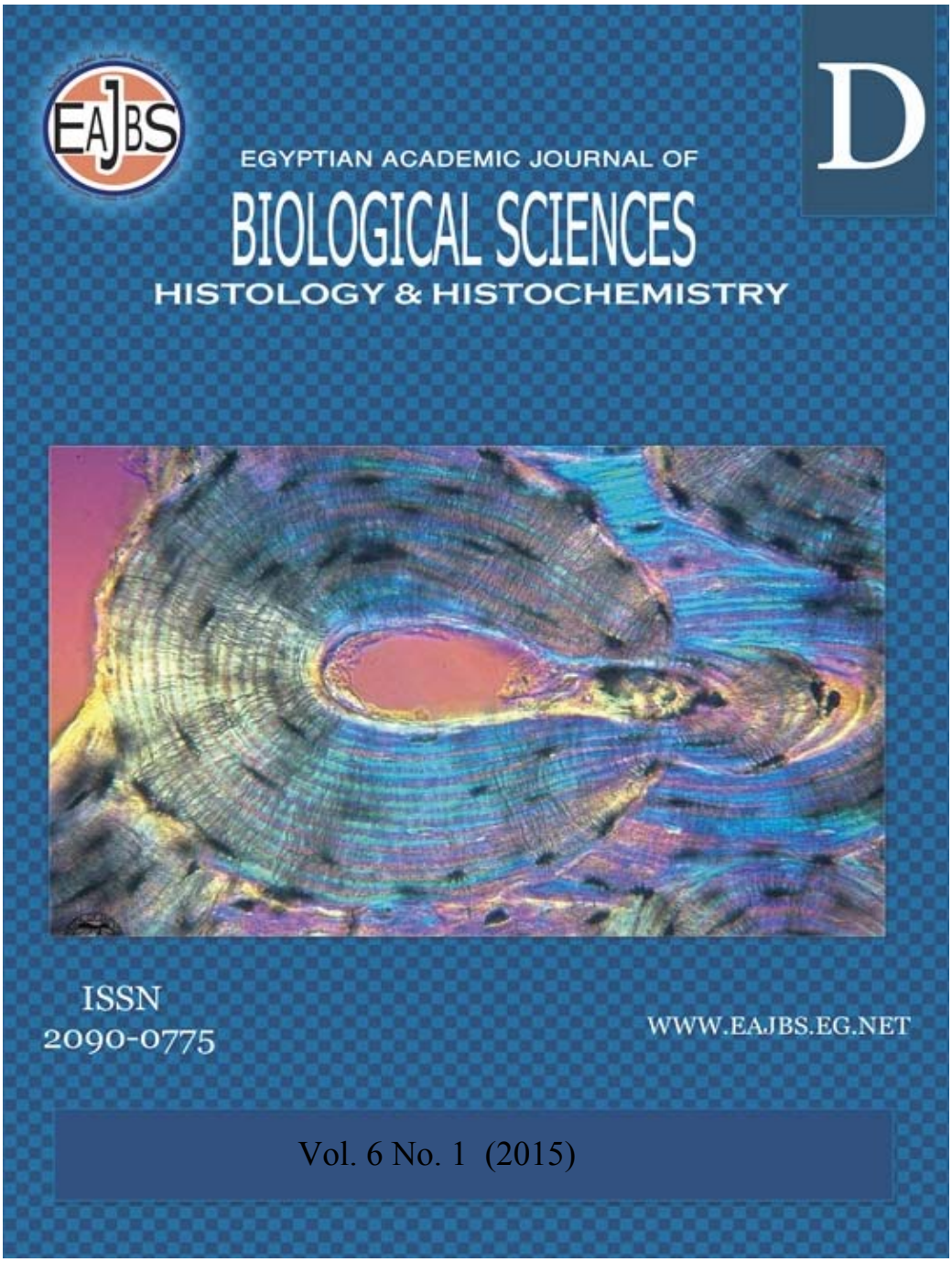

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Histological and ultrastructural studies of spleen in an amphibian animal. An evolutionary prospective

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ABSTRACT

The histological and ultrastructure of spleen of the maculated toad, *Bufo regularis* (amphibians) were studied. Splenic structure is differentiated into red and white pulps and connective tissue. The red pulp is formed of sinusoids, arterioles, lymphocytes, reticular cells, macrophages, plasma cells and granular leucocytes, as well as thrombocytes in the sinusoids, whereas, the white pulp consists of arterioles surrounded by lymphocytes, reticular cells and some plasma cells. Pigment cells were also noticed in red and white pulps.

In conclusion: the discussion of this investigation with other studies of higher vertebrates (amniotes) explained that the organization of the spleen structure in the maculated toad, *Bufo regularis* upgrade from simplicity to complexity and is considered as an intermediate condition between lower vertebrates and those of amniotes.

INTRODUCTION

The spleen represent an organ of no special significance till the mid of the twentieth century. It is closely associated with the circulatory system and play an important role in haemopoiesis and generation of primary immune response, it is functionally to remove older erythrocytes from the circulation and leads to discard the debris from bloodstream (Jakubovsky *et al.* 1990; Zapata and Cooper,1990.; Jakubovsky and Porubsky, 1995; Mebius and Kraal, 2005).

Recently, extensive studies were focused on the structure of the spleen in different mammalian species (Steinman *et al.*,1975; Fukuta *et al.*, 1982; Tanaka, 1990; Connolly *et al.*, 1999; Mebius and Kraal,2005, El-Dawi, 2008; Song *et al., 2012*; Khan *et al.*, 2014). However, in birds, few and sporadic investigations were dealt with general lymphatic tissue (Eldawi, 2008; Song *et al., 2012*; Khan *et al.*, 2014) or certain parts of the spleen (Smith and Hunt, 2004; Nagy *et al.*, 2005).

So far, there are only very few and sporadic studies on the structure of the spleen or lymphatic organs of amphibians. Garcia, *et al.* (1983), Alvarez (1990) and El-Dawi (2008) studied the spleen structure of *Bufo calamita*, *Rana perezi* and *Bufo viridis*, respectively.

Moreover, the cellular components such as the immune function of the lymphocytes and the pigment cells were studied in certain amphibian animals (Obara *et al.,* 1982; Scalia, *et al.*, 2004; El-Dawi, 2008). Scarce attempts have been made to study the splenic in the lower vertebrate, and compared with those of higher vertebrate.

In accordance, the aim of this investigation is planned to study the spleen histological and ultrastructure of the maculated toad, *Bufo regularis,* with special reference to certain immune cells and discuss this structure with those of higher vertebrates. High hope that this study may offer a base knowledge for the evolution of the vertebrate spleen and may be represents a preliminary investigation for further experimental studies in the immune system.

MATERIALS AND METHODS

Animal's collection and dissection:

Ten adult specimens of the maculated toad, *Bufo regularis* (amphibians), were collected from Wadi Bydan & Rowaida at Almadina Almonawara - Saudi Arabia.

In King Fahad Medical Research laboratory (girls department), King Abdul Aziz University, Jeddah, Saudi Arabia, the animals were narcotized by ethyl ether, dissected and the spleen were carefully removed, cut into small pieces and prepared for light and transmission electron microscopy.

Light microscopy (LM):

Pieces of the spleens were fixed in alcoholic Bouin's fluid for 24 hrs., then washed, dehydrated in graded ethanol series, cleared in terpineol. The samples were embedded in paraplast, sectioned at 6 µm thick and mounted on slides. The sections were stained with Harris' hematoxylin and eosin (HE), Mallory stain, and Giemsa stain (Humason, 1979). The sections were examined using Olympus light microscope (LM) and photographed. The measurements of the cell types were determined using an eye piece and slide micrometer.

Transmission electron microscopy (TEM):

Very small pieces of the spleen were fixed in cold (4°C) 2% glutaraldehyde in 3.5 phosphate buffer (pH 7.5) for 2 hours, according to Milloning (1964). After repeated rinses in phosphate buffer, the pieces were post-fixed (2 hours at 4°C) in 1% osmic acid (OsO_4). The samples were then dehydrated, treated with propylene oxide, infiltrated and embedded in Epon 812. Semithin sections were cut with the RMC-MT7 ultra-microtome and stained with toluidine blue. Ultrathin sections (silvery) were cut, using a diamond knife and stained with uranyl acetate and lead citrate. The sections were then examined and photographed using a Joel 1010 Transmission Electron Microscope at the central laboratory at Faculty of Science, Ain-Shams University, Cairo, Egypt

RESULTS

General microscopic structure:

The spleen of the maculated toad, *Bufo regularis*, is reddish small rounded body (about 2.5–3.9 mm in diameter) lying dorsal to the anterior end of the cloaca. The splenic tissue is encapsulated by a thin fibrous connective tissue layer, without trabeculae and shows two pulps; red and white pulps (Fig.1).

The red pulp consists of arterioles and sinusoids reticulated with cords of cells and show two different regions. The larger region found at the periphery and contains sinusoids, arterioles, lymphocytes, reticular cells, some macrophages and a variety of leucocytes. macrophages, reticular & plasma cells and blood capillaries are also noted (Figs. 2-4).

The smaller region scattered in the central part and includes lymphocytes,

The white pulp is found in between the red pulp and characterize by clusters of lymphatic tissue around central arteries. These clusters contain backed lymphocytes, reticular cells and some plasma cells (Figs.3 & 4).

Between the red and white pulps lies a transitional zone of loose connective tissue, few lymphocytes, reticular cells and macrophages (Fig. 4).

Great numbers of pigment cells were observed in white and red pulps and seem to be phagocytic cells (Fig. 5).

Cellular components

Five cell types have been identified in the spleenic tissue. These cells are lymphocytes, macrophages, plasma cells, reticular and pigmented cells (Figs.6-14).

Lymphocytes

The lymphocytes vary in sizes and shapes and measure about 12 -24 μm in diameter. Each lymphocyte has a rim granular pale staining cytoplasm around the nucleus. (Figs. 6-9).

Ultrastructurally, the cell surface of the lymphocyte shows short microvilli and the cytoplasm is loaded by ribosomes. The rough endoplasmic reticulum (RER) are scarce and the Golgi bodies are very small and hardly detectable, as well as the rounded mitochondria are small in number. The nucleus shows dense heterochromatin masses and dense nucleoli also observed. (Figs.8- 9).

Macrophages

The macrophages are abundant in the red pulp and the transitional zone between the white pulp and the red one. Macrophages are variable in shapes and sizes. They measure about 11–39 μm in length and 8–12 μm in width. Their small heterochromatic nuclei appear dark in colour. The cytoplasm is heterogenous containing a variety of granules and vesicles of different sizes (Figs.10 & 11).

The macrophages are characterized by the presence of many vesicles (phagocytic vesicles) and sometimes possess cytoplasmic processes. The cytoplasm contains numerous lysosomes of varying sizes and shapes, as well as rounded mitochondria. The oval heterochromatic nucleus is slightly irregular in shape and contains a large one or two nucleoli. (Figs. 10&11).

Plasma cells

The plasma cells are few in number, small in size (measure about 7- 18 μm in diameter) and are found in the red pulp and transitional zone. They are usually rounded, slightly elongated or polygonal in shape. Each cell has an eccentric rounded nucleus. The cytoplasm is strongly basophilc due to the presence of extensive ribosomes on the endoplasmic reticulum (Figs. 3 & 4).

The plasma cells are characterized by presence of highly developed RER. The cytoplasm contains free ribosomes, Golgi stacks and mitochondria which are hardly detectable. The nucleus is heterochromatic and provided with large nucleolus (Fig. 12).

Reticular cells

The reticular cells are variable in size and measure about 10-35 μm in diameter. The reticular cells are irregularly rounded in shape and surrounded by lymphocytes especially in the white pulp. Ultrastructurally, few mitochondria, short RER and Golgi bodies associated with some lysosomes are occasionally observed. The nucleus is large, irregular in shape and contains a uniform peripheral heterochromatin masses (Figs. 2,4,13).

Pigment cells

The pigment cells are large (measure about 31 μm in diameter). They have many branches extend between the

splenic tissues and cells and characterized by darkly stained cytoplasm. The pigment cells have large heterochromatic nuclei and may appear as lobed structure. The pigments appear as small groups scattered in the cytoplasm (Figs.5 &14).

DISCUSSION

The general structure of spleen in the present investigation, the maculated toad, *Bufo regularis*, indicates that there are no trabeculae in the splenic tissue, similar to that have been reported in the toad (Garcia, *et al.*, 1983; El-Dawi, 2008) and the frog (Alvarez,1990 ; Al-Saffar, 2008). Also, in reptiles (Zapata *et al.,* 1981; El-Dawi, 2008). Even, in birds (Lucas *et al.*, 1954; Hodges,1974; El-Dawi, 2008; Khan *et al*., 2014). However, Bradley and Grahame (1960) stated that sparse trabeculae, were observed in the spleen of the fowl. Whereas, Nasu *et al.* (1992) found poorly developed trabeculae in the dove spleen. On the other hand, in mammalian species, numerous trabeculae pass inwards to the splenic tissue and contains the main blood vessels (Fukuta *et al.,* 1982; Roitt, *et al.,* 1989; Tanaka, 1990; Connolly *et al.*, 1999; Mebius and Kraal, 2005).

The presence of the white and red pulps in *B. regularis*, as well as blood vessels and connective tissue, coincide with that reported in amphibian species (Welsh and Storch, 1982; Garcia *et al.*, 1983; Alvarez, 1990; El-Dawi, 2008). However, Welsh and Storch (1982) stated that the presence of red and white pulps in the spleen of the primitive amphibians (Gymnophiona), does not found in other amphibians, (urodeles).

Both white and red pulps were also reported in reptilian species (Zapata *et al.*, 1981; Borysenko, 2005; El-Dawi, 2008), and birds (Lucas *et al.* , 1954; Hodges, 1974; El-Dawi, 2008; Khan *et al*., 2014), as well as mammals (Jakubovsky and Porubsky,1995; Galindez *et al.,* 2000 & 2003; Mebius and Kraal, 2005).

In *B. regularis*, the present investigation, the red and white pulps are without clear border. Similar to that observed in *B. viridis* (El-Dawi, 2008). However, in *B. calimata* these two areas are clearly distinguishable with clear borders (Garcia *et al.* 1983).

In birds, *G. gallus,* El-Dawi (2008) described clear borders of the pulps. This is in contrast to that in dove and chicken spleens (Nasu *et al.*, 1992) where the white and red pulps could not be distinguished from each other. Most probably, such controversies are due the seasonal changes in which the animals were subjected during the study. Bassioni (1991) confirmed that there were seasonal changes in the development and differentiation of red and white pulps in the spleen of the snake, *Psammophis sibilans*. On the other hand, Roitt *et al.* (1989) found that in mammalian spleen, the white pulp is formed of germinal center and an arteriole surrounded by the lymphoid tissue. Such complexity is greatly different than those of the amphibians, reptiles and birds .

The transitional zone between the red and white pulps, in the present investigation *B. regularis,* simulates in structure to that reported by El-Dawi (2008) in *B. regularis* and to the marginal zone by Garcia *et al.*(1983) in *B. calimat*a. Also, in reptiles, the turtle, *Mauremys cas[pica* (Zapata *et al.*, 1981). Moreover, such marginal zone was reported in many mammalian species (Blue and Weiss, 1981 a & b; Fukuta *et al.,*1982; Roitt *et al.*, 1989; Tanaka, 1990). However, Nakamine *et al*. (1992) distinguished similar zone and perivenous layer in the red pulp only in two odontoceti species. It could be suggested that, there is upgrade complexity in the transitional zone in the spleen from the amphibians toward the advanced amniotes.

The cellular components (lymphocytes, plasma cells, macrophages, reticular cells and other granular leucocytes) of the lymphatic organs are involved in the immune response (Roitt *et al.*, 1989). Moreover, the cells of haemopoietic tissues in fishes similar to those of mammals (Boomker, 1981).

In the present investigation, the main cell types were lymphocytes, macrophages, plasma and reticular cells, as well as pigment cells were also observed. These cells have been extensively described in the amphibian, *B. viridis* and the reptilian, *Eumeces schneideri* (El-Dawi, 2008). All the aforementioned cell types were described in fishes (Boomker,1981), amphibian animals (Welsh and Storch, 1982; Garcia, *et al.*, 1983 & 1985; Alvarez, 1990; El-Dawi, 2008), reptiles (Zapata *et al.*,1981;Borysenko,2005; Aznaurian and Amiryan, 2006; El-Dawi, 2008), birds (Hodges,1974; Khan *et al*., 2014) and mammals. (Roitt *et al*., 1989; Tanaka, 1990). However, the pigments cells were not described in birds and mammals (Hodges, 1974; Roitt *et al*., 1989; Tanaka, 1990; Mebius and Kraal, 2005; El-Dawi, 2008)

The lymphocytes, in the present investigation, were heterogenous in their sizes and morphology. The differences are clearly observed in the nuclear and cytoplasm ratio as well as the degree of cytoplasmic staining with histological dyes. Du Pasquier (1976) stated that in birds and mammals, the lymphocytes are two types, the T- and B- cells which produce antibody. He also stated that in primitive animals, the T- and B-lymphocytes have properties of both mammals and birds.

In the present investigation, the reticular cells were observed in the red and white pulps enclosing the lymphocytes. El-Dawi (2008) reported that in both *B. viridis* and *E. schneideri*, the lymphocytes of different sizes, and plasma cells are abundant in between the branches of the reticular cells in the white pulp. El-Dawi (2008) suggested the reticular cells may have a certain function or role help in the maturation of the lymphocytes. In reptiles, Zapata *et al.* (1981) stated that in turtle, the reticular cells constitute a network enclosing the dendritic cells, macrophages, lymphoblasts and small and medium lymphocytes. On the other hand, In birds, Bradley and Grahame (1960) reported that the reticular cells were densely around the lymphocytes and arteries of the white pulp. Moreover, Du Pasquier (1976), Cooper (1982) and Mebius and Kraal (2005) generally described an intimate relation between the reticular cell branches and the immature lymphocytes in the white pulp more than those of the red one.

The plasma cells in *B. regularis* were found in red and white pulps. Similar observation has been reported in spleen of *B. viridis* and *E. schneideri* but found only in the transitional zone of the *G. gallus* (El-Dawi, 2008). He also added that plasma cells are abundant in white pulp more than that of the red pulp. This was also confirmed by Obara *et al.* (1982) in spleen of toads. Whereas, Zapata *et al.* (1981) stated that in the turtle, *Mauremys caspica*, the mature plasma cells are scare in the white pulp. Borysenko (2005) stated that the splenic white pulp of the anuran and reptilian lizard is the site of maturation of lymphocytes, in contrast with that of mammals.

In the present study the plasma cells usually possess extensive and dilated cisternae of rough endoplasmic reticulum, suggesting capability of producing secretory product, presumably antibody. In toad, golden skink and turtle (El-Dawi, 2008; Borysenko, 2005, respectively) suggested a secretory function of the plasma cells either in red or white pulps

In macrophages of the present investigation were observed in both red

and white pulps and possess different shapes and structures. Similar observation have been reported in toad (El-Dawi,2008).Hodges (1974), Boomker (1981) and Roitt *et al.* (1989), stated that the different types of the granular leukocytes (neutrophils, basophils or eosinophils), and monocytes are macrophages or phagocytic cells depending on the differential staining of their granules.

It is noteworthy to mention that in *B. regularis*, of the present investigation, the pigment cells were observed in both red and white pulps and their branches extend between the splenic tissues. These results coincides to those of toad and golden skink (El-Dawi,2008). Scalia, *et al.* (2004) demonstrated that the pigment cells of amphibians are macrophages: they show an ultrastructurally distinctive morphology, able to phagocytose

It should be noted that, the general organization of the spleen of the amphibians is simpler than that of the mammalians, However, the cellular components show distinct similarities to those of the reptiles and birds (El-Dawi,2008) and the mammals (Boomker, 1981). Cooper (1982) stated that the distinction of the spleen of anurans into red and white pulps are apparent, however, well defined germinal centers in the white pulp equivalent to those of mammalian spleen, are not prominent. On the other hand, Roitt *et al.*(1989) and Tanaka (1990) reported that the white pulp of the spleen of the higher mammalian species is highly organized and complicated more than those of the primitive mammals

In accordance, it is apparent that the organization and evolution of the spleen structure upgrade to the complexity. Thus, the morphological observations on amphibian spleen are considered as an intermediate condition between lower vertebrates and those of amniotes.

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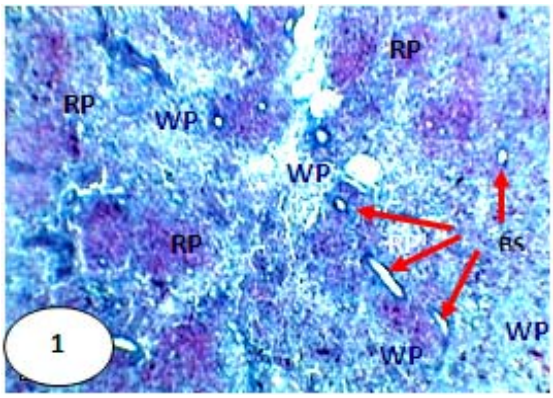
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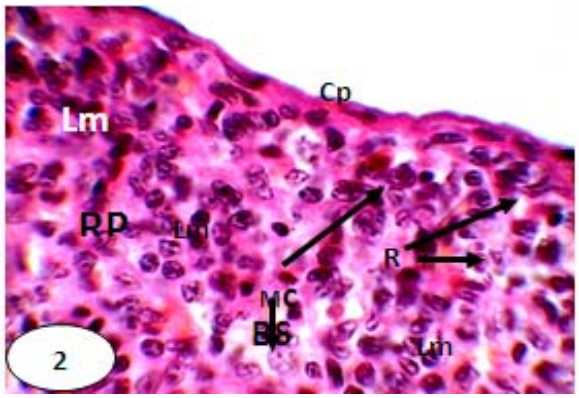
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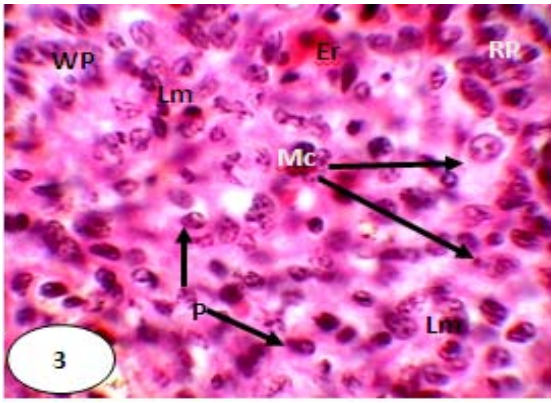
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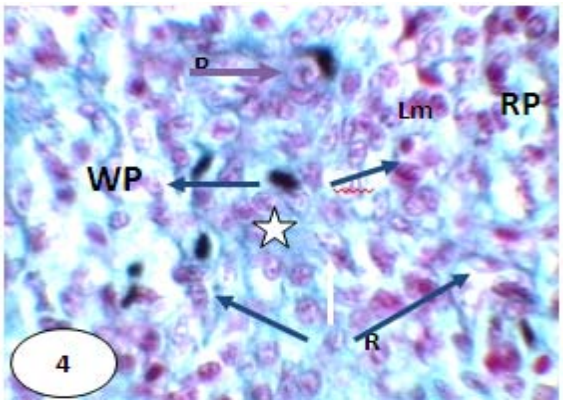
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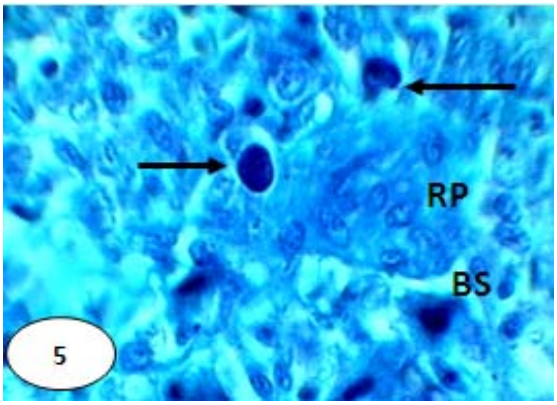
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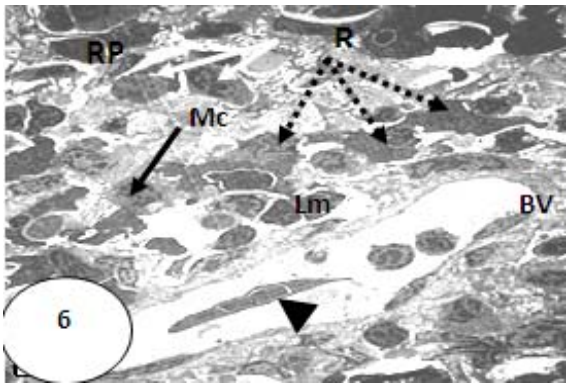
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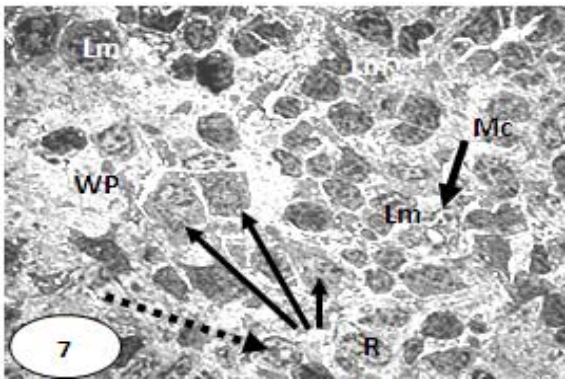
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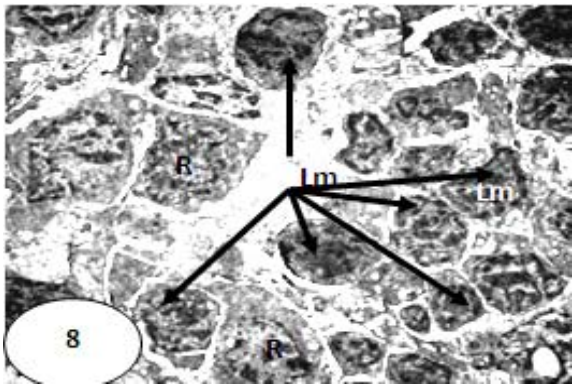
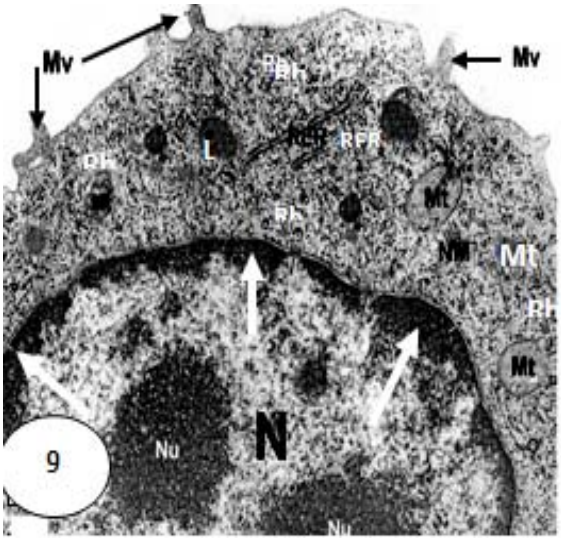
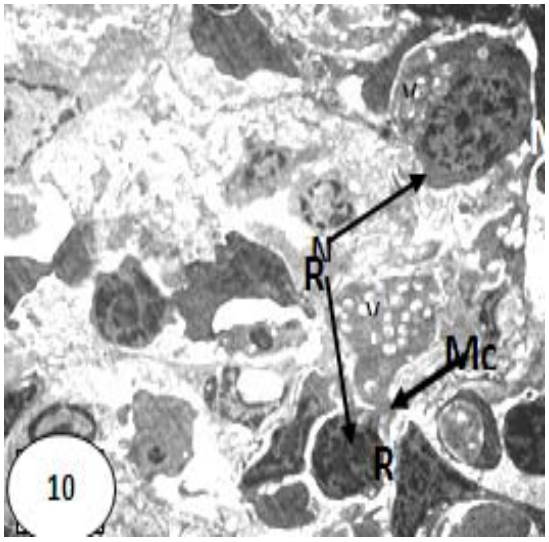
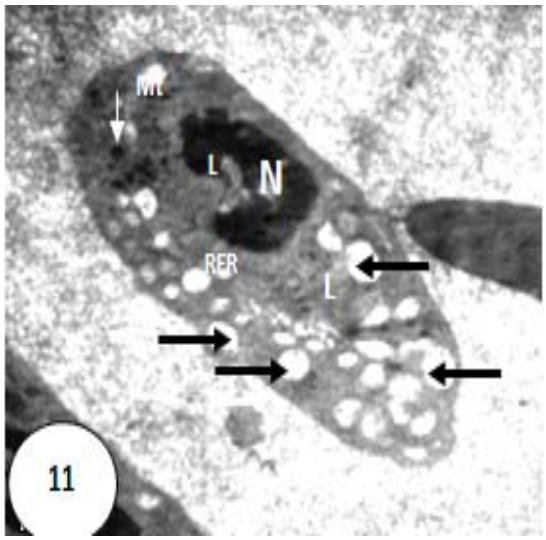
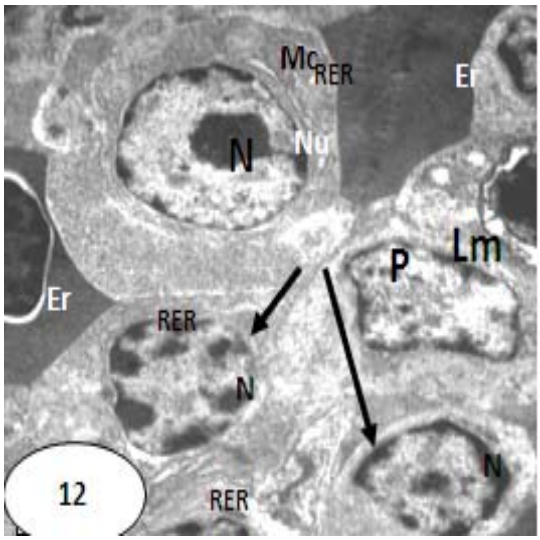
Mc
WP
Lm
R
7

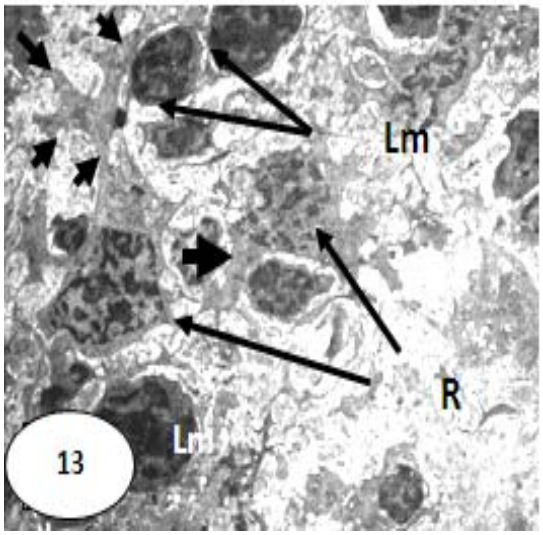

R
Lm
Lm
R
8

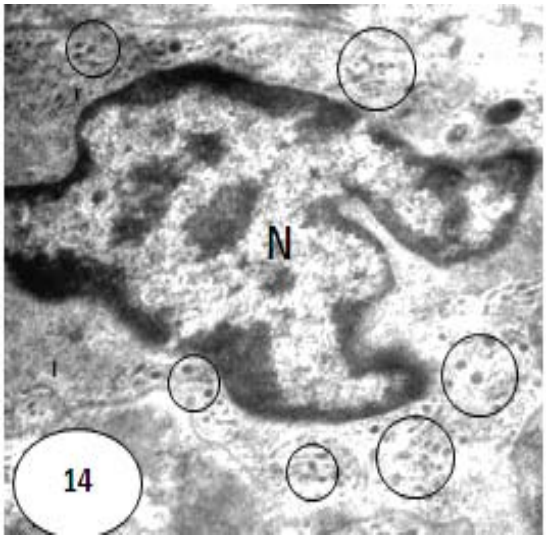

Mv
Mv
L
RER
Mt
N
Nu
9


R
Mc
R
10


Mt
L
N
RER
L
11


Mc
RER
Er
N
Nu
P
Lm
Er
RER
N
N
12
RER


Lm
R
13


N
14

ARABIC SUMMARY

دراسات هستولوجية وتركيبية دقيقة على الطحال فى حيوان برمائى نظرة تطورية

إعتـدال عباس حسن حويت
٢- قسـم الأحيـــاء/ حيـــوان – كلية العـــلوم - جامــعـة الملك عبد العزيـز - جـدة- المملكة العربية السعـودية

تناول البحث دراسة هستولوجية وتركيبية دقيقة لتركيب الطحال فى احدى البرمائيات وهى الضفدعة الرقطاء (بوفو ريجيولاريس). ووجد أن نسيج الطحال يتميز الى مناطق غير محددة وهى اللب الأحمر و اللب الأبيض. وقد وجد أن اللب الأحمر يتكون أساساً من جيوب دموية ، أوعية شريانية ، خلايا ليمفاوية، خلايا شبكية، خلايا بلعومية، خلايا بلازمية، وخلايا حبيبية بيضاء ، بالإضافة الى الصفائح الدموية. أما اللب الأبيض فإنه يتكون أساساً من أحجام مختلفة من خلايا ليمفاوية، خلايا شبكية، خلايا بلازمية .

وقد وجد أيضاً أن هناك منطقة إنتقالية بين اللب الأحمر والأبيض تتكون من خلايا ليمفاوية، خلايا شبكية، بعض الخلايا البلازمية وأحياناً خلايا بلعومية.

وقد لوحظ وجود عدد من الخلايا الصبغية منتشرة فى اللب الأحمر واللب الأبيض. وبمناقشة هذه النتائج مع مثيلاتها فى الفقاريات العليا وجد أن الطحال يتطور تدريجياً الى التعقيد ، وأن الطحال فى الضفدع يعتبر مرحلة وسط بين الحالات البدائية والرهليات الأكثر تقدما.