Effect of Tamoxifen on Histochemical Structure of the Ovary in Adult Albino Rats

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Abstract: Introduction: The harmful effect of the tamoxifen on the ovary have been reported and as the widespread use of tamoxifen as the only antiestrogen available for the treatment of breast cancer has naturally leed to a close reexamination of both the laboratory and clinical toxicology of the drug. Close monitoring of clinical information is extremely important to ensure the safety of patients either on clinical trials or within the approved treatment community. **Aim of the Work:** Is to evaluate the histochemical effects of therapeutic doses of tamoxifen on the ovary of adult albino rats. **Material and Methods:** A total number of 45 adult female albino rats were used in this study for 30 days as follows: Group 1 (Control group):15 animals served as control group and orally administrated distilled water, Group 2, (Treated group): 15 animals were given tamoxifen orally through a metal tube in a dose of 0.5 mg/day equal to 20mg/day for human for 15 days. The tamoxifen dissolved in 0.5 ml of propylene glycol, Group 3 (Recovery group): 15 animals were given tamoxifen orally through ametal tube in adose of 0.5 mg/day equal to 20mg/day for human for 15 day and stoped for the same period. the tamoxifen dissolve in 0.5 ml of propylene glycol**.** At the end of the experimental study, the ovarian tissue taken for histochemical structure. **Results: C**omparing with control group the it was found that the ovaries of adult treated and recovery animals were apparently smaller in size and weight the surface epithelium was formed one layer of small attenuated cells with narrow tunica albuginea underneath. The granulose cells were apparently fewer in number with small darkly stained nuclei with no apparent sign of activity. **Conclusion:** The observations of the present work indicate a possible causal relationship between ovarian affection and ingestion of tamoxifen. This can be avoided by close medical observations using ultrasonography for ovaries the drug should not be used for more than three to six cycles and stopped for at least three cycles before reuse.

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Key Words: Tamoxifen, ovary, Fertility.

1. Introduction

Tamoxifen is a non-steroidal triphenylethylen derivative with predominant anti-oestrogen activity ***(1)*****.** It has long been in widespread use in the management of established breast carcinoma, perceived as safe, effective, and with negligible side effects, ***(2)*****.** It has recently been advocated as of potential value in breast cancer prevention and its efficacy as a preventive agent is currently being evaluated in asymptomatic women deemed to be at increased risk of breast cancer ***(3).*** Tamoxifen is known to have varied biological effects ranging from complete estrogen antagonism to pure estrogen agonism depending upon its concentration, the sex of the animal, and the target organ (**4**).

In humans and rats, tamoxifen is predominantly antiestrogenic with residual estrogenic activities ***(5)***.

Tamoxifen has been widely used in the management of breast cancer, originally introduced as an alternative to surgical endocrine ablation in disseminated breast cancer. Tamoxifen rapidly gained acceptance not only in the advanced cases but also in early stage breast cancer ***(5).*** Tamoxifen is the only compound known to prevent breast cancer incidence in healthy women ***(6)***.

Although an oestrogen antagonist in the breast, tamoxifen is structurally closely related to the synthetic oestrogen diethylstilbestrol ***(1)***. The widespread use of tamoxifen as the only antiestrogen available for the treatment of breast cancer has naturally led to a close reexamination of both the laboratory and clinical toxicology of the drug. Close monitoring of clinical information is extremely important to ensure the safety of patients either on clinical trials or within the approved treatment community. The past years has seen increased patient and physician awareness about the possibility of tamoxifen-induced carcinogenesis ***(7).***

2. Material and Methods:

Material:

1. Experimental animals:

A total number of 45 adult female albino rats were used in this study. They were obtained from the animal house, Alazhar university Their body weight varied from (150 -200 gms), maintained in the animal house in normal conditions and adequately fed,

generally accepted standard diet and provided with sufficient tap water.

2. **Chymical Material:**

Tamoxifen:

The trade name of tamoxifen is nolvadex. It was obtained from Astra Zeneca company. Tamoxifen was obtained from nolvadex tablets 20 mg.

Methods:

The animals were divided into three groups: -Group 1 (Control group): 15 animals served as control group and orally administrated distilled water **-Group 2(Treated group):** 15 animals were given tamoxifen orally through a metal tube in a dose of 0.5 mg/day equal to 20mg/day for human for 15 days. The tamoxifen dissolved in 0.5 ml of propylene glycol **(Stygar, 2003).-Group 3 (Recovery group):** 15 animals were given tamoxifen orally through ametal tube in adose of 0.5 mg/day equal to 20mg/day for human for 15 day and stoped for the same period. the tamoxifen dissolve in 0.5 ml of propylene glycol **(8)** The dose calculated according to (***9)*** formula. The control, treated and recovery animals were sacrificed on the metestrous or diestrous 1st stages of the estrous cycle (maximum cellular proliferation) **(10).** Small specimens were taken from the ovary of the control, treated and recovery animals. They were fixed in boun's fluid and neutral formaline. After proper fixation the specimens, were dehydrated, cleared and embedded in parftin wax and were cut at athickness of 5-7, microns for histological and histochemical staining by light microscope. The following histological and histochemical staining were used **Heamatoxyline and Eosin**: for general histological structure. **Masson's Trichrome**: stain for collagen fibers. **Orcienstain**: for elastic fibers. **Feulgien reaction**: for DNA. All methods were applied according to **(11) Light microscopic study**. Preparation of the ovary specimen for light microscopic study 1**.** The spicemen were fixed in 70% alcohol and kept refrigated at four degree centigrate for one day.2. The spicemen were dehydrated successively in ascending grade of alcohol (80 %, 85 % and 90 %); each solution for one day and finally one hour in absolute alcohol for complete dehydration.3. The spicemen were cleared in xylol for one day then put into melted soft paraffin wax.4. Embedding was performed in hard paraffin wax overnight in an oven 50 C.5. Paraffin blocks were prepared and slices of seven microns thickness were made.6. Before staining, the sections were dewaxed and stained using the following stains for light microscopic examination: **Heamatoxyline and Eosin**: for general histological structure. **Masson's Trichrome**: stain for collagen fibers. **Orcienstain**: for elastic fibers. **Feulgien reaction**: for DNA.

Haematoxylin and Eosin Stain (12) Method:1. The deparaffinization of sections was done through changes of xylol and hydration was done through descending grades of alcohol.2. The sections were stained with haematoxylin for ten minutes.3. Bluing in tape water was carried out for tow to three minutes.4. Counter stain in 1 % solution of eosin for one minute was done.5. Rinsing rabidly in distilled was carried out.6. Dehydration, clearing and mounting in canada balsam were performed. **Result:** Nuclei were stained blue and cytoplasm were stained red. **Masson's Trichrome stain Preparation:**1. Cytoplasmic stain:-1% ponceau de xylidine in 1% acetic acid -1% acid fuchsin in 1% acetic acid 2. Differantiation and mordant:-1% phosphomollybidic acid in distilled water. 3. Fiber stain:-2% mythyl blue in 2% acetic acid. **Method:-**Sections were taken dowen to water.-The nuclei were stained with Weigert's iron haematoxyline for 10 minute.-The sections were then washed in water.-Differentiation of the nuclear stain was done with 5% HCL in 70% alcohol.-Washing was then carried out in the tap water and rinsed in distilled water.-The red cytoplasmic stain was used for 5-10 minutes.-Rinsing was then performed using distilled water.-1% phosphomolybdic acid was used for differentiation until collagen was decolorized, while the muscle fibers, red blood cells and fibrin remained red.-Rinsing was then done in distilled water.-Counter staining was done in aniline blue for 2-5 minutes.-Washing in acedic acid for at least one minute was performed.-dehydration in ascending grade of alcohol, clearing in xylene and mounting in canada balsam was done. **Results:-**Muscles, RBCs, fibrin and cytoplasmic granules: red.-Collagen, reticulin, amyloid and mucin: blue.

Feulgen stain (11) is a staining technique discovered by Robert Feulgen and used in histology to identify chromosomal material or DNA in cell specimens. It is darkly stained. It depends on acid hydrolysis of DNA, therefore fixating agents using strong acids should be avoided. **Methods:**1. The specimen is subjected to warm (60°C) hydrochloric acid.2. Then the specimen is subjected to Schiff reagent. 3. Counterstained with Light Green SF yellowish. 4. It is dehydrated with ethanol.5. Cleared with xylene. 6. Mounted in a resinous medium. **Results:** DNA should be stained red.

Orcienstain (11) Methods:1. Decerate section and hydrate to distilled water. 2. Stain in Alcian Blue, 1% for 20 minutes. 3. Rinse in two changes of distilled water 4. Place in 1% oxalic acid for 2 minutes.5. Wash in running tap-water for 1 minute. 6. Rinse in two changes of distilled water. 7. Dip several times in 70% alcohol. 8. Immerse in **Orcein stain** for 10 - 90 minutes. 9. Rinse in several changes of distilled water. 10. Dehydrate in 100% ethyl alcohol, two changes. 11.

Clear in xylene, three changes. **Results:** Elastic fibers is purple.

3. Result

Histopathological results: A- Control group: The ovary of the adult rat appeared as a small bean shaped body composed of a surface epithelium covering fibrous stroma. Within the fibrous stroma, ovarian follicles at different stages of maturation were observed, including primary, growing and mature Graaffian follicles. The most mature follicles appeared nearer to the surface epithelium Fewatretic follicles and corpora lutea were observed in the ovarian stroma. The medulla consists of a richly vasculoraized loose connective tissue. The surface epithelium of the adult ovaries was formed of one layer of cubical cells, separated from the underlying ovarian stroma by the tunica albuginea. Corpus luteumand blood capillaries can be identified in the ovarian medulla**.** position of oocyte was located in the centre of primary and growing follicles whereas in Graaffian follicles, the oocytes were pushed to one pole of the follicle In mature follicles, the granulosa cells composed of three parts, the first was corona radiata, surrounding the 'oocyte external to zonapellucida, the oocytes were apparently large cells with pale cytoplasm and eccentric nuclei. In both growing and mature follicles, the oocytes were completely surrounded by a homogenous eosinophilic material forming the zonapellucida. The second was the peripheral granulosa cells, laying under cover the theca cells and lined the cavity of the follicle, while the third was the cumulus oophorus, the oocytes-zona-corona radiata complex to the peripheral granulosa cells **(Fig. 1).** It was observed that, the corpus luteum appeared as a large ovoid or rounded body surrounded by capsule of interstitial cells, it usually contains central cavity. Two types of cells could be identified in the mature well developed corpus luteum; the first was **theca lutein** cells which occupy mainly the peripheral zone. It is fusiform fibroblast-like cells with oval central or peripheral nuclei. The second type of cells was the **granulosa lutein** cells. **(Fig. 1).** Using feulgen reaction for demonstration of DNA revealed the positive reaction in the nuclei of the granulosa cells of the mature graaffianfollicles while a slight positive reaction is detected in the nuclei of the luteal cells **(Fig. 2).** The elastic fibers in the control ovary are meager. So no abnormal distribution of elastic fibers **(Fig. 3)**. Few strands of collagenous fibers are normally present under the surface epithelium, few collagenous fibers are observed around corpora lutea, follicles and blood vessels **(Fig. 4)**

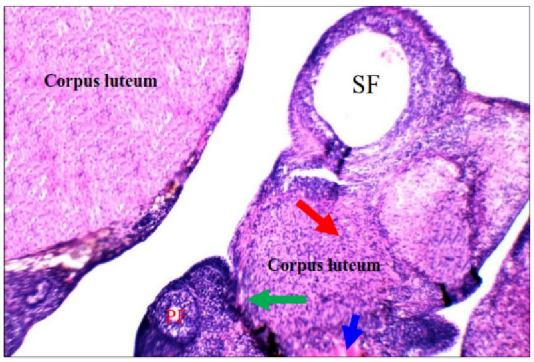

Fig 1: A photomicrograph of the ovary of adult rat the control group showing primary follicles (PF), secondary follicle (SF), corpora lutea with theca lutein cells (green arrow) and granulosa lutein (red arrow) and highly vascular stroma (blue arrow) (H & E X 400)

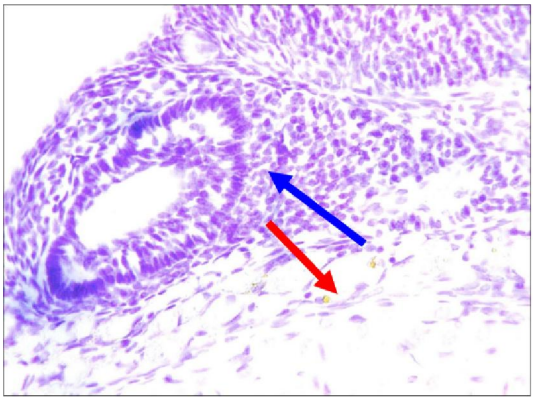

Fig 2: A photomicrograph of the ovary of adult rat the control group showing**;** ovarian tissue showing marked positivity (+++) of follicular and peri-follicular cells (blue arrows) in a faint background (red arrow) (Feulgen stain X 400)

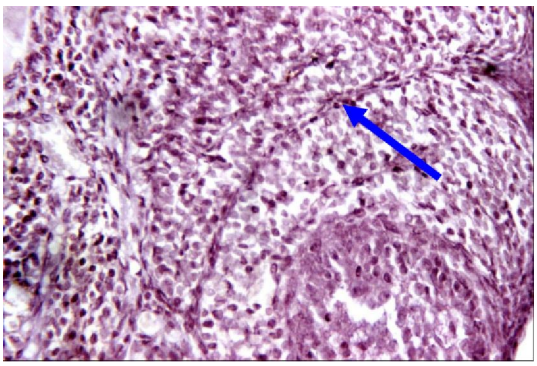

Fig 3: A photomicrograph of the ovary of adult rat of thecontrol group showing**;** ovarian tissue negative for orcein stain (Orcein stain X 360)

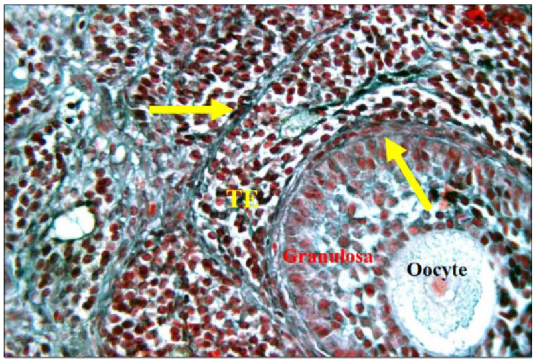

Fig 4: ovarian tissue negative for Masson trichrome stain, note, the average distribution of collagen fibers (yellow arrows) (Masson trichrome stain X 360)

B- Treated group:

Sections in the ovaries showed that the epithelium was formed of one layer of cells irregular in shape. They appeared small and attenuated in comparison with those of the control animals and separated from the ovarian stroma by a narrow tunica albuginea. The granulosa cells in mature ovarian follicles appeared smaller in size and poorly luteinized with darkly stained nuclei showing no apparent signs of activity. Manyatretic follicles were noticed in the ovaries of this group of animals. These follicles were characterized by vacoulation of the oocyte cytoplasm, disappearance of zonapellucida, pyknosis of the nucleus and degeneration of granulosa cells. The corpora lutea in the ovaries appeared slightly smaller in size than control. Most of their cells were degenerated with ill distinct cell boundaries. The main histological changes in the ovary after treatment with tamoxifen were the apparent decrease in the number of the developing follicles and corpora lutea and increase in the number of atretic follicles. The granulosa cells show small dense nuclei and acidophilic cytoplasm. While other follicles shows detachment of the ovum from the surrounding granulosa cells, with abnormal arrangement of the corona radiata cells. The cytoplasm of the ovum appears acidophillic and shrunken There was a mild to moderate increase in the number of atreticanovulatory follicles and their cells exhibit deeply stained nuclei and pale vacuolated cytoplasm **(Fig. 5).** Moderate intensity of the feulgen reaction was observed in the nuclei of in follicular and peri-follicular cells **(Fig. 6).** There is also abnormal distribution of elastic fibers which appears thick and fragmented **(Fig. 7).** After treatment with tamoxifen, changes were observed in the amount and distribution of collagenous fibers in stromal and peri-follicular regions (**Fig. 8).**

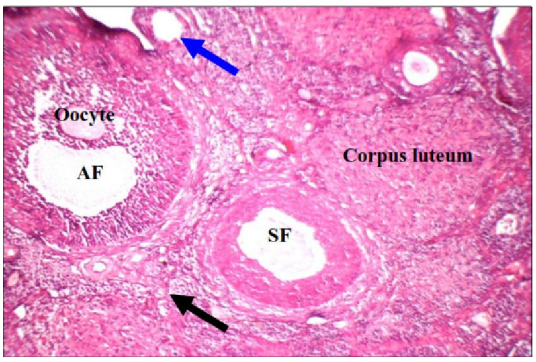

Fig 5: A photomicrograph of the ovary of adult rat post treatment group high power view of previous slide showing atretic follicle with atrophied lining (blue arrow), secondary follicles with necrotic lining (SF), antral follicles (AF), and corpora lutea in edematous stroma (black arrow) (H & E X 400)

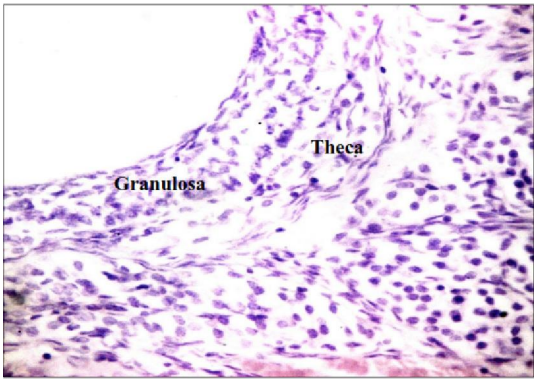

Fig 6: A photomicrograph of the ovary of adult rat post treatment group showing moderate positivity (++) of granulosa and theca cells (Feulgen stain X 400)

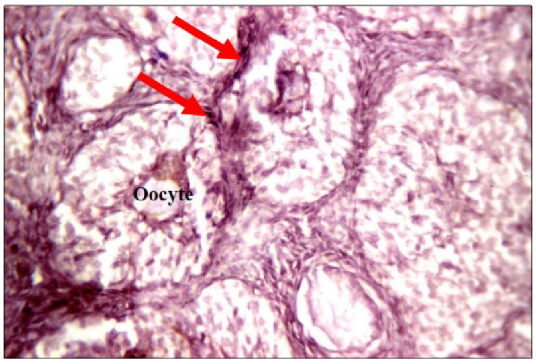

Fig 7: A photomicrograph of the ovary of adult rat post treatment group showing; ovarian tissue positive for orcein stain and the elastic fiber appear thick and fragmented (red arrows) (Orcein stain X 360)

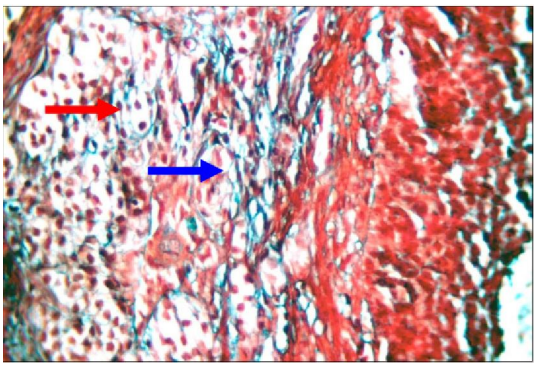

Fig 8: A photomicrograph of the ovary of adult rat post treatment group showing; ovarian tissue positive (+++) for Masson trichrome stain, note, excess stromal (red arrow) and peri-follicular collagen fibers (blue arrow) (Masson trichrome stain X 360)

C. Recovery group:

The follicles show detachment of the ovum from the surrounding granulosa cells**.:** Manyatretic follicles were noticed in the ovaries of this group of animals, edematous stroma and congested blood vessels The growing follicles show large follicular cavities and the granulosa cells reduced to form a few layers. These growing follicles are numerous and look like cysts**.** There is apparent decrease in the number of the developing follicles and corpora lutea and with atrophied epithelial lining of the follicles Most of the corpora lutea cells were degenerated with ill distinct cell boundaries **(Fig. 9).** Moderate intensity of the feulgen reaction was observed in the nuclei of follicular and peri-follicular cells **(Fig. 10)** There is also abnormal distribution of elastic fibers which appears thick and fragmented **(Fig. 11).** After treatment with tamoxifen, changes were observed in the amount and distribution of collagenous fibers in stromal, peri-follicular and blood vessels **(Fig. 12)**.

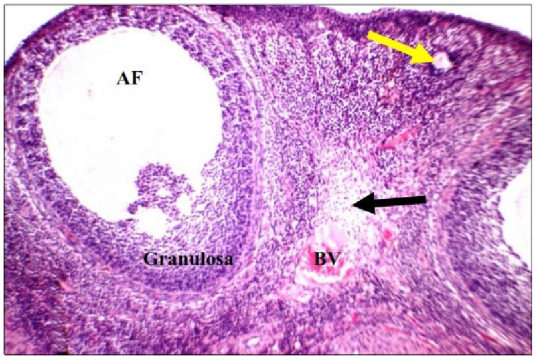

Fig 9: A photomicrograph of the ovary of adult rat recovery group showing; high power view of previous slide showing atretic follicle (yellow arrow), antral follicle (AF) in edematous stroma (black arrow) showing congested blood vessels (BV) (H & E X 400)

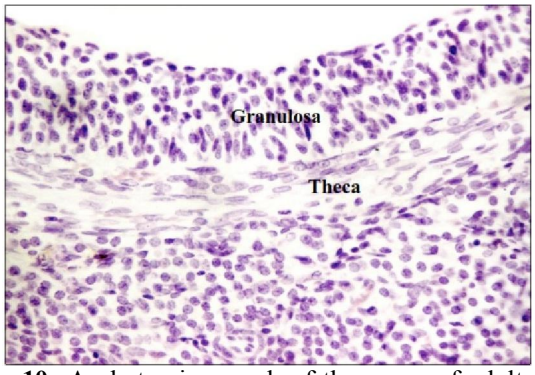

Fig 10: A photomicrograph of the ovary of adult rat recovery group showing; ovarian tissue with mild positivity (+) of granulosa and theca cells (Feulgen stain X 400)

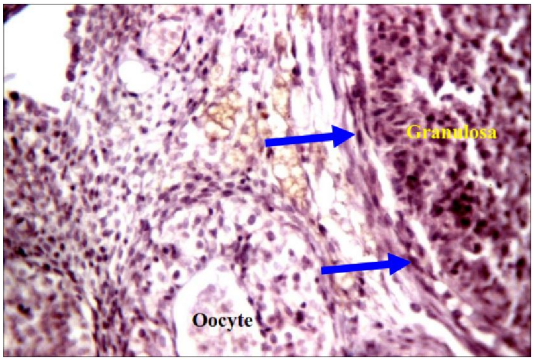

Fig 11: A photomicrograph of the ovary of adult rat recovery group showing**;** ovarian tissue positive for orcein stain, note; elastic fibers appears thick and fragmented (blue arrows) (Orcein stain X 360)

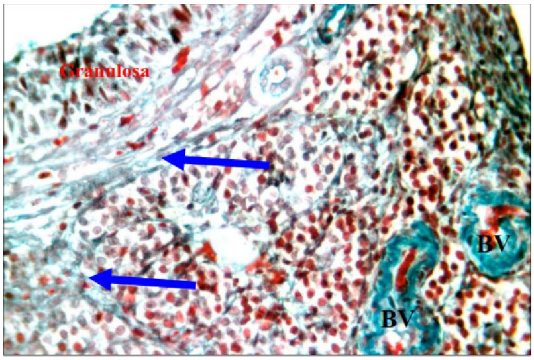

Fig 12: A photomicrograph of the ovary of adult rat recovery group showing; ovarian tissue positive (++) for Masson trichrome stain, note, excess stromal and peri-follicular collagen fibers (blue arrows) as well as in blood vessel wall (BV) (Masson trichrome stain X 360)

4. Discussion

Tamoxifen is a synthetic non-steroidal antiestrogen and it is one of the most effective drugs for treatment of breast cancer through its ability to

antagonize estrogen-dependent growth by binding estrogen receptor (Ers) and inhibiting proliferation of mammary epithelial cells ***(13).*** However, tamoxifen has estrogen agonist effects in other tissues such as bone and endometrium due to liganded ER activating target genes in these types of cells, which may lead to development of endometrial cancer ***(14).*** More recently tamoxifen is used for chemoprevention in women at high risk of developing ER-positive breast cancer. A high risk of developing breast cancer may be secondary to age, early menarche, a family history of breast cancer or a personal history of benign breast disease ***(15).*** In the present study the ovary of the control animals is formed of cortex and medulla. The cortex contains the ovarian follicles at different stages of maturation. Alarge mature follicle is present deep in the cortex which exhibit layers of granulosa cells and the internal cavity. The ovum surrounded by a well developed zonapellucida and corona radiata. These follicles surrounded by two layers of theca cells. The corpus luteum is a large rounded structure surrounded by connective tissue capsule formed mainly by collagen fibers. Numerous blood capillaries are observed between the cells. These results are in accordance with the results of ***(16)*** reported that the corpus luteum of all mammals is formed of at least two steroidogenic cell types, large originate from granulose cells and small luteal cells. Also the corpus luteum contains numerous blood capillaries and surrounded by C. T. capsule contains collagen fibers. Treatment with tamoxifen led to moderate decrease in the number of developing follicles and corpora lutea with a moderate increase in the number of atretic follicles. These results are similar to those described by ***(17)*** who found that tamoxifen treatment led to suppression of ovulation and lack of corpora lutea. ***(18)*** demonstrated these effect of tamoxifen on the ovary explained by disruption of the programming of hypothalamic, pituitary ovarian axis function which led to decreased hypothalamic LHRH mRNA expression levels. Also ***(19)*** explained this effect by blockage of preovulatory luteinizing hormone and follicle stimulating hormone (FSH) surges and of suppressed serum progesterone profoundly without changing circulating levels of estrogen. In the present study, tamoxifen led to formation of large follicular cavities with reduction of granulosa cell layer and of cystic follicles were observed. ***(20)*** explained the arrest of granulosa cells maturation due to reduction of CAMP production which prevent FSH Stimulation. Increases, in LH receptors, so decrease LH receptor. They reported the possibility of premenopausal and postmenopausal ovarian cyst, formed after TAM treatment, to be treated with gonadotropin releasing hormone agonist. Using feulgen reaction for demonstration of DNA, a strong + ve reaction in the nuclei of granulosa and luteal cells was observed due to the induction of apoptosis in the ovary. This result was in line with ***(21)*** who cited that the apoptotic changes that were detected after tamoxifen treatment due to regulation of growth factors such as P_{53} cadherin/catenin to activate induction of apoptosis. Also ***(22)*** found that tamoxifenanalouges facilitate apoptosis by increasing Bax/Bcl_2 ratio specially in luteal cells. In the present study the ovaries of adult treated animals surface epithelium was formed one layer of small attenuated cells with narrow tunica albuginea underneath. The granulose cells were apparently fewer in number with small darkly stained nuclei with no apparent sign of activity. The corpora lutea were formed of only one type of cells, the lutin cells. Most of these cells appeared degenerated with ill distinct cell boundaries, so tamoxifen markedly reduces fertility and increased the rate of abortion and congenital anomalies in rats. Tamoxifen increases the number of atretic follicles in the ovaries of this group of animals. These follicles were characterized by vacoulation of the oocyte cytoplasm, disappearance of zonapellucida, pyknosis of the nucleus and degeneration of granulosa cells. With accumulation of cytoplasmic lipid droplets, the oocyte disappeared completely, also apoptotic bodies were noticed. Somegranulosa cells of this group showed leutinization, necrosis and keratolysis of their nuclei, this denoting at programmed cell death. Inter follicular stroma showed marked deposition of collagen fibers and irregular shaped stromal cells with irregular pyknotic nuclei if compared with the control group. In the present study, the ovaries of the treated animals were apparently smaller in size than control. Our explanation for these finding is the increase in the number of atretic follicle. A similar result was obtained by ***(23),*** who studied the effects of tamoxifen (0.3 or 3.0 mg/kg body weight/day) for 10 consecutive days on the ovary of a wild rat. He described the effect of tamoxifen on the ovaries was dose dependant. The low dose of tamoxifen decreased the number of non atretic follicles larger than 400 µ in diameter, increased atresia in follicles smaller than 200 µ, while high dose of tamoxifen increased the number of follicles between 200 and 400 µ decreased the number of follicles larger than 600 µ. While (24)in their study, described increase in the numbers of atretic follicles regardless the dose of tamoxifen or size of the ovarian follicle. In the present study, the granulosa cells of ovarian follicles appeared smaller in size and fewer in number. These findings were in agreement with ***(23)*** They reported a decrease in granulosa cell number which appeared poorly lutenized as shown by a decreased granulosa cell cytoplasm to nuclear volume ratio. In addition, treatment withtamoxifen producing irregularity in the estrous cycles associated with

increased cycle length. These results suggest that the tamoxifen induced partial inhibition of ovulation, and this is possibly through its action on follicular growth and atresia mainly in non antral (less than 200 μ) and mature follicles (401-600 μ). Similarly, ***(25)*** stated that tamoxifen treatment in hypophysectomized rats resulted in a significant ($P< 0·05$) reduction (23·1 ± 7·6 oocytes/rat) in ovulatory response decrease in ovarian weights and serum progesterone concentrations. They suggest that tamoxifen exerts direct ovarian anti ovulatory and oestrogen-antagonist actions. In the present study, the corpora lutea appeared smaller than normal and containing one type of cells, the luten cells, which appeared degenerated with indistinct cell boundaries. These results were in agreement with ***(26)*** who reported a decrease in the mass of corpus luteum tissue in rats through a direct inhibitory action. Similar results were observed in guinea pig by (***27)*** their explanation for these changes was that tamoxifen induces luteolysis in corpora lutea and the luteal cells appeared pale with pyknotic nuclei. However, (***28)*** proved that the histological structure of corpus luteum in human ovary was normal in tamoxifen induced ovulatory cycles. This reinforced the idea of (***29)*** that oestrogen-agonist and antagonist activities of tamoxifen depend on the animal species and the time of exposure. Follicular degeneration was reported during treatment withtamoxifen in the form increased number of atretic ovarian follicles this was in agreement with ***(30)***

5. Conclusion

The observations of the present work indicate a possible causal relationship between ovarian affection and ingestion of tamoxifen. This can be avoided by close medical observations using ultrasonography for ovaries the drug should not be used for more than three to six cycles and stopped for at least three cycles before reuse. When the tamoxifen is ineffective in the treatment of anovulation, human menopausal gonadotropin (hMG) administration is typically selected. We describe the use of tamoxifen regimen that was successful in increasing the effectivenessof ovulation induction.

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