Flutamide Induces Xenoestrogenic Environment in Rat testes: Light and Electromicroscopic Evaluation

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Abstract: Objectives: To evaluate the toxic effects of prenatal flutamide exposure on testicular structure and ultrastructure of pubertal rats. Materials and Methods: 40 newly born male albino rats were divided into: Control group: included 10 offspring whose mothers were injected subcutaneously by vehicle only from the 10th gestational day until birth and Study group: included 20 offspring whose mothers were injected by flutamide in a dose of 5 mg /day dissolved in vehicle from the 10th gestational day until birth. When rats reached the adult age, they were sacrificed and both testicles were removed and prepared for light microscopic (LM) and electron microscopic (EM) examination. Results: LM examination of treated animals' specimens showed seminiferous tubules containing few numbers of germ cells, spermatogonia and spermatocytes with degenerated material in the cavity, but contains no sperms. Part of the seminiferous tubule contained edematous fluid in between the germ cells and the lumen of the tubule contains debris of degenerated cells. EM examination showed little number of degenerated primary spermatocytes and Sertoli cells with multiple vacuoles in their cytoplasm, but no sperms were detected in lumen. Higher magnifications showed degenerated Sertoli cells with multiple vacuoles in its cytoplasm and degenerated spermatogonia. In other parts of the seminiferous tubules, Sertoli cells appeared deeply infolded (lobulated nucleus) with vacuolated cytoplasm. Degenerated spermatogonia with rounded nuclei and patches of heterochromatin appeared resting on irregular basal lamina. Degenerated spermatocytes with shrunken nuclei were also seen. Basal lamina was distorted with collagen deposits. Sertoli cells cytoplasm contained scanty mitochondria with loss of its cristae. Conclusion: Prenatal exposure to flutamide has deleterious effect on adult testicular function as regards spermatogenesis with proved ultrastructural changes and pregnant females should be instructed to avoid exposure to flutamide either as occupational exposure or as therapeutic use.

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Keywords: Flutamide, prenatal exposure, ultrastructure changes

1. Introduction:

Androgen receptor plays a critical role in control of male sexual differentiation. During mammalian sex differentiation, the androgens; testosterone and dihydro-testosterone, produced by fetal/neonatal male during sexual differentiation are critical factors in the male phenol-type (**Gray *et al.*, 1998**). The differentiation of the Wolffian structures, e.g., the epididymis, vas deferens and seminal vesicles appear to be testosterone-mediated, while masculinization of the prostate and external genitalia are controlled by the more potent androgen; dihydrotestosterone. These developmental events may be influenced by endocrine disruptor-exposure via an androgen receptor-mediated mechanism in which endocrine disruptor acts either as a hormone agonist or antagonist (**Gray *et al.*, 2000**).

Environmental, anti-androgenic compounds have been recognized as endocrine disruptors because of their hormone-like activities. Anti-androgenic chemicals have the potential to interfere with male reproductive development and function in human and animals. The endocrine disruptors are thought to act via many mechanisms, such as by decreasing androgen synthesis, exerting effects on the pituitary-gonadal axis and/or blocking the androgen receptor. The consequences of these actions may cause abnormal hormonal regulation and gene expression (**Sharpe, 2006, Metzdorff *et al.*, 2007**).

Flutamide (4'-nitro-3'-trifluoro-methyl-isobutyranilide) is a potent non-steroidal androgen receptor antagonist (**Kassim *et al.*, 1997**). Flutamide acts by inhibiting the uptake and/or binding of dihydrotestosterone to the target cell receptor, thus interfering with androgen action. Flutamide is well absorbed orally and extensively and rapidly metabolized to its active metabolite, 2-hydroxyflutamide, and excreted almost entirely by the kidneys (**Xu and Li, 1998**).

In male patients who display androgen excess, flutamide is used therapeutically to treat androgen-dependent prostate cancer, where it prevents androgens from binding to androgen receptors in the prostatic gland and in prostatic cancer cells (**Javier *et al.*, 2001, Zhang *et al.*, 2005**). It is also used in young women who suffer increased androgen production, such as hirsutism, acne and seborrhea (**Thiboutot & Chen, 2003, Sahin & Kelestimur, 2004**).

The present study was designed to evaluate the toxic effects imposed on testicular structure and ultrastructure of pubertal rats by prenatal flutamide exposure.

2. Materials and Methods

The present study comprised 60 albino rats (20 males & 40 females) with weight range of 250-300 grams. Rats were purchased from the laboratories of Ministry of Agriculture, and kept under standard conditions, temperature 20°C, humidity 60% and 12-hs day/night cycle, and maintained on standard diet and free water supply till the end of study.

Males and females rats were kept in separate animal cages, under the prevailing atmospheric conditions and maintained on a balanced diet (bread, barely, carrots, lettuce, milk) and fresh-water supply. Each female was placed over night with one or two males and vaginal smear was examined, next morning, for presence of sperms and that day was considered the first day of gestation.

The pregnant female rats were divided into two groups: Control group: included 10 pregnant female rats and were injected subcutaneously by the propylene glycol vehicle only (10 mg/day) from the 10th gestational day until birth. Study group: included 30 pregnant female rats and were injected subcutaneously by flutamide dissolved in propylene glycol vehicle from the 10th gestational day until birth. Flutamide was given in a dose of 5 mg /day which was documented by **Normadiah *et al.* (1997)** to not interfere with testicular descent.

The offspring rats were left to grow under the same environmental conditions until they reach the standard adult weight and age when they reach the puberty (72 days after pears. Males were collected and isolated, and when reached the adult age (16-20 weeks), they were sacrificed after halothan inhalation and killed by decapitation. Both testicles were removed and standard paraffin sections were prepared and stained with haematoxylin and eosin (**Harris, 1980**) for light microscopic examination.

For electron microscopic analysis, two control and 4 flutamide-treated rats were perfused with 5% glutaraldehyde in 0.1 M cacodylate buffer. A systematic uniform random sampling scheme (**Wreford *et al.*, 1995**) was used to select three wedges of perfusion-fixed testis, which were postfixed in osmium tetroxide/ potassium ferrocyanide, block stained in uranyl acetate, and embedded in Epon araldite (**McLachlan *et al.*, 1994**). One-micrometer sections were prepared from each wedge and stained with toluidine blue for examination of tubule stages. An area containing approximately three or four round tubule cross sections with tubules in stages VII and/or VIII was selected for further examination by electron microscopy. Ultrathin sections were then prepared and stained using standard techniques.

3. Results

Light microscopic examination of specimens obtained from control animals showed normal highly convoluted seminiferous tubules with normal epithelium and interstitial spaces. The seminiferous tubules were lined by stratified germinal epithelium and Sertoli cells. The germinal epithelium is arranged in rows and consisted of spermatogonia, primary spermatocytes, spermatids and spermatozoa. Large number of sperms with the head embedded in the cytoplasm of the Sertoli cells and the tails directed to the lumen of the tubules, (Figure 1). The interstitial tissue between the seminiferous tubules containing the Leydig cells appeared in the form of clusters of polygonal cells throughout the interstitium, (Figure 2).

Electron microscopic examination of control specimens showed pale type A spermatogonium which appeared ovoid in shape, resting on the basal lamina with large ovoid nucleus having finely granular chromatin, one or two peripheral nucleoli attached to the nuclear membrane and some mitochondria in its cytoplasm, (Figure 3). Part of the seminiferous tubules contains dark type A spermatogonia with spherical nuclei containing patches of heterochromatin arranged near the nuclear membrane, lying on the basal lamina. Myloid cell with spindle-shaped nucleus was seen, (Figure 4). Part of the seminiferous tubule appeared containing primary spermatocyte with signet ring mitochondria. Sertoli cells appeared containing numerous mitochondria, vacuoles and head of sperms in its cytoplasm. The basal lamina is also seen, (Figure 5). Another part of seminiferous tubule contained primary spermatocytes with centrally located rounded nucleus and rounded spermatid with acrosomal vesicles and Golgi complex near the vesicle. Tails of the sperms were seen in the lumen and the heads in the wall of the tubule. Higher magnification showed spermatids with acrosomal cap on the nucleus and cytoplasm contains two centrioles, mitochondria and microtubules (Figs. 6 & 7). Higher magnification of part of seminiferous tubule showed elongated sperm with elongated nucleus, acrosomal cap, centrioles, flagellum and mitochondrial sheath (Fig. 8).

Light microscopic examination of specimens taken from treated animals showed seminiferous tubules containing few numbers of germ cells spermatogonia and spermatocytes. The cavity contains degenerated material with no sperms and dark basement membrane and interstitial tissue, (Fig. 9). Seminiferous tubules appeared containing pale

type A and dark type A spermatogonia and degenerated primary spermatocytes (Fig.10). Part of the seminiferous tubule contained Sertoli cells, spermatogonia, degenerated spermatocytes and edematous fluid in between the germ cells and the lumen of the tubule contains debris of degenerated cells (Fig. 11).

Electron microscopic examination of specimens obtained from treated animals showed pale type A spermatogonium with elongated nucleus and peripheral nucleolus. Little number of degenerated primary spermatocytes and Sertoli cells with multiple vacuoles in their cytoplasm were detected. Myloid cell with elongated nucleus was seen. No sperms in the lumen were detected (Fig. 12). Higher magnifications showed degenerated Sertoli cells with multiple cytoplasmic vacuoles, degenerated spermatogonium, Myloid cells with elongated nuclei and Leydig cells with pyramidal nuclei, (Fig. 13). Degenerated Sertoli cells were seen on the basal lamina with swollen nucleus and large cytoplasmic vacuoles and few mitochondria were seen (Fig.14). In other parts of the seminiferous tubules Sertoli cells appeared deeply infolded (lobulated nucleus) with their cytoplasm contains multiple vacuoles. Degenerated spermatocytes with shrunken nuclei were also seen (Fig. 15). Degenerated spermatogonia with rounded nuclei and patches of heterochromatin appeared resting on irregular basal lamina (Fig.16). In other parts of seminiferous tubules spermatogonia appeared with elongated nuclei, peripheral nucleolus, resting on distorted basal lamina with collagen deposits. Sertoli cells cytoplasm contained scanty mitochondria with loss of its cristae. Thick basal lamina with high collagen fiber was also seen (Figs. 17 & 18). Parts of interstitial tissue contained Leydig cells with elongated nuclei and one pyramidal nucleolus and its cytoplasm contains multiple vacuoles (Fig.19).

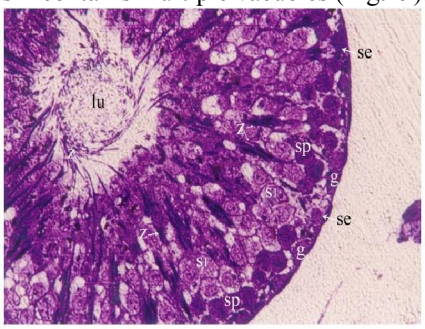

Figure (1): Cross section of the control adult rat testis showing: the semineferous tubule containing all types of germ cells; Spermatogonia (g), spermatocytes (sp), rounded spermatids (s1), large number of sperms (z) with the head embedded in the cytoplasm of the Sertoli cells (se) and the tails directed to the lumen of the tubule (lu), (Hx & E x400).

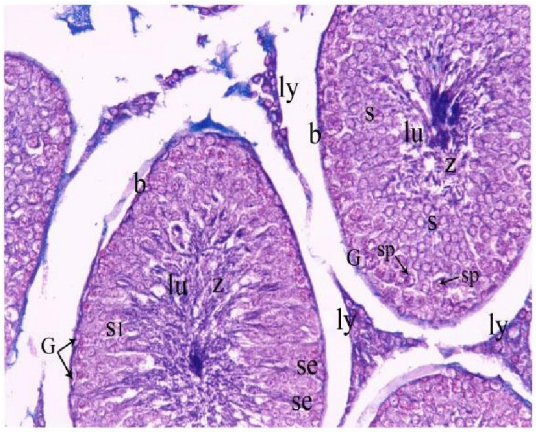

Figure (2): Cross section of control adult rat testis showing: the semineferous tubules containing marked number of spermatocytes (sp), spermatogonia (G), Sertoli cells (se) on the basement membrane (b), spermatids (s1) and sperms (z) in the lumen (lu). Interstitial tissues between tubules contain cluster of Lydeig cells, (Hx & E x200).

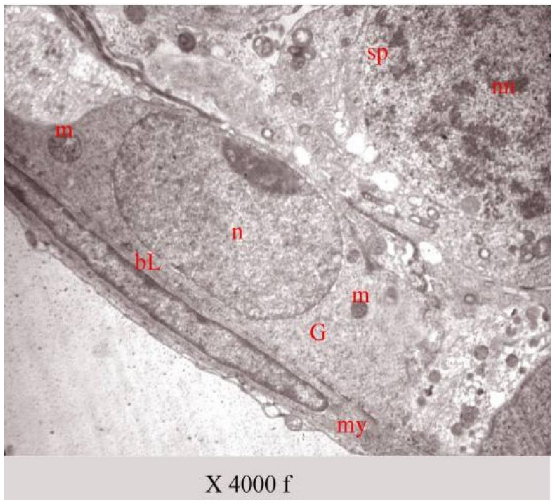

Figure (3): Electron micrograph of adult control rat testis showing: Part of the seminiferous tubule containing pale type A Spermatogonia (G), rested on the basal lamina (bL) with elongated nucleus (n), peripheral nucleolus and its cytoplasm contains numerous mitochondria (m).The myoid cell (my) with spindle shape nucleus is also seen. Notice primary spermatocyte (sp) with rounded nucleus (nn). (x4000).

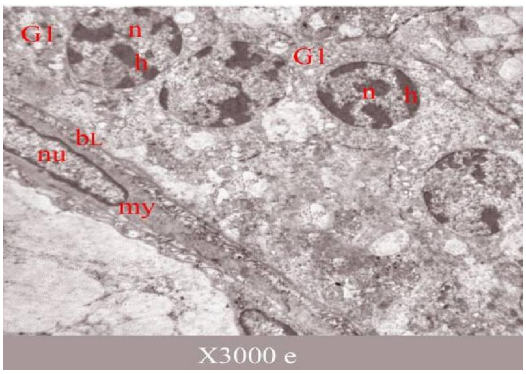

Figure (4): Electron micrograph of an adult control rat testis showing: Part of the seminiferous tubule contains dark type- A spermatogonia (G1) with spherical nuclei (n) containing patches of heterochromatin arranged near the nuclear membrane (h), lying on the basal lamina (bL). Myoid cell (my) with its spindle shape nucleus (nu) is also seen. (x3000).

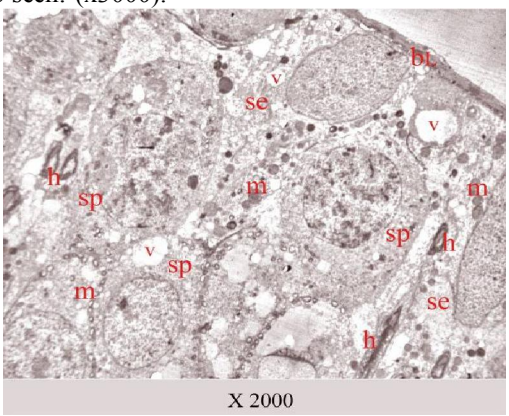

Figure (5): Electron micrograph of an adult control rat testis showing: Part of the seminiferous tubule containing primary spermatocyte (sp) with signet ring mitochondria (m), Sertoli cell (se) containing numerous mitochondria (m), vacuoles (v) and head of sperms in its cytoplasm (h) The basal lamina (bL) is also seen. (x2000).

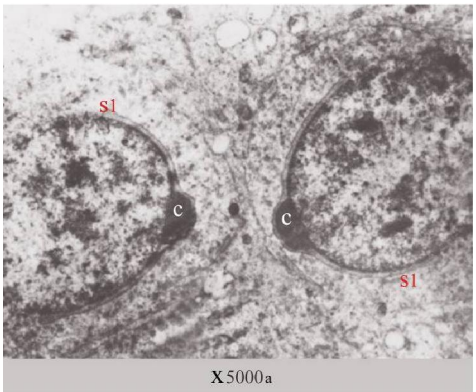

Figure (6): Electron micrograph of an adult control rat testis showing : Part of the seminiferous tubule which contains two spermatids (si) with acrosomal cap (c). (x5000).

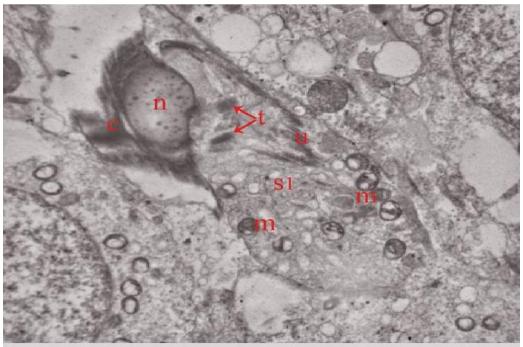

Figure (7): Electron micrograph of an adult control rat testis showing: Part of the seminiferous tubule containing spermatids (si) with acrosomal cap (c) on the nucleus (n). The cytoplasm contains two centrioles (t), mitochondria (m) and microtubules (u). (x6000).

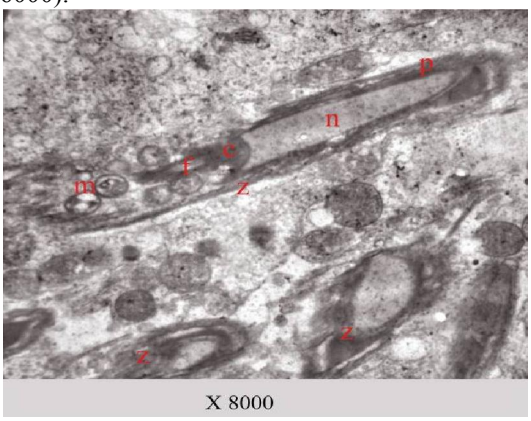

Figure (8): Electron micrograph of an adult control rat testis showing : part of the seminoferous tubule containing elongated sperm (z) with elongated nucleus (n), acrosomal cap (p), centrioles (c). flagulum (f) and mitochondrial sheath (m). (x8000).

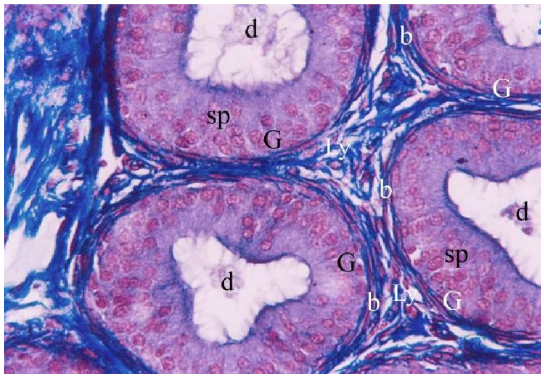

Figure (9): Cross section of a flutamide treated rat showing: The seminiferous tubules containing few number of germ cells spermatogonia (G) and spermatocytes (sp). Also note the cavity contains degenerated material (d), with no sperm and thick basement membrane (b) and interstitial tissue (ly).

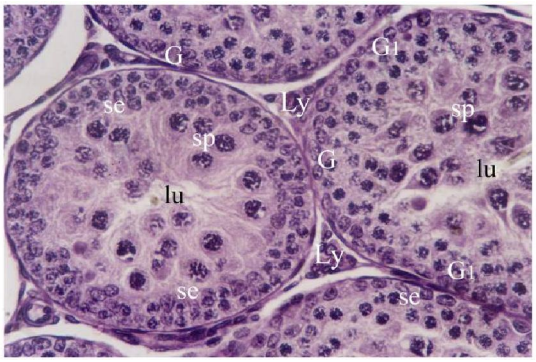

Figure (10): Cross section of a flutamide treated rat showing: The seminiferous tubules containing Sertoli cells (se), pale type-A spermatogonia (G) and dark type-A (Gi) and degenerating primary spermatocytes (sp). Also note the interstitial cells (ly) between the tubules and absences of sperms in the lumen of the tubules (lu).

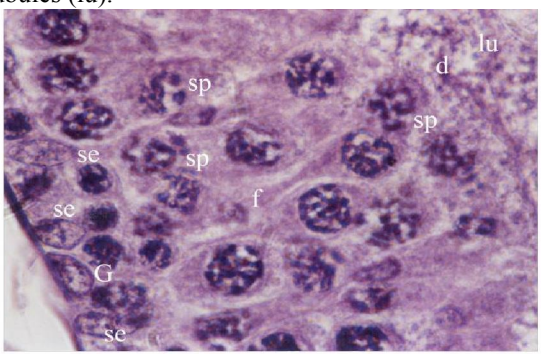

Figure (11): Cross section of a flutamide treated rat showing: Part of the seminiferous tubule containing Sertoli cells (se), spermatogonia (G) degenerating spermatocytes(sp) and edematous fluid (f) in between the germ cells. The lumen (lu) of the tubule contains debris of degenerated cells (d).

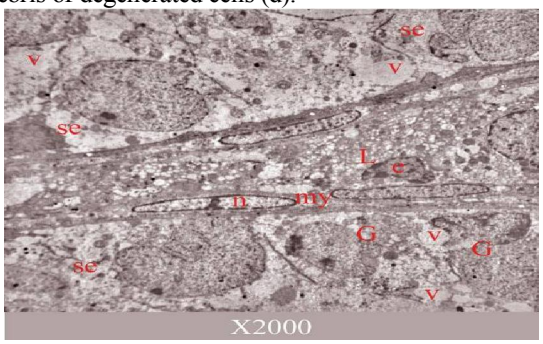

Figure (12): Cross section of a flutamide treated rat showing: Part of the seminiferous tubule contains type A spermatogonia (G) with elongated nucleus (n) and peripheral nucleolus (u). Little number of degenerated primary spermatocyte (sp) and Sertoli cells (se) with multiple vacuoles (V) in their cytoplasm. Also note myoid cell (my) with elongated nucleus (n) and absence of sperm in the lumen (lu) of the tubule.

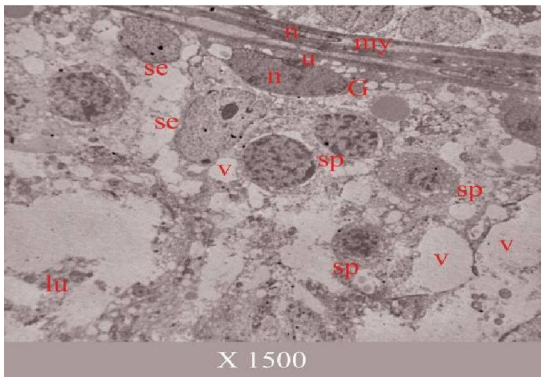

Figure (13): Cross section of a flutamide treated rat showing: Degenerating Sertoli cells (se), with multiple vacuoles (v). in its cytoplasm, degenerating spermatogonium (G). The myoid cell (my) with elongated nucleus (n) and Leydig ceil (L) with pyramidal nucleus (e) are also seen.

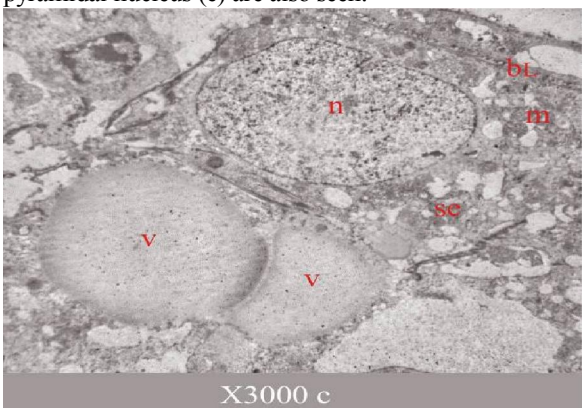

Figure (14): Cross section of a flutamide treated rat showing: Degenerating Sertoli cell (se) on the basal lamina (bL) with swollen nucleus (n), its cytoplasm contains large vacuoles (v) and few number of mitochondria (m).

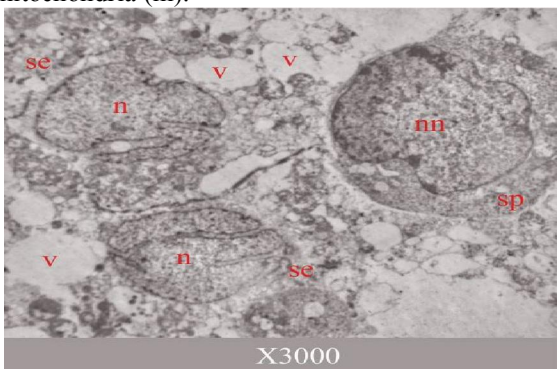

Figure (15): Cross section of a flutamide treated rat showing: The Sertoli cells (se) with deeply infolded (lobulated nucleus) (n). Their cytoplasm contains multiple vacuoles (v). Degenerated spermatocytes (sp) with shrunken nucleus (nn) are also seen.

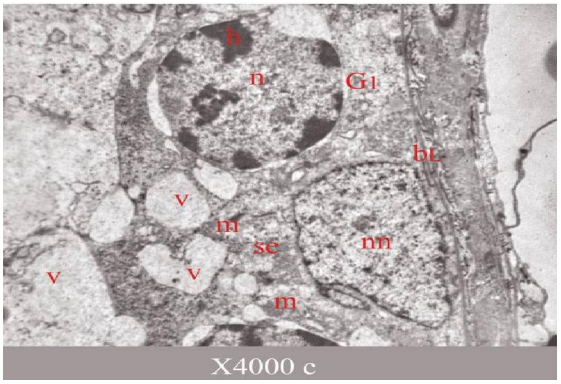

Figure (16): Cross section of a flutamide treated rat showing: Part of the seminiferous tubule contains Sertoli cell (Se) with spherical nucleus (nn). multiple vacuoles in its cytoplasm and few mitochondria (m), also shows degenerated spermatogonium (G1) with round nucleus (n) and patches of heterochromatin (h). The two cells resting on irregular basal lamina (bL).

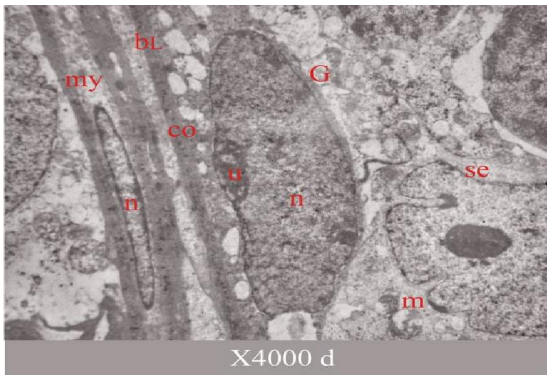

Figure (17): Cross section of a flutamide treated rat showing: Part of the seminiferous tubule containing spermatogonium (G) with elongated nucleus (n) and peripheral nucleolus (u). on a thick and distorted basal lamina (bL) with collagen deposit (co). Also note the Sertoli cell (se)with its cytoplasm containing mitochondria (m) with loss of its cristae and myoid cell (my), with spindle shape nucleus (n)

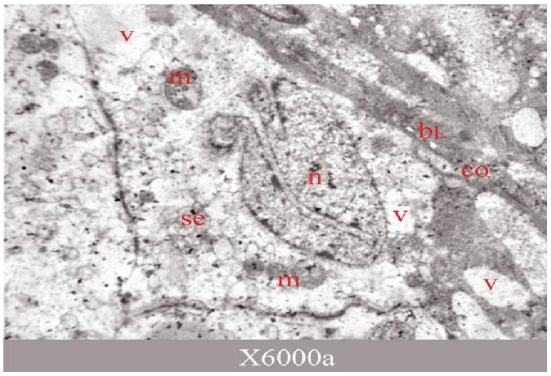

Figure (18): Cross section of a flutamide treated rat showing: The Sertoli cell (se) with its nucleus (n) is highly lobulated and its cytoplasm contain scanty mitochonderia (in) with loss of its cristae and multiple vacuoles (v). Also note thick basal lamina (bL) with high collagen fiber (co). (X 6000).

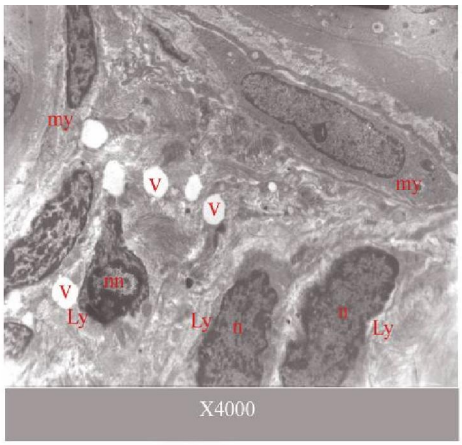

Figure (19): Cross section of a flutamide treated rat showing: Part of the interstitial tissue contains Leydig cells (ly) with elongated nucleus (n) and one pyramidal nucleus (nn) in other cell. Its cytoplasm contains multiple vacuoles(v). Notice myoid cells (my) in the wall of the tubule.

4. Discussion

Spermatogenesis is thought to critically depend on the high intratesticular testosterone levels induced by gonadotropic hormones. Postmeiotic spermiogenesis was blocked by the antiandrogen flutamide, indicating a crucial role of the residual low testicular testosterone level in this process. Extrapolated to humans, it may indicate that abolition of testicular androgen action will result in consistent azoospermia (**Zhang *et al.*, 2003**).

Lee *et al.* (1993) reviewed the coincidence of spontaneous testicular atrophy and its morphological changes in male rats exposed to toxins and reported an incidence of testicular degeneration of 2.5% in rats used for oral toxicity studies and 9.4% in rats used for inhalation studies. This signified that male reproductive organs are vulnerable to toxicity even if not the target organ of the study. This picture is highly amplified with the use of drugs or exposure to xenobiotics with antiandrogen activity (**Kubota *et al.*, 2003**).

Specimens of treated animals showed seminiferous tubules containing few numbers of germ cells, spermatogonia and spermatocytes with degenerated material in the cavity contains, but no sperms were detected. Electron microscopic examination showed little number of degenerated primary spermatocytes and Sertoli cells with multiple vacuoles in their cytoplasm, and no sperms were detected in the lumen. Higher magnifications showed degenerated Sertoli cells with multiple vacuoles in its cytoplasm and degenerated spermatogonia. In other parts of the seminiferous tubules Sertoli cells

appeared deeply infolded (lobulated nucleus) with their cytoplasm contains multiple vacuoles. Degenerated spermatocytes with shrunken nuclei were also seen. Basal lamina was distorted with collagen deposits. Sertoli cells cytoplasm contained scanty mitochondria with loss of its cristae.

These results go in hand with **Spano *et al.* (1996)** who reported eosinophilic globular bodies, sequestered necrotic spermatids, and the germ cell degeneration was associated with cytoplasmic vacuolation of Sertoli cells and markedly decreased maturing spermatids and with **Kinnberg & Toft (2003)** who evaluated the testicular structure effects of flutamide on sexually mature male guppies exposed and reported reduced number of spermatogenetic cysts and an increased number of spermatozeugmata in the ducts and concluded that effects are indicative of a blocked spermatogonial mitosis. Also, **Anahara *et al.* (2004)** found acrosomes and/or nuclei of the spermatids of flutamide treated animals were deformed.

Various mechanisms could explain these destructive effects of prenatal exposure to flutamide on testicular structure. Firstly; flutamide blocks the physiological action of testosterone at androgenic receptor sites and/or alters androgen receptor levels (**Kelce *et al.*, 1997**) and consequently, causes atrophic changes to the seminiferous tubules by depressing the function of Sertoli cells. Furthermore, **Ohsako *et al.* (2003)** reported that subacute flutamide administration for 6 days first affected hypothalamus/pituitary hormone gene expression resulting in altered testicular steroidogenesis and decreased sperm production.

Secondly; flutamide may indirectly induced gonadal impairment and consequently impairs spermatogenesis by alteration of the ectoplasmic specialization, a testosterone-dependent specialized Sertoli cell structure with which step 8 round spermatids is normally associated. Such attribution goes in hand with **Cheng & Mruk (2002)** who reported that the testosterone-dependent loss of step 8 round spermatids may be due to the absence of the ectoplasmic specialization within the Sertoli cell and with **Gye & Ohsako (2003)** who reported that flutamide differentially affects the transcription of tight junction genes, in Sertoli cells, building the ectoplasmic specialization. Also, **Anahara *et al.* (2004)** found the ectoplasmic specialization between the Sertoli cell and spermatids was partially or completely deleted and the stages of the seminiferous cycle were also disarranged after neonatal treatment with flutamide. Thereafter, **Anahara *et al.* (2006a, 2006b, 2008)** reported that flutamide induced actin depolymerization and tyrosine phosphorylation of cortactin at the ectoplasmic specialization in the testis

Thirdly, the detected degenerated Sertoli cells with swollen nucleus and large cytoplasmic vacuoles and few mitochondria in some parts of the seminiferous tubules and in other parts, Sertoli cells appeared deeply infolded, with lobulated nucleus and vacuolated cytoplasm. In addition, degenerated spermatocytes with shrunken nuclei, degenerated spermatogonia with rounded nuclei and patches of heterochromatin appeared resting on irregular basal lamina; these findings give a picture suggestive of apoptosis. This assumption coincided with the work of **Omezzine *et al.* (2003)** that indicated that in utero exposure to the antiandrogen induced, in the rat testes, a chronic apoptotic germ cell death associated with a long-term increase in the expression and activation in germ cells of caspases-3 and -6, two key components in the death machinery. Also, **El-Chami *et al.* (2005)** reported the androgen dependency of Cbl, a proto-oncoprotein known to control several signaling processes and is highly expressed in the testis, as it localizes in pachytene spermatocytes during androgen-dependent stages, is down-regulated upon flutamide exposure, and is up-regulated with testosterone in hypophysectomized rats. **Benbrahim-Tallaa *et al.* (2008)** shown that Sertoli cells originating from 15-day-old rats treated in utero with the anti-androgen flutamide in a dose of 10 mg/kg/d did no longer protect adult germ cells against apoptosis and attributed this to alteration of Sertoli cell functions manifested as decreased levels of several genes mainly expressed in adult Sertoli cells

It could be concluded that prenatal exposure to antiandrogenic drugs as flutamide has a deleterious effect on adult testicular function as regards spermatogenesis with proved ultrastructural changes and pregnant females showed be instructed to avoid exposure to flutamide either as occupational exposure or as therapeutic use.

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