

Histological, Immunohistochemical and Ultrastructural Study of the Epididymis in the Adult Albino Rat.

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Abstract: Very little is known about the basal cells in the epididymal epithelium. Their function is unclear, although they are present in all mammalian epididymal studies. Thirty adult male albino rats were used in this study and were divided into 3 groups: group I (a control group; n =10), group II (injected with Indian ink; n = 10) and group III (exposed to ischemia reperfusion injury; n =10). The animals were dissected and the epididymis samples were fixed and processed for light and electron microscopically studies. Immunohistochemical study was done using monoclonal antibody (mAb) CD₁₄. The basal cells were characterized by slightly heterochromatic nuclei with prominent nucleoli, few organelles and lipofuscin granules. The principal cells appeared large; with large convoluted nuclei and their cytoplasm were rich in lipofuscin granules and multiple vacuoles. Some macrophages in close proximity to the epithelium were structurally similar to the basal cells. The immunohistochemical staining revealed that the basal cells were negative to the mAb CD₁₄. Indian ink injection into the lumen of the epididymis revealed accumulation of dark granules in the cytoplasm of the principal cells with their absence in the cytoplasm of the basal cells. Induction of ischemia reperfusion injury resulted in degeneration of the basal parts of the ductus epididymis and the principal cells while the basal cells appeared resistant to such injury. On the basis of the present findings, a supporting role of the basal cells was proposed while their role in the local immune defense mechanism was put in doubt.

Key words: Epididymis, rat, ischemia reperfusion injury, Indian ink, Basal cells, Macrophages

INTRODUCTION

The epididymis is a highly convoluted tubule which connects the testis to the vas deferens. It is the site for maturation and storage of spermatozoa (Beu *et al.*, 2009). The epididymal epithelium was reported to be formed of four major cell types: principal, basal, clear, and halo cells (Cooper, 1986; Robeire and Hermo, 1988). Domeniconi *et al.* (2007) added two other cell types to that classification; apical cells and dark cells. They reported that cells other than the principal cells did not appear to possess any specific ultrastructural features.

The principal cells are the most abundant cells in the epididymis (Leung *et al.*, 2004). They had been described to play a major role in synthesis, secretion and absorption within the epididymis (Cooper, 1986). This was explained by their rich contents of organelles (Hamilton, 1990). The later author added that tight junctions have been demonstrated between the principal cells forming the blood-epididymis barrier and this barrier is critical for prevention of autoimmune response against the antigenic post-pubertal germ cells. Leung *et al.* (2004) reported that although those cells other than the principal cells had not been studied to the same extent as the principal cells, yet their presence was essential for the integrated function of the epididymis.

The basal cells are the second most common cell type after principal cells in the epididymis (Leung *et al.*, 2004; Domeniconi *et al.*, 2007). While the structure and function of the principal cells had been extensively studied, the function of the basal cells was unclear (Robeire and Hermo, 1988). Some studies suggested a major role for the basal cells in regulating the electrolyte and water transport by the principal cells through local formation of prostaglandins (Leung *et al.*, 2004). Other studies suggested a supportive role for the basal cells (Hinton and Palladino, 1995). The basal cells had also been *proposed* to possess a protective role against reactive oxygen species thus protecting the maturing spermatozoa (Andonian and Hermo, 2003).

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The halo cells were found throughout the epididymis. They were believed to play a role in the immunological barrier of the male reproductive tract (Veri *et al.*, 1992; Palacios *et al.*, 1993).

The clear cells participated in the uptake of the luminal components and the disposal of cytoplasmic droplets detached from spermatozoa (Abe and Takano, 1989; Kasper and Stosiek, 1989).

The apical cells were found to be fewer in number than any other cell type. They demonstrated a wide apical portion and narrow stem extending to the basal lamina (Marta and Risley, 1986). The authors added that the migratory cells had also been demonstrated crossing the epithelium in various regions of the epididymis and were thought to be intraepithelial lymphocytes.

The major cells involved in immune responses are the monocytes, macrophages, and T & B lymphocytes (Arrighi and Domenighini, 1993). Both lymphocytes and macrophages had been demonstrated within the mammalian male reproductive tract (Veri *et al.*, 1992).

The aim of the present study was to describe the cells in the epididymal epithelium and the possible similarity between the basal cells and the interstitial macrophages. The phagocytic role of the basal cells and their response toward ischemia reperfusion injury were also investigated.

MATERIALS AND METHODS

Animals:

Thirty male albino rats aged 12 months were used in this study. The rats were housed at under normal room temperature (22°C). They had free access to food and water. The animals were divided into 3 groups: group I (control group; n = 10), group II (injected with Indian ink; n = 10) and group III (subjected to ischemia reperfusion injury; n = 10).

Tissue Preparation for Light Microscopically Study:

The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnotol; Steris Laboratories Inc., Phoenix, AZ). The epididymis samples were fixed with Bouin's solution for 48 hours, dehydrated, and embedded in paraffin. Sections of 5 µm were cut, mounted on glass slides and processed for hematoxylin and eosin staining.

Immunostaining (According to Xu et al., 2003):

Immunohistochemical staining of the sections was done using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Primary antibodies used for the immunocytochemical staining of cells present in the epididymal epithelium and interstitial tissue were mouse anti-rat CD₁₄ (Serotec USA, Washington, DC). They were used at a dilution of 1/100; this antibody recognizes a cytoplasmic antigen in monocytes and most macrophages. Briefly, the sections were blocked by incubation with diluted normal serum for 30 min at room temperature. The sections were incubated for 18 hours at 4°C with primary antiserum diluted in PBS. The sections were then incubated for 30 min with appropriate (anti-goat, mouse, and rabbit) diluted biotinylated secondary antibody solution and incubated for 30 min with Vectastain Elite ABC reagent. Finally, sections were incubated in diaminobenzidine (DAB) solution (Vector Laboratories) until stain developed, rinsed in water, and counterstained with hematoxylin and eosin.

Electron Microscopy (According to Yeung et al., 1994):

After dissection of the connective tissue, a segment of the corpus epididymis was cut into small pieces, fixed in 4% glutaraldehyde then washed in phosphate buffer and post fixed in 1% osmium tetroxide. Fixation was followed by dehydration and embedding in epoxy resins. Semithin sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate then examined and photographed using transmission electron microscope Philips CM 10 electron microscope.

Indian Ink Injection (According to Itoh et al., 1998):

This was done to demonstrate the epididymal cells with potential phagocytic activity. India ink was diluted 1:4 with distilled water. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital. After incision of the scrota, injections of 10 µl Indian ink into the parenchyma of the ventrocaudal quadrant of the testis and into the proximal area of the epididymis using a 29-gauge needle were performed. The animals were sacrificed after 30 minutes and specimens from the epididymis were removed and processed.

Induction of Ischemia Reperfusion Injury (According to Pálffy et al., 2006):

Torsion of testis (randomly right or left) was simulated by 5-fold clockwise rolling of testis. After 30 minutes of ischemia, the blood flow was restored by five folds counterclockwise rotation (detorsion-DT, reperfusion phase). In this process, the increased oxygenation of ischemic tissue induced oxidative stress. After another 30 minutes of reperfusion, the specimens from the epididymis were removed and processed.

RESULTS AND DISCUSSIONS

Results:

The cytoplasm of the principal cells demonstrated brown stained lipofuscin granules with numerous vacuoles (Fig. 1) and multiple basophilic granules mainly in a supranuclear position (Figs.1, 2). They rested on the basement membranes and their cytoplasm was relatively more electron dense than that of the basal cells (Figs. 4, 5). Their cytoplasm showed also lysosomes, some lipid granules, few myelin figures, scarce mitochondria and multivesicular bodies (Fig. 7). Their nuclei appeared elongated, oval and sometimes convoluted (Fig. 5). Finally, they showed no reaction against CD₁₄ antibodies (Figs. 9, 10).

The basal cells looked rounded or oval and rested on the basement membranes. The majority of the cells had oval indented nuclei occupying most of the cytoplasm. These nuclei appeared slightly heterochromatic with peripheral arrangement of the chromatin (Figs. 4-6). The mitochondria were few in number and globular in shape but sometimes elongated and situated near the nuclei. Few electron dense granules; mostly lysosomes had also been demonstrated in addition to some vacuoles of different sizes (Fig.6). Accumulation of brown stained lipofuscin granules was demonstrated very close to the nuclei (Fig. 1). Similar to the principle cells, the basal cells showed no reaction against CD₁₄ antibodies (Figs. 9, 10).

The halo cells appeared rounded or polygonal in shape with central rounded to oval slightly indented nuclei with prominent nucleoli. The nuclei appeared surrounded by large portions of the cytoplasm compared to those of the basal cells. They had a very clear, electron lucent cytoplasm. The cytoplasm possessed few organelles and few electron dense granules (Figs. 1, 7). Similar to the principle and basal cells, brown uniform lipofuscin granules could also be demonstrated in their cytoplasm (Fig. 3) and they showed no reaction against CD₁₄ antibodies (Figs. 9, 10). The halo cells were situated directly above the basement membrane (Fig.12).

The interstitial macrophages were found to be large and oval cells. Each cell had a single large nucleus with few basophilic cytoplasmic granules (Fig. 3). The nuclei might appear segmented or multiple (Fig.14). The cells appeared to lie in close vicinity to the basement membrane and near the blood vessels. Multiple, large cytoplasmic vacuoles could also be demonstrated within these cells (Fig. 8). In the majority of the semithin sections, they appeared similar in morphology to the basal cells (Fig. 13). A positive reaction toward CD₁₄ antibodies was demonstrated by large interstitial cells that showed irregular nuclei (Figs. 9, 10). Such cells thought to be the interstitial macrophages.

After Indian Ink Injection:

Granules of Indian ink could not be demonstrated within the cytoplasm of the basal, halo and interstitial macrophages cells. However, surprisingly, the Indian ink granules had been demonstrated in large number within the basal parts of the principal cells (Figs. 11, 12).

After Ischemia Reperfusion Injury:

After ischemia reperfusion injury, degeneration was observed within the basal parts of the ductus epididymis and the interstitium. The cells of the epididymal epithelium appeared separated from each other and the blood vessels appeared dilated and congested (Fig.15). The principal cells showed degeneration at their basal as well as infra-lateral surfaces. The interstitium became more cellular and showed multiple cells near the basement membrane. Such cells had large and irregular nuclei (Fig. 16). Meanwhile, the basal cells appeared intact with prominent nuclei and were firmly adherent to the basement membrane (Fig. 17).

Discussion:

Very little is known about the basal cells in the epididymal epithelium. Their function is unclear, although they are present in all mammalian epididymal studies.

Yeung *et al.* (1994) reported that the number of the basal cells increased towards the direction of the corpus epididymis and decreased towards its caudal end. Such findings couldn't be proved in the current study. The cytoplasm of the basal cells showed the presence of few organelles in the form of scanty mitochondria and lipid globules. Yeung *et al.* (1994) attributed these lipid-like droplets to be specific for the human epididymis.

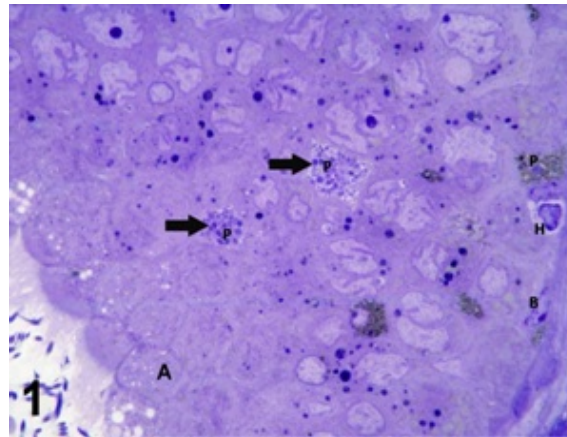


Fig. 1: A photomicrograph of a semithin section in the epididymis of a control rat (group I) showing clusters of brown lipofuscin granules inside the cytoplasm of the principal cells (P) and in a basal cell (B). There are multiple basophilic granules (arrows) associated with vacuoles inside some principal cells. Note the presence of a halo cell (H) and the numerous vacuoles in the apical cells (A) near the lumen. (Toluidine blue x 1000).

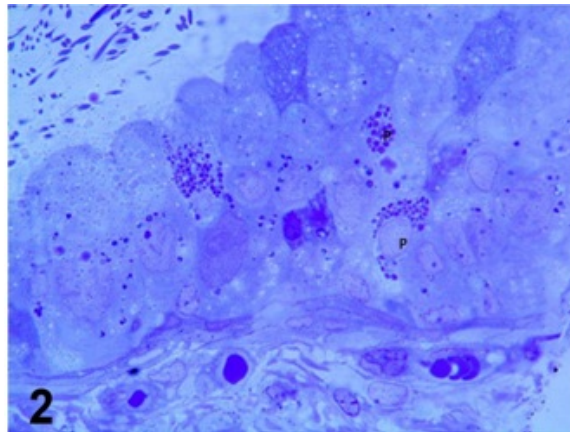


Fig. 2: A photomicrograph of a semithin section in the epididymis of a control rat showing clusters of dense darkly stained basophilic granules inside the principal cells (P) mainly in a supranuclear position. (Toluidine blue x 1000).

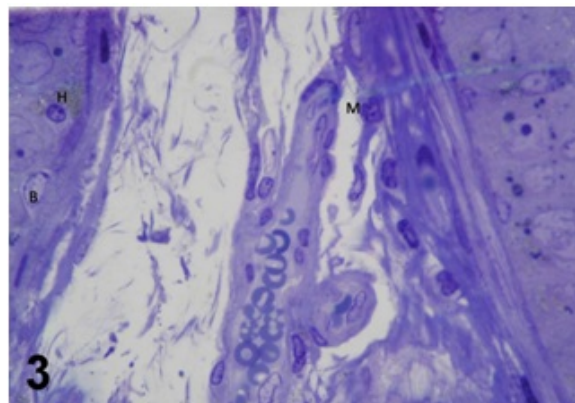


Fig. 3: A photomicrograph of a semithin section in the epididymis of a control rat showing a halo cell (H) with a rounded nucleus having a clear nucleolus and a cytoplasm filled with brown stained lipofuscin granules. Note a basal cell (B) with a large oval nucleus and a macrophage (M). (Toluidine blue x 1000).

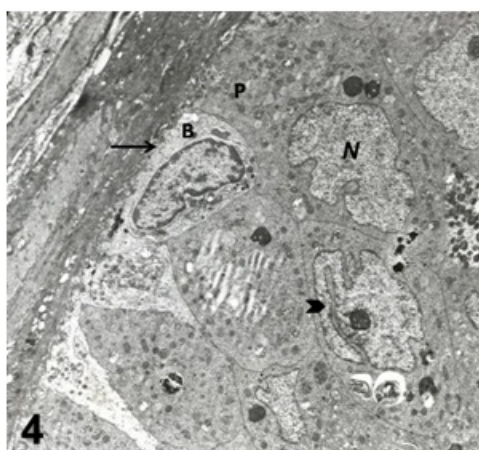


Fig. 4: A photomicrograph of a section in the epididymis of a control rat showing a basal cell (B) resting on a clear basement membrane (arrow) having an indented oval nucleus with peripheral chromatin. The basal cell (B) resting side by side to the principal cells (P). The principal cells show indented nuclei (N) with one nucleus appeared greatly convoluted (arrowhead). (Uranyl acetate x 10000).

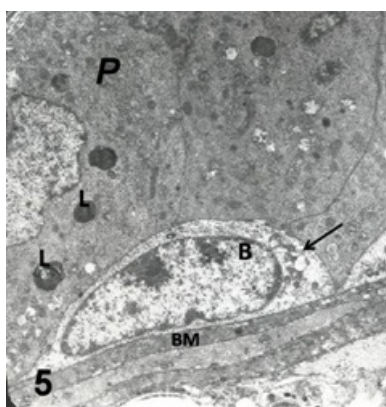


Fig. 5: A photomicrograph of a section in the epididymis of a control rat showing a basal cell (B) resting on a clear basement membrane (BM) and having a heterochromatic oval nucleus with clusters of chromatin and few electron dense granules. Note vacuoles lying beside the nucleus (arrow). A principal cell (P) shows an oval nucleus and contains large electron dense granules (lysosomes) (L). (Uranyl acetate x 10000).

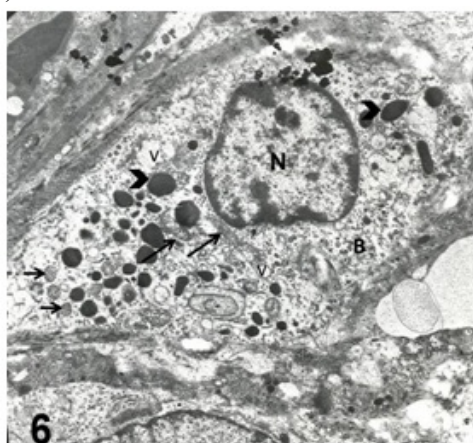


Fig. 6: A photomicrograph of a section in the epididymis of a control rat showing a basal cell (B) with a rounded hyperchromatic nucleus (N) and peripheral chromatin. The cytoplasm shows few organelles in the form of dispersed globular mitochondria (short arrows) and few elongated, large mitochondria close to the nucleus (long arrows). Multiple vacuoles (V) and electron dense granules mainly lysosomes (arrowheads), on both sides of the nucleus can be seen. (Uranyl acetate x 10000).



Fig. 7: A photomicrograph of a section in the epididymis of a control rat showing a principal cell (P) with an oval indented nucleus and large lysosomal granule (L), a large multivesicular body (short arrow) and some myelin Figures (long arrows) in the cytoplasm. Note also the presence of a halo cell (H) with rounded nucleus having peripheral chromatin with prominent nucleolus and a very clear cytoplasm containing few organelles. (Uranyl acetate x 10000).

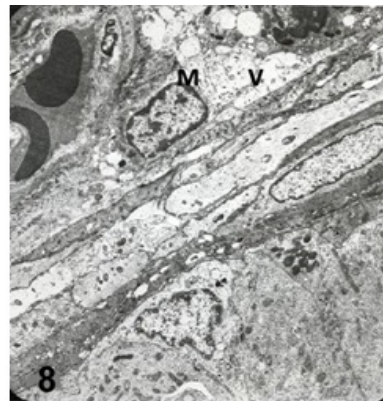


Fig. 8: A photomicrograph of a section in the epididymis of a control rat showing a large macrophage (M) with many large cytoplasmic vacuoles (V) and a heterochromatic nucleus.(Uranyl acetate x 10000).

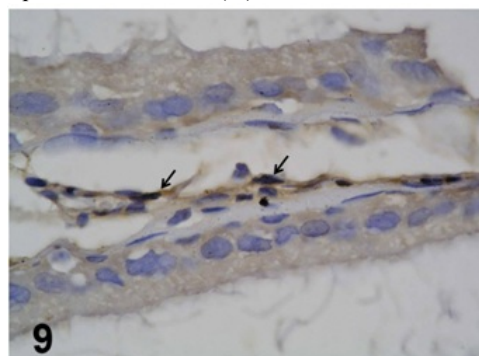


Fig. 9: A photomicrograph of a section in the epididymis of a control rat showing the immunohistochemical reaction. Note the brown immunostaining of macrophages (arrows). (CD₁₄ immunostaining with counterstain of hematoxylin X400).

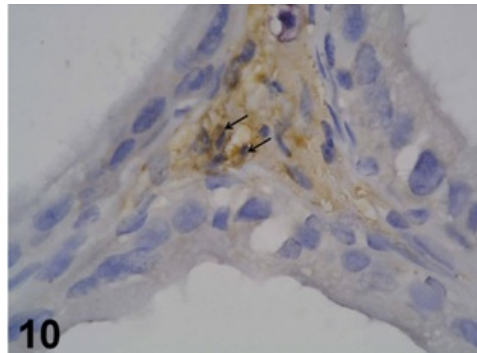


Fig. 10: A photomicrograph of a section in the epididymis of a control rat showing the immunohistochemical reaction. Only the interstitial cells are stained (arrows). (CD₁₄ immunostaining with counterstain of hematoxylin X400).

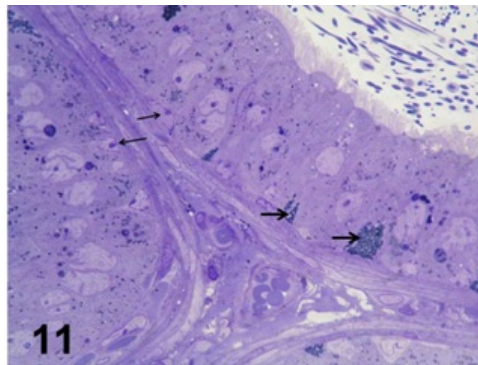


Fig. 11: A photomicrograph of a semithin section in the epididymis of a rat of group II showing that the basal cells are devoid of Indian ink granules (thin arrows) meanwhile, the basal parts of principal cells are rich in Indian ink granules (thick arrows). (Toluidine blue x 1000)

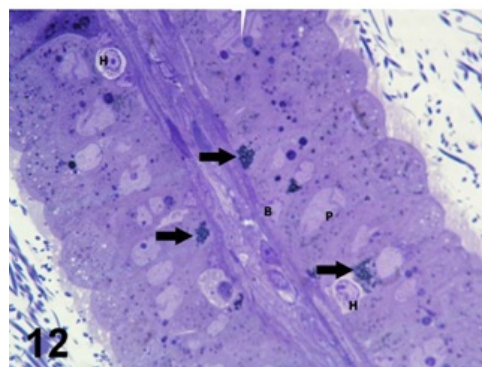


Fig. 12: A photomicrograph of a semithin section in the epididymis of a rat of group II showing the different types of cells; basal (B), principal (P) and halo (H) while clusters of the India ink (arrows) in some principal cells. Note the increased number of halo cells. (Toluidine blue x1000).

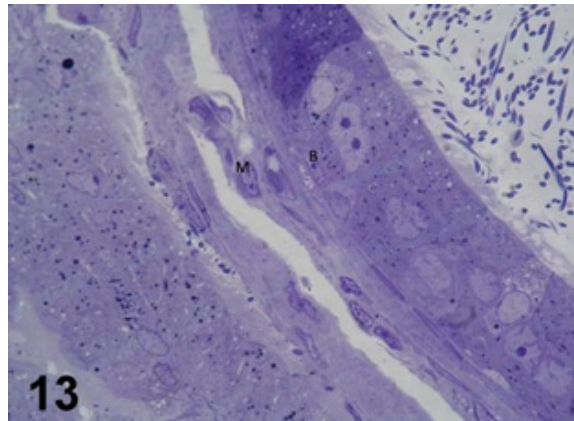


Fig. 13: A photomicrograph of a semithin section in the epididymis of a rat of group II showing an interstitial macrophage (M) with irregular nuclei and prominent nucleoli. Note the similarity between the interstitial cells and the basal cells (B). (Toluidine blue x1000).

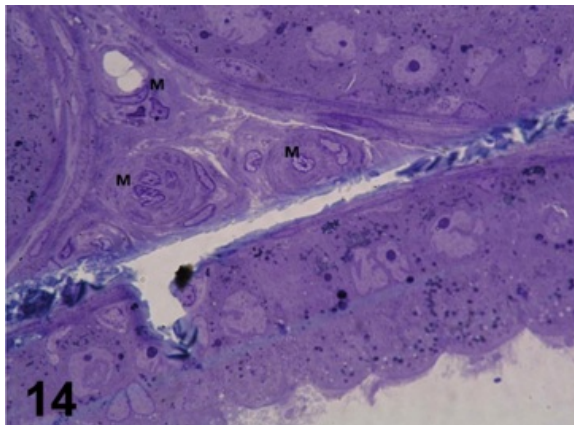


Fig. 14: A photomicrograph of a semithin section in the epididymis of a rat of group II showing a collection of large macrophages (M) with segmented or multiple nuclei. (Toluidine blue x 1000).

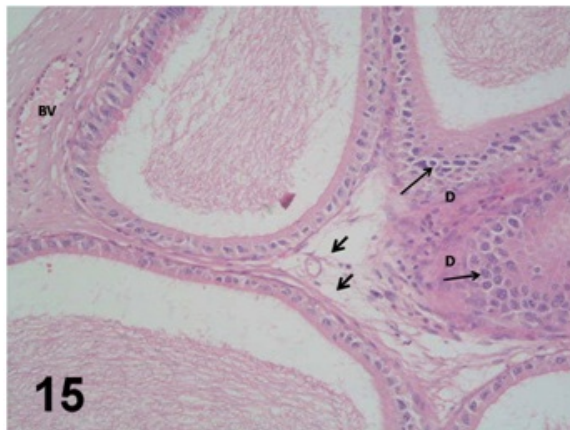


Fig. 15: A photomicrograph of a section in the epididymis of a rat of group III showing degeneration of the basal parts of the ductus epididymis (D) and the interstitium (short arrows). The cells of the epididymis are separated from each other (long arrows) and the blood vessels (BV) are dilated and congested with margination of leukocytes. (Hematoxylin and Eosin x 200).

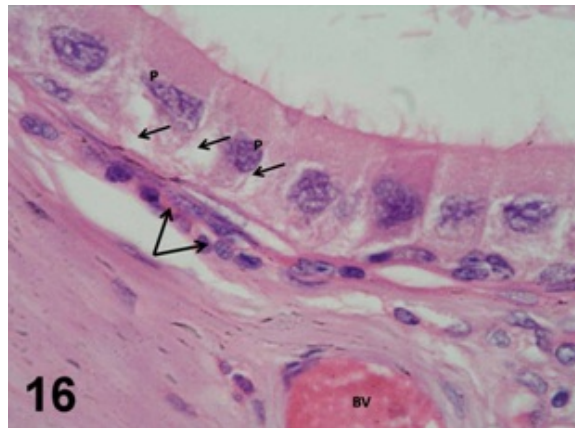


Fig. 16: A photomicrograph of a section in the epididymis of a rat of group III showing degeneration in the basal and lower lateral walls (short arrows) of the principal cells (P). Note the presence of multiple cells in the interstitium near the basement membrane of the ductus epididymis. The cells are large with large and irregular nuclei (long arrows). Note a blood vessel (BV) is dilated and congested. (Hematoxylin and Eosin x 1000).

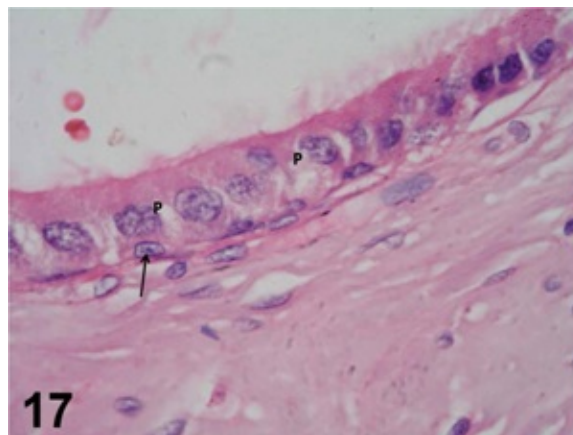


Fig. 17: A photomicrograph of a section in the epididymis of a rat of group III showing the principal cells (P) with evidence of degeneration in the form of vacuolation and a basal cell with an evident nucleolus resistant to degeneration (arrow). (Hematoxylin and Eosin x. 1000).

The principal cells were found in the present study to be the most numerous cell types within the epididymal epithelium. This finding was in agreement with many authors (Marta and Risley, 1986; Leung *et al.*, 2004).

Many vacuoles were detected in the cytoplasm of the principal cells. This finding was previously demonstrated by Domeniconi *et al.* (2007). Aire *et al.* (2008) described those vacuoles as a single large heterogeneous lipid droplet of unknown function that was characteristically situated immediately proximal to the nucleus. They also described variable cytoplasmic vesicles present in all cytoplasmic levels not only supranuclear with coalescent pattern and referred that to the lysosomal activity of the principal cells.

The cytoplasm of the principal cells was found to contain few organelles in the current study. However, Al-Amawi *et al.* (2007) described the cytoplasm of the principal cells to be full of numerous supranuclear mitochondria and abundant rough and smooth endoplasmic reticulum.

Numerous lipofuscin granules had been demonstrated in the cytoplasm of both the basal cells and principal cells in the present study. These granules often appeared in the supranuclear area of the principal cells and were larger than those found in the infranuclear portion. These findings were consistent with the findings of Hermo and Demelo (1987); Cooper *et al.* (1988) ; Yeung *et al.* (1989) who found the lipofuscin granules to be located between the basal and principal cells and suggested transfer of material between both cell types. Their presence might be a specific feature related to protein secretion and transcytosis between the adjacent

principal cells as Domeniconi *et al.* (2007) reported. The previous authors described the ingestion and digestion of material in epididymal epithelium by the basal cells to be a primary immunological function. Such local immune defense was relevant in view of occurrence of transcytosis across the epididymal epithelium.

Wang and Holstein (1983) identified macrophages in all segments of the epididymal epithelium from the caput till its caudal end while Yeung *et al.* (1994) found an increase in macrophage number toward the corpus and a decrease toward the caudal end of the epididymis. Such findings couldn't be proved in the current study. The macrophages were found in the present study to possess indented nuclei most probably to accommodate the cytoplasmic granules. This was in agreement with Yeung *et al.* (1994).

The macrophages were found in the present study in the interstitium situated close to the basement membrane and hence close to the basal cells which were lying abut to the basement membrane. On the other hand, Yeung *et al.* (1994) found that the macrophages were present within indentations of epithelium where the basal cells might be absent. Such finding raised out the hypothesis that some of the basal cells that were loaded with lipofuscin granules moved out of the epithelium to be replaced by peritubular macrophages. However this hypothesis was never proved. The previous authors also reported an intermediate form of macrophages /basal cells that contained large lipofuscin or phagocytic vesicles and were not abutting the basement membrane except by narrow stalk.

Ultrastructural similarity between the basal cells and macrophages was observed in the current study. The presence of multinuclei in the macrophages was a characteristic feature in the current study that helped to differentiate between those two cells. Abe and Takano (1989) reported that there was no confusion between the basal cells and macrophages; the former rested on a clear basement membrane and possessed sparse granules and desmosomes with the adjoining cells while the later had phagocytic inclusions and migrated between cells.

The above mentioned debate about the ultrastructural similarity between the basal cells and macrophages raised out the hypothesis that macrophages could be either progenitors of the basal cells or they might be a method of renewing the epididymal cells by helping the turning over population of the basal cells that were loaded with debris. Such explanation postulated a possible significance of epididymal cells in immunological aspects of fertility.

The ability of both basal cells and macrophages to ingest and digest cell debris was attributed to their immunocompetent function in preventing presentation of any antigen of sperm cell origin to the immune system (Hermo and Demelo, 1987; Cooper *et al.*, 1988; Yeung *et al.*, 1989).

The difference in immunostaining patterns in epididymis may be related to the regional difference in function. In the present study, the macrophages demonstrated positive immunoreactions to antibodies raised against the CD₁₄ while the basal cells did not. This is contradictory to the findings of Yeung *et al.* (1994) who reported selective immunoreactivity of the basal cells towards the CD₁₄ marker. Such finding supposed a role for the basal cells in the local immune defense mechanism in the epididymis. Lacking of expression of the CD₁₄ marker on the surface of the basal cells in the present study might adversely affect the postulations that the basal cells are immunocompetent cells in the epididymis. This also made their origin from the immunocompetent macrophages questionable.

Indian ink was used in the present study to demonstrate epididymal cells with potential phagocytic activity. Surprisingly, Indian ink granules were demonstrated within the cytoplasm of the principal cells. Similar finding was previously reported by Moniem and Glover (1972). Meanwhile, no granules were found within the cytoplasm of the basal cells. These findings might put the immunological function of the basal cells in doubt and in the same time raised the possibility of the principal cells to possess an immune function.

Induction of ischemia reperfusion injury in the present study was associated with degeneration of the principal cells. However, the basal cells appeared to be resistant to such deranging stimulus. This finding might highlight their supporting function for the epididymal epithelial cells and may post their ability to repopulate the epithelium following degeneration. Ozturk *et al.* (2007) reported that rats exposed to ischemia reperfusion injury had demonstrated a significant disorganization of the epithelium and loss of microvilli in the epididymal tissue.

Ischemia reperfusion was also associated with vascular dilatation and congestion with margination of leukocytes. In addition, there was an increase in the number and size of macrophages which appeared to have multiple nuclei. Vascular congestion and margination of leukocytes were also observed by Lysiak *et al.* (2001) who reported an increase in the number of neutrophils adhering to testicular subtunical venules after torsion repair/reperfusion and increased neutrophil recruitment to the testis.

The halo cells were also seen more frequently after ischemia reperfusion which was a parallel finding to the increased leukocyte migration out of blood vessels. Veri *et al.* (1992) and Palacios *et al.* (1993) postulated that the halo cells to be lymphocytes or monocytes and they added that these cells played a role in the immunological barrier of the male reproductive tract.

In conclusion, the present study opens the door for further studies concerning the immunological role of basal and principal cells of the epididymis. A supporting role for the basal cells has been observed. The principal cells may have a role in phagocytosis of particles within the epididymis. Further studies are needed to support such observations. It is also recommended to extend this study to include fresh human specimens to study the relation between sperm number and form and the histological features of epididymal cells in different age groups to assess the role of principal/basal cells in sperm maturation following ischemic injury.

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