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RESEARCH ARTICLE

MORPHOLOGICAL CHARACTERIZATION OF HEMOCYTES AND HISTOLOGICAL STRUCTURE OF THE HEMOPOIETIC TISSUE "WHITE BODY" OF THE CUTTLEFISH "SEPIA OFFICINALIS" (CEPHALOPOD:SEPIIDAE).

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Abstract

The present study focused on the morphological characterization of hemocytes of *Sepiaofficinalis* and the histological structure of its hemopoietic organ (white body). Haemolymph was collected from the branchial heart with syringe then blood smears were prepared and stained with Haematoxylin& Eosin (H&E).White body was dissected and fixed in Kahl's fixative for histological studies also other pieces were fixed in glutardehyde for the preparation of semithin and ultrathin sections. Three main types of hemocytes were identified by light microscope: Hyalinoytes, granulocytes and agranulocytes.The histological structure of hemopoietic organ (white body) of cephalopods revealed that it is a multilobed organ enclosed by a thin fibrous connective tissue capsule that consists of collagenous fibers. Hemocytes within the white body were enclosed in a supportive connective tissue fibers that form a network of small unites in which hemocytes are grouped in clusters.

Introduction:-

Cephalopods are the largest members in phylum Mollusca. They include nautilus, octopus, squid and cuttlefish. Cephalopods differ from other molluscs in that they have a closed circulatory system that consists of a central heart, two branchial hearts, and a system of blood vessels throughout the tissues (Castillo *et al.*, 2015).

Cephalopods are economically and medically important; they are very important in fisheries, act as host for many parasites that can be transmitted to human such as *Anisakis* sp. and also used as an ecotoxicology model so understanding of their immune system is very important (Hochberg, 1990; Le Pabic *et al.*, 2014).

Hemocytes are the cellular components of the haemolymph but they are also present in many sites such as vascular tissues and the connective tissue (Loker, 2010). They play an important role in the internal defense and also function in wound repair, digestion, transport and excretion of nutrients (Chu, 2000)

Molluscan hemocytes can be distinguished into at least two main types, the granulocytes and the agranulocytes (Hine, 1999; Castellanos-Martínez *et al.*, 2014). The two main types of hemocytes could be classified into many sub-types according to many criteria such as their shape, cell size and granularity (Pila *et al.*, 2016).

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Nomenclatures of cephalopod blood cells differ by various researchers. For example some researchers referred to the cephalopod blood cells as leukocytes (Bolognari, 1951), amebocytes (Wells, 1978) or hemocytes (Cowden and Curtis, 1974, 1981).

The process that is responsible for the production of new blood cells to the circulation and the tissues is called haematopoiesis (Pila *et al.*, 2016). In coleoid cephalopods, including octopus (*Octopus vulgaris*, *O. briareus*), cuttlefish (*Sepia officinalis*) and squid (*Euprymna tasmanica*), the site of haematopoiesis is the white body (Pila *et al.*, 2016).

The white body is located in the orbital pits behind the eyes, it is composed of many lobes and covered with a connective tissue layer. The haemopoietic elements in the white body exists as folds that extend deeply into its lumen which form a series of interconnecting sinusoids filled with mature hemocytes or prohemocytes (Cowden and Curtis 1974, Salazar *et al.*, 2015; Castillo *et al.*, 2015).

The present study aimed to identify the different hemocyte types and sub-types of *Sepia officinalis* and to study the histological structure of the hematopoietic organ (white body).

Materials and Methods:-

Collection and identification of Samples:

Female Specimens (n=3) were collected from Red sea around the aqua area and then kept in National Institute of Oceanography and Fisheries – NIOF- Red sea branch for 1-2 days.Then transferred to the laboratory of zoology department at Assuit University in air-pumped tank. Specimens were identified according to FAO species catalogue (Jereb and Roper, 2010).

Collection and Staining of Haemolymph:

Specimens were dissected; the ventral mantle was opened to expose the branchial hearts. Haemolymph was collected from the branchial heart with syringe then blood smears were prepared and stained with Haematoxylin& Eosin (H&E).

Histological and Histochemical studies:

White body was dissected and fixed in Kahl's according to Awad (1999) for histological studies. 7 micron paraffin transverse serial sections were prepared and stained with Haematoxylin& Eosin (H&E) and Masson's trichrome stain for histological and histochemical examination.

Transmission electron microscopy:

Small pieces of the white body were fixed in 2% glutaraldehyde. Semithin and Ultrathin sections were prepared according to the protocol of transmission electron microscope unite, Assuit University. The obtained ultrathin sections were examined by transmission electron microscope (JEOL TEM 100 CXII) at 80 kv and photographed.

Results:-

Classification of Hemocytes:

Blood smears of *Sepia officinalis* were examined by light microscope. A total of 1596 blood cells (hemocytes) were counted. The hemocytes can be classified into three main types: hyalinocytes, granulocytes and agranulocytes.

Hyalinocytes (19% of total cells counted):

These cells have a very little amount of agranular or granular basophilic cytoplasm. They may be rounded, triangular, spindle shape or oval (Fig.1a-e).

Granulocytes (71 % of total cells counted):

Granulocytes were distinguished into three main types according to the staining affinity of their cytoplasm and granules:

Granulocytes with acidophilic granules and cytoplasm (54 % of total cells counted):

Nuclei in this type vary in their shape and position. Cells may have one nucleus (uninucleated) or have two or three nuclei (multinucleated). The nucleus in the uninucleated hemocytes has different shapes (Rounded, Oval, or kidney

shape), different positions (central or peripheral) and has one, two or three lobes (Fig. 2). The multinucleated cells have two or three nuclei with rounded or triangular shape (Fig. 3).

Granulocytes with acidophilic cytoplasm and basophilic granules (16% of total cells counted):
These cells may be rounded (Fig.4a, b), oval (Fig.4c) or pear (Fig.4d) in shape.

Granulocytes with basophilic cytoplasm and acidophilic granules (1% of total cells counted):
In this type the acidophilic granules are grouped in a peripheral position in the cytoplasm. It may have one bilobed peripheral nucleus (Fig. 5a, b) or two central nuclei (Fig.5c).

Agranulocytes (10% of total cells counted) have two sub-types:
Agranulocytes may have acidophilic or basophilic cytoplasm as follow:

Agranulocytes with acidophilic cytoplasm (7% of total cells counted):
they may be uninucleated cells (rounded, oval, Irregular or club-shaped cells with rounded or oval nucleus) (Fig. 6) or multinucleated cells with two or three nuclei (Fig.7).

Agranulocytes with basophilic cytoplasm (3% of total cells counted):
they have different shape (rounded or oval). Their nuclei may be rounded or oval (Fig. 8).

Agranulocytes with acidiophilic or basiophilic cytoplasm may exhibit blunt pseudopodia (Fig. 6f), (Fig. 9a). While, agranulocytes with basiophilic cytoplasm may exhibit filopodia (Fig. 9b, c).

One or more vacuoles may be present in the agranulocytes (Fig. 10a,b). Some hemocytes may contain or secrete refractile material (Fig. 10 c-f) or may contain inclusion bodies (Fig. 10g, h). Cell fragments were recorded as in figures 6f and 8a.

The previous results revealed that the predominant hemocyte type is the granulocytes that have acidophilic granules and cytoplasm especially those with kidney shape nucleus.

Histological structure of the white body (the hematopoietic organ):
The white body is amultilobed organ, with white color, located in the orbital pit behind the eye (Fig.11)

Examination of serial sections of the white body by the light microscope showed that it consists of several lobes which are separated anteriorly and gradually interconnected posteriorly (Fig.12). The white body is enclosed in a thin layer of connective tissue capsule which is composed of collagenous fibers (Fig. 13a). Histologically there are two types of lobes. The first type consists of sex layers (Fig. 13b); the first layer is supported by a net of connective tissue fibers which enclosed the hemocytes within it (Fig. 13c, d). The second and the fourth layers are composed of compact layer of connective tissue (Fig. 13e, f). The third and fifth layers are composed of patches of supportive connective tissue fibers where hemocytes are attached (Fig. 13b, e, f). The sixth layer lines the lobe and is composed of connective tissue fibers which have a reticular appearance (Fig. 13b).

The second type is composed of clusters of hemocytes and few connective tissue fibers (Fig. 14a, b).

Examination of the hemocytes that located in the white body by the transmission electron microscope showed that hemocytes may be granulated or agranulated and may have oval, rounded or kidney shaped nucleus. The hemocyte that contain oval shape nucleus is the predominant type, while hemocytes with kidney shaped nucleus are very rare in the white body (Fig. 14).

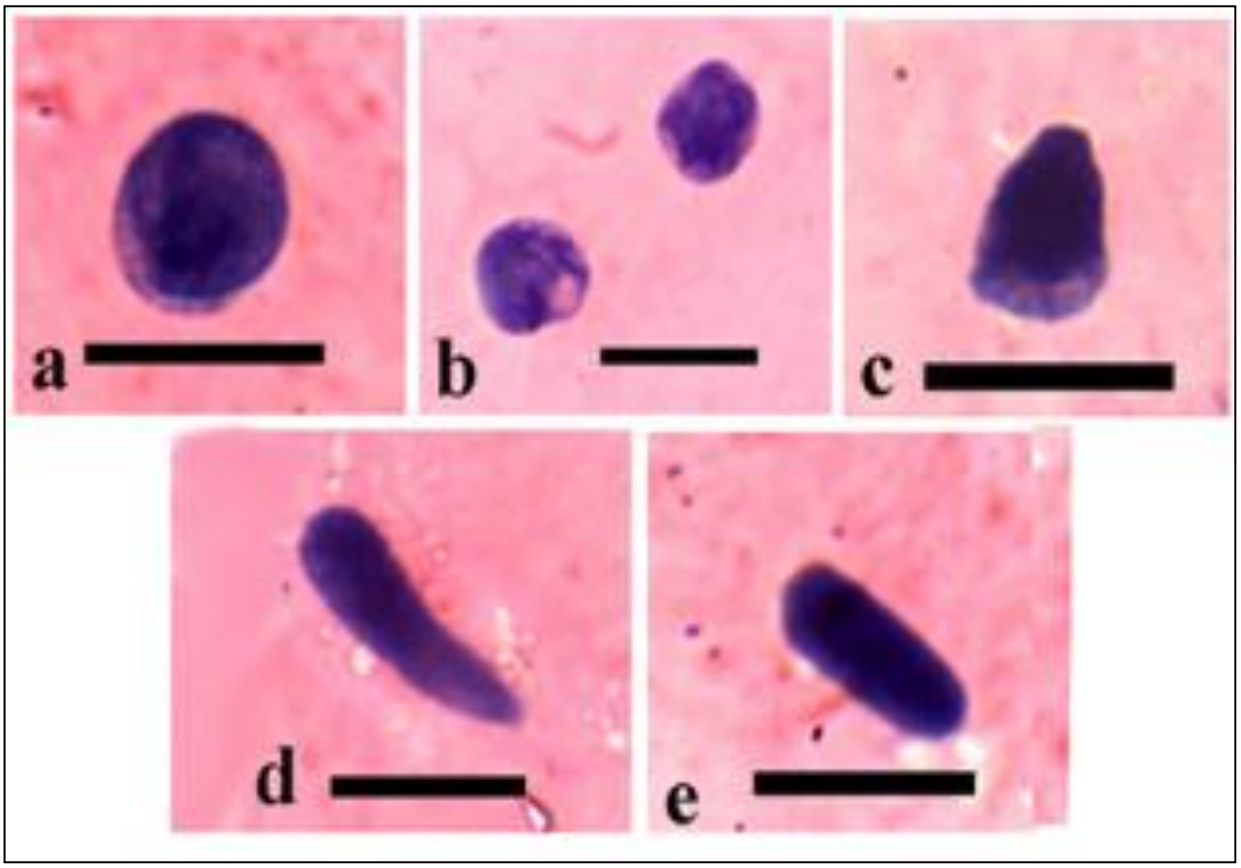

Fig.1:-A photomicrograph of hyalinocytes with its different shapes: rounded (a and b), triangular (c), spindle shape (d) or oval (e). Scale bar =10µm.

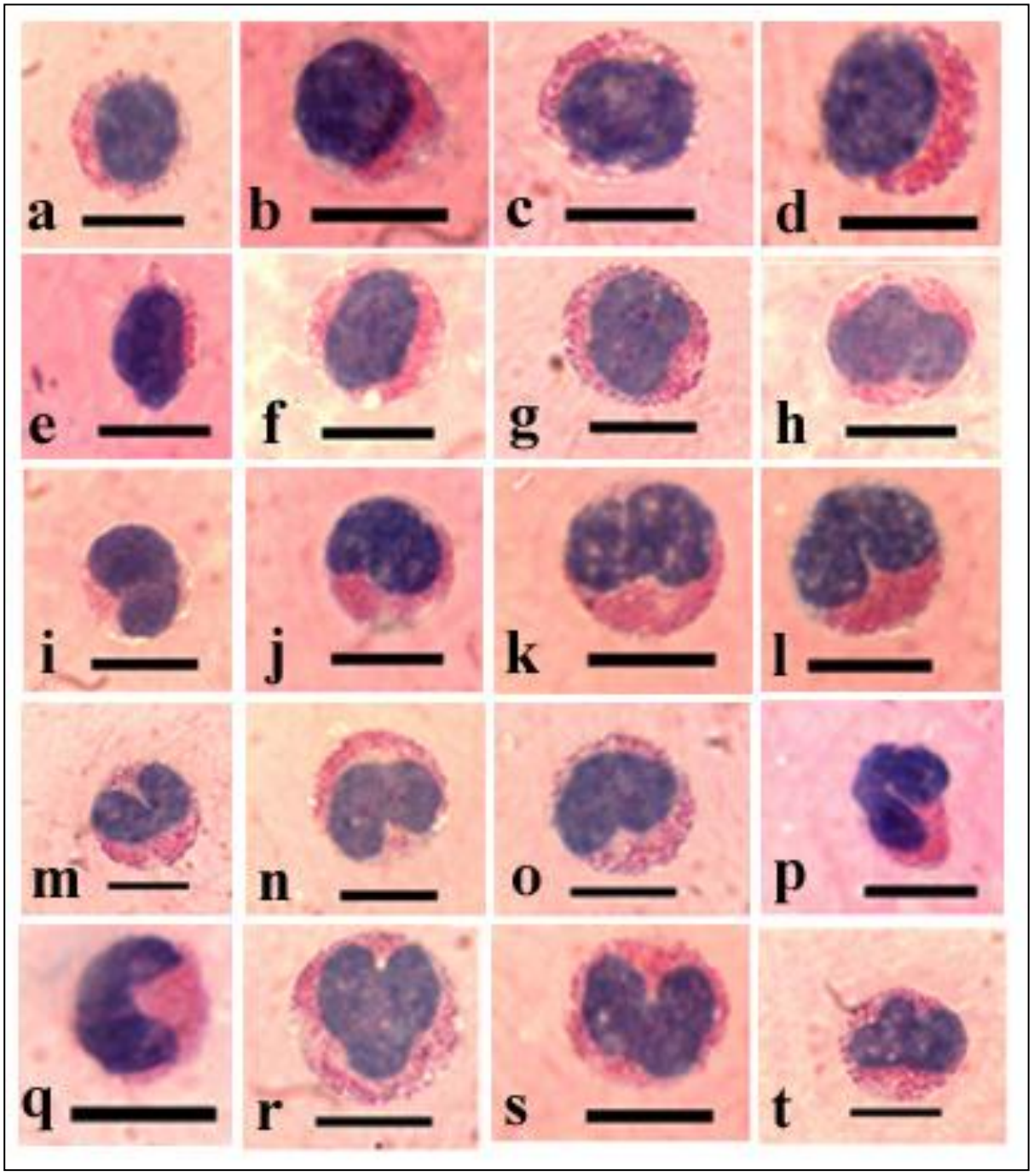

Fig.2: A photomicrograph of granulocytes (with acidophilic granules and cytoplasm) showing different shapes of uninucleated granulocytes. The nucleus of these cells may have one (a-k), two (l-p) or three lobes (r-t). The nucleus has different shapes (rounded (a-c), oval (d-f), oval with central restriction (g, i-k), irregular (h), U-shape (l-p) or kidney (q) shape). The nucleus located either centrally (g,h,r) or peripherally as the remainder.Scale bar =10µm.

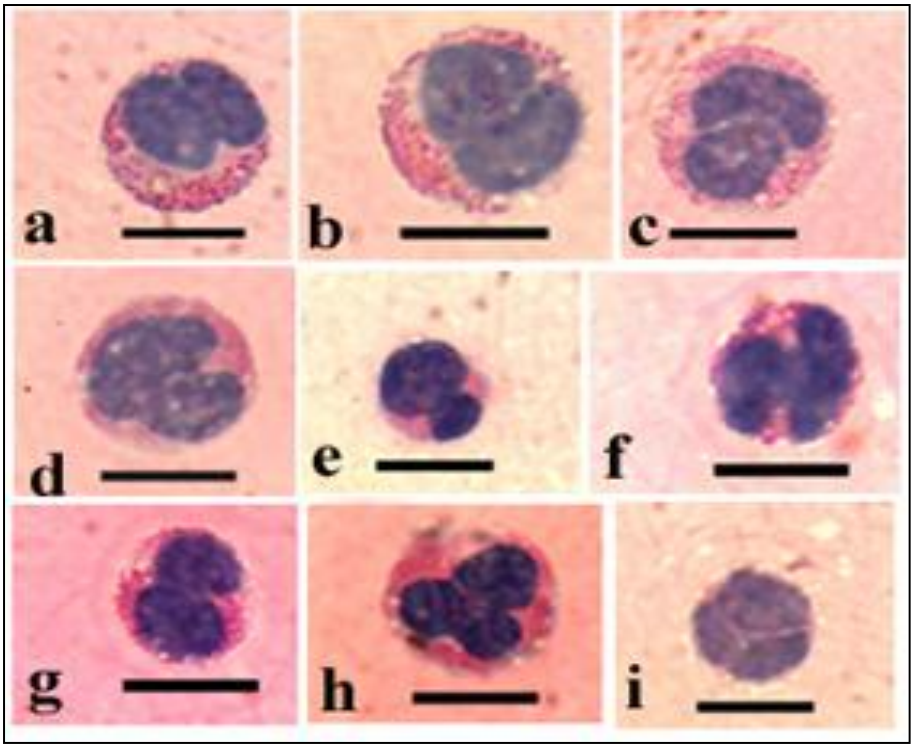

Fig.3: A photomicrograph of granulocytes (with acidophilic granules and cytoplasm) showing different shapes of multinucleated cells which have two (a-g) or three nuclei (h and i). Scale bar =10µm.

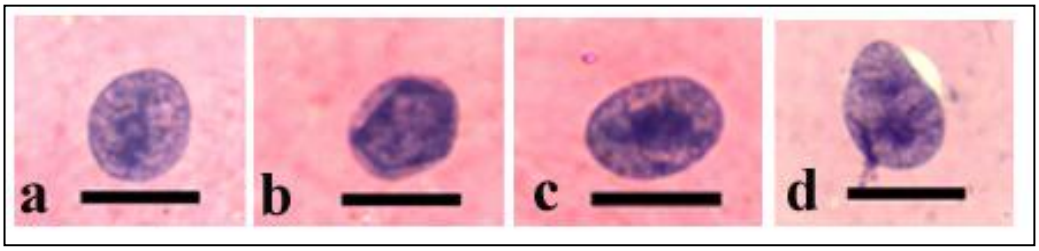

Fig.4: A photomicrograph of granulocytes (with basiophilic granules and acidophilic cytoplasm) with different shapes: rounded (a,b), oval (c) or pear (d) shape.

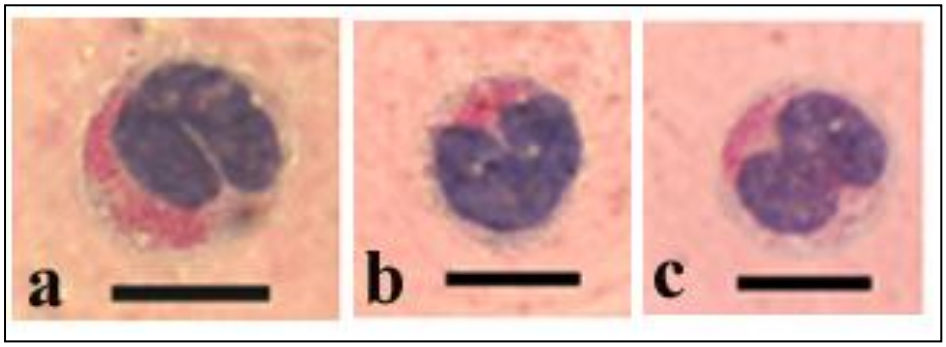

Fig.5: A photomicrograph of granulocytes (with acidophilic granules, which grouped peripherally, and basiophilic cytoplasm). It may have one bilobed peripheral nucleus (a, b) or two central nuclei (c). Scale bar =10µm.

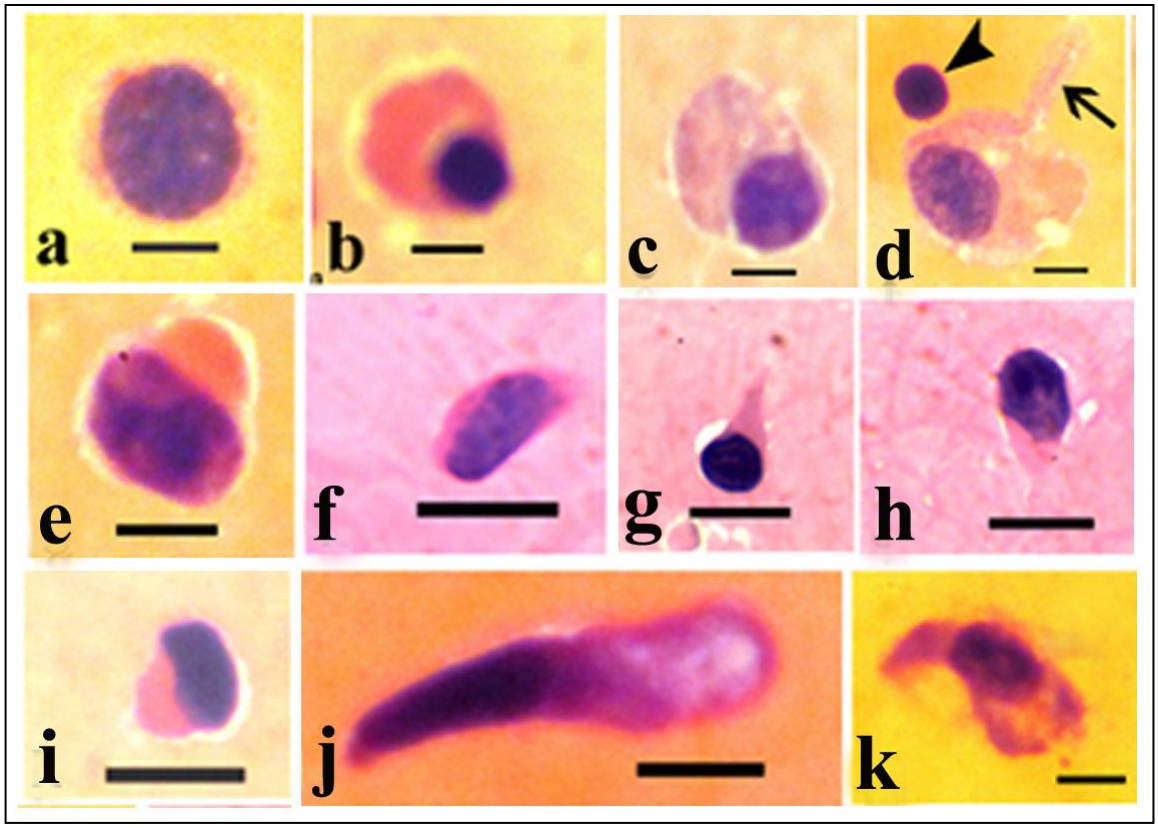

Fig.6: A photomicrograph of uninucleatedagranulocytes with acidophilic cytoplasm. Cells vary in their shape: rounded (a), pear (b) ,oval (c-f), triangular (g-i), club-shaped (j,k). Nucleus located either centrally (a) or peripherally (b-k). arrow refers to blunt pseudopodia, arrow head refers to cell fragment. Scale bar: (a-e and i-k) =50µm, (f-h) =10µm.

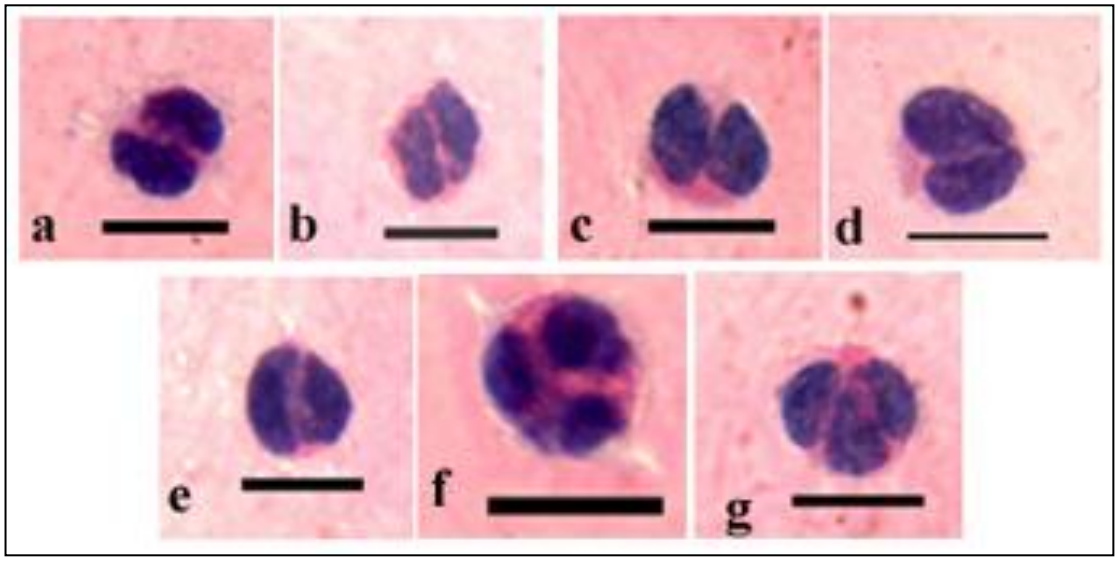

Fig7: A photomicrograph of multinucleated agranulocytes with acidophilic cytoplasm. These cells with two (a-e) or three nuclei (f and g).Scale bar =10µm.

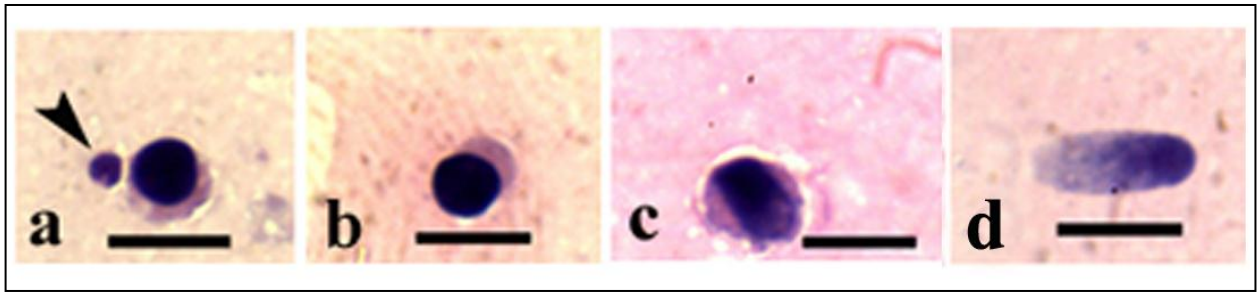

Fig.8: A photomicrograph of various shapes of agranulocytes with basiophilic cytoplasm, rounded (a and b) or oval (c and d) shape. arrow head refers to cell fragment**.** Scale bar =10µm.

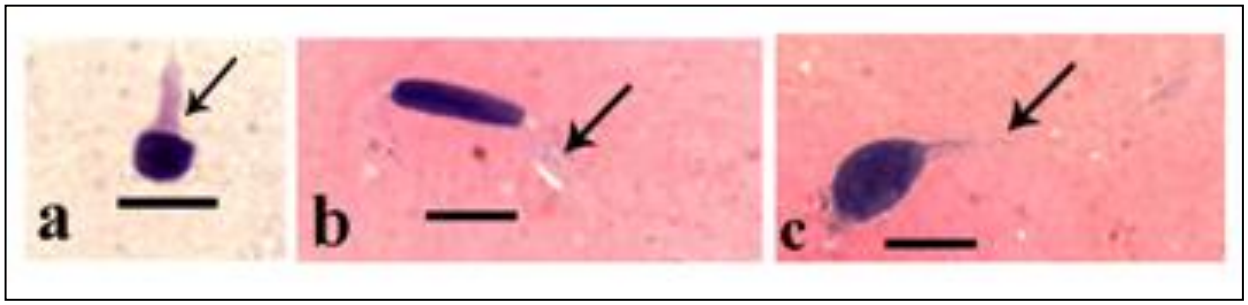

Fig.9: A photomicrograph of agranulocytes with basiophilic cytoplasm, arrow refers to blunt pseudopodia (a) or filopodia (b) and (c).Scale bar =10µm.

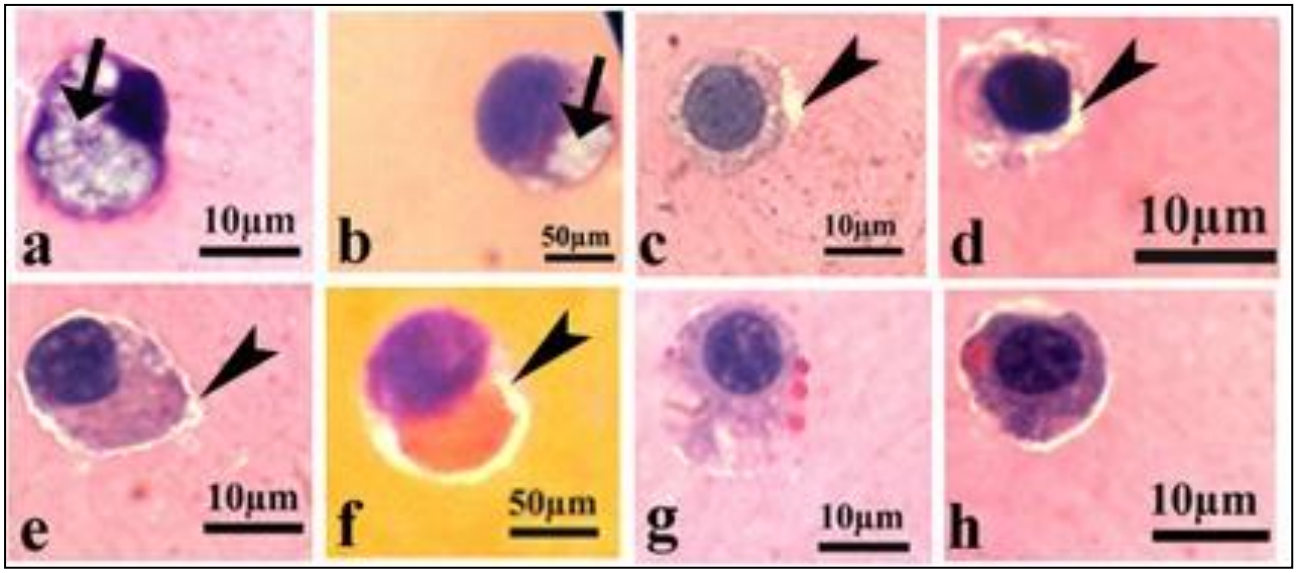

Fig.10: A photomicrograph of agranulocytes with one or more vacuoles (arrow in **a,b**). Hemocytes containing refractilematerila (arrow head in c-d) or secrete refractile material (arrow head in **e** and **f**) or may contain inclusion bodies (**g** and **h**).

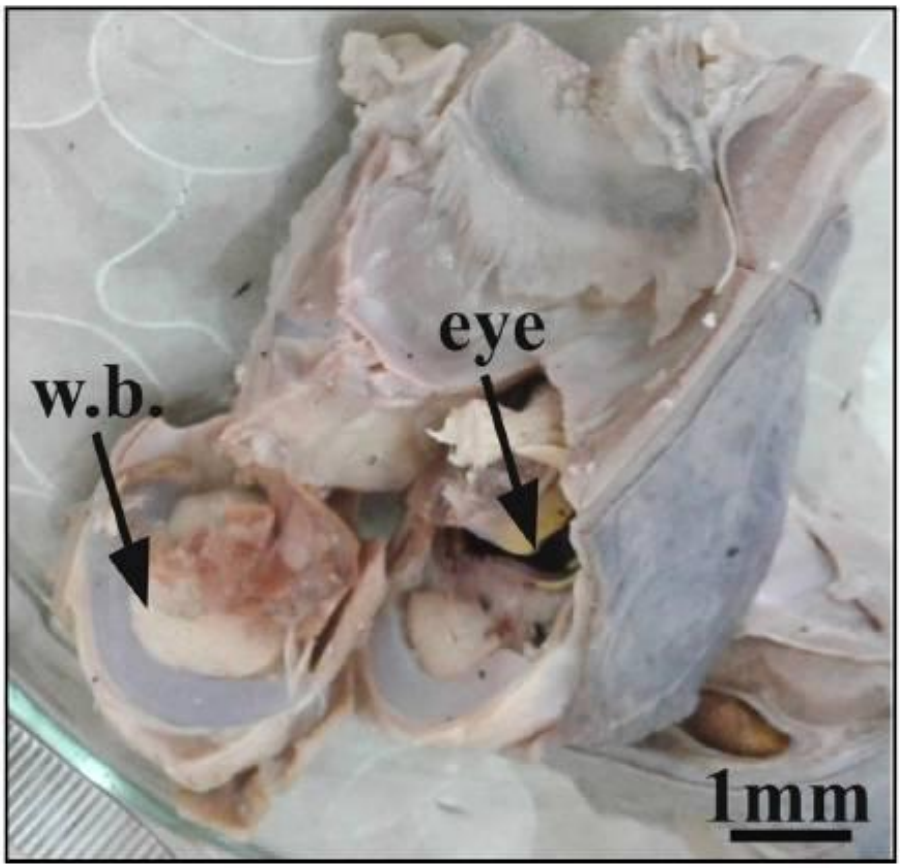

Fig. 11: Photograph of dissected white body of *Sepia officinals* showing its white color and its position behind the eye.

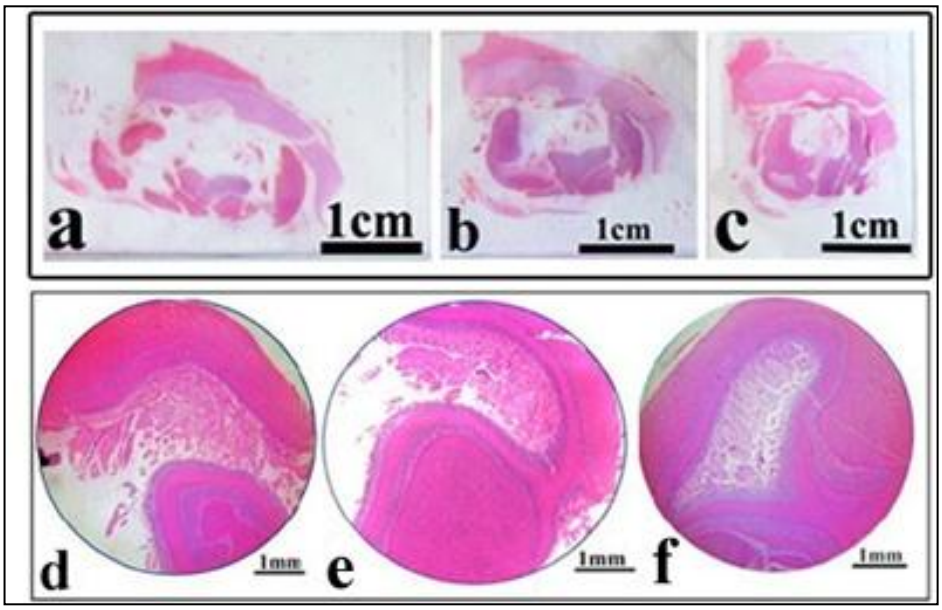

Fig. 12:-Photographs of serial sections of the white body (stained with H&E) showing**:** lobes in the anterior region are separated (a) then gradually connect to each other (b and c).d, e and f are photomicrographs of enlarged portions from a, b and c respectively.

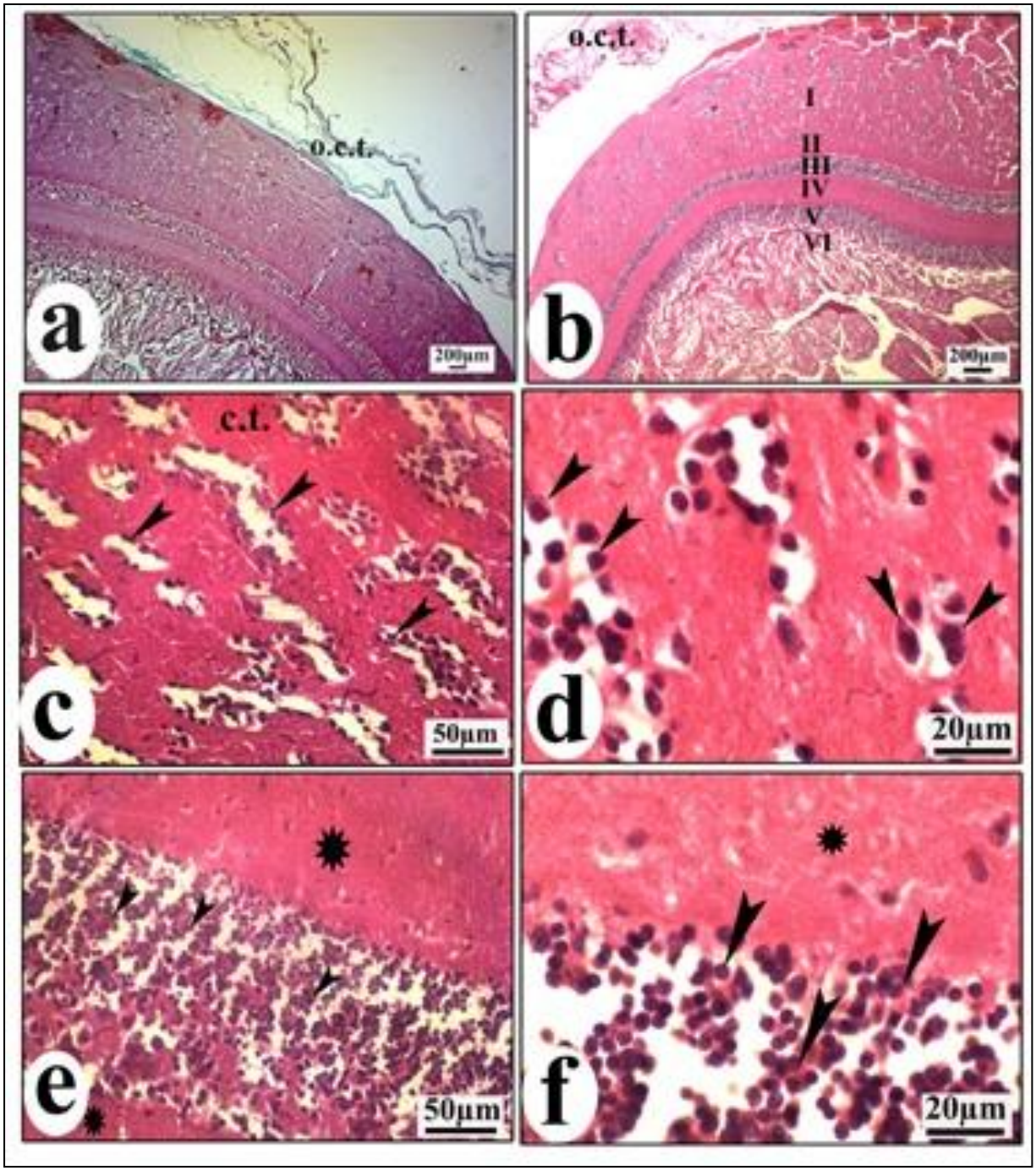

Fig. 13: A photomicrographs of transverse sections of the white body showing: the outer connective tissue capsule (o.c.t.)(a) (stained with Masson's trichrome), the first type of lobes consisted of sex layers (I-VI) (b) (stained with H&E), Enlarged portions from layer I showing hemocytes (arrow head) which supported by connective tissue (c.t.) (c,d),compact layer of connective tissue fibers in layers II, IV (star) and hemocytes attached to connective tissue fibers (arrow head) (e,f) (b-f stained with H&E).

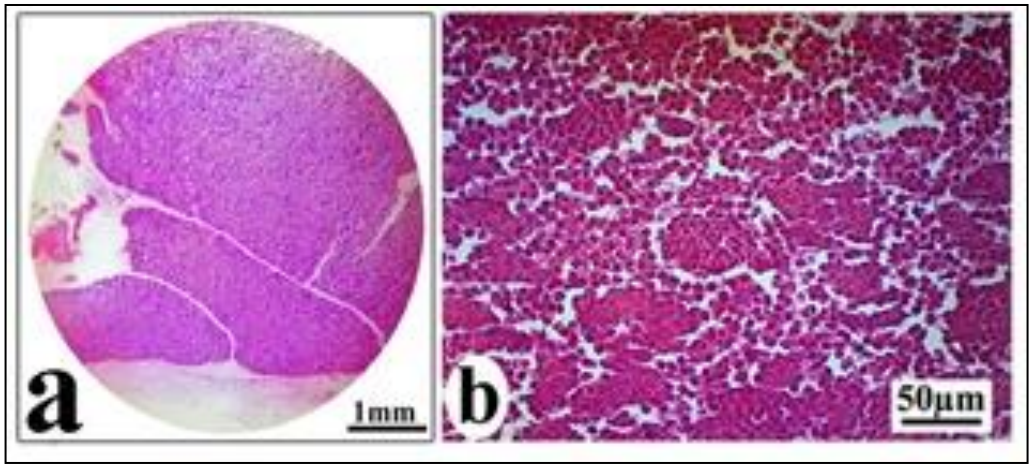

Fig. 14: A photomicrographs of transverse sections of the white body showing the second type of lobes which consists of aggregated hemocytes (**a,b)** (stained with H&E).

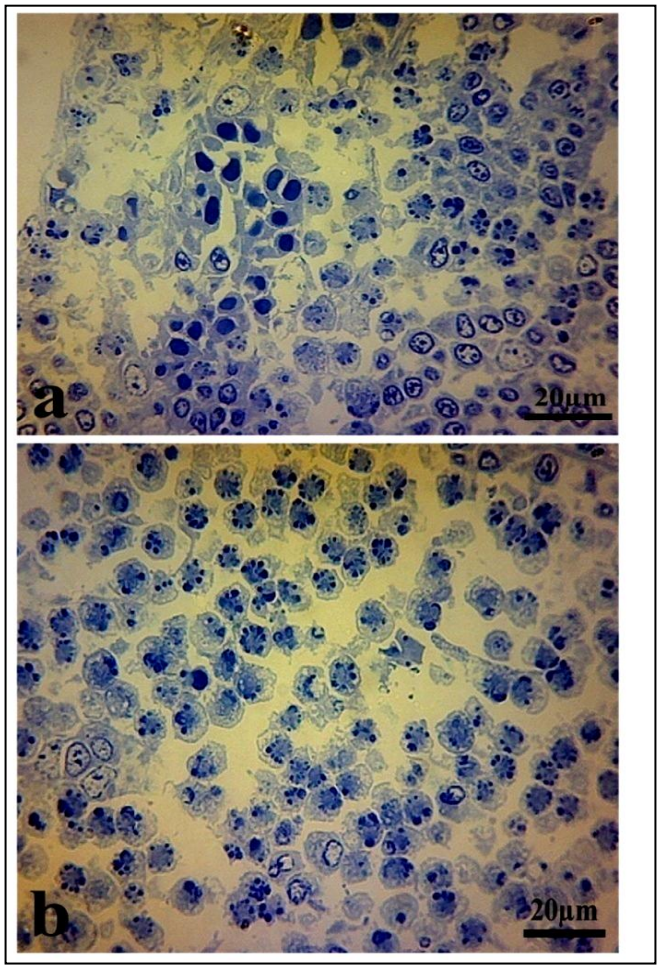

Fig. 15:-A photomicrographs of semithin sections of the white body of showing the different shapes of the haemocytes.

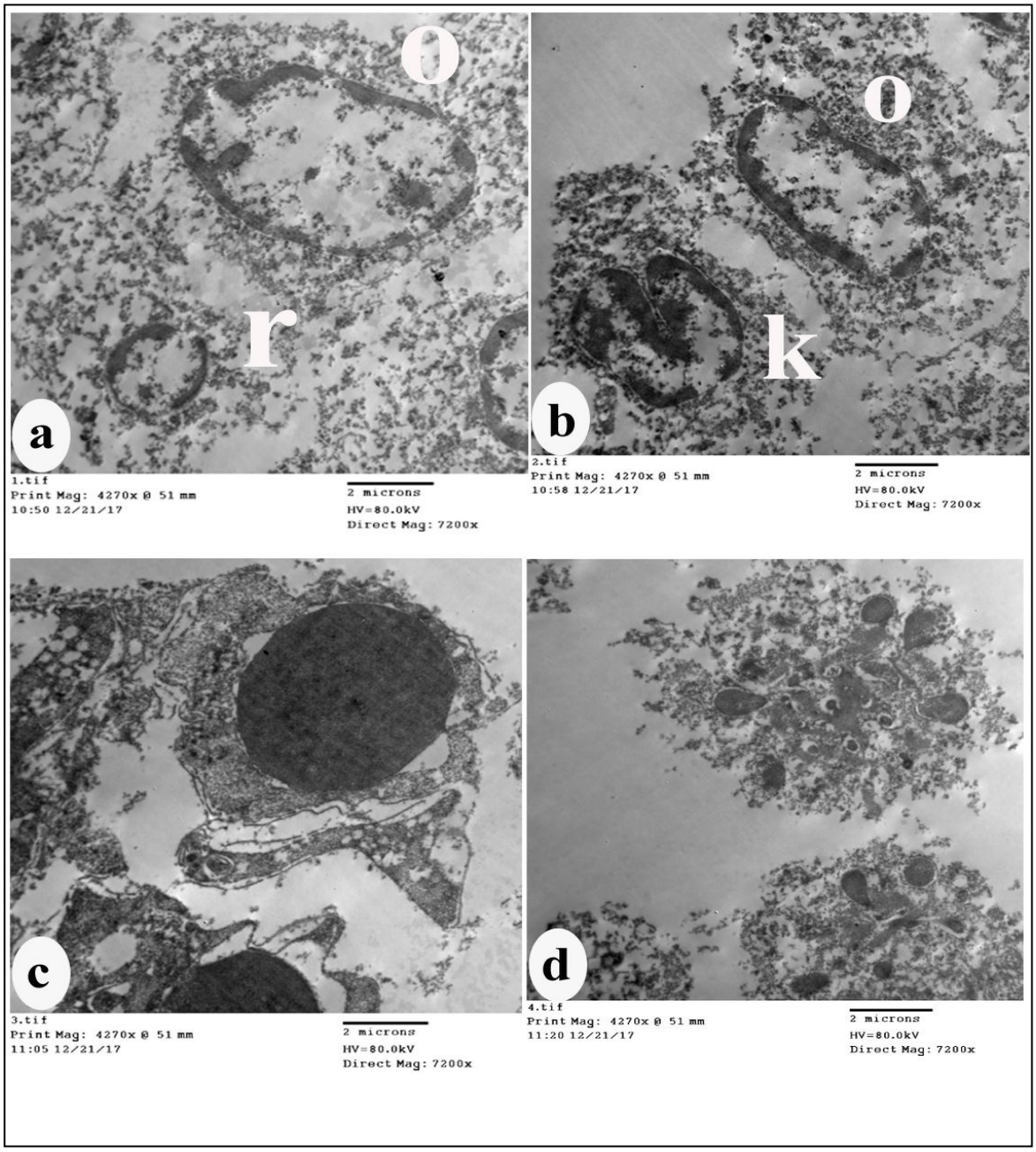

Fig. 16:-A photomicrographsof ultrathin sections of the white body showing the haemocytes with its different shapes nucleus: oval (o), rounded (r) or kidney shapes.

Discussion:-

1. Characterization of molluscan hemocyte population is very important to evaluate their ability to respond against environmental stress or pathogens (Hégaret *et al.*, 2003).
2. In the present study the main criteria which used for the classification of hemocytes are: the presence or absence of cytoplasmic granules, the ability of the cytoplasm and granules (if the latter is present) to be stained by acidophilic or basophilic stains, the shape of the cell and the nucleus and the ability to form pseudopodia.
3. In the present work hemocytes of *Sepia officinalis* were studied and classified into three main types: Hyalinocytes, granulocytes and agranulocytes. Granulocytes with kidney shape nucleus are the predominant cell type. Hyalinocytes may be granulated but differ from granulocytes in that they have a very small amount of

cytoplasm. Both granules and cytoplasm may be acidophilic or basophilic. Cell fragments were recorded in the present study.

4. Malek and Cheng (1974) reported that molluscanhemocytes can be distinguished into agranular cells and large granular cells. The latter can be classified according to their granules into granulocytes with acidophilic, basophilic, refractile granules or the three types of granules may be present in the same cell. Also in the present study some hemocytes which contain or secrete refractile material were recorded.
5. In *Biomphalaria glabrata*, only granular cells were recorded (Matricon-Gondran and Letocart 1999), while agranular cells were recorded in *Littorinalittorea* (Gorbushin and Iakovleva 2006).
6. Donaghy *et al.* (2010) recorded the presence of two main types of hemocytes in two edible gastropod species (the disk abalone and the spiny top shell): granulocytes and hyalinocytes. The latter have no or few granules.
7. Cowden and Curtis (1981) and Malham and Runham (1998) reported the presence of a single cell type of hemocyte in cephalopods. These cells have several cytoplasmic inclusions and u-shaped nucleus which look like the mammalian monocytes.
8. Only one cell type (granulocytes) was recorded by Kondo *et al.* (2003) in the circulating hemolymph of *Octopus vulgaris*, *O. ocellatus* and *O. minor*. They reported the presence of three types of granules in the hemocytes of *Octopus vulgaris*; eosinophilic, small or large basophilic, rod-shaped basophilic granules. The first and second types of granules were also detected in the present work .
9. Le Pabic *et al.* (2014) studied hemocytes morphology in *Sepia officinalis* and reported the presence of a single cell type with lobate, large nucleus, slightly basophilic cytoplasm that contains acidophilic granules and may contain lucent vesicles in some cells.
10. Castellanos-Martinez *et al.* (2014) recorded the presence of two morphologically different hemocytes in the cephalopods hemolymph. The first cell type was large and had many granules. They reported that this cell type was rounded, with an abundant cytoplasm, u-shaped, eccentric nucleus, and has many pseudopodia; the second cell type was lesser in number, size, and granulation, had fewer pseudopodia, irregular in shape and had a rounded nucleus.
11. However, three hemocyte types were recorded in the study of the blood cells of *Octopus vulgaris*; hyalinocytes, granulocytes and haemoblast-like cells (Salazar *et al.*, 2015).
12. The present study agrees with Salazar *et al.* (2015) in the presence of more than one hemocytes type which differs in their shape, presence or absence of cytoplasmic inclusions and shape of their nuclei but differ from that of Castellanos-Martinez *et al.* (2014) in that the number of cells that produce pseudopodia is very small.
13. Troncone *et al.* (2014) identified three hemocyte types in octopus; granulocytes, hyalinocytes and haemoblast-like cells. The granulocytes are able to extend pseudopodia. Hyalinocytes are round to ovoid in shape and might contain granules in their cytoplasm. Haemoblast-like cells have a small size.
14. Phagocytosis is the most important defense mechanism in invertebrates. Hemocytes of mollusks are known to efficiently phagocytose foreign material such as bacteria and cell debris (Malek and Cheng 1974). In the present study few phagocytic cells were recorded.
15. In the present work, two types of pseudopodia: blunt pseudopodia and filopodia could be formed in agranulocytes. Also one or more vacuoles may be present in this type of hemocytes. This result agrees with Helal and Abd El-Maksoud (1999) who mentioned that infected *Limnaea natalensis* snails possess hemocytes with many inclusions and produce many long, often branched, pseudopodia.
16. The result of Sminia (1981) revealed that granulocytes and hyalinocytes are phagocytic, but granulocytes were found to be much more active. Malek and Cheng (1974) revealed that all molluscan haemolymph cells are capable of pseudopodia formation.
17. In squid and octopus, the white body is considered to be an immune organ mainly due to the fact that blood cells, or hemocytes, are known to be present in high numbers and in different developmental stages. Hence, the white body has been described as the site of hematopoiesis in cephalopods (Salazar *et al.*, 2015).
18. The present study and Claes (1996) found that the white bodies of *Sepia officinalis* are located in the optic sinuses. They are white in color. Salazar *et al,.* (2015) described the white body as a multilobed organ and also the present study and the study of Cowden and Curtis (1974) showed that each white body is consisted of several interconnected lobes.
19. Bolognari (1951) revealed that the white bodies of many European species of cephalopod are composed of two lobes. Claes (1996) found that the white bodies of *Sepia officinalis* are composed of two main lobes of different size. Both lobes are divided into several secondary lobes and a large number of small lobules were recorded they give a glandular appearance to the organ.

20. In the present study two types of lobes with different histological structure were recorded. One of these lobes consists of histologically different layers which suggested that each layer perform different function as explained by Claes (1996).
21. In the present work the white body enveloped by a thin fibrous connective tissue capsule. This result agrees with Bolognari (1951) as he reported that the white bodies of several cephalopod species are covered with a thin layer of connective tissue.
22. The present work studied the different types of circulating hemocytes and also deals with the histological structure of each layer of the hematopoietic organ (white body) of *Sepia officinalis.*
23. Two major haemocyte developmental stages have been identified in the white body of *O. vulgaris*, *O. briareus*and in two unidentified squid species: haemocytoblasts and leukoblasts (Pila *et al.*, 2016). Haemocytoblasts are found in the reticulum of the white body's lobes, and give rise to leukoblasts, which are characterized by a reduced cytoplasmic volume and nuclear size compared to their progenitors (Ford 1992).

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