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Original Article

Ultrastructural Features of Eutopic Endometrium in a Rat Model of Endometriosis

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ABSTRACT

Endometriosis is a chronic recurrent disease that is relatively common. Diagnosis is difficult and often delayed. Current treatments are inadequate with unacceptable side effects and multiple surgeries may be needed. Abnormal eutopic endometrium may play important role in endometriosis-associated infertility. This study aimed to examine the ultrastructural changes in eutopic endometrium in a rat model of surgically induced endometriosis. Endometrial tissue was removed from rats in surgical endometriosis induction group (n = 10), sham operated (n = 10) and non-operated control (n = 10) groups in the diestrus phase of the estrus cycle. They were studied with light, transmission and scanning electron microscope as well as morphometric analysis. Eutopic endometrium in surgically induced endometriosis showed pseudostratified epithelium, vacuolated columnar cells alternated with dark cells. The stroma was edematous exhibiting dilated, congested blood vessels. The mean endometrial mucosal depth and surface epithelial height significantly increased. Ultrastructurally, most luminal epithelial cells showed vacuolation. Mucous secretory granules were surrounded by dilated rough endoplasmic reticulum cisternae. Mitochondria, glycogen deposits and vesicles with electron dense cores were observed. The nuclei were highly euchromatic. Well defined microvilli were noticed with evident apical tight junctions. Scanning electron microscope revealed flattened and structurless surface epithelium with apparent decrease in the number of pinopodes. A different response to sex hormones in different parts of eutopic endometrium was observed. Ultrastructural features of estrogen dominance or progesterone resistance in the eutopic endometrium might account for inappropriate cyclic changes occurring in the disease.

1. Introduction

Endometriosis is defined as the presence of, estrogen-sensitive, endometrial glands and stroma outside the uterus that is associated with inflammatory response [1]. It is a chronic disorder characterized by pelvic pain and subfertility [2]. Endometriosis appears to be one of the most common benign gynecological proliferations in premenopausal women [3].

No permanent cure for endometriosis has been found. Symptom relief is the primary goal of existing treatment options, which may be pharmacological or surgical [4]. Complicated network of etio-pathologic processes have

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been targeted with the aim of interrupting the mechanisms by which endometriosis develops [5]. However, none of these processes has prevailed [6]. The reason maybe because as one mechanism is interrupted the other networking pathways take over and the disease survives. Conceivably, a more orchestrating pathway needs to be knocked down for the whole process to be arrested [7]. Experimental animal models play an important role in understanding the nature of the disease [8]. The existing controversies concerning the pathophysiology of endometriosis and its influence on pregnancy and vice versa demonstrate the need for research in laboratory animals [9]. Recent studies was done on the structural and biochemical changes [10], endometrial nerve fiber [11], endometrial angiogenesis [12] and endometrial receptivity in endometriosis [13]. Previous studies made morphological and functional comparison between the ectopic and eutopic endometrium in pregnant rats [8]. They found that decidualisation and pinopode expression of eutopic endometrium in surgically induced endometriosis relatively decreased and adequate endometrial morphology would not always display normal endometrial receptivity.

The purpose of the present study is to investigate the ultrastructural changes in eutopic endometrium of non pregnant rats.

2. Materials & Methods

2.1. Animals and Experimental design

Adult female Wistar rats, purchased from Animal House, Assiut University, weighted 200-250 g were maintained under 12:12 lighting conditions. Animals were housed in stainless steel cages and had free access to food and tap water *ad libitum* for the duration of the study. Experimental study groups consisted of surgical endometriosis induction group (n: 10), sham operated (n: 10) and non-operated control (n: 10) groups of 6-8 months old rats.

2.2. Induction of experimental endometriosis

Surgery is performed aseptically to autotransplant small pieces of uterine horn to the peritoneum of the pelvic cavity and the mesentery of the small intestine, as in published studies [14,15]. Rats were anaesthetized with 300 mg/kg chloral hydrate [16]. Skin on the ventral aspect was cleaned with 70% alcohol. Midline laparotomy was performed on the lower abdomen using a clean surgical technique and the left uterine horn was removed. The excised horn was immersed in a sterile small petri dish containing Ham's F-12 medium with 100 U/ml penicillin and 100 Pg/ml streptomycin, warmed to 37 °C. Then the horn was opened longitudinally and cut into 4 smaller, roughly equal-sized fragments after peeling away the outer layer of myometrium. Two uterine fragments will be sutured onto the peritoneum with a 4-0 vicryl suture, and the remaining 2 are sutured onto the arterial cascades of the small intestine. The mesentery of the small intestine is spread out on wet gauze such that blood vessels are clearly visualized, and each piece of uterus was sutured to a vessel using 4-0 vicryl suture. The second group which represents the sham operated animals had adipose tissue autografts sutured onto their peritoneal surfaces. The peritoneal cavity is lavaged with 1 to 2 ml of the antibiotic-supplemented Ham's F-12 medium at the end of surgery. Then the midline incision is closed with a 4-0 braided silk suture. Animals were allowed to recover completely before returning them to their cages. Thereafter, they were maintained with food and water, and observed daily. The incision line was inspected daily for signs of dehiscence and bleeding. After surgery,all rats were administrated with penicillin of 40 000 U/day IM for 2 days to prevent infection [16]. A month later, the animals were sacrificed in the distrous phase of the cycle by cervical dislocation. The endometriotic implants and the right uterine horns were excised. The endometriotic implants were fixed in 10% formol saline, dehydrated, cleared, and embedded in paraffin. Sections for light microscopy were cut (5-μm-thick) and stained with H&E [17]. The right uterine horns were cut into two halves;

For transmission electron microscopic examination(TEM): Half of the specimens were cut into small pieces,fixed in 2-3% glutaraldehyde for 2 hours then post fixed in osmium tetra oxide. Semithin sections (0.5–1 μm) were cut, stained with Toluidine blue to be observed in the light microscope and to select specific areas to be used for thin-sectioning. Ultrathin sections (500–800A) were prepared from selected areas in semithin sections, mounted on copper grids, and contrasted with uranyl acetate and lead citrate [18]. They were subsequently examined and photographed using transmission electron microscope (JEOL100 CX Japan) at 80 kV at the Assiut University Electron Microscopic Unit.

For scanning electron microscopic examination (SEM): The other half of specimens were cut, open longitudinally to expose the uterine luminal epithelium. The uteri were rinsed gently with phosphate buffer saline (PBS) to remove surface debris and immersed in a fixative solution of 2% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer, pH 7.2, for two hours .A graded series of alcohols was used for dehydration and liquid carbon dioxide was used to dry the specimens. Dried specimens were mounted on aluminium stubs, fixed in place with colloidal silver and sputter coated with gold [19]. A Jeol (J.S.M-5400 LV; Japanese Electron Optic Laboratory) was used to view the specimens. Photographs were taken at 15 kV at Assiut University Electron Microscopic Unit.

Morphometrical and Statistical study: The endometrial mucosal thickness is estimated by measuring the distance between the tips of the epithelial cells to the muscle layer using an image analyzing system software (Leica Q 500 MCO, Gremany) in the Histology department, Faculty of Medicine, Assiut University. The mean height of the surface epithelium was also performed using 0.5 μm semithin sections stained with Toliudine blue at 40Xmagnification. For each section, the measured parametes are estimated as the mean value of six determinations. Data are reported as means + SD. Statistical analysis was done through a student's (t) test to compare the means between the different groups. The P value was calculated using SPSS program version 19 (SPSS INC., Chicago, Illionois, USA)

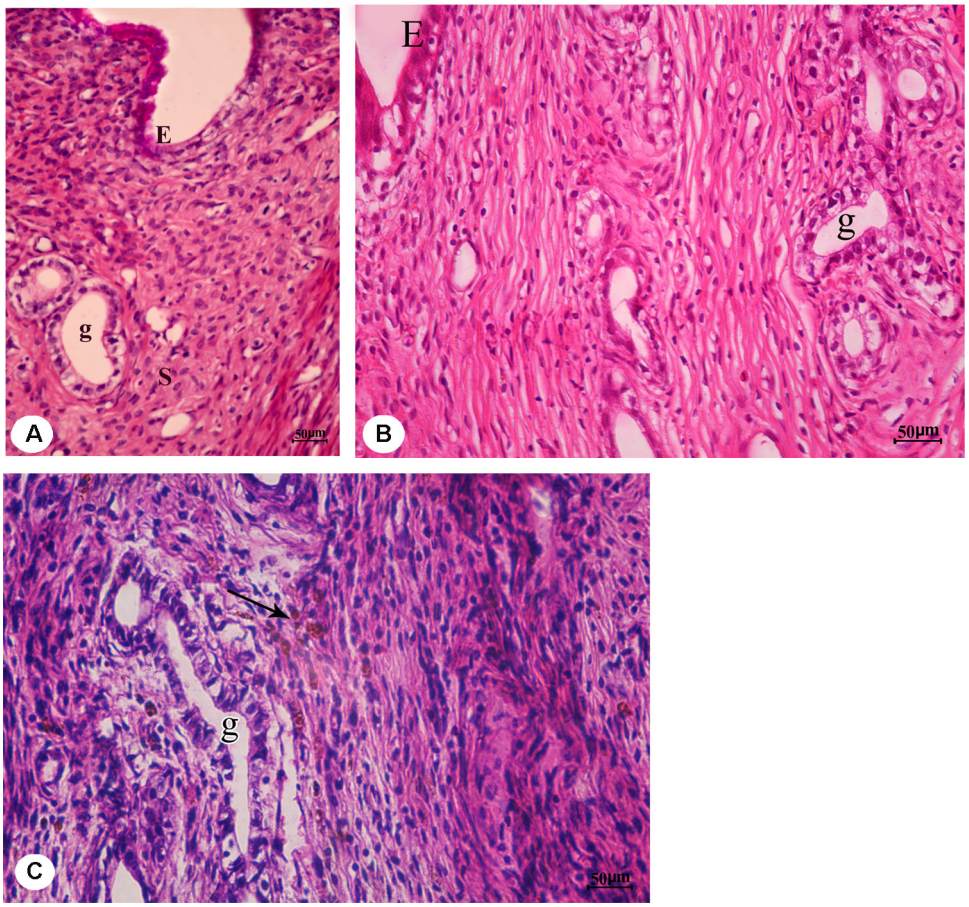

Fig. 1. Photomicrographs of the endometrium from control, sham operated and endometriosis groups showing;
(A) The epithelial lining (E), well-differentiated endometrial glands(g) surrounded by stromal cells(S) and collagen fibers (control group,H&E)
(B): The luminal epithelium (E), and the glandular epithelium (g) are characteristic of the secretory phase (Sham group,H&E)
(C): A photomicrograph of endometriotic lesions in experimental group showing**;** the presence of endometrial glands(g), stroma filled with hemosiderin-laden macrophages(arrow)**.**(endometriosis group,H&E).

3. Results

Five rats died and were excluded from the study (3 of the endometriosis induced group and two of the sham group). Rats from endometriosis induced group died from intestinal obstruction and the remaining two died just after surgical procedures.

3.1. Light microscopic evaluation

Examination of H&E-stained sections of the control and sham operated rats showed that the endometrium was formed of secretory epithelial cells with pale vacuolated cytoplasm. The endometrial glands were well differentiated and appeared circular or elongated. Their lining cells had pale cytoplasm (Fig. 1 A, 1B).Gland-like structures and endometrial-like stroma were found in endometriotic implants of the endometriosis group. Upon high powered microscopy, epithelial cells resembled well-grown low columns. Inflammatory cell infiltration and hemosiderin-laden macrophages were noted (Fig. 1C).

Toluidine blue (TB) stained semithin sections of control rats showed that the endometrium in the secretory phase composed of columnar epithelial cells, lining the uterine lumen and a highly cellular stroma. The surface epithelium showed apically vacuolated cytoplasm and pale oval nuclei. Some cells showed projections upon domed apical surfaces. The stroma contained uterine glands, blood vessels and endometrial stromal cells. Endometrial glands were seen full of secretions aligned with tall columnar cells and foamy supranuclear cytoplasm. Stromal cells were detected as large cells with vesicular nuclei, prominent nucleoli and pale cytoplasm (decidual like cells), flat cells with dark spindle-shaped nuclei and scanty cytoplasm (fibroblasts) and leukocytes with heterochromatic nuclei and deep stained cytoplasm. The numerous endometrial stromal cells are surrounded by a delicate network of collagenous fibers (Fig. 2A).

The rat endometriosis model was characterized by changes in the eutopic endometrium. Sections showed pseudostratified epithelium with certain areas exhibiting hyperplasia of epithelial cells. Some columnar cells showed severe vacuolation and upward migration of their nuclei

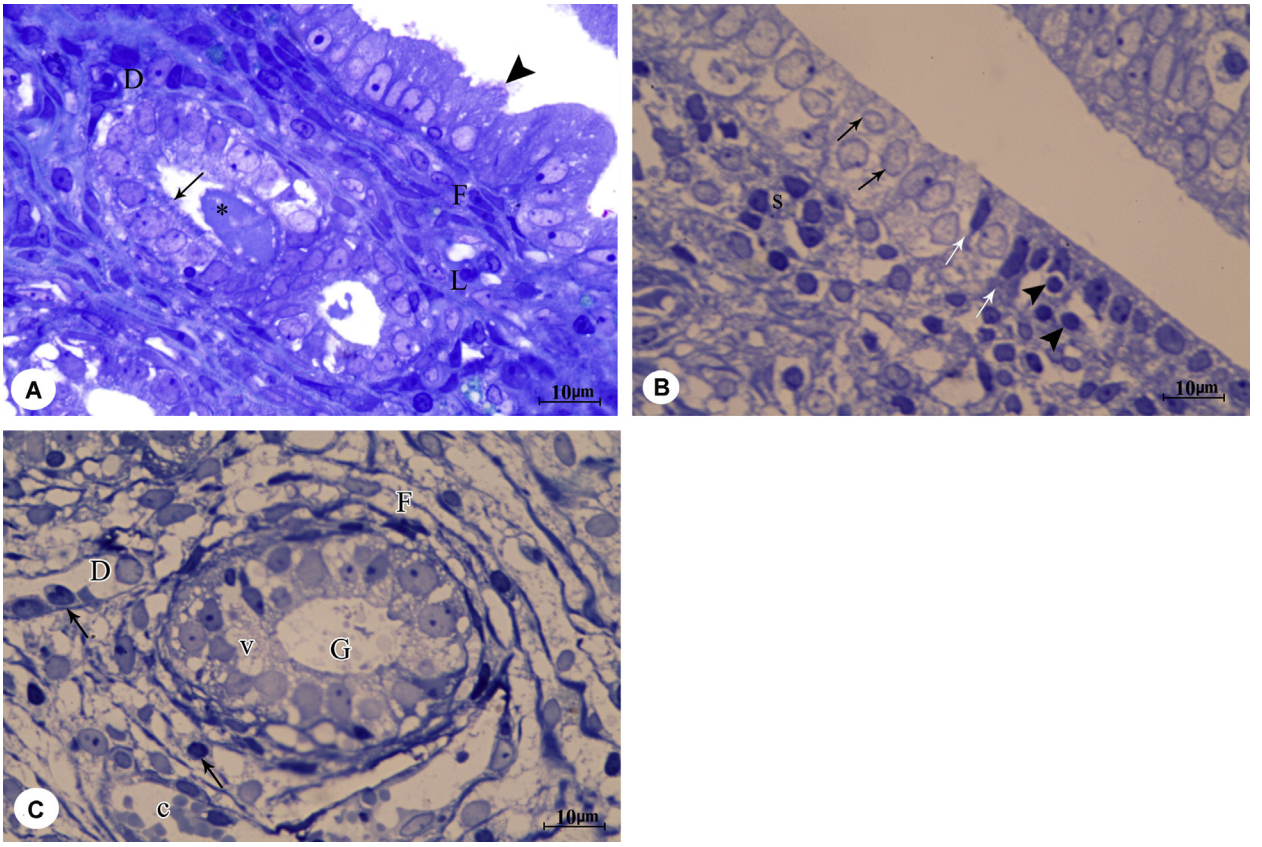

Fig. 2. Histological sections of rat endometrium from control group and endometriosis group showing;
A: Surface columnar epithelial cells with oval vesicular nuclei, cytoplasmic supranuclear vacuolation and apical plasma membrane protrusions (arrow head).The endometrial glands are lined with simple columnar secretory cells with pale nuclei, vacuolated cytoplasm and prominent brush border (↑).The glandular lumen is filled with secretion (*).The stroma shows decidual like cells with large pale nuclei (D), spindle shaped fibroblasts (F) and lymphocytes (L).
(Control group, T.B)
B: Eutopic Luminal epithelium shows pseudostratification, severe vacuolation and upward migration of their nuclei (↑).Pyramidal shaped dark cells (White arrow) and intraepithelial lymphocytes (▲). Some stromal cells show small darkly stained nuclei and vacuolated cytoplasm(S). (Endometriosis group, T.B)
C: Eutopic endometrial glands (G) lined with columnar epithelial cells with large pale nuclei and markedly vacuolated cytoplasm (v). The cellular stroma shows dilated, congested blood vessels (c), many decidual-like cells(D), spindle-shaped nuclei of fibroblasts (f), and leukocytes (↑)surrounding the gland. (Endometriosis group, T.B).

while other dark cells with elongated dark nuclei are interspersed between them. Intraepithelial lymphocytes were also observed .Stromal cells beneath the surface epithelium show small darkly stained nuclei and vacuolated cytoplasm (Fig. 2B).

Endometrial glands were lined with columnar epithelial cells with large pale nuclei and markedly vacuolated cytoplasm. The stroma is quite edematous exhibiting clear spaces between the stromal cells in addition to dilated, congested blood vessels (Fig. 2 C).

3.2. Electron microscopic evaluation

Examination of the ultrathin sections of control group revealed columnar epithelial cells that bore microvilli .Nuclei were generally regular in outline and euchromatic. Several rough endoplasmic reticulum cisternae were often in close association with mitochondria .Clumps of glycogen deposits and occasional secretory droplets were also evident. Lateral membranes were regular with few interdigitations and tight junctions were found at the apical border (Fig. 3A).

Transmission electron microscopy of the endometrium in experimental group revealed that most epithelial cells showed variable degrees of vacuolation. Their nuclei were highly euchromatic with active large nucleoli .Other dark cells with elongated dark nuclei are interspersed between columnar cells .Mucous secretory granules were seen fused near the cell apex surrounded by dilated rough endoplasmic reticulum cisternae (Fig. 3B).Numerous mitochondria, small glycogen deposits and vesicles with electron dense cores are observed .Well defined microvilli were noticed at the apical cell membrane with evident apical tight junctions only (Fig. 3 C).

The endometrial stroma of the control group showed large lymphocyte with dark heterochromatic nucleus, a spindle shaped fibroblast with euchromatic nucleus surrounded by collagen fibers and decidual like cells with

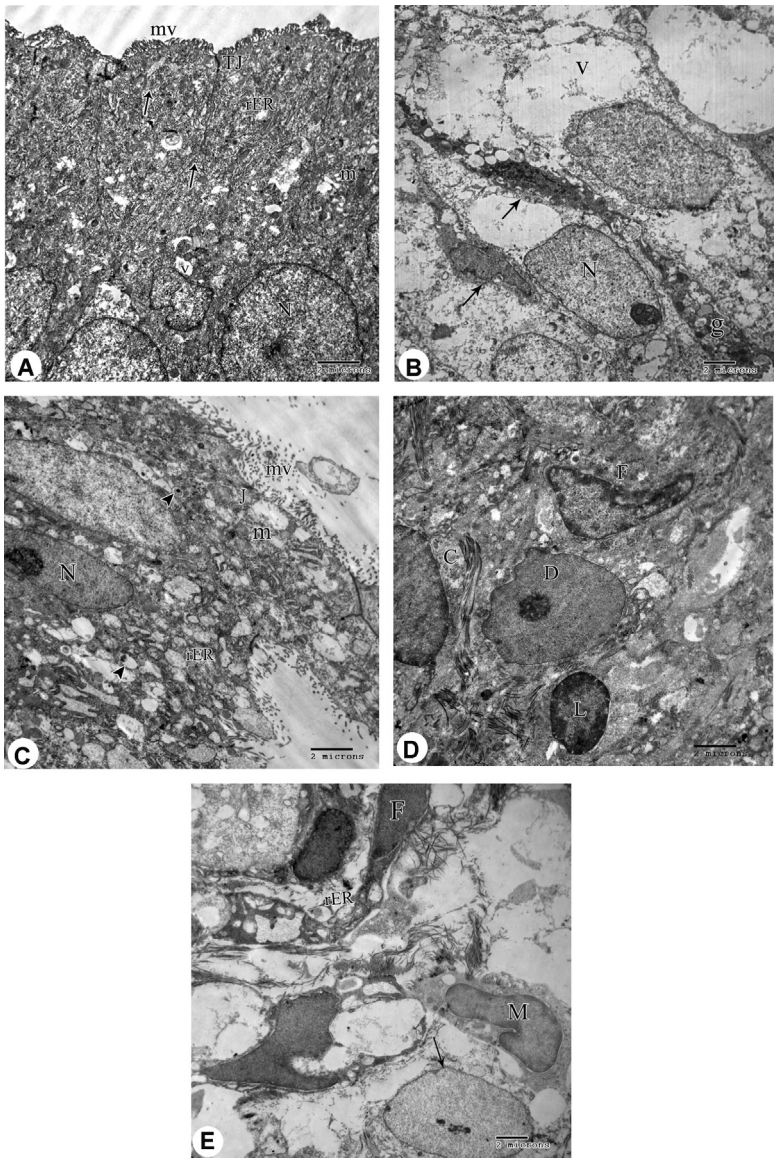

Fig. 3. Transmission electron micrographs of rat endometrium from control group and endometriosis group showing;
A: Luminal epithelial cells with tight junctions between adjacent cells (TJ).The apical cell membrane shows well defined microvilli(mv).The cytoplasm contain large euchromatic nuclei (N), numerous mitochondria (m), rough endoplasmic reticulum (rER), scattered deposits of glycogen granules (↑)and secretory vesicles (v). (Control group)
B: Eutopic columnar cells with severe vacuolation of the cytoplasm (v) and large pale nuclei (N).Other dark cells with elongated dark nuclei are interspersed between columnar cells (↑).Their apical cytoplasm shows fusing electron-lucent secretory granules (g). (Endometriosis group)
C: Eutopic columnar cells showing highly euchromatic nuclei (N) and prominent nucleoli. Apical Surfaces have well defined microvilli (mv).The cytoplasm contains dilated cisternae of rough endoplasmic reticulum (rER), plentiful mitochondria (m) and vesicles with electron dense cores (▲). Lateral membranes were regular with intact junctional complexes (J) (Endometriosis group)
D: Endometrial stroma rich in collagen fibers(C), fibroblasts with euchromatic nuclei (F), Decidual like cell (D) and lymphocyte (L) with heterochromatic nucleus (Control group)
E: Eutopic endometrial stroma contain collagen fibers separating fibroblasts (F) with dark heterochromatic nuclei and dilated cisternae of rough endoplasmic reticulum (rER) .Other stromal cell with large euchromatic nucleus is also shown (↑) .Notice macrophage (M)surrounded by empty spaces (Endometriosis group).

large euchromatic nuclei (Fig. 3D). The endometrial stroma beneath the eutopic epithelium revealed many decidual like cells with euchromatic nuclei and rarified cytoplasm. Macrophages, spindle-shaped fibroblasts with dark heterochromatic nuclei and scattered collagen fibers were also observed in wide intercellular spaces (Fig. 3E).

SEM examination revealed the surface morphology of the endometriurn where the epithelium is densely covered

Table 1
Mean Endometrial Mucosal thickness in different groups.

	Control	Endometriosis	Sham	P-value[1]	P-value[2]	P-value[3]
Mean ± SD	212.70 ± 59.94	507.21 ± 131.58	251.11 ± 44.63	0.000*	0.012*	0.000*
Range	117.0 - 360.0	299.0 - 817.0	183.0 - 320.0			

1: Control vs. Endometriosis
2: Control vs. Sham
3: Endometriosis vs. Sham

Table 2
Mean Epithelial height in different groups.

	Control	Endometriosis	Sham	P-value[1]	P-value[2]	P-value[3]
Mean ± SD	18.44 ± 3.36	34.46 ± 7.01	22.93 ± 4.67	0.000*	0.001*	0.000*
Range	12.3 - 25.6	19.1 - 47.6	15.0 - 30.0			

1: Control vs. Endometriosis
2: Control vs. Sham
3: Endometriosis vs. Sham

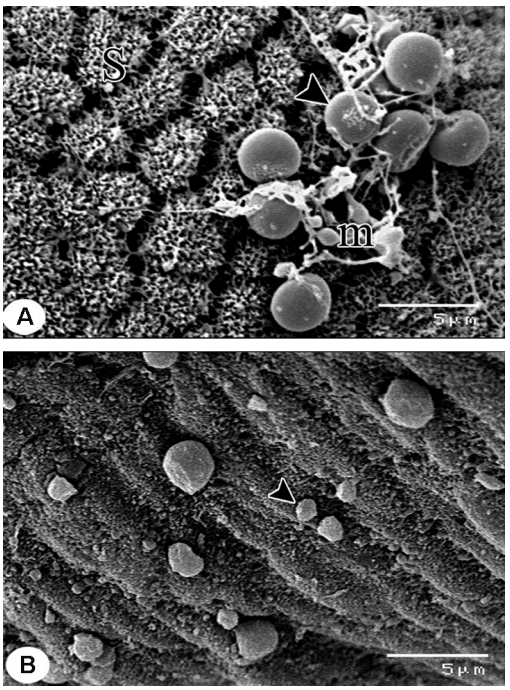

Fig. 4. Scanning electron micrographs of control and endometriosis group showing;.
A: Secretory epithelial cells (S) covered by dense microvilli and fully developed pinopodes (▲) with intervening mucous threads (m).
(Control group)
B: Eutopic endometrial surface appears flat and structurless with apparent decrease in the number of pinopodes (▲).
(Endometriosis group).

with microvilli and groups of mature pinopodes projecting into the uterine lumen (Fig. 4A).While in eutopic endometrium SEM revealed flattened and structurless surface epithelium with apparent decrease in the number of pinopodes (Fig. 4B).

3.3. Morphometric results

Statistical analysis of the mean endometrial mucosal thickness, surface epithelial height of control, endometriotic and sham operated rats were shown in Table 1 and Table 2. There were statistically significant differences found between groups.

4. Discussion

The intent of this study was to obtain an experimental model of endometriosis in rats which permits a morphological description of the eutopic endometrium in non pregnant uterus. There are potential limitations in this rat experimental model. During dissection there were pelvic adhesions which were reported previously [16].There are major differences between human and rat, because rodents do not menstruate and endometriosis does not occur spontaneously in these animals, although the rat model is still widely employed [20] .The rat has a bicornuate uterus, which was an important anatomical feature in this study, as it permitted one of the horns to be resected and transplanted, with the other one remaining intact [9]. Also the short cycle length makes the rat an ideal animal for the investigation of changes occurring during the cycle [21].

In the current work, experimental endometriosis group also revealed significant endometrial depth differences probably due to edematous stroma exhibiting clear spaces between the cells. Previous study [8] explained increased endometrial depth was due to mitotic proliferation.

In the present study, ultrathin sections of eutopic endometrium often showed discrepancies in the same group. Similar histological features in the uterine epithelium of estrogen injected animals were noticed where it showed pseudostratified epithelium [22].In many cases, there was clear evidence of asynchrony between the estimated day of the menstrual cycle and the observed histological/ultrastructural appearances. It was apparent in baboons where many of the affected animals showed a late secretory phenotype in the whole specimen or only part of it, indicating that there was heterogeneity within the endometrium suggesting that there is an aberrant maturation process [23]. Such discrepancies may be a result of the potential impact of one or more factors such as site of endometriosis, stage and severity,fertility history [24]. Schweppe et al [25] previously claimed that complete proliferative development was found only in some

endometriosis patients and full secretory transformation was absent in all specimens. The incomplete morphologic response to cyclic hormonal changes may explain the frequent failure of endocrine therapy.

Sex hormones have a crucial role in the physiopathology of endometriosis, and their effect on eutopic endometrium has been well-established [26].Two forms of estrogen are altered in women with endometriosis, which is significant because estrogen causes endometriosis tissues to grow. Estrone is a weak form of estrogen, and estradiol is a much more potent form of estrogen. Estrone is converted to estradiol and vice versa by two enzymes that work with the help of progesterone. In a normal endometrium, progesterone is able to increase the conversion of the strong estradiol to the weak estrone. Progesterone also suppresses oestrogen receptor. Both actions would lead to a decline in oestrogen actions thereby keeping the growth effects of estrogen on tissues under control [27]. In endometriosis, the enzyme which converts estradiol to the weaker form estrone is not at all found in the endometrial tissue. This causes levels of the stronger estradiol to accumulate. The enzyme which converts weaker estrone to stronger estradiol, however, is fully functional. This results in even higher amounts of more powerful forms of estrogen at the level of the endometrial lesions, causing more and more growth of this tissue outside of the uterus [28].

According to Bulun [29], this finding suggests that eutopic endometrium in these women is resistant to progesterone. This feature is referred as the development of progesterone resistance in both ectopic and eutopic endometrium [30,31]. The action of progesterone on tissue in women with endometriosis may explain why there is a reduction in the antiestrogenic action on tissue [29,32].

The current study showed that the apical cell membrane of eutopic epithelium bore well defined microvilli at some areas. In normal estrous cycle,short regular microvilli are present with progesterone alone, whereas oestrogen alone typically results in long thin regular microvilli [33].As the microvilli appear to be intimately concerned with implantation, any morphological change in the apical cell membrane may interfere with normal trophoblastic-endometrial interactions [34].

The present ultrathin sections revealed also, decidual like cells, macrophages, spindle-shaped fibroblasts and scattered irregular collagen fibers. Likewise previous stromal ultrastructural analysis showed increase in fibroblastic activity and tissue skeleton was also increased for collagen fibers [8]. It had been declared that tissue infiltration of macrophages in the eutopic endometrium in women with endometriosis was significantly higher than in control women [35].

Macrophages have defective scavenger activity liberating cytokines and growth factors which could promote the growth of endometriosis [36]. Recently, it was documented a mutual communication between macrophages and endometrial stromal cells [37]. Macrophages stimulated the expression of genes in endometrial stromal cells that might support the survival of endometrial cells in ectopic sites. Some researchers also found a reduced decidualization capacity in endometrium from women with endometriosis [38,39].

Pinopods are characteristic for mid to late secretory phase endometrium, which exhibit cycle-dependent changes and are most prominent during the putative implantation interval [40]. SEM in the present study revealed flattened and structurless surface epithelium with apparent decrease in the number of pinopodes. Similar researches showed that deep transversal folds, being the main type of fold at estrous stage and longitudinal folds gave the surface a rugged appearance [21]. Assesments regarding the implantation defects in a later study, revealed regressive pinopode like structures ultrastructurally in the experimental endometriosis group while none was observed for sham and controls, which proves the cyclic delay in experimental endometriosis [8].

Pinopode expression is progesterone dependent [41]. A strong correlation between pinopode with plasma progesterone level has been reported in the uterus of humans [41], rats [42] and mice [43]. Estrogen however causes loss of pinopode expression [42]. Therefore, progesterone resistance might be a contributing factor for ultrastructural observations and decreased pinopodes expression.

5. Conclusion

Ultrastructural features of estrogen dominance or progesterone resistance in the eutopic endometrium might account for inappropriate cyclic changes occurring in the disease.

Conflict of interest

The authors declare no conflict of interest.

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