OPEN ACCESS
International Journal of Aquatic Science
ISSN: 2008-8019
Vol. 9, No. 2, 77-84, 2018

Cytological study of *Echinometra mathaei* (Echinoidea: Camarodonta: Echinometra) the Persian Gulf sea urchin

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Received: December-18-2017 **Accepted:** January-14-2018 **Published:** April-02-2018

Abstract: The sea urchins belong to the phylum of Echinodermata, have been founded in the middle or the lowest depth of marine coral reefs. The sea urchins have been used in many different studies for more than one century. As there was no comprehensive study concerning characterization of the Persian Gulf sea urchin, *E. mathaei*, this study was aimed to investigate the cytology of its coelomocytes. Study of cytology was performed using light, scanning, and electron microscopy and flow cytometry as well. Coelomic cavity of sea urchin is filled with coelomic fluid that houses free mobile populations of cells of sea urchin *E. mathaei*, specifically called coelomocytes. They are distinguished to seven types based on their morphological and ultrastructural characteristics including red and colourless spherulocytes, granular spherulocytes, small spherulocytes, vibratile cells, and phagocytic cells. Phagocytic cells exist in two distinct forms; the petaloid and filopodial cell. Flow cytometry analyses showed three cell populations. To our knowledge, this is the first report describing ultrastructural details of the coelomocytes of *E. mathaei*.

Keywords: Sea urchin, *Echinometra mathaei*, Cytology

Introduction

In spite of significant value of the Persian Gulf ecosystems in terms of biodiversity, a few studies were locally conducted on marine animals of its environment. Up to now, no study was documented regarding cell morphology of the Persian Gulf sea urchin, *E. mathaei.*

The sea Urchins belong to the phylum of Echinodermata, a large group of marine basal deuterostomes with 7000 species that originated from Cambrian period. Up to now, about 8000 species of sea urchins (Echinometra genus) have been reported in oceans as common marine invertebrates (Pawson, 2007; Yokota, 2002; Zhou *et al*., 2011).

The body shape in sea urchin is spherical surrounded by sharp spines and hundreds of tube feet provided them the ability to move on rock and soft surfaces. Their mouth composed of five teeth surrounded by a soft tissue (Amarowicz *et al*., 2012).

The coelomic cavity is encompassing gonads, aquatic vascular system, and the gut that are suspended in the coelomic fluid. Coelomic fluid contains different kinds of cell populations. The entity of this fluid is similar to sea water, but has lower salinity and also includes some proteins that are involved in anti-pathogen responses (Smith *et al*., 2010).

In late 1900's, Russian biologist Elie Metchnikoff (1854-1916) noticed that some cells in the perivascular coelom and mesenchymal tissue of echinoderms show the ability to move and engulf inert or even living particles (Metchnikoff, 1891; Silva *et al*., 1995, 1998).

Sea urchins, has partially been reported to have three main coelomocyte cells: phagocytes, vibratile cells and the spherulocytes (Smith, 1981; Chia and Xing, 1995, Smith *et al*., 2006). However, there may be more cell types that have not been discovered yet.

The role of coelomocytes in immunity, wound repair, coagulation, allograft rejection (Hildeman and Dix, 1972) bacterial clearance (Plytycz and Seljelid, 1993; Yui and Bayne, 1983) encapsulation and opsonisation (Clow *et al*., 2004) has been demonstrated. Sea urchins have been used in many different studies like gene expression regulation, molecular embryology, reproductive biology, cellular biology, evolution, population genetics, and toxicology for more than one decade (Bodnar, 2013). Coelomocytes can be used as an indicator of water pollution and surveillance tools in ecological and environmental studies (Kuwahara *et al*., 2009). As there was no comprehensive study concerning characterization of the Persian Gulf Sea urchin, *E.*

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mathaei, this study was aimed to investigate the cytology of its coelomocytes.

Materials and Methods

Sample collection

Ten specimens of *E. mathaei* (Blainville, 1852) weighted from 50-70 g were collected manually at depth of zero to 5 m from coastal waters of Lark island, the Persian Gulf (the South of Iran) (Fig.1) in January 2016. The specimens were kept alive and transferred to the laboratory at Pasteur Institute of Iran. The specimens were maintained in a flow-through aquarium and were acclimatized at least for two weeks prior to experiments as any kind of stress may disturb the homogeneity of cell populations.

Fig.1: Specimen collection area. Larak Island, Persian Gulf (26°51'12" N 56°21'20" E). © National Geographic

Cytology

Isolation and preparation of Coelomocytes

For extraction of coelomic fluid containing cells, the specimens were brought out of water and placed oral side up on a bench. A needle was inserted through the peristome, between the test and the lantern, into coelomic cavity to a maximum depth of 1 cm. About 5.0 to 10.0 ml of coelomic fluid were slowly drawn using a 25 gauge needle without sacrification. CF was mixed with equal volume of EDTA (50 mM) in PBS (0.01 X), as anticoagulant reagent (Arizza *et al*., 2007). The sea urchins were returned to the aquaria.

Light microscopy

For fresh visualizing of living cells, some drops of coelomocytes containing CF suspension (100 µl) were placed onto glass slide and living coelomocytes were quickly examined under light microscopy. Morphology of the cells was viewed on a light microscope in 10 and 40X magnifications.

Surface morphological assay by FE-SEM

Coelomic fluids were collected and centrifuged at 1000 rpm for 1 min at ambient temperature. The pellets were immediately fixed with 2.5% glutaraldehyde (v/v) in 0.1 X PBS (pH 7.4), washed in 0.1 X PBS, and dehydrated in graded series of ethanol (10, 30, 50, 70, 90, and 100%) and allowed to be dried on RT. After that, the specimens were mounted on aluminum stubs and sputter-coated with 10 to 20 nm gold nanoparticles. Observations were made on a FE-SEM instrument (MIRA3, TESCAN Co., Czech Republic).

Evaluation internal part of Coelomocytes by TEM

Coelomic fluids were collected and centrifuged at 1000 rpm for 1 min at ambient temperature. The cell pellets were fixed in 2.5% glutaraldehyde (v/v) in anticoagulant solution for 12 h at room temperature. Coelomocytes were then resuspended in anticoagulant solution, embedded in agar (2.5%), centrifuged at 800 rpm for 5 min and post fixed in 1% osmium tetroxide in cacodylate buffer for 2 h at 4°C. Coelomocytes were then dehydrated in graded ethanol series (50, 70, 90, and 100%) and then embedded in the resin. Ultrathin sections (50–70 nm) were stained in uranyl acetate and lead citrate for 30 and 5 min, respectively. Specimens were observed and photographed using a Zeiss EM900 electron microscope operated at 20 kV.

Study of coelomocyte populations by flow cytometry

To further study of cell populations, flow cytometric analyses were implemented on fresh coelomic fluid using a flow cytometer (CyFlow ML, Partec Co., Germany). Size and granulocity of different cell populations were evaluated by FloMax software in forward (FSC) and side scatter (SSC) channels.

Results

Cytology

Cytology of Coelomocytes regards to classification of the cell populations, and their surface and internal morphology was performed by light microscopy (LM), Field Emission Scanning and Transmission Electron Microscopy (FE-SEM and TEM), and flow cytometry.

Light microscopy

The coelomocytes of CF were classified to seven cell types, based on light microscopy evaluations. These different coelomocytes types were distinguished according to their size, shape, and color under LM in fresh preparations of CF from the sea urchin.

Three basic categories of coelomocytes; i.e. phagocytes, vibratile, and spherule cells were observed (Figs. 2 and 3).

Phagocytes have a round or bean-shaped eccentric nucleus in a vast cytoplasm surrounded by an enormous membrane. There were two categories of phagocytes depending on their morphology and size including petaloid and filopodial cells (Fig. 2). Petaloid phagocytes have lamellipodia that extended from the cell body through all directions (Fig. 2A). Filopodial cells had numerous long filopodia. They have unconvertible filopodial morphology (Fig. 2B). Vibratile cells were small spherical cells with a long flagellum. The cells could swim rapidly through the fluid by their flagellum (Fig. 2C).

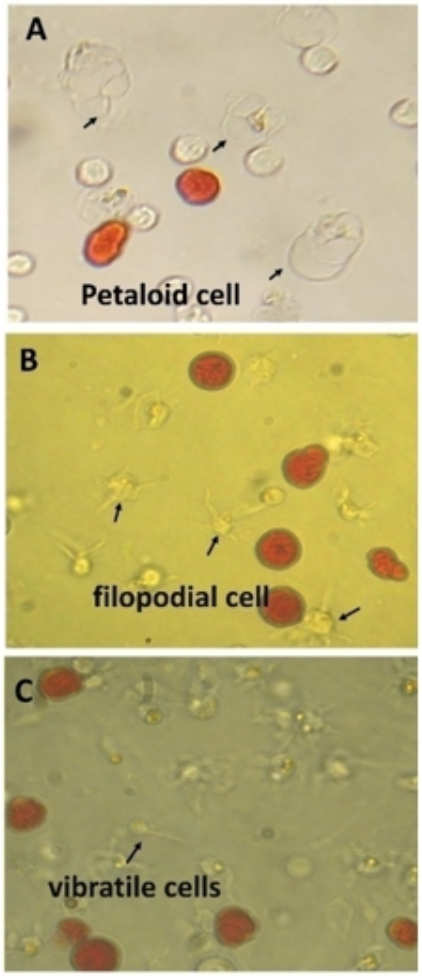

Fig. 2: Morphology of the petaloid (A. 40X), filopodial phagocytes (B. 40X), and vibratile cell (C.40X) in the CF of *E. mathaei*.

Spherule cells were round or ovoid in shape. Cytoplasm of spherule cells was filled with spherical inclusions (granules), obscuring the irregularly-shaped nucleus. The granules in spherule cells were packed tightly against the cell membrane. Based on our observations, spherule cells were sub-categorized to orange granular spherule cells, red spherule cells, colorless spherule cells, and small orange spherule cells (Fig. 3).

Colorless spherulocytes were spherical to oval in shape; their nucleuses were round and often eccentric. Their cytoplasm was slightly flattened and filled with colorless spherules of unknown content. Orange granular spherulocytes displaying around shape in which their round nucleus were located in center or in the periphery of the cells. The cytoplasm was filled with large spherules containing an orange content of unknown composition. Red spherulocytes were spherical to oval in shape. Nucleus was not visible due to the accumulation of red granules in cytoplasm. Small spherulocytes had limited numbers of small and spherical cells were observed (Fig. 3).

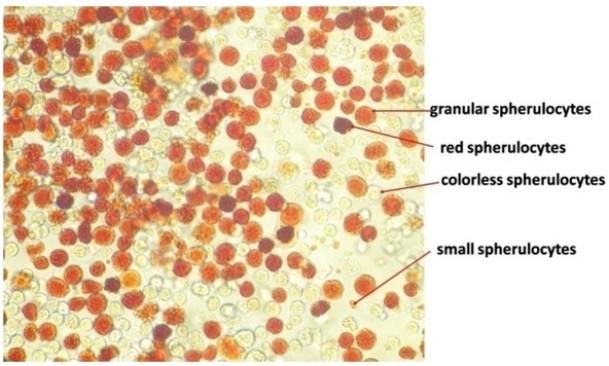

Fig. 3: Spherule cells in CF of *E. mathaei.* Four types of coelomocytes including 'orange granular spherule cells', 'redspherule cells', 'colorless spherule cells', and 'small orange spherule cells' identified and characterized based on their shape and color using light microscopy. 40X.

Evaluation of Surface Morphology of coelomocytes by FE-SEM

Seven types of coelomocytes were recognized on the basis of their size, shape, surface morphology, and the shape and size of their pseudo-legs as well.

Phagocytic cells have several vast or narrow appendices that can be seen at their surfaces. There were two kinds of recognizable phagocytic cells which were different in number and shape i.e. petaloid and filopodial cells. Petaloid cells had different lamellipodia extended in all orientation from the cell body (Fig. 4A). Filopodial cells had different mendacious legs which were distributed orderly in their margins. They had

long filopodia. This kind of filopodia had disorderly distributed in various direction of the cell (Fig. 4B). Vibratile cells have relatively long pseudopodia that were located in one pole of the cell (Fig. 4C).

Spherulocytes were spherical cells but due to enjoying many small spherules in their cytoplasm, their surfaces were not smooth. They were four spherulocytes based on their size, shape, and color including small, red, colorless, and granular spherulocytes.

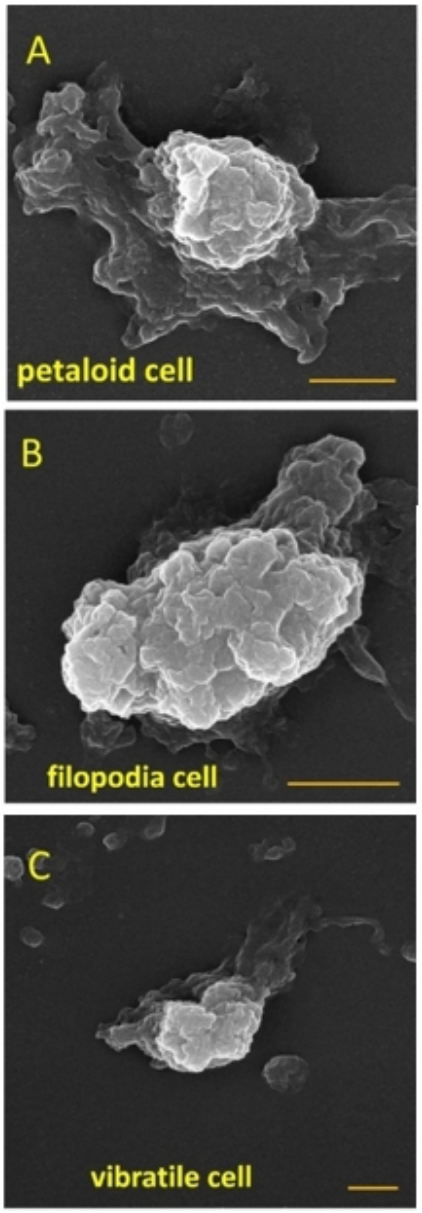

Fig. 4: Scanning electron micrographs of coelomocytes of *E. mathaei*. A) Petaloid cell; B) Filopodial cell; C) Vibratile cell. Scale bars: 2 µm.

Small spherulocytes were small cells and among other coelomocytes are few in number. They were seen with a relatively smooth surface (Fig. 5A). Red spherulocytes are spherical in shape containing small spherules which entirely filled the cells. Their surfaces were significantly rough (Fig. 5B). Colorless spherulocytes had moderately rough surface and were slightly smaller than red ones (Fig. 5C).

Granular spherulocytes were round or oval in shape and had a remarkable rough surface (Fig. 5D). Small pseudo-legs were seen in the periphery of red, colorless, and granular and seen in low density.

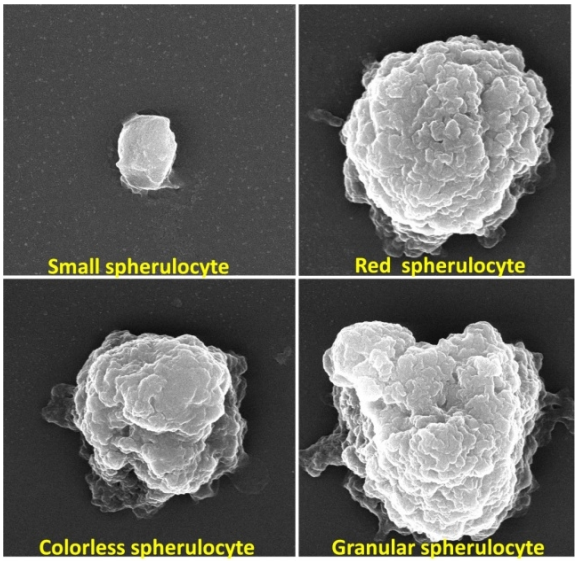

Fig. 5: *E. mathaei.* Scanning electron micrographs of coelomocytes of the sea urchin. Four types of coelomocytes are seen in difference size. A) Small spherule cells; B) Red spherule cells; C) Colorless spherule cells; D) Granular spherule cells. Scale bars: 2 µm.

Evaluation of internal part of the coelomocytes by TEM

Internal organelles and compartments of the coelomocytes were studied in detail with TEM. Seven types of aforementioned cells were seen in TEM and differentiated from each other based on their size, shape, the density and size of their granules, and also morphology of nucleus and nucleolus.

Petaloid cells had a petal-shaped cytoplasm with remarkable pseudopodia. The nucleus was heterochromatin with highly dense nucleoli (Fig. 6A). Filopodial cells had filiform cytoplasmic appendices that had been expanded in different directions. They had segmented euchromatin nucleus without visible nucleolus (Fig. 6B). A vibratile cell contains many large hyaline vacuoles and a few granular dense bodies. A few Smooth Endoplasmic Reticulums (SERs) were seen para-centrically adjacent to a Golgi system. Nucleus and flagellum were not seen in this section (Fig. 6C).

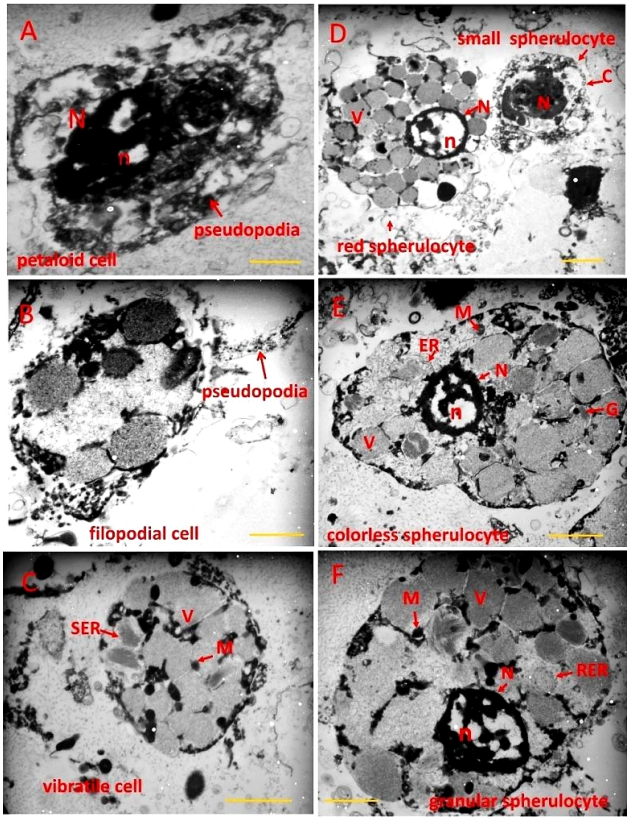

Fig. 6: *E. mathaei's* coelomocytes morphology demonstrated by TEM. Seven types of coelomocytes are identified and characterized. Abbreviations: N, nucleus; n, nucleolus; G, Golgi complex; V, vacuole; RER, Rough Endoplasmic Reticulum; SER, Smooth Endoplasmic Reticulum M, mitochondria. Scale bars: 2 µm.

Spherulocytes were spherical or ovoid cells with a relatively peripheral nucleus. These cells were filled with numerous and various granules of different sizes, which can be divided into four classes including small, red, colorless, and granular spherulocytes based on the density and shape of the granules and the placement of the nucleus in the cell (Fig. 6).

Small spherulocytes were the smallest coelomocytes. In the original form, they are spherical cell with a large nucleus filling almost all of the cell's space. In the image taken by an electron microscope, there was a large nucleus and a thin cytoplasm being limited to the edge of the cell. Aggregate and homogeneous materials were visible in the cell. Some small dense nucleoli were seen within the nucleus (Fig. 6D).

Red spherulocytes were spherule cells with a relatively large regular nucleus that was compressed to the edge of the cell, which is a characteristic of this type of cells. Chromatin was relatively dense in which sometimes the nucleolus was observed. The cytoplasm contains many spherules of various sizes and dense electron content. In some cells, sometimes there are empty vacuoles in the cytoplasm. Some mitochondria, a well-developed Golgi complex and sometimes pseudo legs are found (Fig. 6D).

Colorless spherulocytes were elliptical or circular cells with a peripheral nucleus in which the cytoplasm had lots of large vacuoles filled with fairly or moderately electron dense materials. It is also possible to see some of the developed vesicles from the Golgi apparatus as well as the endoplasmic and mitochondrial network (Fig. 6E).

Granular spherulocytes were ovoid or circular cells with irregular large nuclei drawn to the cell's environment in which sometimes the nucleoli were visible. The cytoplasm of these cells contained multiple secretary granules with fairly or moderately dense electron content. A large number of mitochondria and a well-developed Golgi complex were found (Fig. 6F).

Study of coelomocyte populations by flow cytometry

Coelomocytes were analyzed by flow cytometry to find possible population of the cells. Three groups were determined reference to their relative size and granulocity.

The population R1 contained many small cells that are related to phagocytes and other small spherulocytes. The population R2 showed medium size cells which might be an accumulation of colorless coelomocytes. The cells in the population R3 were the largest one and had the most granulocity comparing to others corresponds to red and granular spherulocytes (Fig. 7). The frequency percent of the cells in R1, R2, and R3 were 80.43, 6.3, and 13.25 %, respectively.

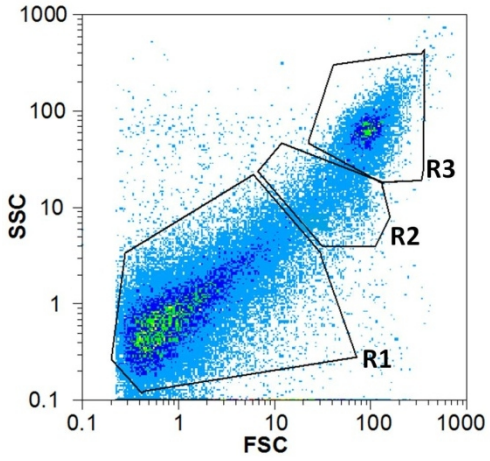

Fig. 7: Flow cytometry of coelomocytes of the sea urchin *E. mathaei*.

Discussion

In this study we tried to characterize the Persian Gulf *E. mathaei* in terms of the cytology of coelomocytes. The coelomocytes were circulating cells in the coelomic fluid that fills the body cavity of echinoderm. Several types of coelomic cells had been described in echinoderms (Chia and Xing, 1995; Edds, 1993). Different studies clearly showed the vast diversity of cells present in the coelomic fluid of echinoderm. However, absence of a standard reference among groups and particularly, differences in terminologies and even specimen preparation protocols, had led to existing heterogeneity.

We have described, for the first time, at least seven types of coelomocytes in *E. mathaei* including red and colorless spherulocytes, granular spherule-ocytes, small spherulocytes, vibratile cells, and phagocytic cells as observed using light microscopy, SEM, and TEM. Some cell types of *E. mathaei* were almost similar to other echinoid species that reported in the previous studies (Gross *et al*., 2000; Smith *et al*., 2006; Arizza *et al*., 2007; Smith *et al*., 2010). Coelomocytes were assumed to be the main effectors of the echinoderm immune system (Gross *et al*., 1999). Studies on echinodermal immune responses carried out by Metchnikoff were the onset of comparative cellular immunology (Metchnikoff, 1893; Gross *et al*., 1999). This effort led to the Nobel Prize award for him and Paul Ehrlich in 1908 for their groundbreaking works on echinodermal models.

We categorized the phagocytes cells to two major types; one with numerous long filopods, and the other cell with bladder-like and veils of cytoplasmic extensions.

Vethamanvy and Fung in 1972 observed two morphological conversion of the phagocyte in *Strongylocentrotus drobachiensis* at different physiological states (Vethamanvy and Fung, 1972). Edds in 1984 has suggested that in *S. droebachiensis*, phagocytes can transform from petaloid to filopodial form spontaneously (Edds, 1984). According to our findings, vibratile cells in *E. mathaei* were round, highly motile, and flagellated that contained large cytoplasmic granules. Vibratile cells were small spherical cells with a long flagellum. We did not find any witness of phagocytosis by the vibratile cell, similar to Vethamanvy and Fung in 1972, however Liebman in 1950 offered phagocytic flagellated cells in *Arbacia punctulata* (Liebman, 1950). Vethamany and Fung in 1972 described ultrastructure of a vibratile cell in *S. drobachiensis*. Vibratile cells move via lashings of the flagellum (Johnson, 1969; Bertheussen and Seljelid, 1978). It is thought that the vigorous movement may assist the circulation of the coelomic fluid within the body cavity (Smith, 1981). The vibratile cell has been seen to discharge mucoid substances in vitro (Bertheussen and Seljelid, 1978) and these substances gelled soon after release. These authors, therefore, suggested that the vibratile cells have the same functional properties as platelet does in vertebrate's blood. However this hypothesis will require further investigations.

Spherule cells exist in all of the echinoderms (Smith, 1981). They have special cytoplasm that filled with many large spherical inclusions. Our observations generally agree with other reports (Deveci *et al*., 2015, Xing *et al*., 2008), although our findings suggest that these cells in *E. mathaei* could be classified to at least four different morphological types including red , small , colorless, and granular spherule cells. TEM and SEM evidences support this difference.

The common specifications of the red and colorless spherule cells that observed in *E. mathaei* are similar to those defined for other Echinoidea (Liebman, 1950; Johnson, 1969; Bertheussen and Seljelid, 1978; McCaughey and Bodnar, 2012; Queiroz and Custodio, 2015). Our results about the general features of the red spherule cells are similar to those previously explained for holothurians (Andrew, 1965) and for *S. drobachiensis* (Vethamanvy and Fung, 1972).

Our results indicated that red spherule cells are distinguished cell type since the spherical electron-dense granules are unlike those observed in the granular spherule cells. These cells are able of low amoeboid motion and likely possess the pigment 'echinochrome A'. The existence of this pigment in red coelomocytes has been previously reported (Koltsova *et al*., 1977; Lee *et al*., 2014). In addition, these cells have a well expanded rough endoplasmic reticulum explicitly indicating active metabolism. Instead of the red pigment of the red spherule cells, colorless spherule cells have numerous vacuoles in their cytoplasm. In *E. mathaei*, the cytoplasmic spherules were seemingly empty with a slightly central electron dense material. Inversely, in *S. purpuratus* and *H. erythrogramma* most spherules were filled by a highly electron-dense material (Heatfield and Travis, 1975; Dheilly *et al*., 2011). The observation of cytoplasmic spherules containing slightly electron dense material

in *E. mathaei* is in accordance with the granules reported in *S. droebachiensis* (Vethamany and Fung, 1972).

According to our observations, small cells contained central nuclei and scanty cytoplasm. They were the less abundant and smallest circulating coelomocytes. In their basic form, they were spherical with a single nucleus that nearly fills the entire cell space although Johnson *et al*. did not mention this kind of cells in her different studies (Johnson *et al*., 1970).

Granular spherule cells were very distinct from the other types of spherulocytes. Their spherules were well specified and their dense nucleuses were ever more visible. These cells have similar morphology to the "granular spherulocyte" observed in *Eucidaris tribuloides* (Queiroz and Custodio, 2015) and "green spherulocyte" observed in *A. punctata* (Liebman, 1950).

FCM plot for coelomocytes showed a similar distribution for all FCM analyses in echinoderms (Xing *et al*., 2008). As there is very little previous works on cytology of coelomocytes using FCM, we tried gating different populations. As no clear separation between subpopulations was observed on FCM plots, flow cytometry as a method for the sorting of different cell types of coelomocytes of *E. mathaei* has proved to be controversial.

Acknowledgements

It is our immense pleasure to acknowledge Mr. H. Aghazadeh for his kindly contribution in SEM and TEM sample preparation. We hereby thank Mrs. M. Ramezani for her kindly contribution in the photography of TEM sections.

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