, suggesting a phagocytic function. Granulocytes I are usually round to oval and rich in cytoplasmic organelles like RER, mitochondria, ribosomes, multifarious granules and small vesicles, pointing towards synthetic and secretory functions. Granulocytes II are elongated or oval, cytoplasm contained organelles like mitochondria, RER, ribosomes, vacuoles, Golgi bodies with inflated cisternae and distinctly large, dense granules. Semigranulocytes are round to oval cells with ribosomes, vacuoles, RER cisternae, mitochondria and small dense granules throughout the cytoplasm. Moreover, SGC with a large vacuole and endocytosed materials were observed, probably involved in endocytosis of foreign materials. We propose that the information generated from the present study will be useful for the aquacultural practises of freshwater decapod crustaceans.
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KEY WORDS**:** agranulocyte, granulocyte, semigranulocyte, ultrastructure, freshwater crab.

Ультраструктура и классификация гемоцитов пресноводного краба *Cylindrothelphusa steniops* (Crustacea: Decapoda)

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РЕЗЮМЕ: В настоящем исследовании используются методы световой и электронной микроскопии для выяснения морфологических и ультраструктурных деталей профиля гемоцитов пресноводного краба *Cylindrotelphusa steneops*. Гемоциты классифицировались на основе формы клеток, характеристик ядра, внешнего вида цитоплазматических органелл, псевдоподий, а также природы гранул. Мы идентифицировали четыре типа

клеток, которые включают агранулоциты (Ag), гранулоциты I (GI), гранулоциты II (GII) и семигранулоциты (SGC). Агранулоциты были овальными или неправильной формы клетками, лишенными гранул в своей цитоплазме. Они имели выраженные псевдоподии и фагосомоподобные структуры в цитоплазме, что предполагает фагоцитарную функцию. Гранулоциты I обычно имеют круглую или овальную форму и богаты цитоплазматическими органеллами, такими как RER, митохондрии, рибосомы, разнообразные гранулы и небольшие везикулы, что указывает на синтетические и секреторные функции. Гранулоциты II удлиненные или овальные, цитоплазма содержит органеллы, такие как митохондрии, RER, рибосомы, вакуоли, тельца Гольджи с раздутыми цистернами и отчетливо крупными плотными гранулами. Семигранулоциты представляют собой круглые или овальные клетки с рибосомами, вакуолями, цистернами RER, митохондриями и небольшими плотными гранулами по всей цитоплазме. Кроме того, наблюдались SGC с большой вакуолью и эндоцитированными материалами, вероятно, участвующими в эндоцитозе чужеродных материалов. Мы предполагаем, что информация, полученная в ходе настоящего исследования, будет полезна для аквакультуры пресноводных десятиногих ракообразных.
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КЛЮЧЕВЫЕ СЛОВА: агранулоциты, гранулоциты, семигранулоциты, ультраструктура, пресноводный краб.

Introduction

In decapod crustaceans, cellular defence is well coordinated by circulating hemocytes. Crustaceans possess an open circulatory system, where nutrients, oxygen, hormones, and hemocytes are distributed via hemolymph. The hematopoietic tissue and hematopoietic stem cells are responsible for the production and supply of hemocytes. The most important function of the hemocytes in the defence mechanisms is the recognition and removal of non-self- matters (Sritunyalucksana *et al*., 1999). This function is primarily achieved by several defense mechanisms that become activated depending on the pathogen's characteristics. When foreign material is recognized by hemocytes, they eliminate or sequester invading pathogens through phagocytosis, encapsulation, and secretion of lysosomal enzymes and bacteriostatic substances (Söderhäll, Smith, 1986; Hose *et al*., 1987).

In crustaceans, the classification of hemocytes is one of the most debated points, because many researchers have identified different cell types in various species. Mostly, the classification schemes are based on either morphological aspect using light or electron microscopic studies or by employing cytochemical assays to understand cytochemical properties of the hemocytes (Matozzo, Marin, 2010; Parrinello *et al.*, 2015, Cheng *et al*., 2018). Recently, monoclonal antibody technique was used to study the types of hemocytes in certain crustaceans (Kumar *et al*., 2012). It is demonstrated that these techniques provide accurate information about structural and biological functions of hemocyte types. However, the resulting nomenclature varies and the classification becomes controversial. In many crustaceans, the classification scheme is generally based on the presence or absence of cytoplasmic granules (Martin, Graves, 1985; Latha *et al*., 2017). Based on this criterion, three hemocyte types were recognized which include agranulocytes also defined as hyalinocytes, semigranulocytes and granulocytes. A further level of complexity ascends if we study the number, size and composition of granules by special microscopic techniques like fluorescence and electron microscopy or by electrocytochemical method. Through renewed research on the morphology of various crustacean hemocytes, we can develop a uniform terminology for naming cell types.

In recent years, several methods have been used to characterize hemocyte types from various crustaceans. Zhou *et al.* (2018) characterized three hemocyte types from mud crab *Scylla paramamosain* Estampador, 1950 by flow cytometry and morphological studies like

cytochemical staining and electron microscopy. Matozzo and Marin (2010) carried out morphological study of hemocytes from the crab *Carcinus aestuarii* Nardo, 1847 by using light microscopy and differing cytochemical assays. Cheng *et al.* (2018) analysed the hemocytes from four crustaceans using a laser scanning confocal microscope, flow cytometry, western blotting, and transmission electron microscopy. Deyashi and Chakraborty (2022) classified hemocyte structural types in freshwater crab by employing light microscopy, electron microscopy and flow cytometric analysis. Koiwai *et al.* (2021) used single-cell RNA sequencing and revealed nine types of hemocytes of *Marsupenaeus japonicus* (Spence Bate, 1888) based on their transcriptional profiles. Parrinello *et al.* (2015) studied hemocytes of *Cancer pagurus* Linnaeus, 1758 and *C. borealis* Stimpson, 1859 using light and scanning electron microscopy.

To understand the appropriate functional mechanisms of immunity of crustaceans, it is essential to classify and characterise the structure of circulating hemocytes in the hemolymph. Considerable efforts have been invested in studying the types, morphology, structure and function of hemocyte populations in various economically important marine and estuarian decapods. Clare and Lumb (1994) described the fine structure of hemocytes and hemopoietic tissue and phenoloxidase (PO) activity in the blue crab *Callinectes sapidus* (Rathbun, 1896). Hose *et al.* (1990) reported hemocyte classification scheme integrating morphology, cytochemistry and function in three decapods; *Loxorhynchus grandis* Stimpson, 1857, *Homarus americanus* (H. Milne Edwards, 1837) and *Panulirus interruptus* (Randall, 1840). Alvarez and Chung (2015) reported hemocyte prophenoloxidase system plays a central role in the melanization and sclerotization particularly in wound healing in crustaceans. Estrada (2016) employed fluorescent lectin-binding assays and cytochemical reactions to identify, specificity and distribution of carbohydrate moieties and presence of several hydrolytic enzymes in hemocytes of white-leg shrimp *Litopenaeus vannamei* (Boone, 1931). Reports of morphological, fine structural and functional aspects of hemocytes of freshwater crabs are relatively sparse (*Paratelphusa masoniana* (Henderson, 1893) by Rakesh *et al.*, 2013; *Travancoriana schirnerae* Bott, 1969 by Latha *et al.*, 2017; and *Varuna litterata* (Fabricius, 1798) by Deyashi and Chakraborty, 2022) when compared to that of marine and estuarine counterparts. Thus, the present study has been undertaken to evaluate the fine structure of hemocytes of an endemic freshwater crab, *Cylindrotelphusa steniops* (Alcock, 1909). This freshwater crab species is abundant in the paddy fields of Kannur (Kerala, India) and usually residing in deep burrows constructed near to water channels in paddy fields.

Material and Methods

The adult intermoult crabs of carapace width 3.5 to 4 cm were collected from the paddy fields of Peravoor, Iritty (11.8962°N, 75.7342°E) in Kannur district of Kerala, during May 2023. The crabs (N=15) were collected weekly (the experiment was repeated thrice) by hand picking method from the burrows and maintained in the laboratory under near-natural conditions and were fed clam meat and puffed rice for seven days. The crabs were maintained in three plastic basins containing filtered water and fed with balanced meal twice in a day; water in the basins was replaced in every 12 hours.

Hemolymph (2 ml) was collected by puncturing the walking legs and transferred to an eppendorf tube containing 3% glutaraldehyde in 0.1 M sodium cacodylate buffer as fixative. After proper fixation, the sample was centrifuged at 1000 rpm for five minutes. The pellets were further fixed in 3% glutaraldehyde and washed in buffer, post fixed in 1% osmium tetroxide and washed in buffer. The pellet was dehydrated in graded series of graded alcohol (50–100%), cleared in propylene oxide, infiltrated in propylene oxide and epoxy resin and finally embedded in siliconized rubber mould with epoxy resin.

Embedded mould was kept in an incubator at 60 degrees for 48 hours and blocks were cooled and used for sectioning. One-micron thick sections were cut using ultra microtome (Leica ultra cut UCT) with glass knife and stained by toluidine blue (1%) (for light microscopic studies). Ultra-thin sections (below 100 nm) were produced by ultra microtome (Leica EM UC7) with diamond knife (DD atome). Ultrathin sections were collected on copper grids and stained (Double metallic) by uranyl acetate and Reynold' s solution (sodium citrate + lead nitrate). Ultrathin sections were observed under TEM (Philips Tecnai T 12 spirit) and photographed, analysed for fine structural details. The semithin section were observed under a Nikon Eclipse Ni-U Research microscope, photmicrographed and observed for light microscopic studies.

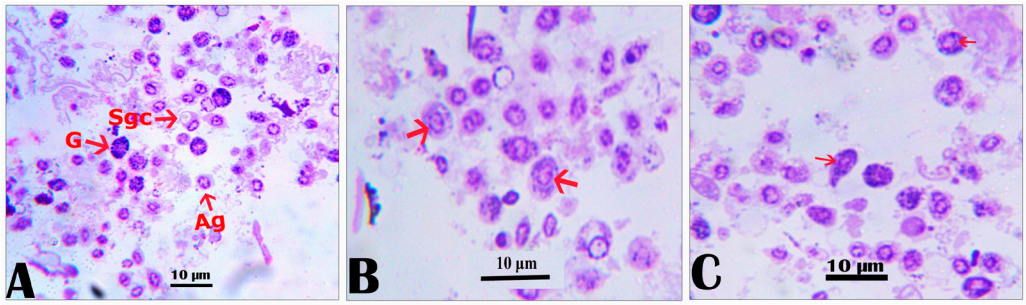

Fig. 1. Light micrograph depicting haemocytes of *Cylindrotelphusa steniops* stained with toluidine blue. A — light micrograph showing agranulocytes, granulocytes and semigranulocytes; B — light micrograph of granulocytes showing thick chromatin patches (arrows indicate granulocytes with thick chromatin patch inside nuclei); C — granulocytes with thin chromatin patches (arrows indicate granulocytes with thin chromatin patch inside nuclei);
Abbreviations: Ag — agranulocyte; G — granulocyte; Sgc — semigranulocyte.

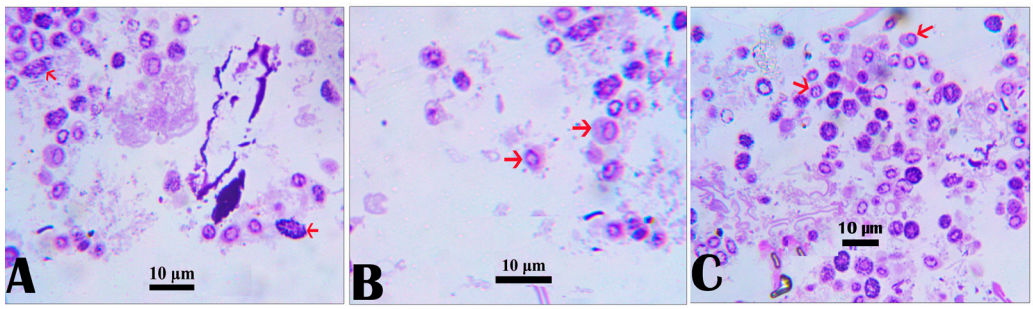

Fig. 2. Light micrograph exhibiting additional features of granulocytes, semigranulocytes and agranulocytes. A — granulocytes containing large number of granules in the cytoplasm (arrows indicate granulocytes with granules obscuring nucleus); B — light micrograph showing features of semigranulocytes (arrows indicate nucleus and cytoplasm of semigranulocytes); C — light micrograph indicating the details of agranulocytes (arrows indicate the thick chromatin patch of agranulocytes).

Results

Light microscopy

Based on the light microscopic observations, hemocytes of *C. steniops* are classified into three types — granulocytes, semigranulocytes and agranulocytes (Fig. 1A). In semithin stained sections, granulocytes were oval or roughly round cells with centric or eccentric nuclei and cytoplasmic granules. The granulocytes showed many large blue granules when stained with toluidine blue. Their nuclei possessed both thick and thin blue patches of chromatin (Fig. 1B and C). In some granulocytes, granules completely obscure the nucleus (Fig. 2A). Semigranulocytes are round or oval cells with purple cytoplasm (Fig. 2B). The nuclei contained thin patches of chromatin attached to the nuclear membrane (Fig. 2B). Their cytoplasm granular in nature. Agranulocytes are oval or irregular cells without granules. Their nuclei exhibited thick blue patches of chromatin content (Fig. 2C). The granulocytes represented approximately 54% of the total circulating hemocytes while agranulocytes and semigranulocytes comprised 25 and 21%, respectively.

Ultra microscopy

Based on the ultrastructural observations, four types of hemocytes were identified and classified as agranulocyte (Ag), granulocyte I (GI), granulocyte II (GII), semigranulocytes (SGC) (Fig. 3).

Agranulocytes

Agranulocytes were oval or irregular shaped cells, (10.91µm×7.54µm) and exhibited nucleo-

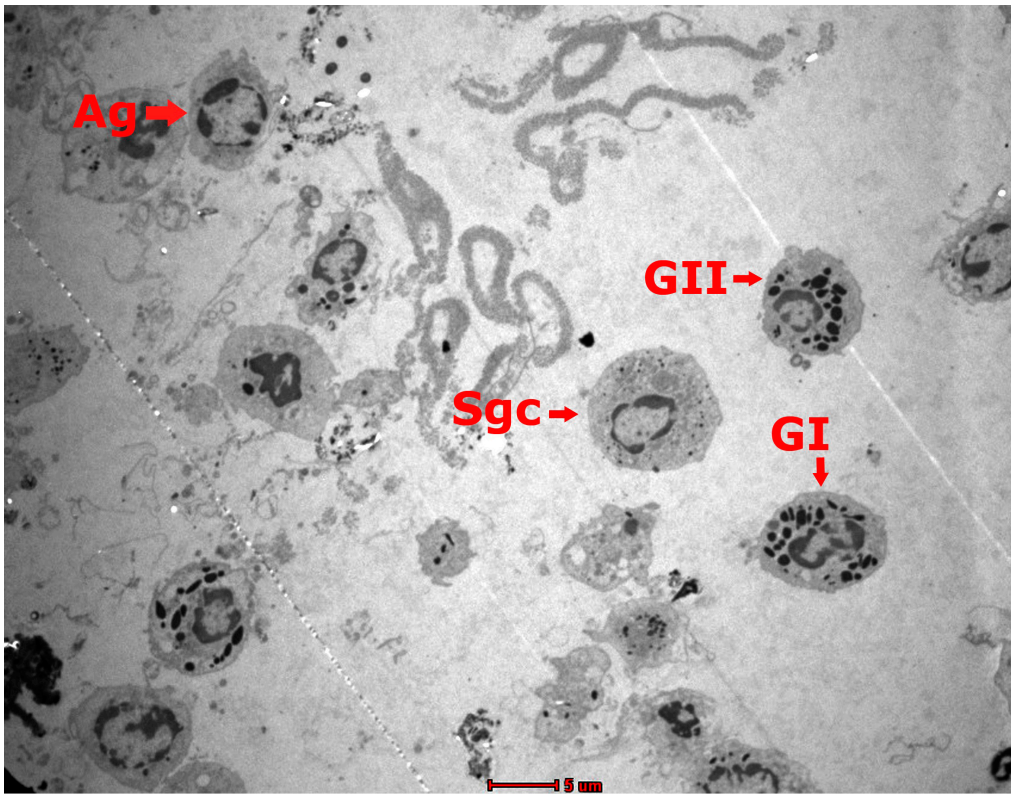

Fig. 3. Transmission electron micrograph of hemocyte types.
Abbreviations: Ag — agranulocyte; GI — granulocyte I; GII — granulocyte II; Sgc — semigranulocyte.

cytoplasmic ratio (NPR) (0.53). The nucleus (4.91×4.27μm) was round to oval and possessed a smooth, distinct nuclear envelope (Fig. 4A). The inner nuclear membrane displayed discontinuous patches of dense heterochromatin and the granular chromatin content was scattered in the centre of the nucleus. Agranulocytes were generally devoid of granules but rarely one or two granules were spotted in their cytoplasm (0.18–0.27 μm) (Fig. 4A). The cytoplasm contains various cell organelles such as RER, mitochondria, vacuoles and ribosomes. RER cisternae concentrated towards perinuclear region. Many lysosomal compartments were evident inside the cytoplasm. They had prominent pseudopodia and several phagosome-like vacuoles were observed in the peripheral region of the cytoplasm (Fig. 4A). Exocytosis like activities were observed (Fig. 4B).

Different Granule Types in the Haemocytes

Depending on the size, shape and density, six granule types were distinguished in the hemocytes which include Type I, Type II, Type III, Type IV, Type V and Type VI (Fig. 5A–F).

Type I

These are small to large, **round** (diameter 0.45–0.9 μm) granules with a homogenous dense matrix (Fig. 5A). Type 1 granules were typically encountered in GI, GII and SGC and it present abundantly in GI.

Type II

These are **oval**-shaped (0.36×0.27 — 0.81× 0.54 μm) granules of homogenous dense matrix (Fig. 5B). It is frequently occurred in GI, GII, and SGC.

Type III

These are **elongated** (0.45×0.18 — 1.81× 0.72 μm) granules of homogenous dense matrix with limiting membrane (Fig. 5C). It is spotted in GI, GII, and SGC. It is abundant in GII and rarely present in SGC.

Type IV

It is **kidney** shaped (0.45×0.27 — 0.72×0.18 μm) granules with electron dense matrix (Fig. 5D). It is common in GI.

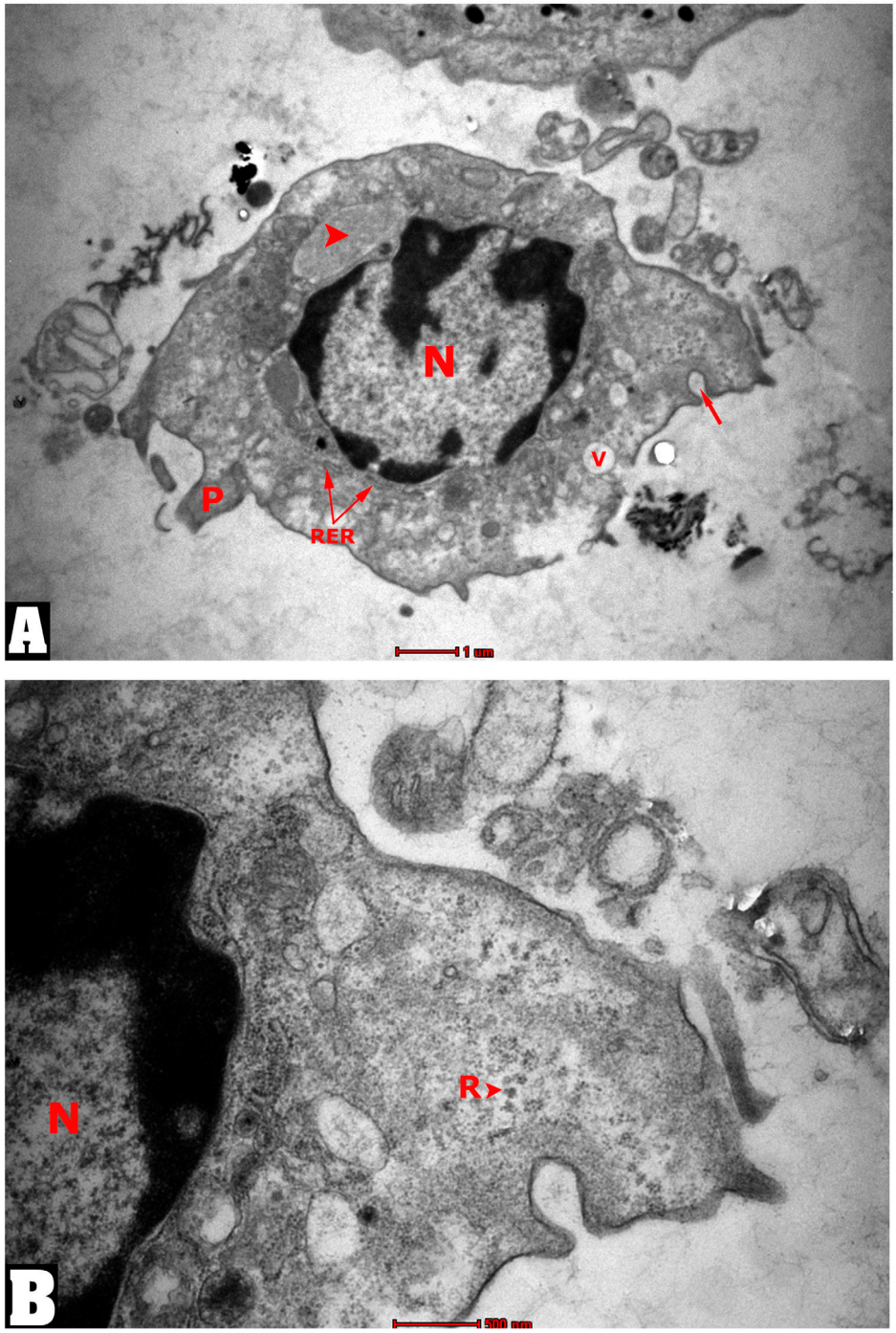

Fig. 4. Ultrastructural images of agranulocytes. A — agranulocytes illustrating large nucleus and cytoplasmic organelles; B — agranulocytes with prominent pseudopodia and exocytosis like activity.
Abbreviations: N — nucleus; P — pseudopodia; RER — rough endoplasmic reticulum; R — ribosomes; V — vacuole; arrow indicates exocytosis like activity in Ag; arrow head indicates phagosome like vacuoles.

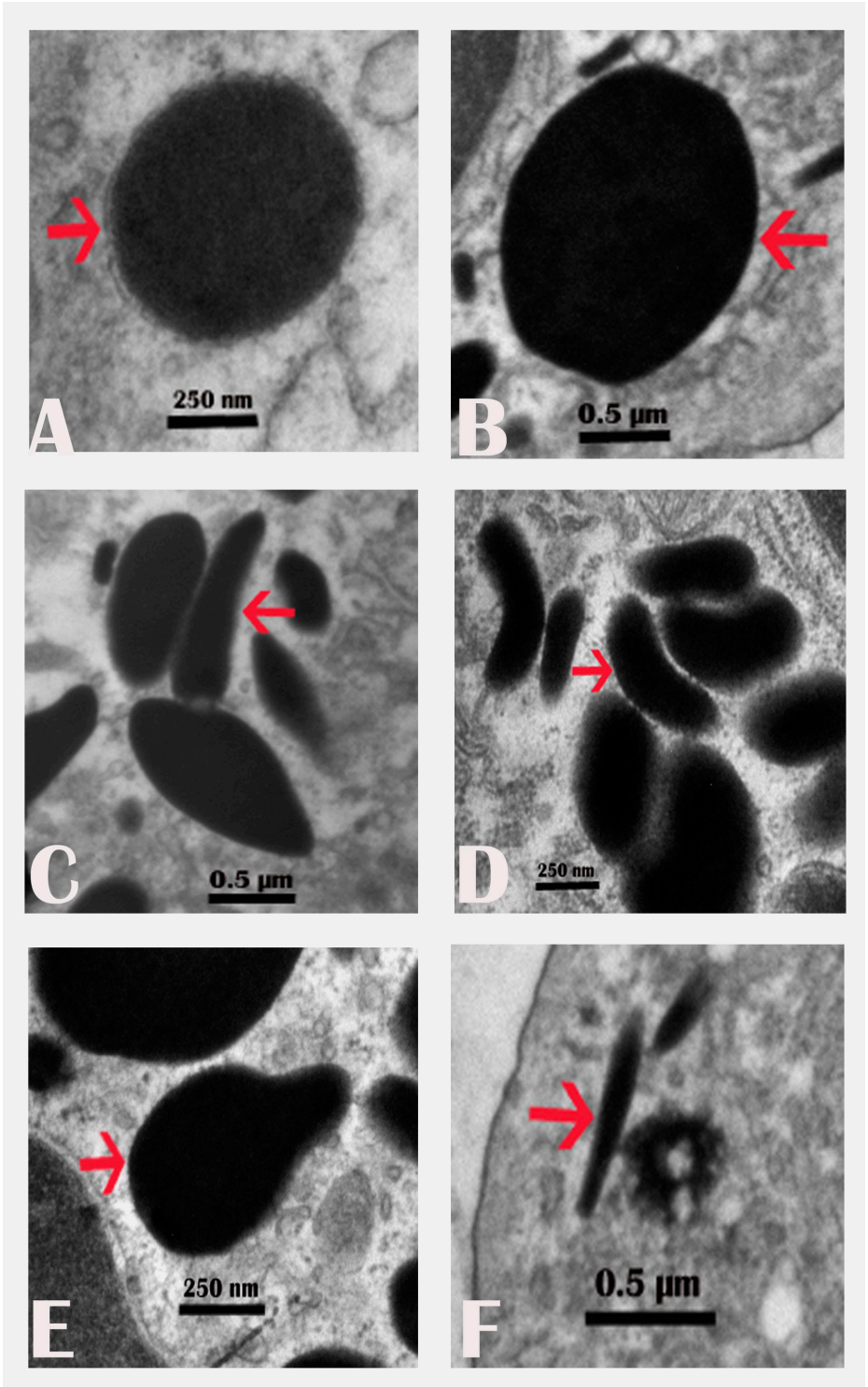

Fig. 5. Different granule types in hemocytes of *Cylindrotelphusa steniops*. A — type I; B — type II; C — type III; D — type IV; E — type V; F — type VI.

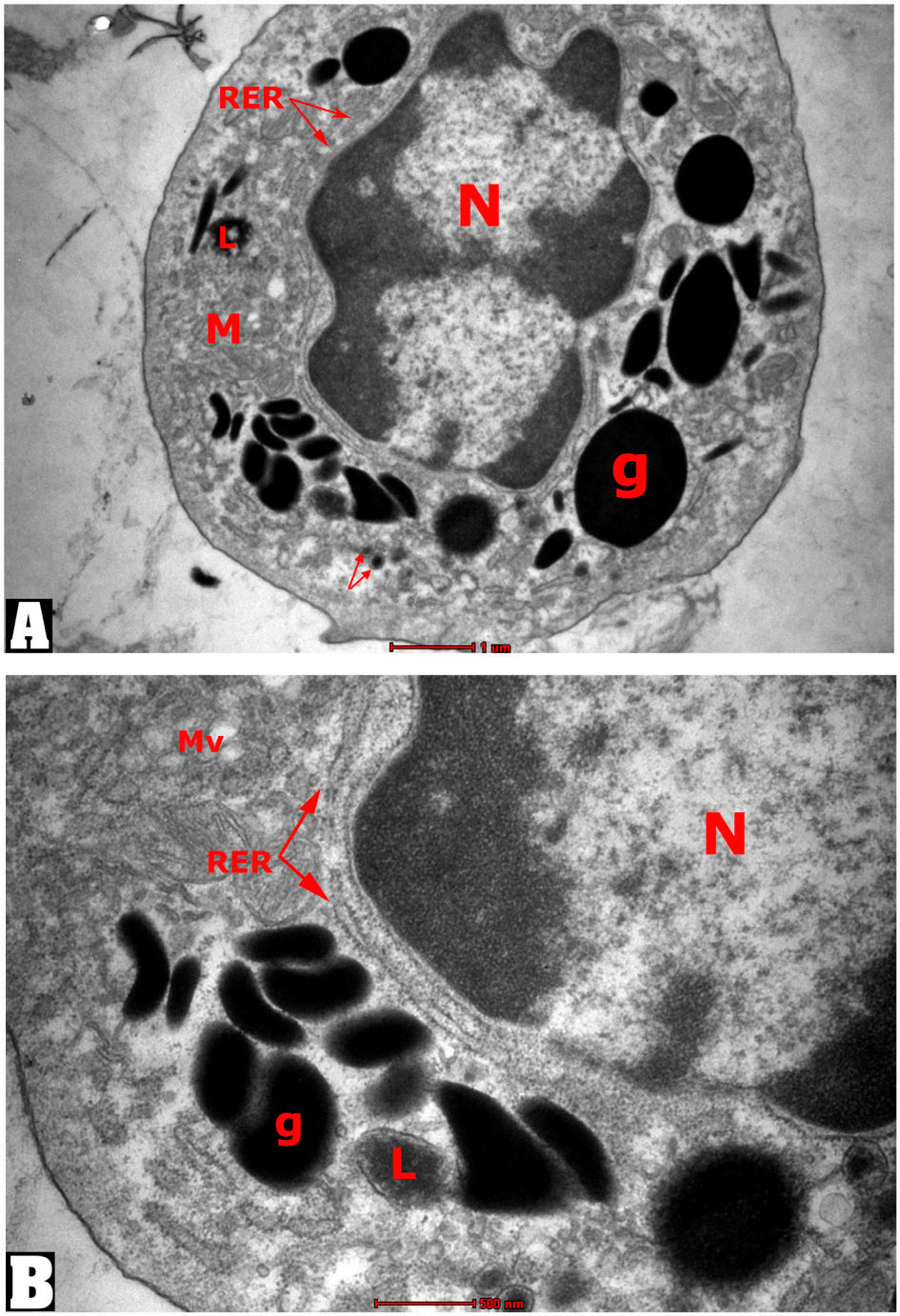

Fig. 6. Fine structural features of granulocyte I. A — granulocyte I displaying cytoplasmic organelles; B — high magnification of cytoplasm of GI showing multivesicular bodies and lysosome like inclusions.
Abbreviations: g — granule; L — lysosomes; M — mitochondria; Mv — multivesicular bodies; N — nucleus; RER — rough endoplasmic reticulum; arrows indicate vesicles probably originating from SER.

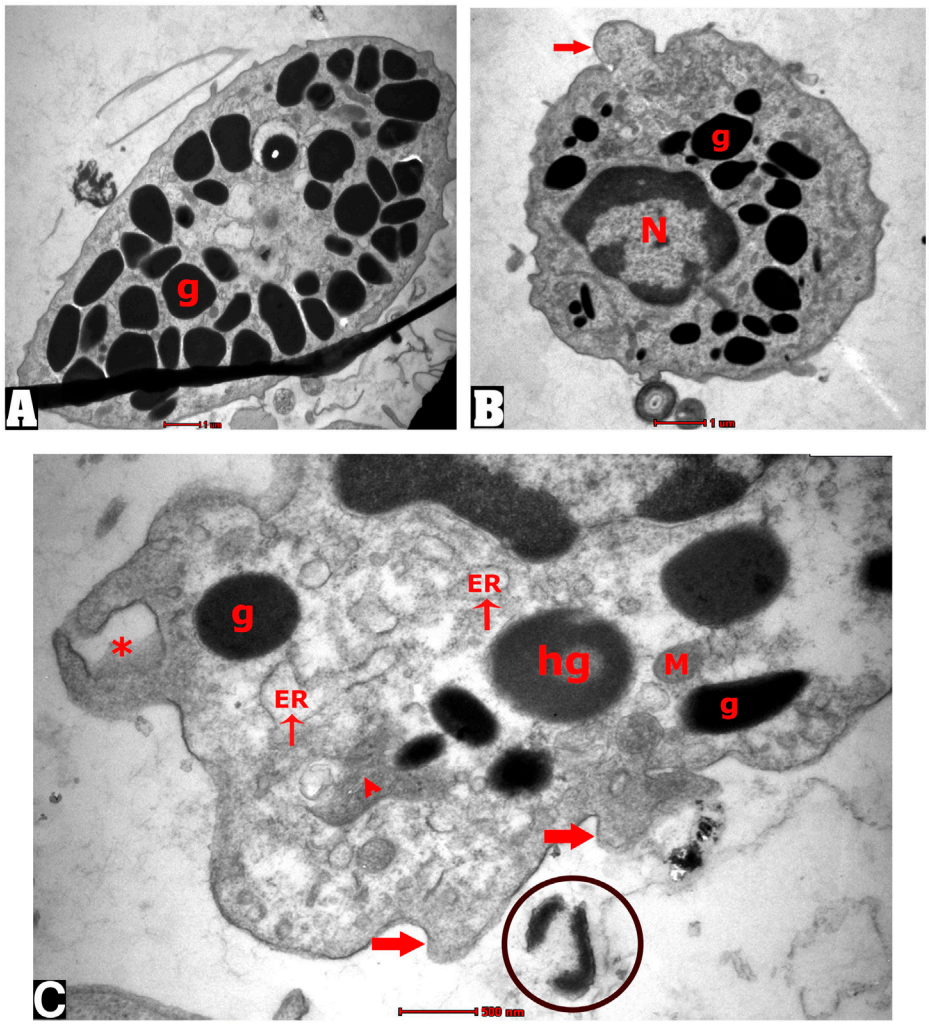

Fig. 7. Electron micrograph of granulocyte II. A — granulocyte II fully filled with granules which obscure nucleus; B — granulocyte II with acentric nucleus, granule types and bulbous pseudopodia; C — granulocyte II undergoing apoptotic like process with cell membrane blebbing and degenerating cytoplasmic organelles (arrow head indicates mitochondrial degeneration; asterisk indicates plasma membrane invagination with amorphous electron lucent contents; in circle indicates a large granule probably released out of GII).
Abbreviations: ER — endoplasmic reticulum; g — granule; hg — heterogenous granule type; M — mitochondria; arrow in figure B indicates bulbous pseudopodia projection; right arrows indicate cell membrane blebbing and degeneration; upward arrows indicate inflated RER cisternae.

Type V

These are **tear** shaped (0.72×0.36 — 0.91×0.54 µm) granules with homogenous dense matrix (Fig. 5E). It is numerous in GI.

Type VI

These are **rod** shaped (0.45×0.18 — 1.27×0.18µm) less dense granules (Fig. 5F). Common in GI.

Granulocyte I

Granulocytes I are usually round to oval cells (9.27×7.36 µm) and showed an oval or irregular, centric or eccentric nuclei (5.54×2.72 µm). Ultrathin sections of granulocytes I showed smooth and continuous nuclear envelop and heterochromatin appeared as thick dense patches. Large number of RER cisternae was seen surrounding

the perinuclear region (Fig. 6A). The cytoplasm also contained organelles like mitochondria, ribosomes, granules and small vesicles, probably originating from SER (Fig. 6A). Mitochondria were located around the nucleus and in the peripheral region close to the plasma membrane. The main distinctive feature is the presence of numerous round, oval, elongated, kidney, tear, and rod-shaped electron dense cytoplasmic granules, often seen surrounding the nucleus. Kidney shaped (type IV) and rod shaped (type VI) granules are frequently observed in the cytoplasm. Multivesicular bodies and lysosome like inclusions were observed (Fig. 6B).

Granulocyte II

Elongated or oval cells (14.45×7.54 µm) with centric or eccentric nuclei. The cytoplasm contained organelles like mitochondria, RER, ribosomes, vacuoles, Golgi bodies with inflated cisternae and electron dense and lucent vesicles. Granulocyte II carried distinctly large, dense, granules of different shapes and sizes (Fig. 7A, B). Type III (elongated) granules were mostly evident. The granules generally possess a homogenous electron dense content, but sometimes exhibit an heterogenous internal structure, made up of electron dense and electron lucent areas. Occasionally, they can emit small bulbous pseudopodial structures (Fig. 7B). TEM features of certain granulocytes II showed events of endocytosis where plasma membrane is deformed to become an invagination, encompassing an amorphous pattern of electron lucent content (Fig. 7C). Moreover, cell membrane showed blebbing and their cytoplasm was notable with inflated RER cisternae, mitochondrial degeneration and vacuolation (Fig. 7C). Degranulation process through the release of electron dense granules in the extracellular medium was also observable in GII (Fig. 7C). We have also noted that some of the GII are undergoing apoptosis like events because of the attack of an unknown pathogen.

Semigranulocytes

These are round or oval cells (9.09×8.81 µm) with oval, nearly centric nucleus. Irregular patches of less dense heterochromatin were adhered to the inner nuclear membrane (Fig. 8A). The cytoplasm contained organelles such as, ribosomes, vacuoles, small dense granules, RER cisternae and mitochondria (Fig. 8A, B). Both large and small mitochondria were present throughout the cytoplasm indicating their role in energy production. One of the prominent features of SGC is the presence of small dense granules which are distributed throughout the cytoplasm and granules are usually smaller than those of GCs (Fig. 8A, B). Specifically, accumulation of small dense granules was observable in the periphery of cell, close to plasma membrane and a few granules were found in the extracellular region (Fig. 8C). These cells had filopodia like structures (Fig. 8A) and the presence of large vacuole is an important feature of SGC (Fig. 8C). Inside the large vacuole, endocytosed materials could be seen (Fig. 8C).

Discussion

The fine structural details of circulating hemocytes in *C. steniops* showed four cell types: agranulocyte, granulocyte I, granulocyte II and semigranulocytes. TEM observations of hemocyes in *C. steniops* were similar to the reports of studies carried out in other decapod species. Parrinello *et al*. (2015) analysed the types and cell features of hemocytes in *C. pagurus* and *C. borealis* by using light microscopy and scanning electron microscopy. Based on the cell size, number and quantity of cytoplasmic granules and nucleocytoplasmic ratio, they have identified granulocytes, semigranulocytes and hyalinocytes. By employing light microscopy and differing cytochemical assays, Matozzo and Marin (2010) characterized hemocytes as hyalinocytes, granulocytes and semigranulocytes from the crab, *C. aestuarii*. Ultrastructural observations of circulating hemocytes in *Cherax quadricarinatus* (von Martens, 1868) depicted three types, granulocytes, semigranulocytes and hyalinocytes (Wentao *et al*., 2017). Notably, in our previous (Anjali, Smija, 2025) and present studies, we have identified four different circulating hemocytes in freshwater crabs *Arcithelphusa cochleariformis* Pati et Sudha Devi, 2015 and *C. steniops* and based on the results obtained, we adopted this nomenclature i.e., granulocytes I, granulocytes II, semigranulocytes and agranulocytes. We found similarities in the fine structural details of circulating hemocytes in *A. cochleariformis* and *C. steniops.*

In the present study, agranulocytes of *C. steniops*, generally devoid of granules and resem-

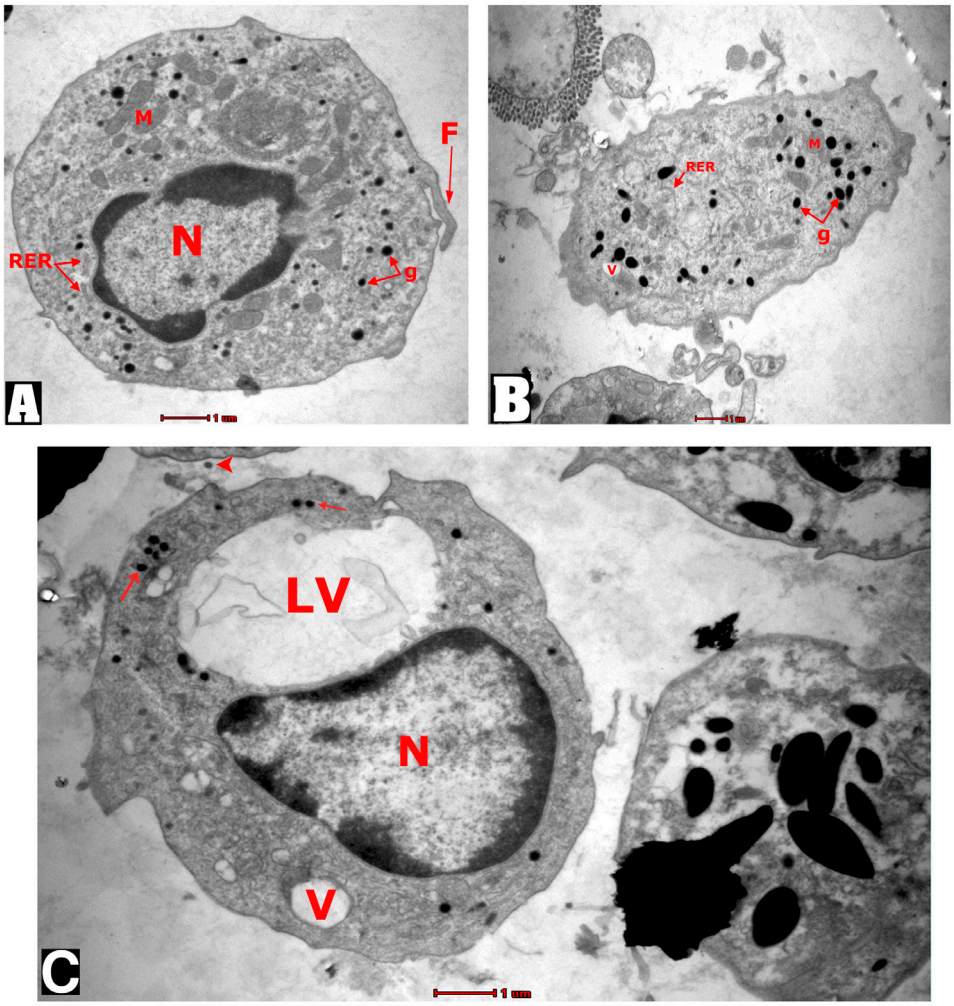

Fig. 8. Electron micrograph showing cytoplasmic details of semigranulocytes. Arrows indicate accumulation of small dense granules in cell periphery close to plasma membrane; arrow head indicates small dense granule in extracellular region. A — semigranulocyte with cytoplasmic organelles and polymorphic mitochondria; B — semigranulocyte with abundant small dense granules and RER cisternae dispersed throughout the cytoplasm; C — semigranulocyte with large vacuole and endocytosed materials.
Abbreviations: F — filopodia; g — granule; Lv — large vacuole, M — mitochondria; N — nucleus; RER — rough endoplasmic reticulum; V — vacuole.

bled hyaline cells reported in other crustaceans (Latha *et al*., 2017; Deyashi, Chakraborty, 2022). In our study, agranulocytes were morphologically and structurally very different from granulocytes and semigranulocytes. Agranulocytes had large round nucleus and cytoplasm contained RER, clumps of ribosomes, phagosomes and vacuoles and are able to emit pseudopodia. It is suggested that they are mainly involved in phagocytosis as evident from their prominent pseudopodia and phagosome-like vacuoles observed in the cytoplasm. Phagocytic capability of hemocytes were analyzed in various invertebrate species like molluscs, insects and crustaceans (Canesi *et al*., 2002; Perez, Fontanetti, 2011; Ray *et al*., 2013). Unlike vertebrates, where multifarious immune cells are acting as phagocytes (Varki, Gagneux, 2012), in invertebrates like crustaceans, insects

and molluscs, only certain hemocytes have largely involved in phagocytic activity. In decapod crustaceans such as freshwater crayfish *Astacus astacus* (Linnaeus, 1758), hyaline cells or agranulocytes are primarily phagocytic in nature (Smith, Söderhäll, 1983). Correspondingly, Anjali and Smija (2025) identified phagocytic functions for agranulocytes in the freshwater crab *A. cochleariformis*. Conversely, agranulocytes exhibited less phagocytic activity and are less copious in most bivalve species (Jauzein *et al*., 2013). In molluscs, the capacity for phagocytosis of granulocytes is evidently greater than that of hyalinocytes (Mao *et al*., 2020) and hyalinocytes have more devoted functions related to clotting and wound healing process (de la Ballina *et al*., 2020). As mentioned above, our very recent results (Anjali, Smija, 2025) give further evidence that agranulocytes are an important component in the innate immunity reactions of freshwater crabs, especially involved in the phagocytic activity in the system.

In our present study, we have observed two different types of granulocytes with diversity in size, shape, number, and electron density of cytoplasmic granules and organelles. Granulocytes I showed large number of RER cisternae, surrounding the perinuclear region and their cytoplasm also contained organelles like mitochondria, ribosomes, granules and small vesicles containing materials, probably originating from SER. Perhaps, these indicate their high metabolic activity and pointing towards synthetic and secretory functions. Similar observations regarding the role of granulocytes were reported by many authors in crustaceans. In decapod crustaceans, granulocytes are important in the synthesis of humoral factors for example enzymes involved in proPO cascade, antimicrobial molecules and clotting factors (Tassanakajon *et al*., 2013, Havanapan *et al*., 2016; Qin *et al*., 2019). After further wide-ranging examination (light microscopy and electron microscopy), we have identified granulocyte II and their cytoplasm contained organelles like mitochondria, RER, ribosomes, vacuoles, Golgi bodies with inflated cisternae and electron dense and lucent vesicles. In addition, their involvement in phagocytosis is evidenced by the presence of bulbous pseudopodial structure. The functioning of hemocytes as phagocytes differs among various crustaceans, and they even differ in their phagocytic capability when encountering different pathogens (Li *et al*., 2021). In crayfishes, together GCs and SGCs perform phagocytosis function, but they aim dissimilar foreign materials (Li *et al*., 2021). In lobsters, Hose *et al*. (1990) reported that the granulocytes are intricated in defence against foreign material by phagocytosis and encapsulation. In the present study, certain granulocytes II were observed with cell membrane blebbing, inflated RER cisternae probably indicating vacuolation of RER cisternae, mitochondrial degeneration and vacuolation. Similar morphological changes were noted in the hemocytes of the insect *Spodoptera exigua* (Hübner, 1808) after the bacterial infection and these changes were comparable to that of apoptotic changes during programmed cell death (Park *et al*., 2005). They observed cell membrane blebbing, nuclear membrane degeneration and presence of apoptotic vesicles at 4–8 hours of infection. Subsequently, shape of the hemocytes were lost and also vacuolation of the endoplasmic reticulum was observable at 12 hours of post infection (Park *et al*., 2005). Cui *et al.* (2020) found that in *Fenneropenaeus chinensis* (Osbeck, 1765), virus infected granulocytes undergo apoptosis and they have stronger apoptotic response to white-spot syndrome virus infection than hyalinocytes. The results of the study also demonstrate that the apoptotic response of hemocytes was largely influenced by the virus infection and the differences in apoptotic functions of granulocytes and hyalinocytes to virus designate variation of antiviral mechanisms between two types of hemocytes (Cui *et al*., 2020). If we delve deeper into the results of the above mention study, we could examine differences in the immune responses of hemocyte types to WSSV infection. All these statements, paired with our data may imply the key role of granulocytes II in apoptosis like process. Thus, we assume that granulocytes play an essential role in freshwater crab immune reactions towards invaded pathogens.

In the present study, the size, shape and density played an important role in categorizing granules into six types in the hemocytes *C. steniops.* Similarly, Latha *et al*. (2017) reported six granule types in the hemocytes of *T. schirnerae*. By contrast, Martin and Graves (1985) identified small granule and large granule hemocytes in penaeid shrimps, based solely on the presence or absence and relative size of granules. Deyashi

and Chakraborty (2022) also reported small granule and large granule hemocytes based on various cytomorphological features in *V. litterata*. In our study, large- electron dense type II granules and less numerous type I were frequently observed in both granulocytes I and II. We have observed degranulation (both type I and type II granules) and release of components from the GII and from these observations, we assume that granulocyte II may be the possible cell type that stores prophenol oxidase and other potent enzymes important in innate immune reactions. In general, exposure to live microorganisms or microbial components induced degranulation and release of components of prophenoloxidase system from hemocytes of many other decapods (Sritunyalucksana *et al*., 1999; Xian *et al*., 2016). In our study, small electron dense type I, II and III granules were often encountered in the cytoplasm of SGC. Furthermore, degranulation and release of granules were also monitored in SGC, possibly indicating the release of enzyme phenoloxidase, required for melanization process. This is consistent with the observations made in other decapod crustaceans, in which phenoloxidase activity has been observed after exposure to microbial pathogens (Johansson, Söderhäll, 1985; Sricharoen *et al*., 2005)

In our study, one of the prominent features of SGC is the presence of small dense granules which are distributing throughout the cytoplasm. The SGC with large vacuole exhibited degranulation of the small dense granules as evidenced from their reduced number in the cytoplasm also we could see some endocytosed materials inside the large vacuole. These results may indicate the immune response of the SGC against exposed unknown foreign materials. We expect that certain factors released through the degranulation might induce endocytosis of foreign materials by semigranulocytes. This is consistent with the observations made by Söderhäll and Hall (1984) who demonstrated laminarin and LPS induced degranulation of hemocytes with small granules in the crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823). Söderhäll and Hall (1984) reported that hemocytes on exposure to fungal pathogen, leads to the activation of proPO system. Certain proteins of the proPO system are attached to foreign surfaces, like fungal hyphae and it is suggested that these attaching proteins are responsible for the opsonic function of the hemocytes in crayfish. Another study demonstrates that on adhered cells, these adhesion proteins stimulate a multicellular response, like encapsulation. They also found to activate phagocytosis in *Carcinus maenas* (Linnaeus, 1758) hyaline cells (Aspan, Söderhäll, 1991; Cerenius, Söderhäll, 2004). Furthermore, LPS-activated degranulation and release of proteins of the proPO system from hemocytes leads to increased phenoloxidase activity in black tiger shrimp (Sritunyalucksana *et al*., 1999; Xian *et al*., 2016). In general, several studies recommend that the innate immune system is quickly triggered after immune challenges (Wang, Wang, 2013; Kulkarni *et al*., 2021). We find that further cytochemical investigations are required to ascertain the definite roles of hemocytes in the immune processes of *C. steniops.*

Conflicts of interest

The authors declare that they have no conflicts of interests.

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