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Histological Changes in the Endometrium of Female Albino Rat Uterusunder the Effect of Clomiphene Citrate (CC)

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Abstract: Introduction: Clomiphene citrate (CC) is a synthetic estrogen receptor modulator that excites or suppresses estrogenic responses regarding tissue category. CC acts as a super-ovulatory drug prescribed in female infertility managing. Though, pregnancy rates after CC treatment are little, usually due to the induced morphological alterations of the uterus caused by the usage of this medication. **Aim of the work:** This work was planned to study the histological changes albino rat endometrium under the effect of CC treatment. **Materials and methods:** Twenty one adult female albino rats were distributed into three groups. Group I was the control group. Group II received 1 mg/kg/day CC for 4 days, while group III was left to recover after receiving CC. Uterine H & E, periodic acid–Schiff (PAS), and caspase3stained sections were subjected to light microscopic and image analyzer examinations. The measured records underwent statistical analysis. **Results:** CC treated animals exhibited increase in the uterine wall thickness with increase in the height and width of the endometrial folds, dilated lumen with patches of hypertrophied surface epithelium (endometrial hyperplasia) and pseudo-stratification, vacuolated cytoplasm and vesicular nuclei with prominent nucleoli. Majorrise in the PAS ocular concentration of the lining epithelium was noticed, but caspase reaction zoneratio displayed a significant alteration in treated groups paralleled to the control group. **Conclusion:** CC treated female albino rats are liable to embedding interference and pregnancy failure due to histological alterations of the normal uterine endometrium.
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Key words: Clomiphene Citrate, uterus, Infertility, apoptosis, endometrium, ovulation.

1. Introduction:

Chemically, CC (resemblingtamoxifen) is a non steroidaltriphenylethylene derivative that displays both estrogen agonist and antagonist belongings. Overall, estrogen agonist properties are obvious only when endogenous estrogen levels are very little. Else, CC acts merely as an inexpensive estrogen antagonist **(1, 2)**. CC treatment changed the morphology of the luminal epithelium of rat endometrium which may affect implantation of the zygote. It is a synthetic estrogen receptor modulator that stimulates or suppresses estrogenic responses according to tissue type. CC acts as a super-ovulatory drug used in female infertility management. However, proportions of pregnancy after CC treatment are low, most frequently due to the induced morphological alterations of the uterus caused by the use of this drug. Although CC is a harmless, effective oral drug, it has been known to have comparatively collective anti-estrogenic side effects on endometrial and cervical mucus. In addition, CC usage results in a rise the frequency of abortions and multiple pregnancies **(3)**. CC acts as a first-line of practice for introduction of ovulation in anovulatory infertile women and used for ovarian hyperstimulation, as measure of an in vitro fertilization process **(4)**. Nevertheless, CC has numerous side effects, asovarian expansion, vasomotor flares, nausea, vomiting, breast distress, headache, irregular vaginal bleeding, visual symptoms, weight increase and dyspnea. It has likewise been stated that CC encourages acute pancreatitis **(5, 6)**. CC may raise uterine cancer threat, with higher doses due to rise of estradiol levels **(7).**

Aim of the work:

This work intended to study the histological deviations created by usage of CC by mouth citrate on endometrium female albino rat.

2. Materials and Methods:

Clomiphene citrate (CC):

CC was achieved in tablet form (Clomid 50 mg; Sanofi–Aventis Pharma Australia Pty Ltd, Sydney, New South Wales, Australia); it was liquefied in refined water. The calculated dosage was given by mouthvia a stomach hose.

Albino rats:

Twenty one adult nulliparous female Sprague Drawly rats were selected for this research with an average weight was 180-200 grams. Animals were housed in 3 separate cages, 7 rats each, in the Anatomy department, Faculty of medicine, Tanta University. They were accustomed for one week prior to the study. They were kept under regular animal house situations and were maintained on normal diet, water and libitum. All animals were adapted to management during a-5 day period before the trial. The experimental measures were permitted by the Research Ethics Committee (REC) in the use of experimental animals of Tanta University, faculty of medicine, quality assurance unit (protocol approval code no. 32554/09/18).

(1) Group I (the control group): consisted of 7rats which were given 1ml of distilled water by mouth via a stomach hose for 4 days. On day five, vaginal smears these rats were obtained and these swabs were examined with light microscope. Smears cell types were recognized to differentiate the estrus phase of each. Five rats were found in the diestrus stage of their cycle and they were anaesthetized with ether killed.

(2) Group II (CC group): consisted of 7 rats and were treated by CC in a dose of 1 mg/kg/day for 4 days by mouth via a stomach hose during the diestrus stage of their menstrual cycle. One day next to this management, rats were anaesthetized with ether killed **(8)**.

(3) Group III (the recovery group): consisted of 7rats and were given CC as previous group (group II). This group was not exposed to further process for a period of 7 days, afterwards this group animals were anaesthetized with ether killed.

Soft tissue specimens:

After completion of the trial, animals were anesthetized with ether. The abdominal walls of rats were cut and the uteri were recognized and separated. They were immersed in 10% formalin, dried, cleared, and underwent paraffin infiltration. The slices were sectionedat 5 micron (μm) and stained with the hematoxylin and eosin (H & E) which is the basis of anatomical pathology identification. H & E technique colors the nucleus and cytoplasm opposing colors to distinguish cellular constituents **(9)**. Other sections were subjected to periodic acid–Schiff (PAS) stain for mucus substances examination **(10)** and all stained sections were examined by light microscope.

Caspaseimmunostaining:

The paraffin-embedded tissue slices were de-paraffinized, hydrated, and prepared for antigen recovery in 10 mmol/l citrate buffer (pH 6) for 10 min. Stain was done by polyclonal rabbit antihuman CPP32 (1:200 titer) and a Vectastain ABC peroxidase rabbit IgG detection kit with 3-amino 9-ethyl carbazole as the chromogen. Counterstaining was done with hematoxylin. Dye was bleached by dealing with 0.3% NH_4OH for 3 min. Slices were dried out then attached with Permount **(11)**.

Image analysis:

Slices were inspected using a Leica Qwin DFC290 HD image analyzer (Leica Wetzlar, Germany). Uteri of all groups were subjected to thickness of the lining epithelium and the cell count of the coating epithelium calculation in a static area size of five haphazard slices for every group. Moreover, the optical concentration of PAS-stained slices and the zone percentage of caspaseimmunostained slices of the uterine lining epithelium were documented by 10 non-overlapping areas at amplification of × 400 per slide from five slides of every animal slices that were designated haphazardly. Calculated records were conveyed as mean ± SD **(12)**.

Statistical analysis:

Measureable records were assessed with mathematical Set for Social Sciences software (SPSS, V 13.0) with the help of t-test. Standards of P less than 0.05 were measured statistically important **(13)**.

3. Results:

Histological results

With light microscopy, uterine sections control rat (group I) could be recognized by its slit like narrow lumen, folded endometrium and thick muscle layer (myometrium). the uterine wall consisted of three stratums: the inner mucosa (endometrium), the middle muscular layer, and the outer serosa. The mucosa was thrown into longitudinal folds, made of simple columnar epithelium and the corium of connective tissue called lamina propria containing uterine glands (Figs 1 & 2). The surface epithelial columnar cells lining the uterine lumen showed basal oval nuclei and vacuolated cytoplasm, while the uterine glands in the lamina propia may be either simple or branched tubular glands. Both glands are lined with simple columnar cells with basal oval nuclei. The corium also contains numerous blood vessels and phagocytic cells (Figs. 3, 4 & 5). Uterine sections of CC treated albino rat group (group II) showed increase in the uterine wall thickness with increase in the height and width of the endometrial folds and dilated lumen with patches of hypertrophied surface epithelium (endometrial hyperplasia) and pseudo-stratification with vacuolated cytoplasm, vesicular nuclei with prominent nucleoli (figs.6 & 7). CC treated sections also showed a

densely cellularstroma, epithelial esinophilic infiltration with prominent esinophilic brush border of surface epithelial cells. They also exhibited increased thickness of sub-epithelial endometrial glands with vacuolated cytoplasm and pyknotic nuclei. Increased thickness of surface epithelial cells with vacuolated cytoplasm and basal nuclei with prominent nucleoli (figs.8 & 9). Uterine sections of the recovery group (group III) comparable to the control exhibited dilated lumen and smooth luminal surface of the endometrium with absent folds. The lining epithelium presented vacuolated cytoplasm, hyperplasia of luminal epithelial cells with pyknotic nuclei. Thickness of the lamina propria and muscularis and dilated and congested blood vessels were observed. Uterine glands of these sections also revealed pyknotic nuclei and vacuolated stromal and glandular epithelial cells (Figs. 10, 11, 12, 13). In PAS-stained sections, the epithelium of the first (control) and the third (recovery) groups displayed mild PAS-positive reaction, which looked heavy in the free border of luminal epithelial cells (Figs.14 & 16). The epithelium of the CC treated group showed moderate PAS-positive reaction that was heavier in the free and basal borders of luminal epithelium (Fig. 15).

Immunohistochemical results:

The immunohistochemical slices of the first (control) rat uteridisplayed moderate caspase reaction in its luminal surface lining and uterine glandular epithelium with an apparent stronger in the luminal epithelial cells (Fig. 17). Sections of CC group (group II) presented robust (strong) caspase reaction in its lining and uterine glandular epithelium (Fig. 18), whereas the third (recovery) group displayed moderate (modest) caspase reaction in its coating epithelium (Fig. 19).

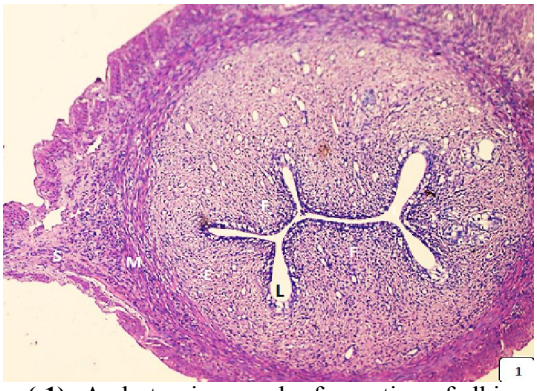

Fig. (1): A photomicrograph of a section of albino rat uterus of the first (control) group displaying layers of its wall includes endometrium (E), myometrium (M) and serosa layer (S). The endometrium is thrown into longitudinal folds (F). A small slit like lumen (L) can be observed. (H & E. X 100)

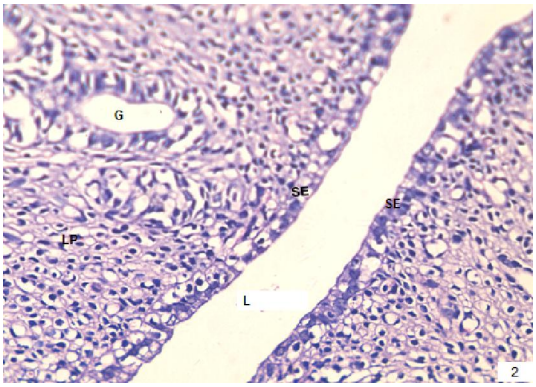

Fig. (2): A photomicrograph of a section of rat uterus of first (control) group presenting uterine lumen (L) lined with surface epithelial columnar cells (SE) and the corium of connective tissue called lamina propria (LP) containing uterine glands (G). (H & Ex400)

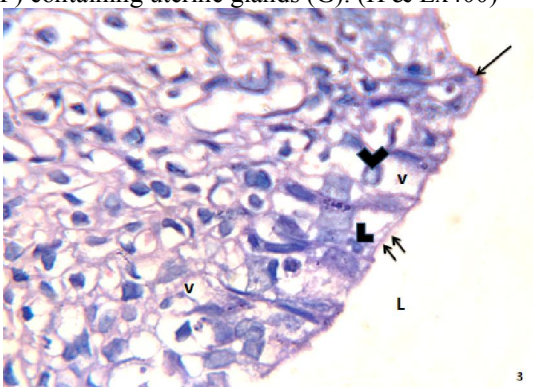

Fig. (3): A magnified photomicrograph of the previous section of rat uterus of the first group displaying the surface epithelial columnar ciliated cells (arrows) lining the uterine lumen (L) with basal oval nuclei (arrow heads) and vacuolated cytoplasm (v). (H & Ex1000)

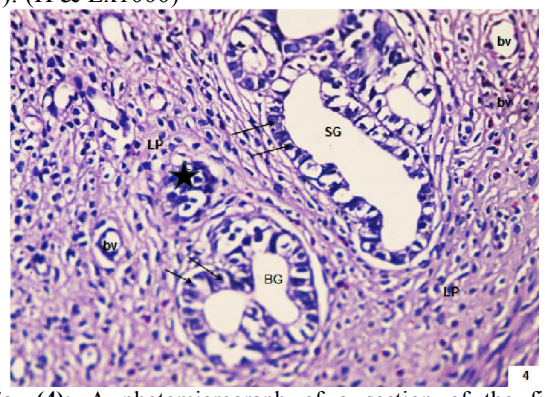

Fig. (4): A photomicrograph of a section of the first (control) group viewing the corium of connective tissue (LP) containing uterine glands, which may be either simple (SG) or branched tubular (BG) glands. Both glands are lined with simple columnar cells with basal oval nuclei (arrow heads). The corium (LP) also contains numerous blood vessels (bv) and macrophage (phagocytic) cells with rounded nucleus and abundant inclusions (star). (H & E x400)

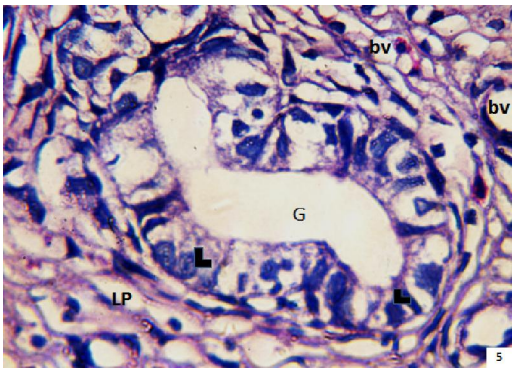

FIG. (5): A magnified photomicrograph of the previous slide of rat uterus of the first (control) group presenting the corium of connective tissue (LP) containing uterine glands (G) lined with simple columnar epithelium with basal oval nuclei (arrow heads). It also shows numerous blood vessels (bv). (H & E x1000)

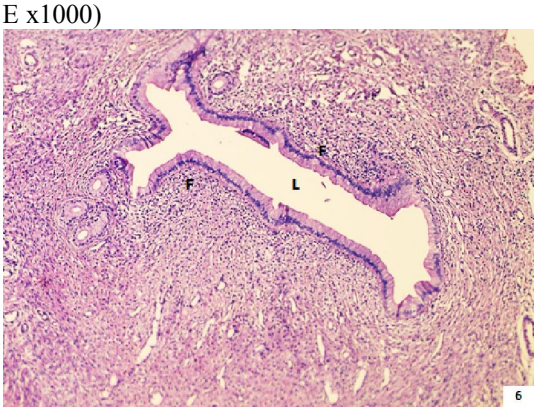

Fig.6: A photomicrograph of uterine slice of CC-treated albino rat group (group II) showing increase in the uterine wall thickness with increase in the height and width of the endometrial folds (F) and dilated lumen (L). (H & E, X 100)

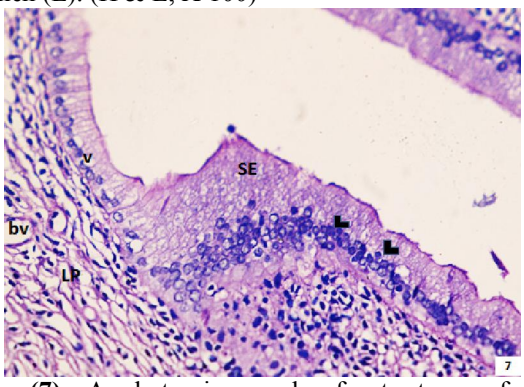

Fig. (7): A photomicrograph of rat uterus of the second group (CC group) showing a patch of hypertrophied surface epithelium (endometrial hyperplasia) (SE) and pseudo-stratification with vacuolated cytoplasm (v), prominent nuclei with prominent nucleoli (arrow heads) and cellular lamina propria (LP) with blood vessels (bv). (H & E x400)

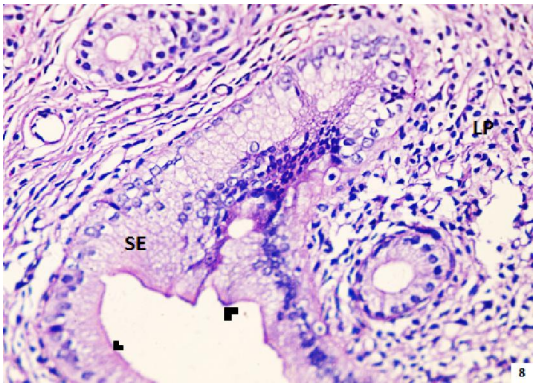

Fig. (8): A photomicrograph of the previous slice of rat uterus of CC group the stroma is densely cellular, (LP), epithelialesinophilic infiltration with prominent esinophilic brush border (arrows) of surface epithelial cells (SE). (H & Ex400)

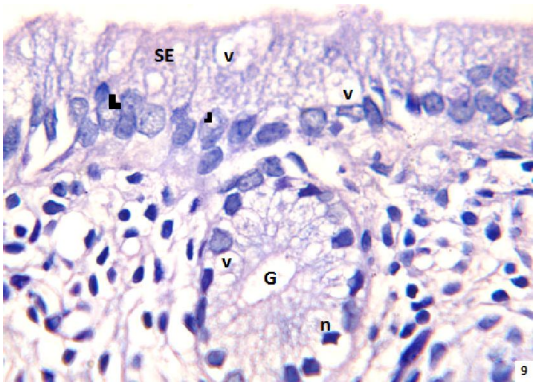

Fig. (9): A photomicrograph of a section of rat uterus of the CC group (group II) showing increased thickness of sub-epithelial endometrial glands (G) with vacuolated cytoplasm (v) and pyknotic nuclei (n). Increased thickness of surface epithelial cells (SE) with vacuolated cytoplasm (v) and basal nuclei with prominent nucleoli (arrow heads). (H & Ex1000)

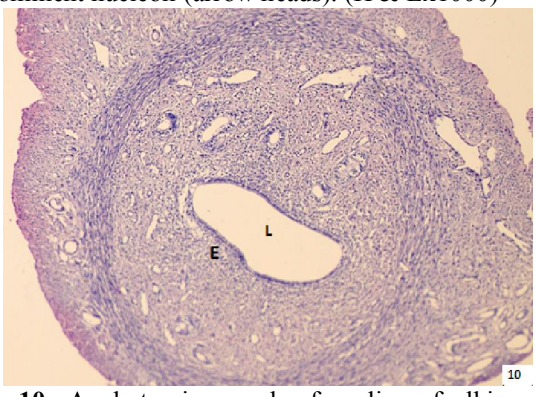

Fig.10: A photomicrograph of a slice of albino rat uterus of the third (recovery) group viewing uterine wall comparable to the control, dilated lumen (L) and smooth luminal surface of the endometrium (E) with absent folds. (H & Ex100)

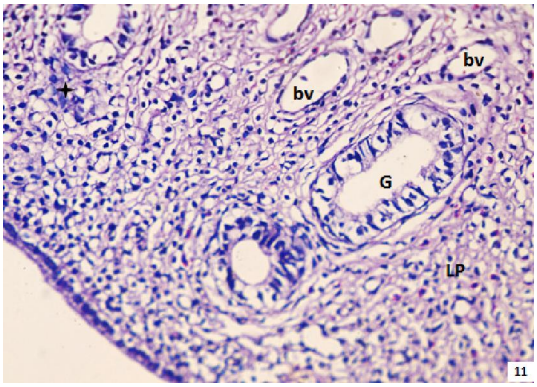

Fig. (11): A magnified photomicrograph of the previous section of rat uterus of the third (recovery) group presenting dilated blood vessels (bv) with less dense stoma (LP), macrophage (star) near blood vessels and dilated glands (G). (H & E x400)

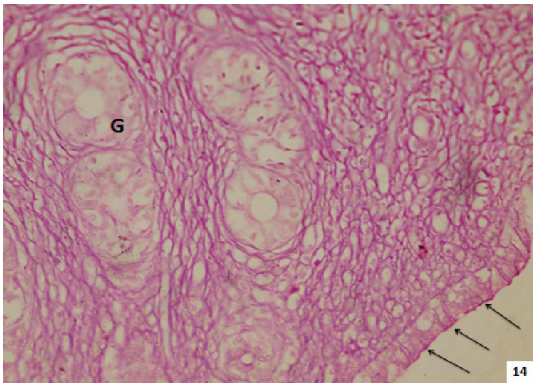

Fig.14: A photomicrograph of rat uterus of the first (Control) group presenting mild PAS positive reaction of the luminal and glandular epithelium (arrows) which is stronger in the free border of luminal epithelial cells (arrow head). (PASx400)

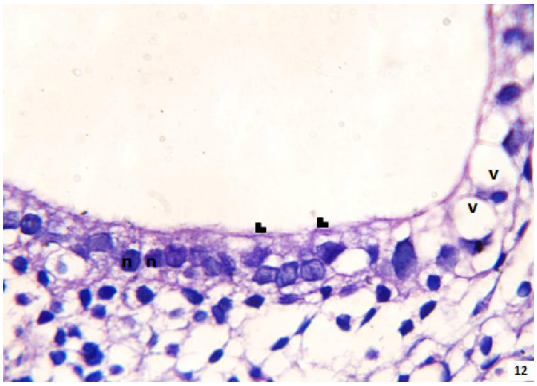

Fig. (12): A magnified photomicrograph of the previous slice of rat uterus of the recovery group (group III) displaying surface epithelial cells with eosinophilic brush border (arrow heads), vacuolated cytoplasm (v) and basal vesicular nuclei (n). (H & E x1000)

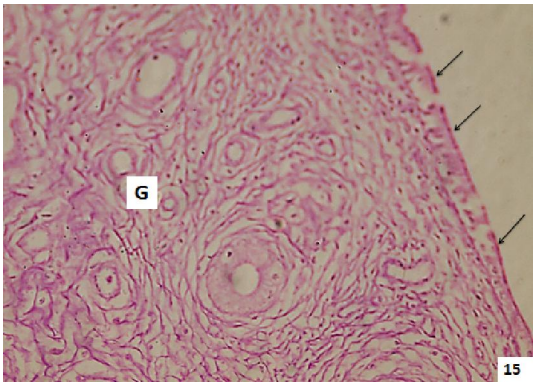

Fig.15: A photomicrograph of rat uterus of the second (CC treatment) group viewing moderate PAS positive reaction in both luminal epithelial and glandular epithelium (G) which is stronger in the free and basal borders of luminal epithelial cells (arrow heads). (PASx400)

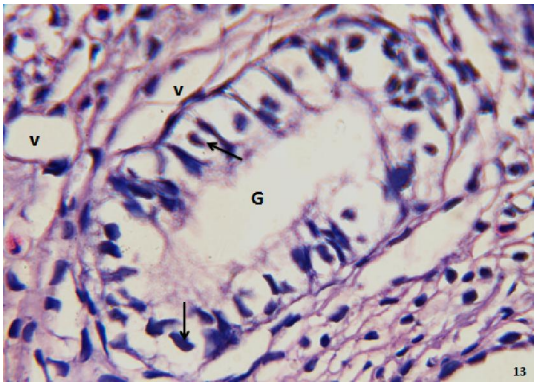

Fig. (13): A magnified photomicrograph the previous section of rat uterus of the third (recovery) group viewing uterine glands (G) with pyknotic nuclei (arrows) and vacuolated stromal and glandular epithelial cells (v). (H & Ex1000)

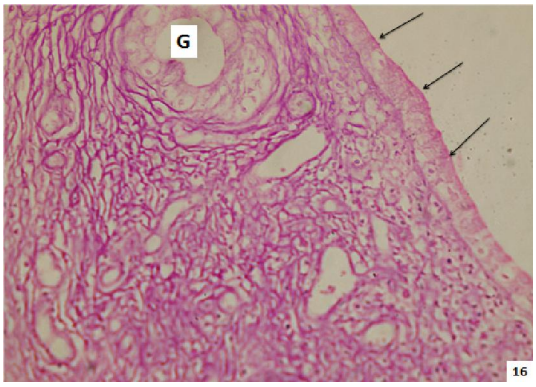

Fig.16: A photomicrograph of a slice of rat uterus of the third (recovery) group presenting mild PAS positive reaction in both luminal and glandular epithelium (G) which is stronger in the free border of luminal epithelial cells (arrows). (PASx400)

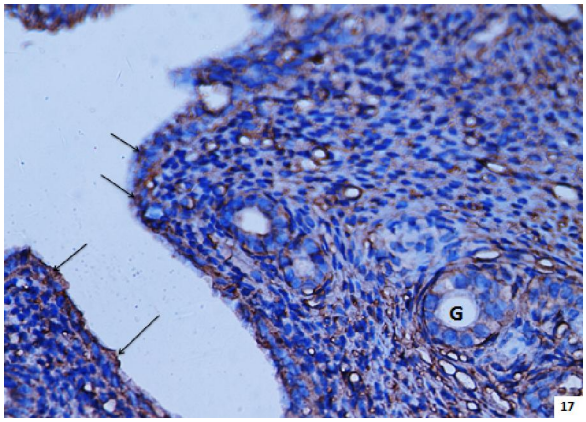

Fig. (17): A photomicrograph of a Slice of rat uterus of the first (control) group present in gmildimmune-staining of surface (arrows) epithelial and glandular (G) cells. (Caspase x400)

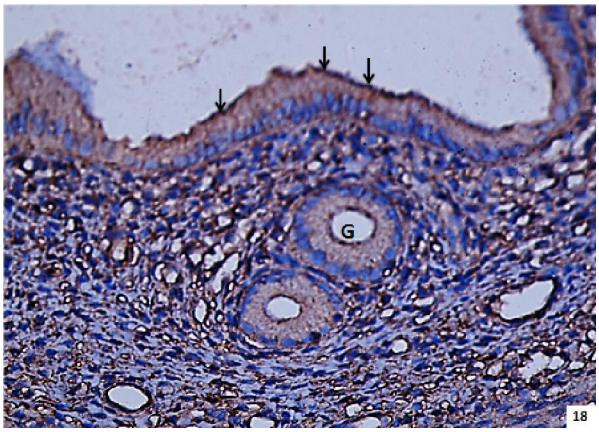

Fig.18: Photomicrograph of a Slice of rat uterus of the second (CC treated) group viewing marked immune-staining in surface epithelial (arrows) and glandular (G) cells. (Caspasex400)

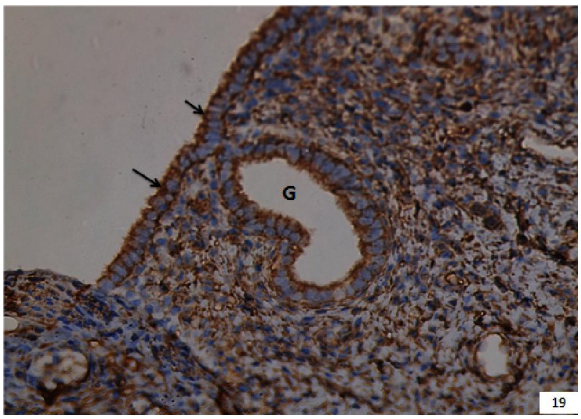

Fig. (19): A photomicrograph of rat uterus of the third (recovery) group presenting moderate immune-staining of surface epithelial (arrows) and glandular cells (G). (Caspase x400)

Quantitative and statistical results:

Regarding epithelial thickness and count, irrelevant rise remained distinguished in the second (CC treated) and the third (recovery) groups compared with the control group. Concerning PAS ocular concentration of the lining epithelium, important rise in the dignified records from CC group linked by the first (control) group (Table 2). Paralleled to the first (control) group sections, the caspase response zone ratio of the luminal epithelium showed important rise in the second (CC treated) group slices besides an irrelevant rise in the third (recovery) group slices (Table 3).

Table (1). Epithelial count thickness and thickness, (Hx & E/ uterus):

	Group1	Group 2	Group 3
Epithelial count	63.67 +9.95	166.83+23.26	67.66+12.59
P value 0.000*	0.000#		
Epithelial thickness	28.09+6.35	298.92+224.4	14.88+3.17
P value 0.001*	0.000#		

#Significant P value linked with clomiphene citrate (Group 2)
* Significant P value linked with control (Group 1)

Table (2): Epithelial periodic acid–Schiff ocular density:

	Control	Clomiphene citrate	Recovery
Means ± SD *P*	0.274±0.025473	0.231±0.013703 0.000*	0.24±0.011738 0.058

* Significant P value linked with control (Group 1)

Table (3): Epithelial caspase reaction area percent

	control	Clomiphene citrate	Recovery
Means ± SD *P*	11.581±5.890143	45.95±8.534 0.000*	28.693±7.147 0.002

* Significant P value linked with control (Group 1)

4. Discussion:

Endometrium is a compound, active tissue consisting of epithelial cells, both luminal and glandular, enclosed by supportive stromal cells, forming the inner stratum of the uterus. The main task of the uterus is to support fertility. The endometrium is the layer critically concerned with getting an embryo, enabling imbedding, decasualization and assisting pregnancy development until placentation. Fruitful gestation creation needs an endometrium that is receptive to blastocyst implantation and prepared to decasualization process, depending on hormonal control that permit pregnancy formation through the period of the menstrual cycle recognized as the space of receptiveness **(14)**. Endometrial receptiveness to embryo embedding is a compound procedure that includes the ovary, endometrium and embryo. The collaboration between the ovary and the endometrium supplies the hormonal impulse for beginning a successful gestation. Estrogen and progesterone hormones act in harmonization to stimulate the expression of energetic molecules vital for embryos to be implanted **(15)**. In this research, CC treated group exhibited plenty of histopathological changes in the form of increase in thickness and count of surface luminal epithelium of rat uterine sections with vacuolated cytoplasm and basal vesicular nuclei. There was also an increase in thickness of sub-epithelial endometrial glands with vacuolated cytoplasmand pyknotic nuclei. Some researchers reported that insufficient uterine synchrony was verified which led to criticalcon sequence as well as damaged implantation in gestations succeeding ovarian stimulation when compared with spontaneous ones. These results suggest that CC changed the appearance of the luminal epithelium, supposing that these cellular fluctuations may affect embedding **(16)**. Our results clearly indicated that few macrophages with a rounded nucleus and cytoplasm containing abundant inclusions nearby various blood capillaries and glands. The count of these peri-glandular and perivascular macrophages is restricted and they do not display any multiplying. The numbers and spreading of decidual macrophages specified that this group of cells does not play a chief controlling role in the achievement of pregnancy. **(17)**. **(18)** stated that most of the macrophages in the stroma traveled nearby to, and became sloping side ways the basal lamina. Most eosinophils were overcome by macrophages. In this issue, we discussed the collaboration of epithelial cells with endometrial stromal cells explaining the acidophilic free border of surface epithelial cells in CC treated animals. **(19)** Recorded that CC administered on the day of coupling to guinea-pigs could result ingestation failure due to interference with the endometrium. Such a compound inter action of mechanisms might arise in females treated with CC to encourage ovulation and aid to clarify low rates of pregnancy in certain circumstances. **(20)** Stated that PAS was observed in the uterine epithelium, discoloration the luminal cytoplasm of the non-ciliated cells. Additionally**, (21)** recorded that neutral mucin content was detected in PAS stained sections. They decided that the neutral mucin content was exaggerated by the epithelial locality and that it was greater in the basal than in the apical areas of the surface epithelial cells and accumulation of glycogen was at the cell base of the glandular epithelium in the endometrium. **(22).** Indicated that macrophages showed PAS-positive reaction in the endometrial stroma in greatest circumstances. In this research, the PAS-positive reactions were noticed in the apical parts of the surface and glandular epithelia in the second (CC treated) group uteri. Also, the strength of the PAS-positive response in the glandular epithelium was higher in the CC treated group, compared to the control and recovery group. Caspase enzymes were included in several phases of apoptosis, from the primary signal to the final lysis of cellular constituents; thus they were used as an indicator of apoptosis **(23)**. The immunohistochemically stained uterine sections of CC-treated rats of the current research showed major rise in caspase reaction area ratio of the lining uterine epithelium. This conclusion is in accordance with **(24)**, in which those authors reported that apoptosis arose over a mitochondria-dependent signals path. **(25)** Reported that CC did not disturb the regular apoptotic action seen at embedding, but did change the appearance of the luminal epithelium, suggesting that these cellular alterations could affect fruitful embedding.

Conclusion:

This work recommends that CC oral management for 4 days at a dose of 1 mg/kg/day to female albino rats may cause interference with embedding and pregnancy failure due to histological alterations of the normal uterine endometrium.

Acknowledgements:

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