CHAPTER – IV

ELECTRON MICROSCOPIC STUDIES ON EPITHELIAL CELLS AND SPERMATOZOA OF CAUDA EPIDIDYMIS IN ALBINO RATS TREATED WITH OCIMUM SANCTUM LEAVES
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INTRODUCTION

The epididymis of the mammalian male duct system has attracted the attention of investigators because of its pivotal role in sperm maturation and as an extragonadal site which may help in the control of fertility without impairing libido and potentia. Further, the regional, anatomical and functional differences recorded in several mammalian species formed the basis for further studies on the subject (Hinton, 1980; Brooks, 1983; Abe et al., 1983, 1984; Orgebein-Crist, 1986; Gopal Dutt and Suresh, 1988; Koe and Lee, 1993; Yeung et al., 1994; Hermo et al., 1994).

Sun and Flickinger (1979) proposed a scheme of cellular differentiation in the rat epididymal epithelium, in the rat, the postnatal development of the epididymis can be divided into three phases: 1) the undifferentiated period from 1 to 15 days; (2) the period of cellular differentiation from days 16 to 44; and (3) the period of growth extending from days 45 to 3 months. From birth up to day 5, the epididymis remains quiescent. Following cellular differentiation, narrow cells appear on day 16, the principal cells and basal cells on day 28. The narrow cells persist only in the initial segment, while in the middle and terminal segments they disappear by day 35, when light cells appear. Evidence suggests that the narrow cells are the precursors of light cells in the middle and terminal segments. The differentiation of the epididymal epithelium completes when the halo cells appear. Subsequently, Sun and Flickinger (1980) have studies by autoradiography the proliferating activity of the cells in rat epididymis during postnatal development. Results have shown that the columnar cells appear to be precursors to the principal and basal cells. As expected, the terminally differentiated light cells are not mitotically active, where as columnar cells (undifferentiated) exhibit high level of proliferative activity. Cytologically the epididymis attains advanced stage of development even before the sperm arrival. It is noteworthy that
in rat, rabbit and rhesus monkey (Leeson and Leeson, 1964; Alexander, 1972; Setty and Jehan, 1977), morphological differentiation of the caput epididymides precedes that of the cauda epididymides; the caput histological differentiation is completed several days before sperm entry, while in the cauda this occurs long after spermatozoa reach this segment (Setty and Jehan, 1977).

**Gross Anatomy**

The epididymis is composed of a single, long, highly coiled duct closely applied to the testis and embedded in a variable amount of adipose tissue of the epididymal fat pad. Based on shape and position on the testis, the epididymis is conventionally divided into three regions viz., caput, corpus and cauda. According to Glover and Nicander (1971) this terminology is misleading since it relates to the gross morphology of the epididymis but not to its microscopic structure. They reported that the mammalian epididymis, histologically and functionally, can be divided into initial and middle segments where sperm maturation takes place and a terminal segment which subserves the function of sperm storage. These segments do not necessarily correspond to the traditional terms of caput, corpus and cauda regions described in several species.

**Zonation**

Variation in the distribution and morphology of cell types have been recognized all along the epididymal duct in a variety of mammals (Hamilton, 1975). Since the epididymal epithelium lacks uniformity in structure, zones have been recognized in a variety of mammalian species. Two histologically distinct zones have been located in the primitive mammal, Echidna (Djakiew and Jones, 1981); five in the mouse (Takano, 1980); six in rat (Reid and Cleland, 1957) and tammar (Jones et al., 1984), seven in guinea-pig (Hoffer and
Greenberg, 1978); and eight in rabbit and human (Holstein, 1969). In addition various subzones have also been identified in the epididymis of rat (Reid and Cleland, 1957). Based on morphological differences, the goat epididymis is divided into five regions; region I and II and the proximal part of region III constitute the head; the distal part of region III and region IV, the body; and region V, the tail (Goyal and Williams, 1991). In some species the epididymal zones may overlap with two functionally divergent zones. The zone VI of the epididymis of Siberian hamster described by Nagy et al., (1982) overlaps with two functionally distinct zones; one located in the distal portion of the middle segment and the other in the proximal portion of the terminal segment according to the divisions described by Glover and Nicander (1971).

Regional differences in relative proportions of the lumen, epithelial height and width of the musculature have been observed in different species. There seems to be little variation in the morphological characteristics and histological features of the initial segment, while much variation is found in other segments. The initial segment in echidna, mouse, rat, hamster, guinea-pig, cat, dog, rabbit, mole, pig, and bull is generally recognized by tall pseudostratified columnar epithelium, long densely arranged stereocilia in a tuft like form and relatively narrow tubules containing small number of sperms. (Nicander, 1957; Suzuki and Racey, 1976; Djakiew and Jones, 1981; and Suresh, 1987). The middle segment is characterized by slender, short and usually bent cilia, abundant pinocytotic vesicles and increase in the luminal spermatozoa (Glover and Nicander, 1971; Nicander and Glover, 1973). The terminal segment is a wide duct, lined by short epithelium with some pinocytotic vesicles, covered by thick muscular coat and its lumen contains high concentration of spermatozoa.
Arterial Supply and Venous Drainage

The major blood supply to the epididymis is from the internal spermatic artery, which in sharp distinction from that of the testis especially in rat lacks convolutions. This branch divides into superior and inferior epididymal arteries whose distribution varies in different parts of the epididymis. The interductoral capillary network is tortuous and dense imparting reddish color to the caput in the rat but decrease in complexity distally in the epididymis (Korman et al., 1973; Hamilton, 1975). There is evidence that in the rat the rate of blood flow through this region is higher than elsewhere along the epididymis (Setchell et al., 1964). In the goat epididymis, the head and tail regions are profoundly supplied with branches of epididymal arteries than in the body (Dhingra, 1978). Arterial and venous branches follow the pattern basically similar to those in the mouse, rat and rabbit (Chubb and Desjardins, 1982). These arteries anastomose all along the epididymis and this arrangement may serve to enhance and stabilize the flow of the blood to the epididymis. Epididymal veins follow routes parallel to those of the epididymal arteries. As in the case of arteries, vein-vein anastomoses are common in all the above mentioned three species. In the rabbit, prominent vascular links join the testis and epididymis (Chubb and Desjardins, 1982). A similar but less conspicuous vascular link is found between the cauda epididymides and testis in rat (Karmano, 1967). The venous plexus that surrounds each artery typically in the rabbit serves to cool the arterial blood by a counter-current heat exchange mechanism (Chubb and Desjardins, 1982). Based on a study of the angioarchitecture of the epididymis of buffalo and sheep, Dhingra (1978, 1980) believes that the mini pampini form plexus of the vein and arteries play an important role in the haemodynamics and thermoregulation of epididymis to prolong the fertile life of spermatozoa.
Innervation

The epididymis in most mammals receives sympathetic and parasympathetic innervation to the smooth muscles commencing at the mid corpus region. It completely lacks somatic innervation. The sympathetic pathway is through caudal mesenteric plexus → hypogastric nerve → middle spermatic nerve via the testicular artery and ductus deferens to the epididymis. The parasympathetic supply to the interior spermatic nerves leading to the epididymis are derived from the nerve trunk composed of both cholinergic and adrenergic fibres, which follow and supply epididymal vessels (El Badawi and Schenk, 1967). These fibres vary in abundance in different epididymal regions. The caput epididymides as in the rat, rabbit and guinea-pig is poorly innervated, whereas in cat and dog, both norepinephrine positive (adrenergic) and acetyl cholinesterase positive (cholinergic) fibres are found (Norberg et al., 1967; El Badawi and Schenk, 1967). In most of the species, the corpus epididymides is rather poorly innervated. The peritubular cholinergic and adrenergic fibres progressively form intricate plexus as the muscular wall of the epididymis increase in thickness distally towards ductus deferens (Baumgarten and Holstein, 1968). The nerve fibres are both excitatory and inhibitory to the smooth muscles of the epididymis.

Musculature

The epididymis possesses characteristic distribution of musculature suited specifically to carry out their functions. Two morphologically distinct types of smooth muscle cells surround the entire length of the epididymal duct. The cells which constitute relatively thin muscular coat enables the slow movements at the proximal region and as the muscular coat increases in thickness proximo-distally, more powerful movements are induced in the distal region at the time of ejaculation (Holstein, 1967).
inactive period in the soft furred field rat (Dechamma and Gopal Dutt, 1980), the
epididymal muscle layer increases in thickness and its connective tissue is seen abundant.
In marked contrast, in the active breeding season, it is characterized by a concomitant
decrease in muscle layer and connective tissue component. Since the fluid transport takes
place even though the flow from the testes is blocked by ligation of ductus efferentes
(Macmillan and Auckland, 1960), it is concluded that the muscular layer is actively
involved in the transport of fluid in the epididymis. This inference is further reinforced by
the evidence that the luminal epithelium is lined with immotile cilia (Hamilton, 1975) and
the transport takes place against increasing hydrostatic pressure from the testis to the caput
and from the caput to the cauda epididymides (Johnson and Howards, 1976). The
contractions of the smooth muscle layer lining the duct provide propelling force for
transporting spermatozoa through epididymis (Baumgarten et al., 1971). These
contractions are peristaltic and the frequency of the electrical activity of the smooth muscle
declines from the caput towards vas deferens (Risley, 1963; Talo et al., 1979).

**Blood-Epididymis Barrier**

The development of a blood-epididymis barrier to provide a specialized
microenvironment for sperm maturation is well documented. Evidence in support of this
view is that the composition of luminal fluid is distinct from that of blood plasma (Setchell
and Hinton, 1981). Ultrastructural studies on the epididymal duct in mammals have clearly
established that various epithelial contacts such as tight junctions, gap junctions, zona
occludens, and desmosomes do not simply allow the molecules to traverse the epithelium
but they do seem to be regulated (Suzuki and Nagano, 1978 a,b; Hinton, 1982). The blood-
epididymis barrier performs a protective role by excluding the entry of immunoglobulins,
many toxic metabolites and environmental agents into the lumen (Hancock, 1981; Hinton, 1982).

Cytology

The epithelial lining of the epididymis in mammals comprises of principal cells, basal cells, narrow cells, clear cells, apical cells and halo cells (intraepithelial lymphocytes/macrophages). These cell types show regional differences in distribution. For example, the principal and basal cells are of common occurrence in zones I to V, the apical cells in zone I and II, the less numerous ‘halo cells’ occur in zone II but rarely in zone III and the clear cells in all the five zones of the wild mouse epididymis (Suresh, 1987).

In the present study, observations have been confined to principal, clear and basal cells which play an important role in sperm maturation, endocytosis, absorbing both exogeneous and endogenous proteins from the lumen and scavenging of the other cell debris which are present in side the epithelial cells.

Principal Cells

The most numerous among the cell types so far described in all mammalian species, are the principal cells. The principal cells are present all along the entire length of the epididymis, through they vary in shape from tall columnar type extending from the basal lamina to the lumen in the proximal region to the squamous type found in the distal region. The principal cells thus decrease in height, increase in width from the proximal to distal segments. Quantitative study showed that among the various cell types the proportion of the principal cells is the highest in the central caput and lowest in the central corpus region of the ovine epididymis (Marengo and Amann, 1990). However, the number of principal cells per unit length of the basement membrane is same in all the regions of the human
epididymis (Regadera et al., 1993). The principal cells have a single round or elliptical nucleus containing granular chromatin and one or more nucleoli. Multinucleate giant cells associated with elevated androgen levels (Kuo and Gomez, 1981).

Ultrastructurally, the principal cells are characterized by well-developed stereocilia and long microvilli borne on the luminal surface. The stereocilia may differ regionally being tall, branched and irregular in the proximal region and short, dense and regular in the distal region. Several coated vesicles, pinocytotic invaginations and vesicles varying in diameter and content are usually observed in the apical region. Small coated vesicles are also often found in the vicinity of the Golgi body. The apical cytoplasm contains circular or oval multivesicular bodies (MVBs) and these vary in their size and content. The large MVBs may contain floucculent or amorphous material or a number of small vesicles (Gopal Dutt, 1999). The presence of microvilli, pinocytotic vesicles, coated vesicles and their ability to internalize fluid phase markers like ferritin, horse raddish peroxidase, thorotраст, etc. (Moore and Bedford, 1979; Flickinger, 1981). Rasweiler and Bedford (1982) have reported that in the rat cauda epididymides, the stereocilia of the principal cells serve as an important route for water absorption by a non-vascular mechanism.

The endoplasmic reticulum is generally well developed in the cytoplasm. Rough ER may exist in different forms. They may be found as flattened lamellae studded with ribosomes or as vesicles studded with ribosomes (Gopal Dutt, 1999). It is possible to find in some species mixed or heterogenous ER as in the adult male mouse described by Flickinger (1979).

A major portion of supranuclear cytoplasm is occupied by an extensive Golgi complex which is composed of parallelly arranged stack of fenestrated cisternae of smooth
surface membranes (Gopal Dutt, 1999). Since the Golgi body is well developed and the RER and ribosomes are abundant, the principal cells are indeed capable of synthesizing protein. Using time course electromicroscopic, radioautography Flickinger et al., (1984) have clearly demonstrated the passage of labelled proteins/ glycoproteins from the RER to the Golgi body and then to coated vesicles, which ultimately release their content into the lumen. Similarly, Nicander and Malmquist (1977) have provided ultrastructural evidence and pointed out that small vesicles, similar to those present in the Golgi area, are involved in the secretion of proteins in the initial segment of the epididymis. The major secretory products of rabbit epididymis are not proteins but glycerylphosphoryl choline, inositol and amino acids (Jones, 1978). The exact mechanism of secretion of such proteins into the lumen is perhaps through small vesicles by exocytosis (Nicander and Ploen, 1979).

**Secretion of Steroids**

The question, whether the epididymal epithelium is truly a site for steroidogenesis has now been resolved. It has been shown in a number of mammalian species including mouse, rat, rabbit and ram, that the epididymis is capable of synthesizing testosterone from acetate or cholesterol (Hamilton and Fawcett, 1970; Hamilton, 1971). Cytochemically, the epididymal epithelium is positive for \( \Delta^\text{3-3}\beta \) HSDH and \( 17\beta\)-HSDH in all the three regions. The reactions for these enzymes in the caudal region is however found to be high (Suresh and Gopal Dutt, 1984). Similar observations have been made in the epididymis of the long-tailed climbing mouse, *Vandeleuria oleracia* (Suresh and Gopal Dutt, 1985). In the rat and ram, the cauda epididymides synthesizes greater amount of testosterone compared to that of the caput epididymides (Hamilton, 1972).
**Phagocytosis of Cytoplasmic Droplet**

One of the important cytological events which occur during sperm maturation is the shedding of cytoplasmic droplets as the sperms pass through the epididymal duct. In bush tailed opossum, there is a clear cut ultrastructural evidence that the principal cells phagocytise and digest the cytoplasmic droplets (Temple-Smith, 1984). This view gains support from the recent finding that the principal cells contain cysteine proteinases such as cathepsins B and H which are involved in the intracellular degradation system. (Tomomasa et al., 1994). The foregoing account lead to the conclusion that the principal cells, like Sertoli cells, are polyfunctional in mammals.

**Basal Cells**

The basal cells vary considerably in size, shape and distribution along the epididymal duct. These cells, though occur sporadically in rat (Hamilton, 1975) are more frequently seen in the wild mouse (Suresh, 1987). The basal cells are usually hemispherical, polygonal or pyramidal or round in shape and their nuclei are elongated and flattened against the basement membrane. Hamilton (1972) described that the shape of basal cells varies profoundly because of their close relationship with the principal cells. The available cytological evidence does not support the contention that the basal cells are involved either in secretion or in absorption. This conclusion is drawn from the observations that the basal cells are numerically less, their location is far away from the lumen and they do not have complex cellular machinery for secretion or absorption (Gopal Dutt, 1999). Veri et al., (1993) and Regadera et al., (1993) have proposed a hypothesis that the basal cells are involved in a scavenging role in a local immune defence mechanism in which antigenic products derived from sperm degradation are phagocytised. The role of
basal cells seems to be imperfectly understood, although these cells are present in all mammalian epididymides studied.

**Clear Cells**

The clear cells, also referred to as light cells or foamy cells by some workers, are remarkably striking in the study of mammalian epididymis. In rat, the early work of Kreth (1965) has shown two types of clear cells, whereas in the material examined by Hamilton (1975) only one type is seen. These cells have not been described at all in the epididymis of rabbit, stallion and bull (Nicander, 1957), mouse (Hamilton, 1975), mole (Suzuki and Racey, 1976), guinea-pig (Hoffer and Greenberg, 1978) and tammar (Jones et al., 1984), monkey (Romos and Dym, 1977) and human (Holstein, 1969). Surprisingly, the clear cells which were earlier reported to be absent in the mouse epididymis (Hamilton, 1975) are now located from the intermediate segment to the tail of the epididymis in the same species (Soranzo et al., 1982). The clear cells are generally found in between the principal cells. They contain distinct ovoid nuclei placed slightly above basal position and contain one or more nucleoli and granular chromatin (Gopal Dutt, 1999). The clear cells have been implicated to holocrine secretory cycle (Martan and Risley, 1963) and as a principal source of glycerylphosphorylcholine (GPC) (Scott et al., 1963).

In impressive cellular feature of the clear cells is the presence of extensive cytoplasmic vacuolation which formed the basis for Reid and Cleland (1957) to divide these cells into different intergrading types. The clear cells in the epididymis of the wild mouse (Suresh, 1987) are characterized by the appearance of prominent supranuclear vacuoles, pinocytotic pits near the cell surface and dense accumulation of lipid or glycolipid bodies in the basal cytoplasm. There is evidence in the case of rat that a glycoprotein secreted in the caput is partly taken up by the clear cells in the cauda.
epididymides (Lea et al., 1978). These cells are also capable of absorbing both exogenous
and endogenous proteins from the lumen. During absorption, the luminal content is taken
up by micropinocytosis and transferred to supranuclear vacuoles (Moore and Bedford,
1979). Hermo et al., (1988) have reported that in the rat, the cytoplasmic droplets in the
sperm first disintegrate into small particles and these are subsequently endocytosed and
digested by the clear cells. Further, in the rat epididymis, it has been reported that the
immobilin protein which serves to immobilize spermatozoa is secreted by the principal
cells of the initial segment and is absorbed by the distal caudal epithelial clear cells by
endocytosis (Hermo et al., 1994).

The epididymis is a novel organ, present only in the amniotes, playing a significant
role in the physiological maturation of the spermatozoa (Robaire and Hermo, 1988;
Cooper, 1992, 1995 a,b, 1998). Such maturation is considered essential for the spermatozoa
to become motile and to fertilize the ova (Cooper, 1990, 1993). The epididymal duct
originated due to the joining of ductuli efferentes at the initial segment, and subsequently
differentiated histologically into caput, corpus and cauda epididymides (Hamilton, 1975;
Robaire and Hermo, 1988). The epithelial lining of the duct also participated in this
differentiation through a decrease in the height of the columnar cells and differences in the
distribution and relative percentage of the various cell types designated as principal,
narrow, clear, basal and halo cells (Reid and Cleland, 1957; Hamilton, 1975; Robaire and
Hermo, 1988; Robaire and Viger, 1993).

The epididymal duct contributes to the physiological maturation of the spermatozoa
by way of secretion of several proteins, glycoproteins and small molecular weight
substances. Modification of the luminal fluid through absorption and secretion of organic
and inorganic ions and fluid, and phagocytosis of dead and defective sperm and debris
from the lumen (Robaire and Hermo, 1988; Hermo et al., 1988) assists in the maturation process. Moreover, through these activities the epididymal duct contributes to a changing luminal micro-environment which is considered essential for the physiological maturation of the spermatozoa (Hinton and Palladino, 1995). However, the role of the various epithelial cell types in the processing of the spermatozoa and the fluid is not fully understood.

The present study is carried out to determine the effect of *Ocimum sanctum* leaves (Benzene extract) on the ultrastructure of the epithelial cells and spermatozoa of the rat cauda epididymis which contribute to the process of sperm maturation by regulating the fluid environment in which the spermatozoa mature.
MATERIALS AND METHODS

Fresh *Ocimum sanctum* leaves were collected from Buddanal forest nursery, which is 30 km away from Dharwad and dried in shade. The dried leaves were coarsely powdered and subjected to soxheltation process to get the benzene extract. Benzene was separated and the extract thus obtained was allowed to dry and stored in a dessicator at 4°C (WHO-Protocol LG-06, 1983). The Benzene extract is then mixed with propylene glycol as required and administered orally (gavage) to the experimental animals.

Adult male albino rats of Wistar strain, 3 to 4 months old and 190 to 200 gm body weight, were acclimatized to the laboratory conditions and received a standard rat pellet diet (Gold Mohar, Hindustan Lever Ltd., Hyderabad) and water *ad libitum*. The rats were divided into two groups comprising five animals each.

**Group I**: The animals were administered 1 ml propylene glycol/ rat/ day for a period of 48 days and served as controls.

**Group II**: The animals were administered by gavage 250 mg/kg body weight of Benzene extract of *Ocimum sanctum* leaves in 1 ml of propylene glycol/ rat/ day for a period of 48 days and served as treated animals.

Twenty-four hours after the last dose the control and treated animals were given mild ether anaesthesia. Fixation by vascular perfusion was carried out intracardiacally, which offers a good preservation of the organs. Perfusion was carried out, using a transfusion set bottle with polythene catheter containing the fluid suspended at about 150 cm above the animal at room temperature, using about 300 ml of 3% gluaraldehyde. The heart was exposed and the ascending aorta cannulated with a polythene catheter of about 1
mm internal diameter through an incision in the left ventricle. Care was taken not to let in air while perfusion was conducted. The right atrium was incised for the out flow of the fixative. Then rate of flow was reduced to 6 to 8 ml per minute. Following perfusion the epididymis were dissected out and its cauda (tail) portion separated and slices of tissue were cut and further fixed in the same perfusion fluid for a period of 2 to 4 hours. The tissues were stored in the sodium cacodylate buffer at 4°C (pH 7.4, 0.1M), washed in buffer and post-fixed in 1% Osmium tetraoxide for one and half to two hours. Then again washed with buffer, dehydrated in alcoholic series for 1 hour gap, stained enblock in 2% uranyl acetate in 90% methanol for 1 hour and cleared in propylene oxide for 10-15 mins. and infiltrated with araldite : propylene (1:1) mixture for over night. Then infiltrated again with fresh araldite (3 changes with a gap of 3 to 4 hours) and embedded in the same media in a beam capsule. The blocks were cut in Leica LKB Broma Ultramicrotome. Ultrathin sections were cut at 100-300 Å mounted on copper grids and stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963). The stained sections were scanned in Jeol-TEM 100 C X II electron microscope for ultrastructural observations.
OBSERVATIONS

Semithin Sections

Semithin sections of the treated group exhibits damaged stereocilia, abundance of vacuoles, including intraepithelial vacuoles of varying size. Many of these vacuoles were positioned in the clear cell, principal cell, and along the length of the epithelium and exhibit exfoliation. The epithelial height was reduced and the tubules devoid of sperms. The intertubular space was increased. The epithelium showed overall decrease in cytoplasmic ground substance; degenerating cells exhibited a characteristic of vacuolization and few of them have disrupted nucleus. The interstitium cells exhibited clearly the characteristics of a degeneration as a result of phagocytosis by macrophages and their nuclei were condensed or had irregular shape which is evident of a complete degeneration of the interstitium cell (Figs.4-6).

Ultrastructural Studies (TEM)

The principal cells (control) had a single round or elliptical nucleus containing granular chromatin and one or more nucleoli. The principal cells were characterized by well-developed stereocilia and long microvilli borne on the luminal surface. Several coated vesicles, pinocytotic invaginations and vesicles varying in diameter and content were usually observed in the apical region. Small coated vesicles were also often found in the vicinity of the Golgi body. The apical cytoplasm contains circular or oval multivesicular bodies and these vary in their size and content. The large multivesicular bodies may contain flocculent or amorphous material or a number of small vesicles. The endoplasmic reticulum was generally well developed in the cytoplasm. Rough E.R. may exist in
different forms. They may be found as flattened lamellae studded with ribosomes or as vesicles studded with ribosomes. A major portion of supranuclear cytoplasm was occupied by an extensive Golgi complex which was composed of parallely arranged stack of fenestrated cisternae of smooth surface membranes (Figs. 1 & 3).

The clear cells were generally found in between the principal cells. They contain distinct ovoid nuclei placed slightly above basal position and contain one or more nucleoli and granular chromatin. The cells characterized by the appearance of prominent supranuclear vacuoles, pinocytotic pits near the cell surface and dense accumulation of lipid or glycolipid bodies in the basal cytoplasm (Fig. 2).

The basal cells vary considerably in size, shape and distribution along the epididymal duct. The basal cells were usually hemispherical, polygonal or pyramidal or round in shape and their nuclei were elongated and flattened against the basement membrane. The shape of basal cells varies profoundly because of their close relationship with the principal cells. The basal cells were numerically less, their location is far away from the lumen and they do not have complex cellular machinery for secretion or absorption. The role of basal cells seems to be imperfectly understood, although these cells are present in all mammalian epididymis (Figs. 1, 3 & 4).

The most obvious effect of *Ocimum sanctum* leaves on the principal cell was a decrease in the number of coated micropinocytotic invaginations on the luminal surface. There was consequent decrease in the number of coated micropinocytotic vesicles. Mitochondria were reduced in size and number. The Golgi apparatus in most cells were regressed. The number of lipid droplets in the principal cells were apparently decreased. The rough and smooth varieties of endoplasmic reticulum exhibit the changes in the
structure. Furthermore, the cisternae contained electron-dense material of different sizes. Deeper in the cytoplasm, there were smaller such membrane bound bodies, were seen associating with the multivesicular bodies. The principal cell reflected the changes in terms of vesicular elements and lysosomal bodies. An interesting observation was that the nucleus was highly indented and decreased in the size and the impact of the treatment was dramatic on the principal cells (Figs.5 & 8).

Micropinocytotic vesicles were rarely seen in the clear cells of the Ocimum treated rats. There was a slight decrease in the number of multivesicular bodies and mitochondria. The number of cytoplasmic vacuoles (CV) was reduced and the density of their flocculent content showed an increase. Coalescences between individual cytoplasmic vacuoles as well as between cytoplasmic vacuoles and micropinocytotic vesicles, which was a common feature in the untreated animals, was much reduced in the treated groups. The electron dense polymorphic granules, that are probably a stage in the formation of the secretory products, showed an obvious decrease in their numbers in the clear cell. A decrease was also evident in the size of lipid droplets in this cell. Autophagic bodies, containing remnants of cellular organelles, become particularly prominent in the perinuclear region of the cells indicating an enhancement of autophagic process within the cell. Size of the multivesicular bodies in the supranuclear cytoplasm increased, and their content appeared heterogenous with dark particulate or filamentous material. The lysosomal bodies increased tremendously. Such bodies were either in the basal cytoplasm or the supranuclear cytoplasm or both. The clear cell increased in the perimeter to such an extent that, in the sections of the cell, they were greater in width than height. Furthermore, in the latter sections of such clear cells could be seen without a nucleus (Figs.6 & 7).
In the *Ocimum sanctum* treated basal cells, the scattered spherical electron dense granules in the cytoplasm was absent. Mitochondria were reduced in number and disturbed. The Golgi regions were less, scattered and appeared widened. The cisternae were less compact. The varieties of rough and smooth endoplasmic reticulum were decreased in number and were disrupted. In the plain of the sections, the nucleus became much elongated than the normal nucleus and the chromatin material in the nucleus was totally absent. The epithelial height was reduced. The vascularity of the organ and other fibroblasts were unaffected (Figs. 5 & 8).

Cross section of tip of the controlled sperm head, anterior portion, mid region and median section of the sperm head were with normal features of perforatorium, plasma membrane, nucleus, acrosome and small vesicle on the ventral surface of the perforatorium, post acrosomal dense lamina and ventral spur. The ventral spur refers to the ventral prominence of the sperm head. Mid region of the sperm head consist of acrosome i.e., bulkier dorsal and lateral regions, perforatorium, outer nuclear membrane and post nuclear cap appear normal. Median sections through the base of the sperm head exhibited well-defined nuclear envelope, basal plate, posterior ring lamellar body and connecting piece of the tail. (Figs. 9 & 10)

Transverse sections of the mid-piece of sperm tail and different portions of principal piece showed normal structures of mitochondrial sheath and fibrous sheath, which including outer dense fibres, axonemal component, central pair, longitudinal column of the fibrous sheath along with their plasma membrane were well defined. In mid-piece of the spermatozoal flagellum, there was spatial arrangement of the 9 outer dense fibres. The centrally located axoneme component was composed of the 9 + 2 arrangement of
microtubules with presence of agranular material between the mitochondrial sheath and outer dense fibres (Figs. 11 & 12).

Longitudinal sections of mid-piece and principal piece of sperm showed well-preserved and normal structure of plasma membrane and internal components. Plasma membrane was intact over the mid-piece and included normal structure of mitochondrial sheath, which contain well preserved mitochondria in compact chain like arrangement throughout the length of the mid-piece. The fibrous sheath of the principle piece with its internal components were well defined (Figs. 10-12).

In the rats treated with *Ocimum sanctum* leaves, the sperm heads exhibited disrupted plasma membrane, acrosomal membrane and surface coating with fuzzy material (Figs. 13 & 14).

Tip of the sperm head showed disruption of plasma membrane and acrosome. Perforatorium (three-pointed star) most of its surface covered by thin portion of the acrosomal sac was condensed. At the anterior portion of sperm head, there was disruption of plasma membrane, acrosome, perforatorium and small vesicle on the ventral surface of the perforatorium which is probably part of the acrosomal system (Fig. 13). Longitudinal section of the caudal portion of sperm heads revealed the disruption of nucleus, perforatorium and loss of acrosomal and plasma membrane along the length of the head (Figs. 13 & 14). Frontal section of the caudal portion of sperm head revealed the disturbance of lamellar body, plasma membrane layer and centrioles. The basal plate, posterior ring and post nuclear cap appeared normal (Figs. 13 & 14).

The transverse sections of mid-piece showed the disruption and degeneration of mitochondrial sheath along the length of the structure. Most of the sections showed
abnormal mitochondrial sheath and loss of plasma membrane. Along the mitochondrial sheath, mitochondria showed the characteristics of disorganization or hypertrophy or commencement of degeneration. Some showed displaced mitochondrial sheath on one side or both sides and there were abnormal pattern of outer dense fibres (Fig. 14). Most of the tail sections showed retention of cytoplasmic droplets around the mid-piece or one side (Figs. 15 & 16).

In transverse sections of the principle piece of different parts of the tail, the rib of the fibrous sheath was disturbed and showed discontinuation in fibrous sheath. Outer dense fibres and axonemal component were normal. Most of the principle pieces were without plasma membrane (Fig. 14).

Longitudinal sections of mid-piece showed loss of plasma membrane. Most of the mitochondria showed hypertrophy or were absent along the length of the tail. Swelling or characteristics of commencement of degeneration were observed in some mitochondria. The principle piece showed discontinuation or was without plasma membrane (Fig. 14). Most of the sections of head and tail were coated with fuzzy material on their surface. (Figs. 13 & 14).
DISCUSSION

Many synthetic and plant derived estrogenic substances have been reported to be good inhibitors of male fertility and affect the androgen dependent organs (Faransworth et al., 1975; Tveter et al., 1975). Recently, plants and their products have been used for regulation of fertility (Atal and Kapur, 1982; Chopra et al., 1986). After the discovery of gossypol, as a potent male antifertility agent (Segal, 1985), much importance is given for screening the antifertility effects of numerous plants.

The principal cell of the epididymal epithelium is concerned with two major functions, viz., endocytosis and secretion. A number of large dense supranuclear located spherical membrane-bound bodies, identified cytochemically as lysosomes, were characteristic of the principal cell (Robaire and Hermo, 1988). Using tracers it was established that such lysosomes were formed due to the association between the pale multivesicular bodies (MVBs) and the small coated vesicles containing hydrolytic enzymes packaged in the Golgi apparatus (Friend and Farquhar, 1967; Jones et al., 1979; Abe et al., 1983). The increase in abundance, as well as the size of the lysosomal bodies, and their appearance in the basal cytoplasm in the principal cell of the initial segment and caput, corpus and proximal cauda epididymis indicate augmented endocytosis.

Vincristine (VCR) treatment, caused degenerative changes in the seminiferous epithelium and the cell fragments thus formed reached the epididymis (Stanley and Akbarsha, 1992 a,b; Stanley et al., 1995; Averal et al., 1995; Akbarsha et al., 1996). It could be suggested that the principal cell is concerned with the removal of such fragments. Moreover, it may be that there is a direct toxicity of the drug on the cellular constituents,
resulting in the triggering of the intracellular homeostatic machinery for scavenging the damaged cellular constituents (Akbarsha and Averal, 1996b).

The extensively developed RER in the basal and perinuclear cytoplasm, sparsely granulated ER in the supranuclear cytoplasm, the large Golgi apparatus formed of many stacks of sacculles and the associated vesicular elements in the supranuclear area are the machinery for extensive protein synthesis and secretion (Robaire and Hermo, 1988; Marengo and Amann, 1990; Araki et al., 1991) and glycosylation of the secretory proteins (Flickinger, 1979, 1985; Francavilla et al., 1983; Holland and Orgebein-Crist, 1988). VCR treatment may augment the secretion of certain specific proteins as reflected in the appearance and accumulation of small coated vesicles, containing secretion granule, in the Golgi apparatus (Akbarsha and Averal, 1998). It is known that such vesicles deliver hydrolytic enzymes to the MVBs (Friend and Farquhar, 1967; Friend, 1969).

The epididymis in general, and the principal cell in particular, is an androgen-dependent (Robaire and Hermo, 1988; Toney and Danzo, 1988; Akbarsha and Averal, 1998). Androgen withdrawal is known to cause extensive changes in the principal cell (Robaire and Hermo, 1988; Akbarsha and Averal, 1998). Thus in the present study, the changes in the principal cell of Ocimum sanctum treated rats may reflect a manifestation of the Ocimum sanctum induced hypoandrogenic status. However, direct action of Ocimum sanctum leaves on the principal cell cannot be excluded. Microtubules constitute a principal component of the tissue matrix system of the epithelial cells (Getzenberg et al., 1990). VCR is a microtubule disrupting agent (Dustin, 1984; Akbarsha and Averal, 1998), and the microtubules of the principal cell may be the target for Ocimum sanctum action. It is possible, therefore, that Ocimum sanctum may cause pathological changes in the epididymal principal cell.
The present study has shown that, the principal cells undergo ultrastructural changes following *Ocimum sanctum* treatment. Among the significant changes observed in the cells are decrease in the number of micropinocytotic vesicles and mitochondria and a reduction in the size of the Golgi apparatus. These findings are consistent with a view that the absorptive function of the principal cell is impaired following *Ocimum sanctum* treatment. These observations are similar to Asha Prakash *et al.*, (1979) following the administration of cryproterene acetate on the rat epididymis.

Clear cells are present in the epithelium of the caput, corpus and cauda of the rat (Robaire and Hermo, 1988). They are characterized by an apical region containing numerous weakly stained vacuoles of various sizes, large supranuclear MVB packed with a dense material and the basal region with the nucleus (Hamilton *et al.*, 1977; Robaire and Hermo, 1988; Toshimari *et al.*, 1990; Robaire and Viger, 1995). The clear cells have an endocytotic role (Moore and Bedford, 1979; Robaire and Hermo, 1988), an activity much greater in the clear cell than in the adjacent principal cell, and the clear cell of the cauda function for the recognition and internalization of the cytoplasmic droplets shed from the sperm (Clermont and Hermo, 1985; Hermo *et al.*, 1988). The results of the present study seem to indicate that in response to *Ocimum sanctum* treatment, the clear cells undergo hypertrophy, hyperplasia and hyperactivity in an attempt to remove the cell debris reaching the ductus epididymal lumen from the testis in the form of residual bodies, Sertoli cell fragments and dead and deformed sperm. These findings are similar to Akbarsha *et al.*, (1996) and Akbarsha and Averals' (1999), observations in vincristine treated rat cauda epididymis.

Evidence for a spermatotoxic effect of VCR, particularly in the context of tubulin-based fibril system in the sperm tail, has been obtained (Akbarsha and Averal, 1996 b).
Such sperm are dead and add to the abundance of cell debris in the epididymal lumen. However, several of the sperm which arrive at the cauda in the VCR-treated rats retain the cytoplasmic droplets (Akbarsha and Averal, 1996b). These sperm are defective and add to the dead sperm and cell debris in the epididymal lumen. On VCR treatment, several spermatogenic cells in the seminiferous tubules are killed, through presumably an apoptotic mechanism. These cells also arrive at the epididymis where they break up into apoptotic bodies and add to the cell fragments in the ductus epididymis (Stanley et al., 1995; Averal et al., 1995; Akbarsha and Averal, 1999). Similar observations have been made in our present study.

Thus, the manifestations in the clear cells on *Ocimum sanctum* treatment are a physiological response involving endocytosis of the cell fragments and dead sperm. Several of the clear cells of *Ocimum sanctum* treated rats were devoid of stereocilia and in such cells the structural integrity of the cytoplasm was almost lost. This was not a fixation artifact because the neighbouring cell types did not reflect such a change. It could be suggested that in as much as the hypertrophy and hyperplasia of clear cells are physiological responses, the subsequent loss of the stereocilia and the structural integrity was a pathological change leading to senescence of the clear cells.

In the clear cell of the *Ocimum sanctum* treated rats, organelles that constitute the secretory apparatus, viz., cytoplasmic vacuoles, electron dense secretory granules and mitochondria exhibit a reduction in their numbers. Also the lipid droplets in the cells of the treated rat are smaller as compared with those in the untreated rats. Further, there is a reduction in the number of micropinocytotic vesicles and multivesicular bodies. These findings are in accordance with the view that both the absorptive and secretory functions of the clear cells are affected following *Ocimum sanctum* treatment.
The above ultrastructural changes, indicative of an impairment of the absorptive and secretory functions of the principal, clear and basal cells, would result in a alterations in the composition of the epididymal fluid which in turn would affect sperm maturation. This contention supports the results of earlier studies (Prasad et al., 1970; Rajalakshmi et al., 1971; Rajalakshmi and Prasad, 1976; Asha Prakash et al., 1979; Akbarsha et al., 1996; Akbarsha and Averal, 1998, 1999).

The experiment also shows that androgens are essential for survival and motility of spermatozoa in the rat epididymis. Cauda region appears to be most favourable site for their survival (Jehan et al., 1973). Sperm motility is an important attribute of sperm quality as there is a good correlation between sperm motility on the one hand and plasma membrane integrity and conception rates on the other (Eliasson, 1971; Okada et al., 1990). The relative distribution of the different morphological types of spermatozoa present in a sample provides the most significant clue to discriminate between fertile and infertile samples (Gopalkrishnan et al., 1992). Morphological abnormalities can be categorized into those related to the head, mid piece or the tail; headless spermatozoa need to be put into a separate category. A high incidence (220%) of headless spermatozoa, usually referred to as “pin heads” is indicative of male infertility (Gopalkrishnan et al., 1989 b).

In the present study, the control animals, showed well defined structures of plasma membrane, acrosome, perforatorium, nucleus, nuclear membrane, basal plate, posterior ring and connecting piece in the anterior portion and median sections of base of the sperm heads. It is known that specialized structural features of the spermatozoon are reflection of its unique functional activities. Authors have discussed the unusual features of the sperm head of different species and suggested that inspite of morphological variations, the main
structures present in the sperm head of mammals are the nucleus, the acrosome, acrosomal cap and the membranous envelops (Harding et al., 1979; Breed, 1984).

Further, a well-defined mid-piece and different parts of principal piece of tail including outer dense fibres, axonemal component, central pair, mitochondrial sheath, fibrous sheath and preserved plasma membrane. The mitochondrial sheath and the outer ring of coarse fibres characterize the mammalian sperm mid-piece (Fawcett, 1975 b; Phillips, 1975 a). It is part of the flagellum, which lies between the neck and annulus and forms the most important site for various metabolic activities of the sperm. Its mitochondrial sheath is believed to be the source of energy for sperm motility. It has been speculated that outer dense fibres might be contractile because of their close association with the axoneme, their coincident appearance during phylogeny with internal fertilization and a concomitant increase in the size of the mitochondrial sheath (Fawcett, 1970). Biochemical studies and other investigations have suggested that the outer dense fibres play an active role in flagellar motion. However, stabilization of outer dense fibre proteins by abundant disulphide cross-linking may give them significant passive elastic properties that serve to stiffen or provide elastic recoil for the sperm tail (Fawcett, 1975 b) and also outer dense fibres provide added strength to protect sperm from damage by shear forces encountered during epididymal transit or ejaculation (Baltz et al., 1990). The function of the fibrous sheath may be to modulate the plane of the flagellar beat. The attachment of doublets 3 and 8 to the longitudinal columns might restrict their participation in microtubule sliding and axoneme binding during flagellar motion. The longitudinal columns themselves might also limit bending of the flagellum in the same plane would not be restricted by these features (Fawcett, 1975 b).
Acrosome contains several enzymes, which are secreted by Golgi apparatus and endoplasmic reticulum and the production of enzymes destined for the acrosome is regulated to some degree by testosterone (Morton, 1975). From histochemical evidence, the presence of carbohydrates or polysaccharides in the acrosome of head of the spermatozoa, which are associated with various enzymatic activities, is indicated (Wislocki, 1949; Leblond, 1950; Leuchtenberger and Schrader, 1950; Schrader and Leuchtenberger, 1951; Onuma and Nishikawa, 1963; Onuma, 1963).

Aladakatti and Nazeer Ahamed (1999) have reported that the morphological changes in the head of spermatozoa in general and the acrosome in particular may have resulted from the alteration in the epididymal milieu of rats treated with *Azadirachta indica* leaf powder and suggested that these changes are probably due to a general disturbance of carbohydrates or polysaccharides present in the acrosome of the sperm head.

The gossypol, isolated from cotton plant, affects the development of the sperm nucleus and acrosome in rats (Chinese team, 1978). Alcoholic extract of *Solanum xanthocarpum* seeds affects the sperm morphology by exhibiting decapitation, acrosomal damage and mid-piece anomalies (Rao, 1988). Crude chloroform extract of *Carica papaya* seed causes the sperm abnormalities such as, sickle shaped head becoming oval at the apical region, broken mid-piece membrane and accumulation of cytoplasmic material in post acrosomal region and mid-piece which results in complete inhibition of fertility in rats (Lohiya and Goyal, 1992). Aqueous seed extract of this plain in mice causes deflagellation, abnormalities in the head region and cytoplasmic droplet and suggested that extract might be causing an androgen deprived effect to target organs resulting in alteration in the internal milieu of cauda epididymis, especially (Chinoy et al., 1995).
Current studies have reported that the aqueous crude extract of *Echeveria gibbiflora* on guinea pig spermatozoa results in the formation of a huge bubble by distension of the plasma membrane and the disappearance of the external acrosomal membrane at the sperm head level (Delgado *et al.*, 1999). Benzene extract of *Ocimum sanctum* leaves in rats induces changes in morphology of the head of the sperm as well as the formation of cytoplasmic droplet in the mid portion of the tail region (Mukhtar Ahmed, 1999). The alcoholic seed extract of *Momordica charantia* causes morphological abnormalities in head of the spermatozoa along with the mid-piece anomalies as indicated by ultra studies in rats (Girmi, 2001).

In the present study, the defects in different parts of spermatozoan head region in *Ocimum sanctum* treated rats, probably have resulted either by interrupting respiratory mechanism or by damaging the spermatozoan plasma membrane or acrosomal membrane complexes. Similar observations have been reported by recent studies with various agents (Wilborn *et al.*, 1983; Tso and Lee, 1981; Aladakatti, 2001).

The defects in the mitochondrial sheath in the mid-piece of sperm is reported in the gossypol treated rats (Oko and Hrudka, 1982; Hoffer, 1983; Nair and Bhiwgade, 1990). Other male antifertility agents are known to produce specific structural changes in the sperm head and axial fibre bundle (Flores and Fawcett, 1972; Eljack and Hrudka, 1979; Lobl and Mathews, 1978; Akbarsha *et al.*, 1996).

In the present study of *Ocimum sanctum* treated rats, the mitochondria of the mid-piece appeared as empty membrane bound spaces without cristae or they are hypertrophied. The plasma membrane surrounding the fibres is also not seen. The fibrous sheath reveals discontinuity in structure and loss of uniformity. According to Fawcett (1975 b), the
mitochondria provide the necessary energy for the maintenance of the function of the outer fibres. In the present study, it may be interpreted that the mitochondrial degenerative changes probably play an important role in the process of necrosis of the outer fibres.

Studies reported that the spermatozoal defects in rat results in the constriction of the mitochondrial sheath, which was observed at the outer dense fibres of the axonemal complex on treatment with cryptoproterone acetate (Bhiwgade et al., 1990). Embelin from the seeds of *Embelia ribes* in rats causes decapitation of spermatozoa and discontinuity of the outer membranous sheath (Gupta et al., 1989). Vincristine, isolated from *Vinca rosea* reveals that the components of axoneme and the outer dense fibrils on one side of the principal piece were missing in flagella of several spermatozoa of rats (Akbarsha and Averal, 1996). Triptolide a diterpine triepoxide isolated from a Chinese medicinal plant, causes changes in the cauda epididymal sperm, which includes head-tail separation, chromatin decondensation of sperm nuclei, complete absence of the plasma membrane of the entire middle and principle pieces, disorganization of the mitochondrial sheath and aggregation of many sperm tails (Huynh et al., 2000). Similar observations as stated above are made in the present study. The spermatozoa of the cauda epididymis of rats treated with *Ocimum sanctum* reveal several abnormalities including abnormal pattern of the outer dense fibres and components of axoneme displaced on one side or both sides in several spermatozoa. It has been suggested that the missing segment of the mitochondrial sheath probably represents a weak point in the structural collar which supports the axial fibre-bundle during contractional wave leading to splitting of the axial bundle and subsequent dislocation of its fibres (Oko and Hrudka, 1982).

In the present study, the extra cytoplasmic material associated with the mid-piece of the spermatozoa was identified as cytoplasmic droplet. Very divergent views have been
expressed regarding the physiological significance of the cytoplasmic droplet. Roberts et al., (1976) have developed a method for the isolation and characterization of the cytoplasmic droplet in the rat, which is believed to be important in sperm maturation in the epididymis possibly through its role in inositol synthesis and metabolism (Eisenberg and Bolden, 1964). The suggestion that the cytoplasmic droplet plays a significant role in the physiology of epididymal spermatozoa is strongly supported by the studies of Van Rensburg et al., (1966), who have found that the live sperm from different parts of caput epididymis showed considerable activity by violent lashing of the tails in all spermatozoa with a cytoplasmic droplet. This activity ceases as soon as the droplet was discharged, suggesting that it is of great significance in sperm activity. Mann (1975) has suggested that lysosomal enzymes of the droplet may perhaps prepare the spermatozoan for the final stage of its maturation.

Several spermatozoa, while leaving the seminiferous tubules, carry the cytoplasmic droplet. It remains in the neck region while in the efferent ducts, near the annulus or at the point of junction between the mid-piece and principle piece while in the initial segment and caput epididymis, at the same level but displaced laterally while in the corpus and it is pinched off on or before arriving at the cauda epididymis. Once detached, the droplet quickly breaks up thereby, releasing its contents, which are endocytosed by the clear cells of the cauda epididymis to be digested by their secondary lysosomes (Hermo et al., 1988). Thus, most of the spermatozoa in the cauda epididymis must be devoid of the cytoplasmic droplet. Ejaculates containing a high proportion of spermatozoa with attached cytoplasmic droplets can be correlated with altered epididymal function and reduced fertility (Cummins, 1973; Bedford, 1976). In the present study, the presence of high proportion of spermatozoa with attached cytoplasmic droplets in Ocimum sanctum treated rats may be due to altered
epididymal function. Similar observations were made in recent studies of progestagen and androgen (Rao and Roy, 1993), Carica papaya (Chinoy et al., 1995), Vincristine (Akbarsha and Averal, 1996), Ocimum sanctum (Mukhtar Ahmed, 1999), Momordica charantia (Girni, 2001) and Azadirachta indica (Aladakatti, 2001) treated rats. Spermatozoa carrying the cytoplasmic droplet would be inhibited in motility and hence, may not fertilize the ova (Hermo et al., 1988).

Hence, in the present study, in the light of the pathological changes viz., disruption of plasma membrane, acrosome, perforatorium, connecting piece of the tails and disorganized or hypertrophied mitochondria along the disrupted mitochondrial sheath, disarrangement of outer dense fibres and discontinuation of fibrous sheath and retention of cytoplasmic droplet, caused by Ocimum sanctum leaves, it is suggested that Ocimum sanctum affects the spermatozoa in the epididymis due to disturbed internal epididymal milieu leading to reduced fertilizing ability of the sperm.