

## The Role of Stem Cells on the Ovarian Failure Induced by Busulfan in Female Albino Rat

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### Abstract

**Background:** Many researches about stem cell therapy in premature ovarian failure treatment have been carried out. The adipose tissue has recently been identified as one of the alternative sources of multipotent resident stem cells in humans. One of the most devastating effects of chemotherapy is the damage of the reproductive system, which in young girls and women younger than 40 years of age is frequently associated with premature ovarian failure. Busulfan has a wide range of using as in a conditioning regimen prior to allogeneic hematopoietic progenitor cell transplantation for chronic myeloid leukemia and in patients undergoing bone marrow transplantation.

**Aim of the Study:** The aim of the study was to evaluate the effect of administration of adipose tissue derived stem cells on the ovarian failure induced by the busulfan in female albino rats.

**Material and Methods:** 70 female 8 weeks old albino rats were divided into 5 groups. Group I (control Group): They consisted of 20 female albino rats. They were received no treatment and served as untreated control. Group II (adipose tissue derived stem cells treated group): They consisted of 20 female albino rats. They were received adipose tissue derived stem cells (AT-ASCs). They received million and half of million units of AT-ASCs/single dose for each animal I.V. Group III (busulfan treated Group): They consisted of 10 female albino rats. In this group the ovarian failure was induced using busulfan therapy by a dose 2mg for successive 4 days. Group IV (busulfan treated, followed by AT-ASCs treated group): They consisted of 10 female albino rats. In this group the ovarian failure was induced by the same regimen as in group III. After one week of chemotherapeutic treatment, the animals received million and half of million units of AT-ASCs/single dose for each animal I.V. Group V (busulfan treated, followed by AT-ASCs treated group): They consisted of 10 female albino rats. The rats in this group received the same regimen as group IV but they were sacrificed 4 weeks after the AT-ASCs injection. Biochemical study: Blood samples were taken from all rats from retro-orbital venous plexus and the serum samples were measured for FSH, LH and serum estrogen level using ELISA technique. Paraffin sections for

female albino rat ovaries were prepared to be stained with H&E and Masson's Trichrome stain for Light microscopic study. Specimens of the ovary were also prepared for electron microscopic study.

**Results:** The AT-ASCs induced definite improvement in the histopathological changes associated with busulfan induced ovarian failure. This improvement was marked one week after AT-ASCs treated but after 4 weeks, there was some regression in this improvement.

**Conclusion:** AT-ASCs transplantation can improve the ovarian damage induced by the busulfan. The need for second dose of AT-ASCs is suggested.

**Key Words:** Stem cells – Busulfan – Female albino rats – Ovary.

### Introduction

**MANY** researches about stem cell therapy in premature ovarian failure treatment have been carried out. Clinical applications of stem cell therapy also have become popular for treating premature ovarian failure. The oocyte and granulosa cells regeneration and the re-establishment of hormone profiles supporting using of stem cells in the improvement and recovery of the damaged ovarian function and fertility [1].

In recent years, interest has rapidly grown in the research field of therapeutic potential of stem cells. Adult mesenchymal stem cells (MSCs) have remarkable plasticity to the extent that they can differentiate into lineages other than the tissue of origin [2].

The adipose tissue has recently been identified as one of the alternative sources of multipotent resident stem cells in humans. The adipose-derived stem cells (AT-ASCs) are a new type of MSCs that are typically abundant in individuals. Cultured AT-ASCs can be differentiated into multiple cell types, including osteoblasts, cartilage cells, adipocytes,

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myocytes, vascular endothelial cells and neurons [3].

The premature ovarian failure (POF) is a mysterious disorder. It is defined by the association of amenorrhea, sex steroid deficiency and elevated levels of serum gonadotropins before the age of 40 years. It has important physical and psychological consequences and is common due to improved survival following treatment for malignancy [4,5].

Accurate estimates of the prevalence of POF are lacking. Spontaneous POF has been estimated to affect 1% of women under the age of 40 years [6]. It is found in 10-28% of women with primary amenorrhea and in 4-18% of women with secondary amenorrhea [7].

Anticancer drugs are unquestionably beneficial as therapeutic agents. However, their side effects on the quality of life of female cancer survivors and their offsprings cannot be ignored. One of the most devastating effects of chemotherapy is the damage of the reproductive system, which in young girls and women younger than 40 years of age is frequently associated with premature ovarian failure [8].

The busulfan is a bifunctional alkylating agent which can create covalent bonds between deoxyribonucleic acid (DNA) strands, interfere with cleavage during DNA replication, and leading to disruption of cell division [9]. The busulfan is used in acute and chronic myeloid leukemia and non-malignant diseases. It is also used to treat blood disorders including polycythemia vera, mucopolysaccharide disorder, sickle cell disease, primary thrombocythaemia and thalassemia [10,11].

So this work aimed to study the effect of administration of adipose tissue derived stem cells on the ovarian failure induced by the busulfan in the female albino rats.

## Material and Methods

This experimental study was done in the Medical Research Center at Ain Shams University Hospitals in the period from September 2016-September 2018. The study included the following:

A- Material:

*Drug:*

The drug used was busulfan (trade name- Myleran): The busulfan was purchased from Excella GmbH, Nurnberger, Germany, in the form of tablets 2mg.

*Animals:*

The study was carried out at the Animal House of Ain Shams University according to the Guidelines for the Care and Use of Laboratory Animals. A total of 80 female albinos rats (8 weeks old-weighting around +100 grams), were used in this experiment. Ten rats used as donors. The remaining 70 rats were used in the experiment. They were housed in hygienic stainless steel cages and kept in clean well ventilated room. They were fed standard pellet diet and were allowed free access to water.

*Experimental design:*

Seventy female albinos rats (8 weeks old), were divided into 5 groups. Groups I, II (n-20) while remaining groups (n-10).

*Group I (control group):* They were received no treatment and served as untreated control.

*Group II (AT-ASCs treated group):* They were received million and half of million units of AT-ASCs/single dose for each animal I.V.

The rats in groups I, II were used for the study at one week and 4 weeks with the corresponding experimental groups.

*Group III (busulfan-treated group):* In this group, the ovarian failure was induced using busulfan therapy at a dose 2mg for successive 4 days. The rats were subjected to the study one week after busulfan treatment to confirm ovarian failure.

*Group IV (busulfan treated, followed by AT-ASCs treated group):* In this group the ovarian failure was induced by the same regimen as in group III. After one week of chemotherapeutic treatment, the animals received million and half of million units of AT-ASCs/animal I.V. through tail vein, single dose. The rats were used for the study one week after the AT-ASCs injection.

*Group V (busulfan treated, followed by AT-ASCs treated group):* The rats in this group received the same regimen as group IV but they were used for the study 4 weeks after the AT-ASCs injection.

B- Methods:

*Induction of ovarian failure:*

Busulfan tablets (2mg) were crushed and dissolved in 2ml distilled water and the rats were given by a gastric gavage 2ml distilled water containing 2mg for each rat for successive 4 days [12]. This dose was calculated according to the human therapeutic dose which is 0.8mg/Kg/day every 6 hours for successive 4 days. Then the rat dose is calculated according to [13] and according to the weight of the rat.

*Isolation & culture of adipose derived stem cells (AT-ASCs) [14]:*

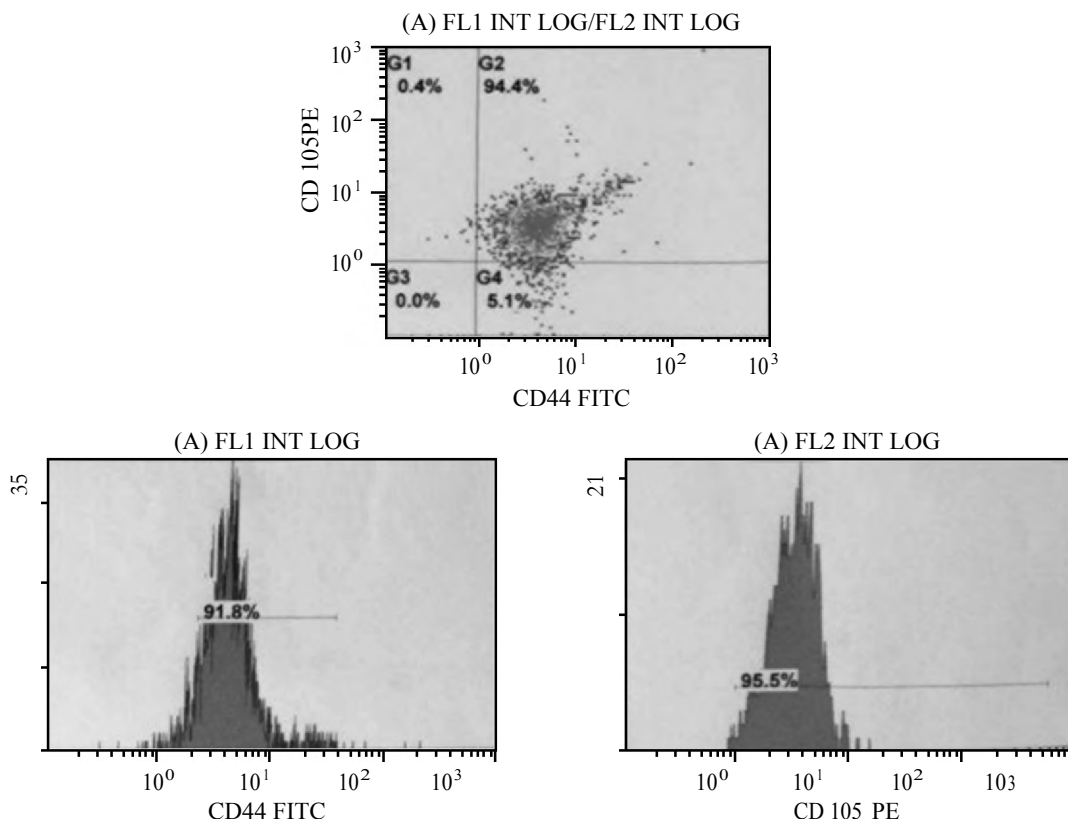
Ten rats were used as the source of ASCs. The harvested inguinal fat pads washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Next, the tissue was minced and digested with 0.1% type I collagenase (Sigma, US) in serum-free medium. The digestion was performed at 37°C for 30 to 60 minutes with gentle agitation. At the end of this procedure, the fat was completely digested and the solution became homogenous. The collagenase was neutralized by adding an equal volume of Dulbecco's Modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) with 13% fetal bovine serum (FBS) (Lonza, Verviers, Belgium) to the solution. The cell suspension was centrifuged at 1300rpm for 5min. Cell pellet was formed at the bottom of the Falcon tube and that pellet was termed the stromal vascular fraction (SVF). The supernatant was carefully removed by pipette leaving the pelleted SVF. The cell pellet was re-suspended in a 10-ml complete culture medium formed of DMEM, 13% FBS, and 1.5%

penicillin streptomycin mixture (Lonza, Verviers, Belgium). The cell suspension was cultured in culture flask 25cm<sup>2</sup> (Easy Flask, Nunc, Roskilde, Denmark) and incubated in CO<sub>2</sub> incubator (Nuair, NU 4950E, Autoflow Water Jacketed CO<sub>2</sub> incubator, USA) at 37°C and 5% CO<sub>2</sub>. The medium was replaced every 3 days, the non-adherent cells were discarded while the attached cells were washed with PBS, and the ASCs expansion was followed up by examination with inverted microscope (Axiovert 100, Zeiss-Germany). Cultured ASCs of passage 0 were used without subculture. Surface-marker expression like CD44 and CD105 was analyzed with flow cytometry. For flow cytometric analysis, adherent cells were detached by treatment with 0.25% trypsin-EDTA, neutralized with FBS-containing culture medium, and disaggregated into single cells by pipetting. The cells were incubated with mAbs for 30 minutes at 4°C, washed twice with PBS, resuspended in 0.5ml PBS, and immediately analyzed by using a flow cytometer (Becton Dickinson, US). The ASCs were prepared in a final concentration of 3x10<sup>6</sup>/ml for injection into each rat using insulin syringe.

Mesenchyma MIX 00000268, LMD: Navios

Institution:  
 Protocol: Mesenchyma MIX 00000268, PRO  
 Listmode Replay: Runtime Protocol  
 Settings File: Altered Compensation  
 Listmode File: Mesenchyma MIX 00000268, LMD

User IO: Flow Lab  
 Acquisition Time/Events: 53.8s/6582 (MANUAL)  
 Instrument SN: AW04107 Software Version: Navios 1.2



Graph (1): Flow Cytometry analysis showed that rat ASCs express CD44 & CD105.

The experimental animals were subjected to the following:

#### I- Biochemical study:

Before scarification, blood samples were taken from all rats of the studied groups by capillary tube from retro-orbital venous plexus in sterile tubes then centrifuged at 1000rpm for 5 minutes and the serum samples were measured for follicular stimulating hormones (FSH), luteinizing hormone (LH), serum estrogen level using ELISA technique at the parasitology center, Faculty of Medicine, Al-Azahr University. This was repeated for all rats just before scarification [15].

#### Statistical analysis of the biochemical study:

In this work, the results of hormonal assay were analyzed statistically to identify the significant differences between the all groups, which were determined by using one-way analysis of variance (ANOVA) followed by Post hoc test. It tested the significance of difference between more than two groups. A (*p*) value less than 0.05 was considered significant. Data were tabulated and represented graphically [16].

#### II- Histological study using:

##### 1- Light microscopy:

The animals were anaesthetized by ether inhalation, placed on supine position and the abdomen was opened by a median incision. One of both ovaries was taken and fixed by immersion in Bouin's solution for 24-48 hours. While the other one was processed for electron examination. After fixation, the ovaries were dehydrated, paraffin-embedded, serially sectioned at 5  $\mu$ m, and mounted on glass microscope slides [17]. This was performed at the histology Department, Faculty of Medicine for Girls, Al-Alzahr University.

The prepared histological slides were stained with the following:

Hematoxylin & Eosin (H&E) stain and Masson's Trichrome stain [18].

##### 2- Transmission Electron Microscopy (TEM):

Ultrastructural study of the ovary from the rats was done through the preparation of semi thin sections (stained with toluidine blue) and ultrathin sections stained with uranyl acetate, lead citrate, examined and photographed by Jeol-JEM-1100 electron microscopy. The ultrathin sections were inserted in the specimen holder. At first, low magnification (X 1000) was used to locate the specimen, and then higher magnification was used to identify the cells and evaluate the findings [18]. This was performed at the Histology Department, Faculty of Medicine for Girls, Al-Alzahr University.

## Results

#### 1- Biochemical results:

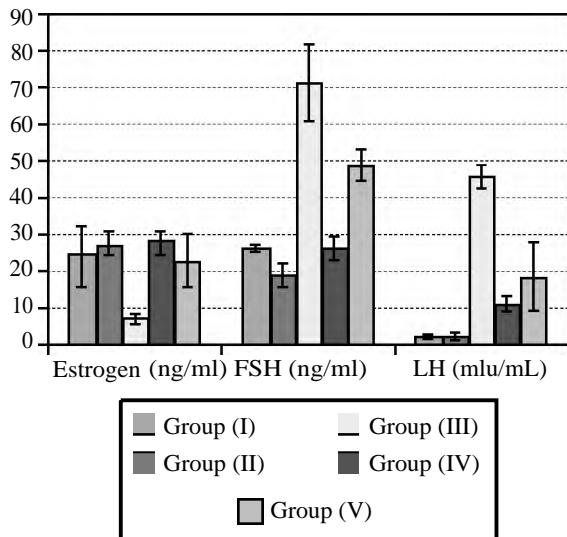
The following biochemical parameters were done to record the effect of AT-ASCs on the ovarian function. The level of FSH, LH and serum estrogen of all studied groups were recorded and statistically analyzed using ANOVA (Table 1,2 & Graph 2). There was a highly significant difference ( $p < 0.001$ ) between all the studied groups, Post hoc test showed a highly significant increase ( $p < 0.001$ ) in FSH, LH levels in the busulfan treated group in comparison with the studied groups but FSH, LH values showed a highly significant decrease ( $p < 0.001$ ) in the AT-ASCs treated group in comparison with the studied groups. The estrogen level was a highly significant decreased ( $p < 0.001$ ) in the busulfan treated group while was a highly significant increase ( $p < 0.001$ ) was reported in AT-ASCs treated group.

Table (1): ANOVA test analysis of the hormone levels (Estrogen, FSH and LH) of the female albinos rats in all the studied groups.

Parameters	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)	ANOVA	<i>p</i> -value
<i>Estrogen (ng/ml):</i>							
Mean $\pm$ SD	24.45 $\pm$ 8.37	27.35 $\pm$ 3.29	7.05 $\pm$ 1.33	28.10 $\pm$ 3.12	22.95 $\pm$ 7.22	9.930	<0.001
Range	17.2-31.7	24.5-30.2	5.9-8.2	25.4-30.8	16.7-29.2		
<i>FSH (ng/ml):</i>							
Mean $\pm$ SD	26.45 $\pm$ 0.87	19.25 $\pm$ 2.94	71.40 $\pm$ 10.51	26.40 $\pm$ 3.70	49.05 $\pm$ 4.33	12.394	<0.001
Range	25.7-27.2	16.7-21.8	62.3-80.5	23.2-29.6	45.3-52.8		
<i>LH (mIU/mL):</i>							
Mean $\pm$ SD	2.20 $\pm$ 0.35	2.12 $\pm$ 1.25	45.65 $\pm$ 2.94	11.05 $\pm$ 2.02	18.15 $\pm$ 9.18	58.943	<0.001
Range	1.9-2.5	1.03-3.2	43.1-48.2	9.3-12.8	10.2-26.1		

Table (2): Post hoc test analysis of the hormone levels (Estrogen, FSH and LH) of the female albinos rats in all the studied groups.

Parameters	Multiple Comparisons (Sig.)				
	(J) Group				
	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)
<i>Estrogen (ng/ml):</i>					
Group (I)		0.433	0	0.326	0.683
Group (II)	0.433		0	0.838	0.239
Group (III)	0	0		0	0
Group (IV)	0.326	0.838	0		0.171
Group (V)	0.683	0.239	0	0.171	
<i>FSH (ng/ml):</i>					
Group (I)		0.403	0	0.995	0.015
Group (II)	0.403		0	0.406	0.002
Group (III)	0	0		0	0.016
Group (IV)	0.995	0.406	0		0.015
Group (V)	0.015	0.002	0.016	0.015	
<i>LH (ng/ml):</i>					
Group (I)		0.403	0	0.995	0.015
Group (II)	0.403		0	0.406	0.002
Group (III)	0	0		0	0.016
Group (IV)	0.995	0.406	0		0.015
Group (V)	0.015	0.002	0.016	0.015	



Graph (2): Mean values (± SD) of hormone levels in the studied groups.

2- Light microscopic results:

Light microscopic examination of H&E ovarian sections of the group I (control group) showed the normal histological structure of the ovary. The ovary was differentiated into outer cortex and inner medulla. The cortex contained different stages of follicular development (primary and secondary follicles), with many graffian follicles bulging into the surface of the ovary.

The multilaminar primary follicle contained large oocyte which is surrounded by more than

one layer of granulosa cells while the secondary follicle was surrounded by many layers of granulosa cells with multiple fluid filled cavities. The graffian follicle was surrounded by many layers of granulosa cells and the corona radiata cells. The antrum appeared between the granulosa cells of the graffian follicle. Large masses of cells bulged into the surface of the ovary giving it the lobulated appearance, these masses called corpora lutea. The medulla of the ovary was formed of connective tissue fibers, many large blood vessels and lymphatic vessels (Figs. 1,2).

Light microscopic examination of ovarian sections of the group II showed the normal histological structure of the ovary which was nearly similar to that of the control (Figs. 1,4). But the medulla contained many large congested and dilated blood vessels (Figs. 4,5).

Light microscopic examination of ovarian sections of group III showed that the ovary became slightly smaller and irregular than the control (Figs. 1,7). The ovarian follicles became haphazardly distributed and their size decreased and became atretic and shrunken follicle. Some mature graffian follicles showed degenerated oocytes with marked reduction in the granulosa cell thickness (Figs. 7,8).

Light microscopic examination of ovarian sections of group IV showed an improvement of the general architecture of the ovary with a remarkable

decreased degenerative changes induced by busulfan, up to be similar to control (Figs. 1,10). Several growing follicles and corpora lutea were observed (Fig. 10). Some mature graafian follicles were nearly similar to control (Figs. 1,2,10,11). The blood vessels still congested (Fig. 10,11).

Light microscopic examination of ovarian sections of group V showed that the general architecture of the ovary was nearly similar to control (Figs. 1,2,13,14) but many corpora lutea were noticed in related to the ovarian follicles and some of the granulosa cells had pyknotic nuclei (Fig. 13,14).

Masson's trichrome stain sections of the ovary of the group I and II showed a normal distribution of the collagen fibers in between the ovarian follicles and in the medulla (Figs. 3,6). Sections examined from group III showed an increased distribution of the collagen fibers in between the ovarian follicles and in the medulla (Fig. 9). Sections examined from group IV and V showed a decreased collagen fiber distribution as compared with group III (Figs. 9,12,15).

### 3- Electron microscopic results:

Electron microscopic examination of the ovarian sections of group I and group II showed the graafian follicle which contains part of the oocyte with a regular and round euchromatic nucleus and regular nuclear envelope. The oocyte was surrounded by zona pellucida, many layers of granulosa cells and the theca folliculi cells. The zona pellucida contained microvilli of the oocyte and cell processes of the granulosa cells (Figs. 16,19). The granulosa

cell was cuboidal in shape with oval, intended and euchromatic nucleus (Figs. 17,20). The theca folliculi cells arranged in many layers and differentiated into theca interna and theca externa, the theca interna cells arranged in more than one layer with oval nuclei while the theca externa formed of flat cells with elongated nuclei (Figs. 18,21).

Electron microscopic examination of the ovarian sections of the group III showed part of graafian follicle which showed segmentation of the oocyte (Fig. 22). The microvilli were hardly seen in the zona pellucida (Fig. 22). Many of the granulosa cells were degenerated and irregularly arranged around the oocyte (Fig. 23). The cells of theca folliculi arranged in many layers, their nuclei were shrunken with marginal chromatin condensation (Fig. 24).

Electron microscopic examination of the ovarian sections of the group IV showed an improvement in the changes that occurred in the oocyte of the graafian follicle. Also the changes in the zona pellucida, granulosa cells and theca cells were improved and became nearly similar to the control (Figs. 16,25). The granulosa cell was cuboidal in shape with oval, intended nucleus like the control one (Figs. 17,26). The architecture of the theca cells became nearly similar to normal. It differentiated into theca interna and externa (Figs. 18,27).

Electron microscopic examination of the ovarian sections of the group V showed a nearly normal architecture of the graafian follicle (Figs. 28,29) but there were some changes in the theca folliculi cells (Fig. 30).

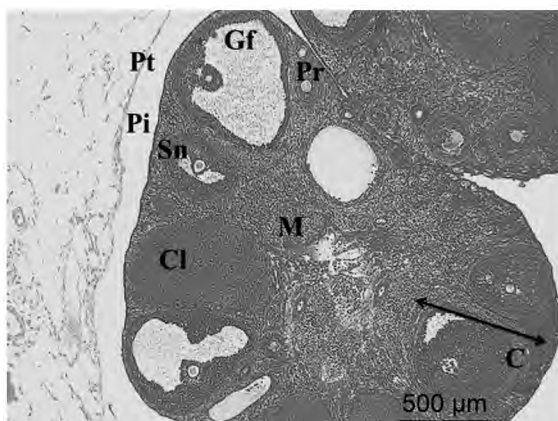


Fig. (1): A photomicrograph of section in the ovary of group I shows different stages of follicular development in the cortex (C) (arrow) primary (Pr), secondary (Sn), graafian (Gf) follicles and corpus luteum (Cl). The medulla (M) shows the blood vessels and lymphatics. The peritoneal capsule (Pt) surrounds the ovary and separated from it by the periovarian space (Pi). (Hx and E X40).

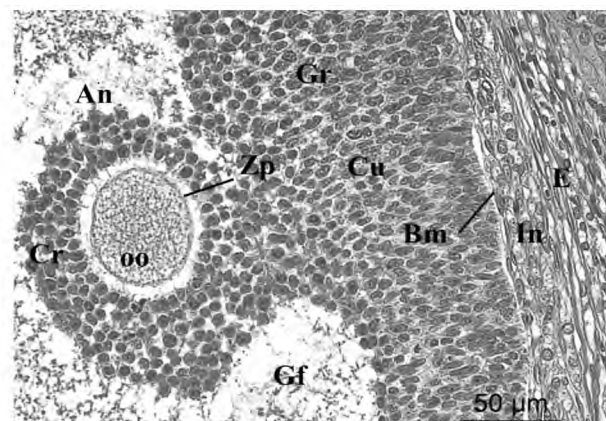


Fig. (2): Higher magnification of the previous Fig. (1) shows the graafian follicle (Gf) which consists of a large oocyte (oo), surrounded by zona pellucida (Zp) and many layers of granulosa cells (Gr). The antrum (An) appears between the granulosa cells. The granulosa cells differentiated into corona radiata cells (Cr) and the cumulus oophorus (Cu). The granulosa cells are surrounded by clear basal membrane (Bm) and the theca folliculi which differentiates into 2 layers: The theca interna (In) and theca externa (E). (Hx and E X400).



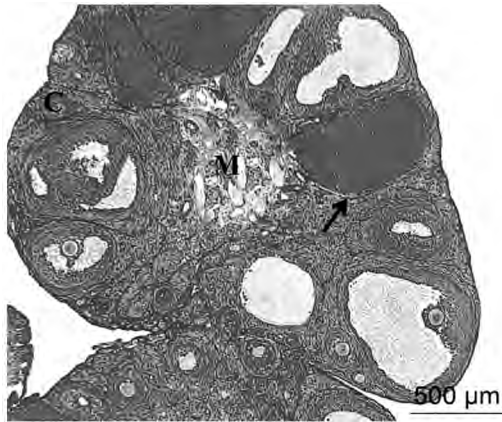


Fig. (3): A photomicrograph of section in the ovary of group I shows the normal deposition of collagen fibers (arrow) between the follicles in the ovarian cortex (C) and in the ovarian medulla (M). (MTC X40).

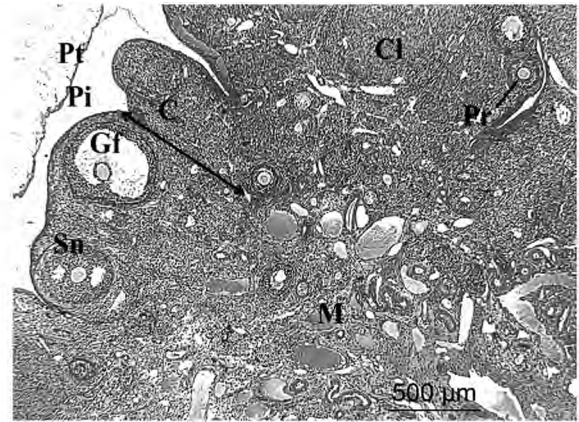


Fig. (4): A photomicrograph of section in the ovary of group II shows different stages of follicular development in the cortex (C) (arrow) primary (Pr), secondary (Sn), graffian (Gf) follicles and corpus luteum (Cl). The medulla contains many large congested blood vessels. The peritoneal capsule (Pt) surrounds the ovary and separated from it by the periovarian space (Pi) (Hx and E X40).

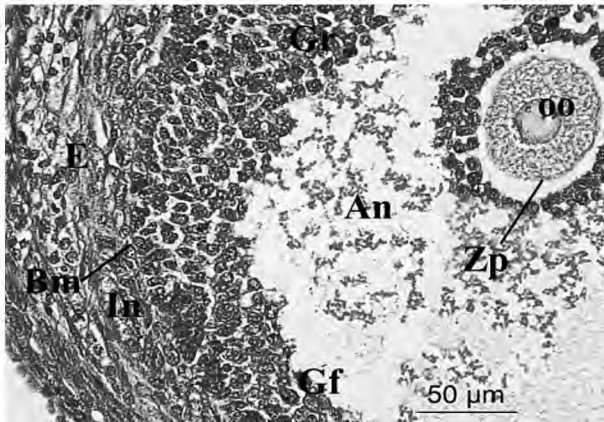


Fig. (5): Higher magnification of the previous Fig. (4) shows the graffian follicle (Gf) which consists of a large oocyte (oo), surrounded by zona pellucida (Zp) and many layers of granulosa cells (Gr). The antrum (An) is noticed. The granulosa cells are surrounded by a clear basal membrane (Bm) and theca folliculi which differentiate into 2 layers: The theca interna (In) and theca externa (E) (Hx and E X400).

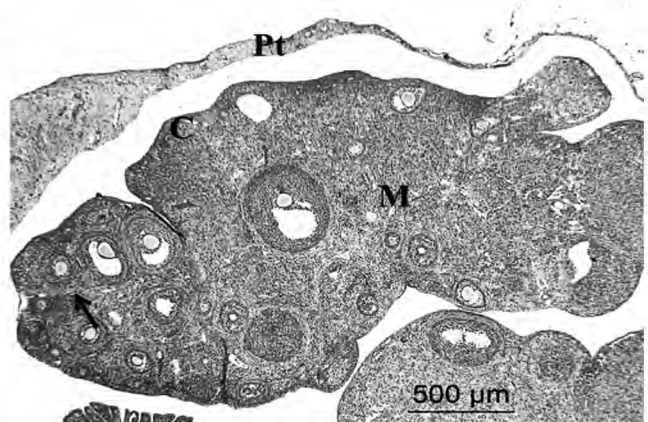


Fig. (6): A photomicrograph of section in the ovary of group II shows the normal deposition of collagen fibers (arrow) between the follicles in the ovarian cortex (C) and in the ovarian medulla (M). The peritoneal capsule (Pt) is noted containing some collagen fibers (MTC X40).

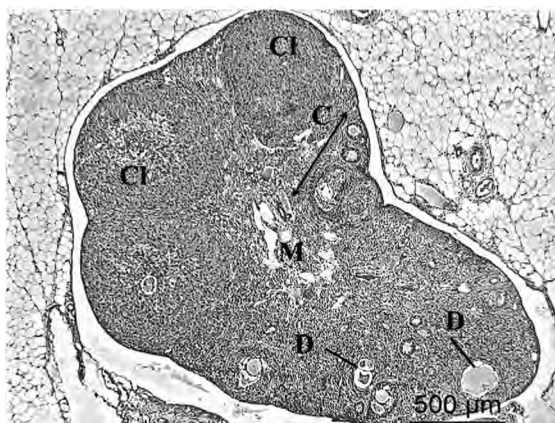


Fig. (7): A photomicrograph of section in the ovary of group III shows the different stages of follicular development in the cortex (C) (arrow), many of them are degenerated (D). Many corpora lutea (Cl) can be detected, some of them are congested. The medulla (M) contains many dilated and congested blood vessels (Hx and E X40).

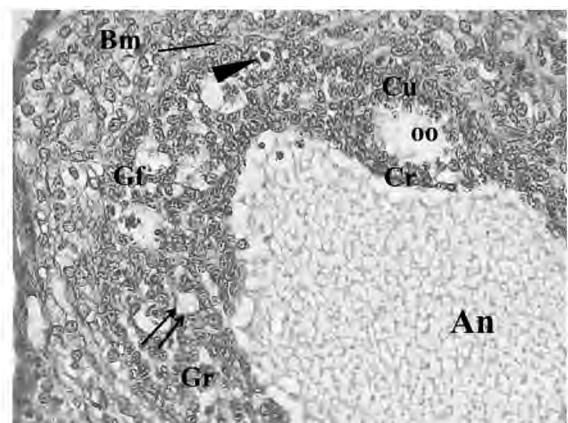


Fig. (8): Apart of pervious Fig. (7) is magnified to show degenerated graffian follicle (Gf) which contains degenerated oocyte (oo) surrounded by the granulosa cells (Gr) which are degenerated and irregularly arranged around the oocyte. Some of the granulosa cells have pyknotic nuclei (head of arrow) and areas of vacuolation of different sizes (2 arrows). They are surrounded by deformed basement membrane (Bm). Notice that the thickness of the granulosa cells decreases. The antrum (An) is noticed. (H and E X400).

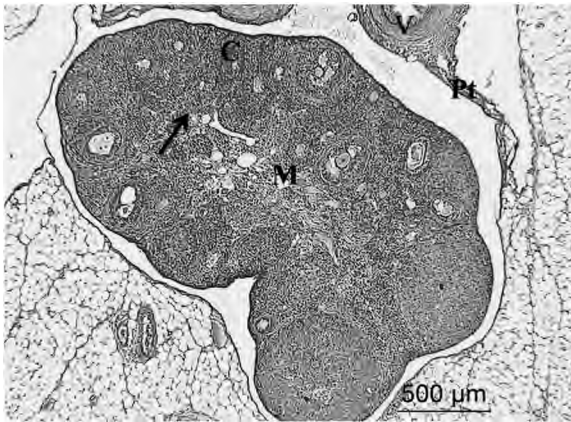


Fig. (9): A photomicrograph of section in the ovary of group III shows increased deposition of collagen fibers (arrow) between the ovarian follicles in the cortex (C) and in the medulla (M). The peritoneal capsule (Pt) is noted containing some collagen fibers (MTC X40).

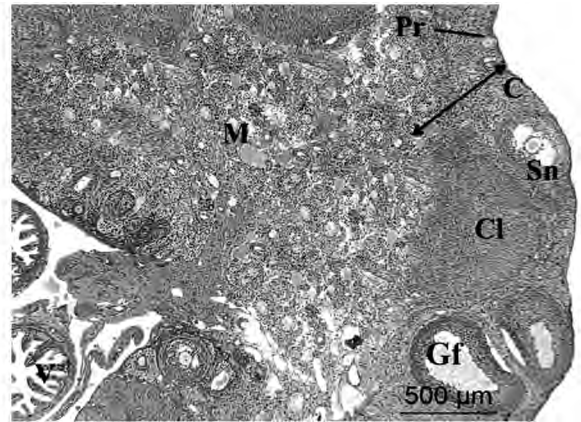


Fig. (10): A photomicrograph of section in the ovary of group IV shows different stages of follicular development in the cortex (C) (arrow) (primary (Pr), secondary (Sn), graffian (Gf) follicles and corpus luteum (Cl)). The medulla contains many congested blood vessels (Hx and EX40).

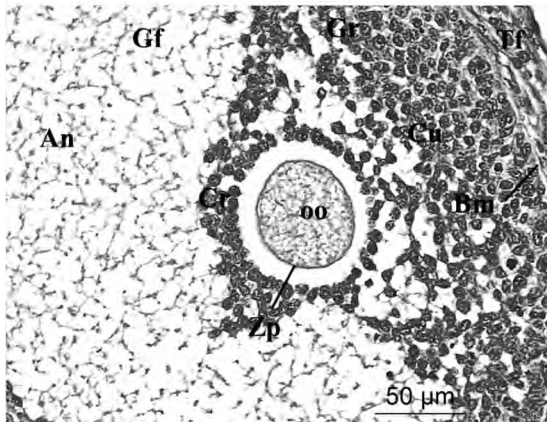


Fig. (11): Higher magnification of the previous Fig. (10) shows the graffian follicle (Gf) which consists of a large oocyte (oo), surrounded by a clear zona pellucida (Zp) and many layers of granulosa cells (Gr). The antrum (An) is noticed. The granulosa cells differentiated into corona radiata cells (Cr) and the cumulus oophorus (Cu). The granulosa cells are surrounded by clear basal membrane (Bm) and the theca folliculi (Tf) (Hx and E X400).

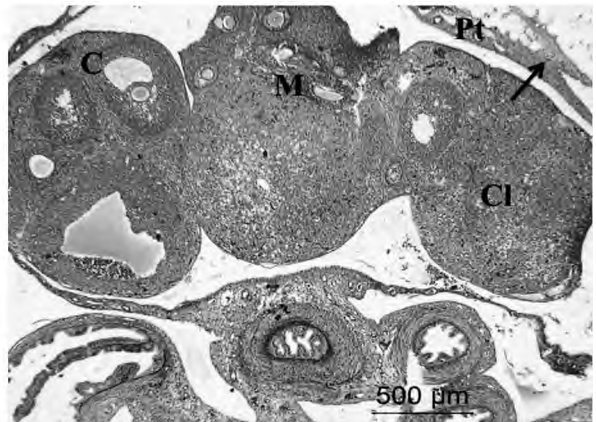


Fig. (12): A photomicrograph of section in the ovary of group IV shows a minimal deposition of collagen fibers (arrow) between the follicles in the ovarian cortex (C) and in the ovarian medulla (M). The peritoneal capsule (Pt) is noted with some collagen fibers (MTC X40).

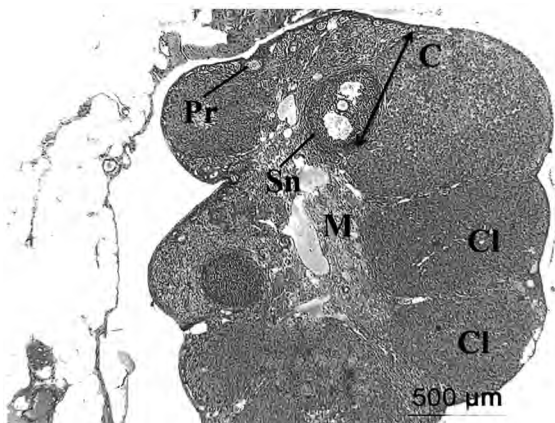


Fig. (13): A photomicrograph of section in the ovary of group V shows different stages of follicular development in the cortex (C) (arrow) primary (Pr) and secondary (Sn) follicles. Notice the presence of increased number of the corpora lutea (Cl) in comparison with the ovarian follicles. The medulla contains many congested blood vessels (Hx and E X40).

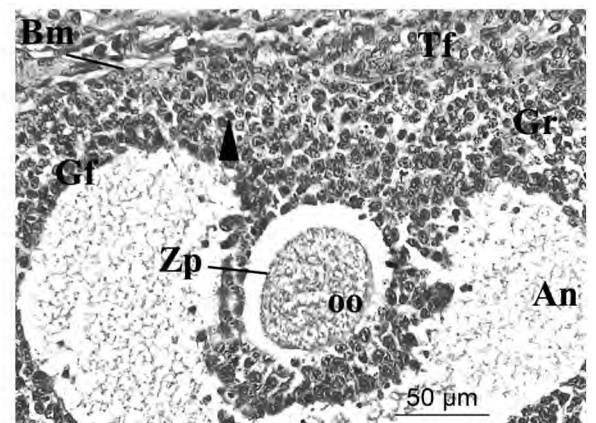


Fig. (14): A part of pervious Fig. (13) is magnified to show the graffian follicle (Gf) which consists of a large oocyte (oo), surrounded by zona pellucida (Zp) and several layers of granulosa cells (Gr). Some of the granulosa cells having pyknotic nuclei (Head of arrow). The granulosa cells surrounded by a basal membrane (Bm) and the theca folliculi cells (Tf). The antrum (An) is noticed. (Hx and E X400).



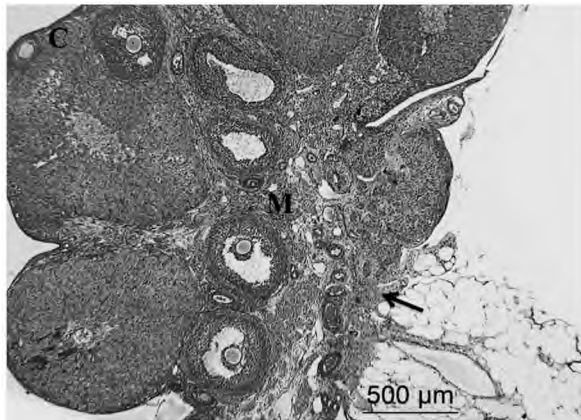


Fig. (15): A photomicrograph of section in the ovary of group V shows a minimal deposition of collagen fibers (arrow) between the follicles in the ovarian cortex (C) and in the ovarian medulla (M) (MTC X40).

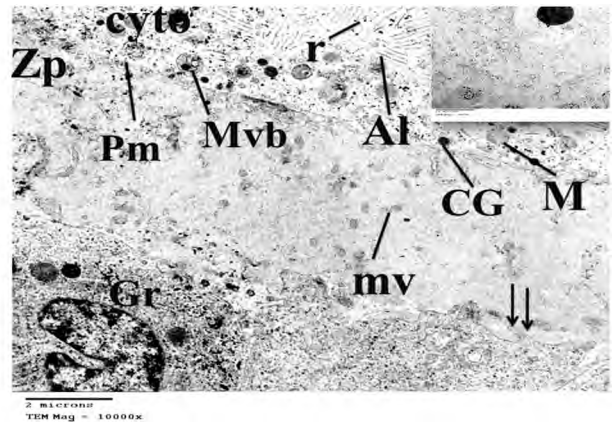


Fig. (16): An electron micrograph of section of a part of the graafian follicle of group I shows a part of the oocyte. The cytoplasm of the oocyte (cyto) which contains abundant cell organelles like mitochondria (M) and annulate lamellae (AL), ribosomes (r), cortical granules (CG) and multivesicular bodies (Mvb). The oocyte has a clear plasma membrane (Pm). Thick layer of zona pellucida (Zp) containing microvilli (mv) of the oocyte and cell processes of the granulosa cells (Gr) (filopodia) (2 arrow). Part of a granulosa cell is noticed (TEM X 10000).

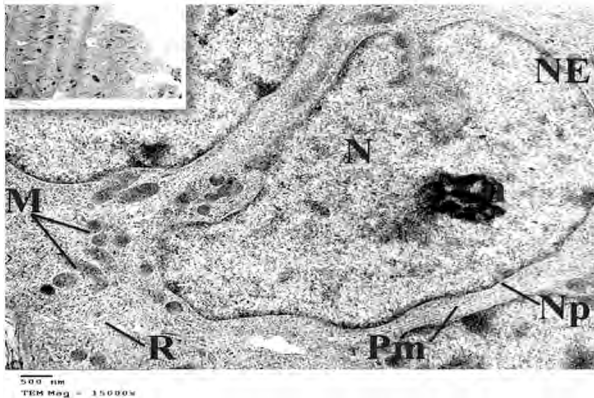


Fig. (17): An electron micrograph of section of a part of the graafian follicle of group I to show the granulosa cell which is cuboidal in shape and surrounded by a clear plasma membrane (Pm). The nucleus (N) is oval, intended, euchromatic and surrounded by a nuclear envelope (NE) that containing nuclear pores (Np). The nucleus also contains electron dense nucleolus (n). The cytoplasm contains mitochondria (M) and rough endoplasmic reticulum (R) (TEM X 15000).

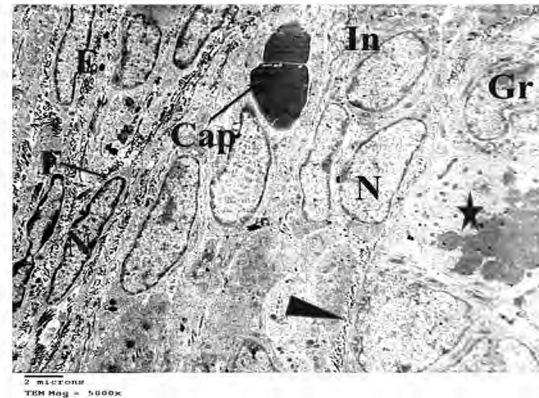


Fig. (18): An electron micrograph of section of a part of the graafian follicle of group I shows the theca folliculi cells which are separating from the granulosa cells (Gr) by the basal lamina (head of arrow). The theca folliculi are differentiated into theca interna (In) and theca externa (E). Blood capillary (Cap) and collagen fibers (F) are seen. one of the cells shows mitotic figure (\*) (TEM X 5000).

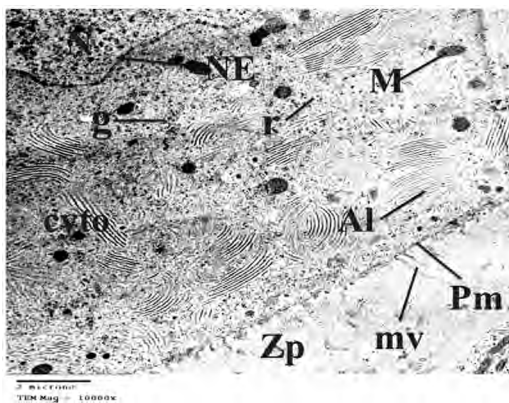


Fig. (19): Electron micrograph of a part of the graafian follicle of group II shows a part of the oocyte which is surrounded by a plasma membrane (Pm) and a zona pellucida (Zp) which contains microvilli (mv). Part of nucleus (N) of the oocyte is observed, surrounded by nuclear envelope (NE). The cytoplasm of the oocyte contains mitochondria (M), annulate lamellae (AL), ribosomes (r) and glycogen particles (g) (TEM X 10000).

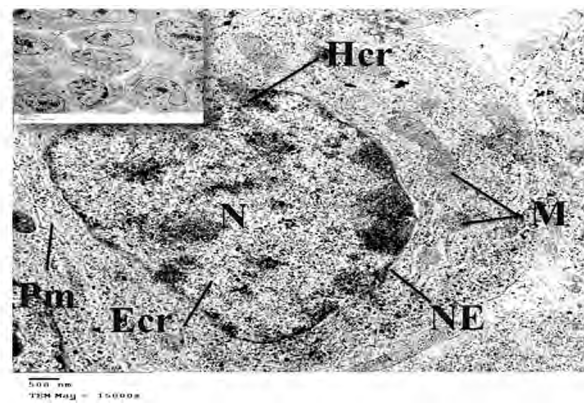


Fig. (20): An electron micrograph of section of a part of the graafian follicle of group II shows one of the granulosa cells which is cuboidal in shape and surrounded by plasma membrane (Pm). The nucleus (N) is rounded, contains two types of chromatin: Heterochromatin (Hcr) and euchromatin (Ecr) and is surrounded by nuclear envelope (NE). The cytoplasm contains many mitochondria (M) of different sizes and shape (TEM X 15000).



Fig. (21): Electron micrograph of section of the graffian follicle of group II shows the theca folliculi cells which are separating from the granulosa cells (Gr) by the basal Lamina (Head of arrow). The theca folliculi differentiate into theca interna (In) and theca externa (E) ((TEM X 5000).

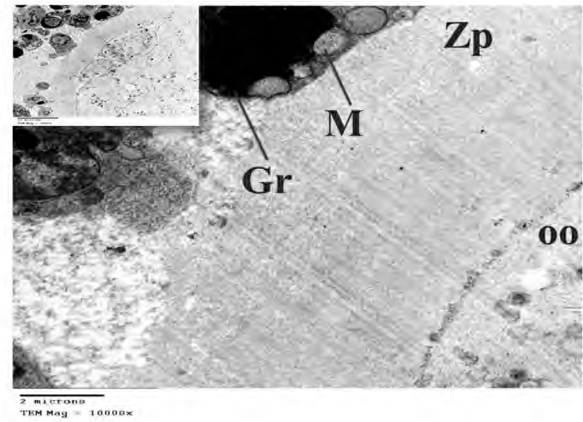


Fig. (22): An electron micrograph of section of the graffian follicle of group III shows part of oocyte (oo) which is surrounding by the zona pellucida (Zp) and the granulosa cells (Gr). The microvilli are hardly seen in the zona pellucida. The surrounding granulosa cells show condensed shrunken nucleus (Pyknotic nucleus) and many mitochondria (M) which become swollen and lose their cristae (TEM X 10000).

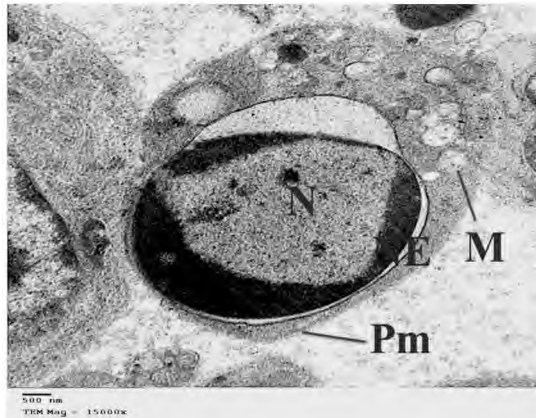


Fig. (23): An electron micrograph of section of the graffian follicle of group III shows one of the degenerated granulosa cells which is characterized by shrunken nucleus (N) with marginal chromatin condensation and dilated nuclear envelope (NE). Its cytoplasm contains are swollen and vacuolated mitochondria (M) . The plasma membrane (Pm) is hardly seen (TEM X 15000).



Fig. (24): An electron micrograph of section of the graffian follicle of group III shows the theca folliculi cells (Tf) which arrange in many layers. Their nuclei (N) are shrunken with marginal chromatin condensation .Their cytoplasm contains multiple vacuoles (V) and lipid droplets (L) (TEM X 5000).

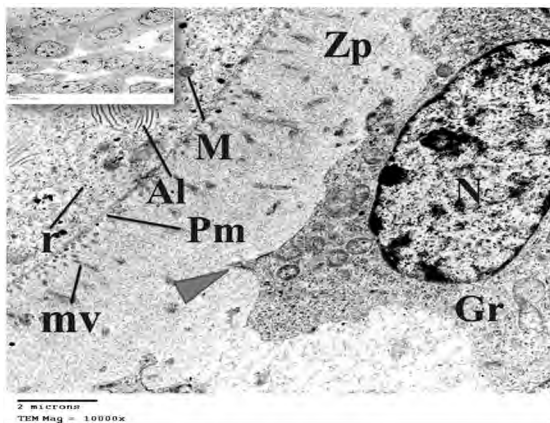


Fig. (25): An electron micrograph of section of the graffian follicle of group IV shows a part of the oocyte. The cytoplasm of the oocyte that contains mitochondria (M), ribosomes (r) and annulate lamellae (AL). The oocyte is surrounded by thick layer of zona pellucida (Zp) containing microvilli (mv) and cell processes (head of arrow) of the granulosa cells (Gr). Granulosa cell (Gr) is noticed with oval nucleus (N). (TEM X10000).

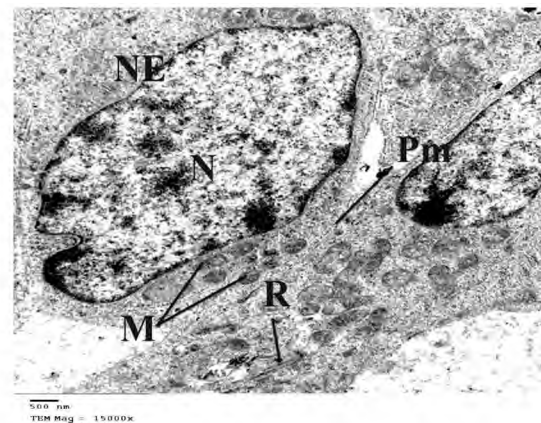


Fig. (26): An electron micrograph of section of the graffian follicle of group IV shows one of the granulosa cells. It is cuboidal in shape and has a plasma membrane (Pm). The nucleus (N) is oval, indented, euchromatic and is surrounded by a nuclear envelope (NE). The cytoplasm contains mitochondria (M) and rough endoplasmic reticulum (R) (TEM X 15000).

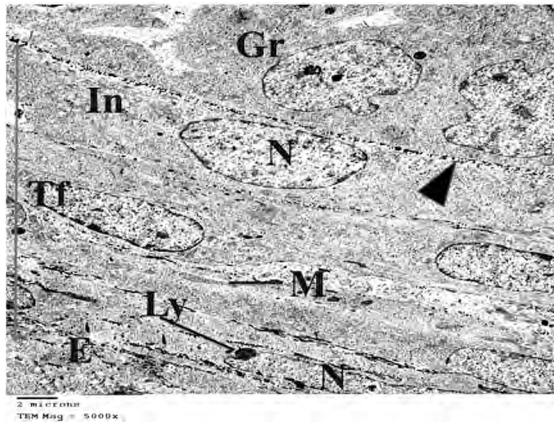


Fig. (27): An electron micrograph of part of graffian follicle of group IV shows the theca folliculi cells (Tf) which are separating from the granulosa cells (Gr) by the basal lamina (arrow head). The theca folliculi differentiate into theca interna (In) and theca externa (E). The Theca interna cells have oval nuclei (N) while Theca externa (E) have elongated nuclei (N). Mitochondria (M) and lysosomes (Ly) distribute thoroughly the cytoplasm (TEM X 5000).

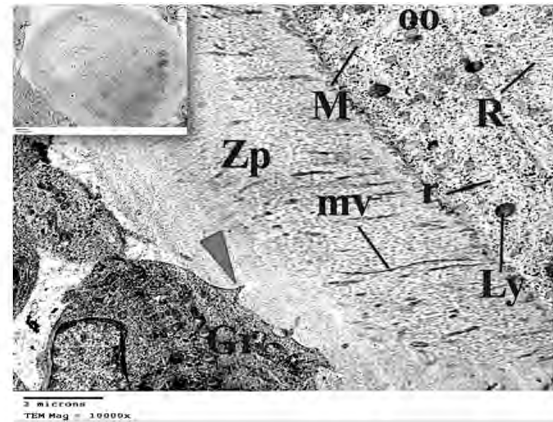


Fig. (28): An electron micrograph of section of the graffian follicle of group V shows a part of the oocyte. The cytoplasm of oocyte (oo) which contains mitochondria (M), lysosome (Ly), ribosomes (r) and rough endoplasmic reticulum (R). Thick layer of zona pellucida (Zp) is seen containing microvilli (mv) and cell processes (head of arrow) of the granulosa cells (Gr). Part of the granulosa cells (Gr) are seen (TEM X 10000).

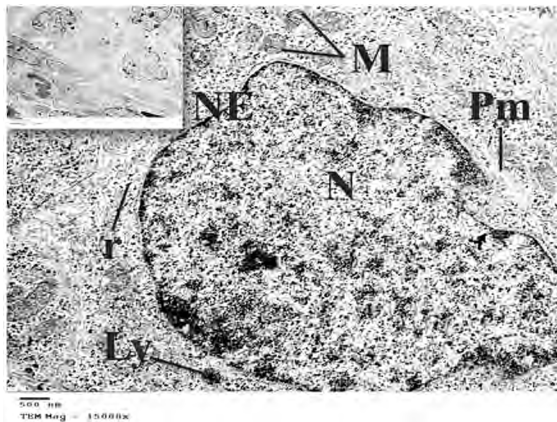


Fig. (29): An electron micrograph of section of the graffian follicle of group V shows one of the granulosa cells. It is cuboidal in shape and has a plasma membrane (Pm). Its nucleus (N) is oval, indented, euchromatic and surrounded by a nuclear envelope (NE). The cytoplasm contains mitochondria (M), lysosomes (Ly) and ribosomes (r) (TEM X 15000).



Fig. (30): An electron micrograph of part of graffian follicle of group V shows the theca folliculi cells (Tf) which are separating from the granulosa cells (Gr) by the basal lamina (arrow head). The theca folliculi differentiated into theca interna (In) and theca externa (E). The Theca interna cells have oval nuclei and contain lipid droplets (L). The cells of theca externa are flat cells with elongated nuclei. Some of the theca externa cells have small electron dense nucleus (2 arrows). Blood capillary (Cap) between theca cells. Lamellar body is observed (green arrow) in the theca interna (TEM X 3000).

## Discussion

The premature ovarian failure is important sequelae of previous exposure to chemotherapy and radiotherapy during childhood and adulthood as mentioned by some authors [19].

The use of stem cells for ovarian regeneration and oocyte production had been proposed as future clinical therapies for treating infertility in women. These findings were in agreement with some authors [8] who reported that the stem cells had attracted interest for their possible use for both cell and gene therapies.

In the present study, the experiment was done by using a rat model of busulfan induced ovarian failure: Confirmation of ovarian failure was assessed in the present study by measurement of serum levels of estrogen, FSH and LH. This came in agreement with a study of some authors [20] who mentioned that the blood tests that used to establish the levels of estrogen, FSH and LH were considered a reliable assessment of reproductive life span of the ovaries and estimation of fertility status.

In this study, a significant increase in FSH, LH levels were reported in the busulfan treated group

but FSH, LH values showed significant decrease in the AT-ASCs treated group. The estrogen level showed significant decrease in the busulfan treated group while a significant increase was reported in AT-ASCs treated group. These findings were in agreement with the authors [21] who explained that the chemotherapeutic agents would damage granulosa cells production. The ovaries produced little to no estrogen in ovarian failure, resulting in loss of the negative feedback system to the hypothalamus and pituitary glands. Thus, the pituitary glands produced elevated levels of FSH and LH.

By light microscopic examination in the current study, a remarkable observation in busulfan treated group was massive loss of follicles and the presence of many degenerated follicles. These findings came in agreement with authors [22] who reported that the busulfan affected the gonadal function in children and adults. In their study, they identified 29 girls who received busulfan in the prepubertal or pubertal period; 26 had signs of ovarian failure.

Also, many authors [23,24] explained these findings and indicated that DNA double-strand breaks were detectable in oocytes of small follicles within cultured mouse ovarian tissue treated with phosphoramidate mustard, the active metabolite of busulfan.

Also some authors [25] confirmed these findings and reported the effect of busulfan on different organs such as the heart, lungs, liver, pancreas and mentioned several pathological changes in the cells as pyknosis of the nuclei and vacuolation of their cytoplasm.

In the AT-ASCs treated group in this study, an improvement of the general architecture of the ovary with remarkable observation of decreased degenerative changes induced by busulfan, nearly similar to control group. The previous results also confirmed by the authors [26] who mentioned that the transplantation of AT-ASCs into the ovaries resulted in the induction of angiogenesis and increased counts of the ovarian follicles.

Also, some researchers [27] confirmed the obtained results and described the role of stem cells, they reported that AT-ASCs were known to secrete a number of cytokines, including high levels of angiogenic growth factors, such as HGF (hepatocyte growth factor), VEGF (vascular endothelial growth factor), PGF (placental growth factor), and TGF- $\beta$ . AT-ASCs, also exhibited moderate expression of FGF (fibroblast growth factor)-2 and Ang (angiopoietin)-1, as well as low levels of Ang-2.

In the present work, congestion and dilatation of blood vessels were noticed in the AT-ASCs treated group. These findings might relate to new vascularization that were in agreement with the authors [28] who said that the MSCs had the ability to differentiate into mural cells (pericytes or smooth muscle cells) *in vitro*. The mural cells made up the surrounding layer of blood vessels and were therefore crucial for stabilization of these vessels. There was some evidence that MSCs express angiogenic factors.

In this study, the AT-ASCs treated group that examined after 4 weeks regression in the improvement of the architecture of the ovary was noticed. These results were in agreement with the authors [27] who counted the ovarian follicles and found that after one week of AT-ASCs administration, the number of ovarian follicles was increased and became near to control. While after one month, the number of ovarian follicles was less than the group that examined after one week.

By electron microscopic examination in the present work, the busulfan treated group showed segmentation of the oocyte. This findings was in agreement with the authors [29] who described the general morphology of segmented oocytes that occur during follicular atresia and reported that nucleated and un-nucleated segments might occur in the same oocyte. Also, the microvilli were hardly seen in the zona pellucida in this research work. This finding confirmed by some researchers [30] who reported that during the follicular atresia, the granulosa cells were very condensed and lost the contact with oocytes or neighboring cells.

In the present work, the busulfan treated group showed that many of the granulosa cells were degenerated and irregularly arranged around the oocyte. These findings confirmed by the authors [31] who noticed shrinkage of the granulosa cells and condensation of the chromatin into dense masses which were signs for degeneration.

In the present work, the busulfan treated group showed remnants of some cytoplasmic organelles of the granulosa cells with dilated and destructed rough endoplasmic reticulum, swollen mitochondria with destruction of their cristae. These findings were explained by some authors [32] who suggested that mitochondrial apoptotic pathway was initiated either by damaging stimuli such as oxidative stress or DNA damage by DNA alkylation.

In this experiment, the AT-ASCs treated group showed general improvement in the changes oc-



curred in the graffian follicles. The changes in the oocyte, zona pellucida, granulosa cells and theca cells were improved and became nearly similar to normal. These findings with in agreement with some researchers [33] who noticed that transmission electron microscope analysis of ovarian tissue in the rat that received stem cells revealed that cell-cell connections via cytoplasmic extensions, cell junctions; zonula occludens, zonula adherens and gap junctions, led to the formation of well-organized ovarian follicles.

Also in this study, the AT-ASCs treated group that examined after 4 weeks, some changes in the theca folliculi cells was noticed. So, in the future, after this research work transplantation of a second dose of stem cells for long term survival was suggested and that was confirmed by other authors [34] who concluded that a second allogeneic hematopoietic cell transplant had an important potential role in treating relapse after failing allogeneic transplant in selected patients.

The findings in this study suggested potential therapeutic effects for ovarian dysfunction by intravenous injection of AT-ASCs. The transplant of those cells may have a role in restoring damaged ovarian tissue. The need for repeated injection of AT-ASCs is recommended.

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## دور الخلايا الجذعية على فشل المبيض المستحدث بعقار البوسولفان في أنثى الجرذ الأبيض

الخلفية: تم إجراء العديد من الأبحاث حول العلاج بالخلايا الجذعية في علاج فشل المبيض المبكر. وقد تم مؤخراً تحديد النسيج الشحمي كواحد من المصادر البديلة للخلايا الجذعية المقيمة متعددة القدرات في البشر. إن تلف الجهاز التناسلي هو واحد من أكثر الآثار المدمرة للعلاج الكيميائي، والذي غالباً ما يرتبط بالفتيات الصغيرات والنساء اللواتي تقل أعمارهن عن أربعين عاماً. يمتلك البوسولفان نطاقاً واسعاً من الاستخدام فمثلاً في نظام التكيف قبل إجراء عملية زرع الخلايا السلفية المكونة للدم لسرطان الدم النخاعي المزمن وفي المرضى الذين يخضعون لزرع النخاع العظمي.

هدف الدراسة: الهدف من الدراسة هو تقييم تأثير إعطاء الخلايا الجذعية المشتقة من الأنسجة الدهنية على فشل المبيض الناجم عن البوسولفان في أنثى الجرذ الأبيض.

المواد والطرق المستخدمة في البحث: تم تقسيم سبعين من الجرذان الإناث (والتي تبلغ ثمانية أسابيع من العمر)، إلى 5 مجموعات. المجموعة (1) (المجموعة الضابطة): لم يتلقوا أي علاج وعملوا كمجموعة غير معالجة وتشمل هذه المجموعة عشرون جرذاً. المجموعة (2): تم إعطائهم الخلايا الجذعية المشتقة من الأنسجة الدهنية. تلقت الحيوانات مليون ونصف مليون وحدة من الخلايا الجذعية عن طريق الحقن الوريدي جرعة واحدة وتشمل هذه المجموعة عشرون جرذاً أيضاً. المجموعة (3) (المجموعة المعالجة بالبوسولفان): في هذه المجموعة تم إحداث فشل المبيض باستخدام علاج البوسولفان بجرعة 2 ملغ لمدة أربعة أيام متتالية وتشمل هذه المجموعة عشرة من الجرذان. المجموعة (4) (عوملت هذه المجموعة بالبوسولفان) ثم الخلايا الجذعية المشتقة من الأنسجة الدهنية: في هذه المجموعة تم إحداث فشل المبيض بنفس النظام كما في المجموعة الثالثة. بعد أسبوع واحد من العلاج الكيميائي، تلقت الحيوانات مليون ونصف مليون وحدة من الخلايا الجذعية المشتقة من الأنسجة الدهنية عن طريق الحقن الوريدي. تمت التضحية بالفئران بعد أسبوع واحد من الحقن. وتشمل هذه المجموعة عشرة من الجرذان مجموعة (5) تلقت الفئران في هذه المجموعة العلاج بنفس نظام المجموعة الرابعة ولكن تم التضحية بها بعد 4 أسابيع من الحقن وتشمل هذه المجموعة عشرة من الجرذان.

الدراسة البيوكيميائية: تم أخذ عينات الدم من جميع الفئران من الضفيرة الوريدية المدارية الرجعية وتم قياس عينات المصل لمستوى هرمون LH، FSH، والأستروجين باستخدام تقنية ELISA تم إعداد قطاعات البارافين لمبيض الفئران وصبغها بصبغات اتش وإي والماسون ترائ كروم للدراسة بالميكروسكوب الضوئي كما تم إعداد عينات من المبيض للدراسة بالميكروسكوب الإلكتروني.

النتائج: أن إعطاء الخلايا الجذعية المشتقة من الأنسجة الدهنية أحدث تحسناً واضحاً في التغيرات النسيجية المرتبطة بفشل المبيض الناتج عن البوسولفان وقد ظهر هذا التحسن واضحاً بعد أسبوع من العلاج بالخلايا الجذعية، ولكن بعد 4 أسابيع هناك بعض التراجع في هذا التحسن.

الخلاصة: يمكن لزرع الخلايا الجذعية المشتقة من الأنسجة الدهنية تحسين ضرر المبيض الناجم عن إعطاء البوسولفان. كما أوضحت النتائج أن هناك ضرورة لجرعة ثانية من الخلايا الجذعية.