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Original Article

Effect of Cyclophosphamide on the Postnatal Development of the Ovary of the Albino Rat

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ABSTRACT

Background: Cyclophosphamide is the most widely used alkylating agent in the treatment of haematological malignancies. On the other hand, cyclophosphamide has adverse effects on the fertility of both sexes.

Aim of the work: The present work was done to study the histological and stereological changes in the developing ovary of the albino rat after a period of treatment with cyclophosphamide and the effect of rehabilitation after stopping the drug.

Materials and Methods: Thirty-six immature female rats (newborn, one week, two weeks, one-and-half month and two- and-half month) were used in this study. They were subdivided into two groups. The control group (n=24) was injected intraperitoneally with dextrose 5%. The experimental group (n= 12) at the age of two weeks, received 100mg/ kg body weight cyclophosphamide intraperitoneally once weekly for four successive weeks. At the end of the treatment period this group was subdivided into two subgroups: subgroup A (treated group) including the rats at the age of one- and-half month and subgroup B (rehabilitated group) including the rats at the age of two-and-half month which received the same drug regimen, but sacrificed after another four weeks without treatment. The specimens of all age groups were processed and stained with Heamatoxylin and Eosin. Other specimens at the age of one- and- half month were processed for semi-thin and ultra-thin examination. In the two age groups (control and treated) morphometeric measurements were performed.

Results: The study showed shrinkage with decrease in the number and the size of the antral follicles, apoptosis and disorganization in the granulosa cell layers. Ultrastucturally, the most important feature observed was the fragmentation and leakage of the cellular nuclear materials of the granulosa cells and their clumping in the antrum.

Morphometeric measurements showed a very highly significant reduction in the average follicular and oocytic diameter of the antral follicles and in the number of the healthy antral follicles while there was high significant reduction in the number of atretic antral follicles. In the rehabilitated group the follicles were less affected. Oocytes were affected in both groups; they were either vacuolated or totally absent. Morphometeric measurements showed highly significant reduction of the average oocytic diameter and in the average number of the granulosa cell layers.

Key Words: Cyclophosphamide, ovarian follicle, ovary, immature rats, granulosa cells.

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INTRODUCTION

Cyclophosphamide is an alkylating agent and of nitrogen mustard derivative that reacts with strong oxidizing agents. It is the most widely used alkylating agent in the treatment of haematological malignancies and a variety of solid tumors *(Demire et al., 1996–a,b; Rao et al., 2005)*.

Cyclophosphamide's therapeutic mechanism of action is to induce DNA damage (*Coppola & Ghibelli, 2000)*. It works through the covalent bonding of the highly reactive alkyl groups of nucleic acids *(Brock et al., 1988; Rooseboom et al., 2004)*. By this way, cyclophosphamide prevents cell division primarily by cross-linking the DNA strands *(Ross, 1962; Colvin et al., 1976; Chabner et al., 2006)* that result in inhibition of DNA synthesis and cell death *(Tew et al., 1996)*.

Cyclophosphamide may be used alone or with other anticancer drugs to treat malignant lymphoma, multiple myeloma, leukemia, non-Hodgkin's lymphoma, Burkitt's lymphoma, carcinoma of the lung, breast, cervix and ovary, mycosis fungoides and neuroblastoma *(Chabner et al., 2006)*. It is also used as an immunosuppressive agent following organ transplant or to treat autoimmune disorders as nephrotic syndrome in children *(Chabner et al., 2001)*.

On the other hand, cyclophosphamide has adverse effects on the fertility of both sexes. In females, the rate of amenorrhea following the chemotherapy varies based on the age *(Meirow et al., 2001)*. Cyclophosphamide exposure prevents folliculogenesis by causing anovulation and results in infertility *(Ray & Potu, 2010)*. Moreover, toxicity can occur through impairment of the follicular maturation and/or depletion of the follicles *(Bines et al., 1996; Blumenfeld, 2002)*.

The aim of this work was to study the morphological and stereological effects of cyclophosphamide on the ovary of the immature albino rat and the ovarian rehabilitation after the withdrawal of this drug.

MATERIALS AND METHODS

In this study, a total number of 36 immature female albino rats were used. The animals were divided into two groups, the control (*n*= 24) and the experimental (*n*= 12). They were obtained from the animal house of Assiut University. Animals were maintained under normal conditions, with free access to food and water in the normal daily light and darkness cycle. The control group included: newborn, one week, two weeks, one-and-half month, two-and-half month old rats. While in the experimental group, animals at the age of two weeks were weighted and received cyclophosphamide dissolved in dextrose 5% intraperitoneally, in a dose equals to 100mg/ kg body weight once weekly for four successive weeks *(Velez et al., 1989)*. At the end of the treatment period the experimental group was subdivided into subgroup A (treated group) including the rats at the age of one- and- half month which sacrificed immediately and subgroup B (rehabilitated group) including the rats at the age of two-and-half month which left for another four successive weeks without treatment then sacrificed.

For light microscopic study, specimens of the two groups (control and experimental) were fixed in Bouin's fluid, dehydrated in ascending grades of alcohol, cleared, embedded in paraffin and serially sectioned (8µm). Sections were stained with haematoxylin and eosin *(Drury & Walington, 1980)*.

Ultrastructural study of the ovary from rats at the age of one-and-half month (4 control and 4 treated animals), were done through the preparation of semithin sections (stained with toluidine blue) and ultrathin sections stained with uranyl acetate, lead citrate, examined and photographed by Jeol-JEM-100 CXII electron microscopy.

In the two age groups (1.5 and 2.5 month), the following parameters were determined from paraffin sections of the tested groups of animals (per an area of 315.9 x 234.7 µm) using image analysis system (Leica Q500): the number of the antral and preovulatoy ovarian follicles (healthy and atretic), the mean diameter of ovarian follicles (antral and preovulatory), the average number of granulosa cell layers in antral, pre-ovulatory follicles and the mean diameter of oocytes.

Statistical analysis of the data was done using the student t-test and the data expressed in means±standard deviation.

RESULTS

Newborn control rat: In control animals, ovarian sections showed only large number of primordial follicles (fig. 1), each consisted of an immature ovum (the primary oocyte) containing large vesicular nucleus which was slightly eccentric with one or two prominent nucleoli. A single layer of flattened epithelial (follicular) cells was surrounding the oocytes. The follicles lay within a dense cortical stroma.

One-week-old control rat: In control animals, the ovary of this age group showed the appearance of more developed type of follicles, the primary follicles (fig. 2). The follicles and the oocytes with their nuclei increased in size. The follicular epithelium here changed into a single layer of cuboidal follicular cells. Some follicles started to be rounded by a stromal capsule called theca folliculi. Some primordial follicles in-between the primary ones were still present.

Two-week-old control rat: In control animals, ovarian sections showed the appearance of growing follicles at variable stages of development (fig. 3), active in mitosis. The primordial follicles were held aside in the peripheral cortical zone underneath the germinal epithelium. A 3rd type of follicles, the secondary follicles or pre-antral follicles (fig. 4) were observed, in addition to the previous types. They were more developed in size and structure. The follicular cells became columnar, the follicular layer instead of being a single layer became more stratified or multi-laminar, forming what is called the stratum granulosum. The surrounding theca folliculi became well apparent. The oocyte increased in size and became surrounded by thin membrane, the vitelline membrane. Their nuclei increased in size and some showed dispersed chromatin.

One-and-half month old rat: In control animals, the ovary became differentiated into peripheral cortical and central medullary zones (fig. 5). The follicles were mainly located into the cortical region. They included the previous three types with much larger pre-antral follicles. The medulla was consisted of connective tissue stroma containing many dilated blood vessels. Atretic follicles were mainly located in the medulla. In some sections the pre-antral follicles increased in size with more granulosa cell layers. The oocytes also increased in size with thick and dispersed nuclear chromatin. They also became surrounded by a well developed vitelline membrane. The vitelline membrane was separated from the stratum granulosum by a potential space called the perivitelline space. At this stage some intercellular spaces were observed (fig. 6). A larger type of follicles was shown called, the antral or tertiary follicles (fig. 7) where the intercellular spaces became confluent to form a single cavity, the antrum. As a result, the granulosa cells became separated. Those which encircled the oocyte forming the corona radiata, while the rest became condensed against the basement membrane. Increased thickness of the theca folliculi was also observed.

In cyclophosphamide-treated animals, the ovarian follicles became haphazardly distributed and their size decreased and became atretic and shrunken follicles (fig. 8). The follicular wall in some follicles became atonic, thus they became attenuated, triangular in shape and disfigured due to compression by neighboring follicles. Marked reduction in the granulosa cell layers and slight thickening of the theca folliculi were observed (fig. 9). Some granulosa cells showed fragmentation and leakage of its contents forming cellular debris while others showed increase in size. Apoptotic granulosa cells were noticed. The oocytes in most of the follicles were affected; where they appeared vacuolated with discontinuity of the vitelline membranes in some areas (figs. 9, 10). Other oocytes were completely absent.

Semi-thin sections of the control animals showed some antral and pre-antral follicles resting on their basement membranes (basal lamina) which separated them from the theca and ovarian stroma. Some atretic follicles were found within the stroma (fig. 11). In some antral follicles, the granulosa cells surrounding the zona pellucida became cuboidal forming the corona radiata (fig. 12).

Semi-thin sections of cyclophosphamide-treated animals showed large number of atretic follicles at an advanced stage of atresia with large number of lipid droplets in-between the shrunken granulosa cells (fig. 13). In some follicles, the phagocytic cells were absent and the degenerated cells appeared to be phagocytosed by the neighboring healthier ones. Others were apparently healthy, but they contained in the antrum, large number of apoptotic cells which were engulfed by the phagocytic cells (fig. 14).

Ultra-thin sections of the granulose cells of the control animals showed large euchromatic nuclei limited by regular nuclear membrane with one or more nucleoli (figs. 15, 16). Their cytoplasm showed lightly-stained oval or rounded mitochondria, well-developed Golgi complexes with well-organized piles, intact rough endoplasmic reticulum and free ribosomes. The granulosa cells were rested on an intact basement membrane (fig. 16) and on the zona pellucida on the other side through which it sent its granulosa processes (fig. 16). Moreover, the zona contained the microvilli of the oocyte and the processes of the granulosa cells (figs. 15, 16). The oocytic cytoplasm contained the same organelles found in granulosal cytoplasm in addition to a variable sized lamellae (cytoplasmic rays) (fig. 17).

Ultra-thin sections of cyclophosphamide treated animals showed pyknotic and very darkly stained granulosa cells with fragmentation of nuclear and cytoplasmic contents and their leakage through the destructed nuclear and the cytoplasmic membranes respectively (fig. 18). These materials became clumped with each other in the antrum. Granulosa cell processes became retracted or lost from the zona pellucida (fig. 19). At the same time, microvilli of the oocyte had also become fewer in number, with most of them terminating just inside the zona pellucida surface. Remnants of the granulosal cytoplasmic organelles were present (figs. 18, 20). They became more randomly distributed throughout the cytoplasm. The rough endoplasmic reticulum became lost, un-identifiable or dilated. Secondary lysosomes containing cellular debris, apoptoic cells and vacuolated rarefied cytoplasm were present in some areas. The basement membrane appeared thicker than that of the control group. The mitochondria became dilated with destructed cristae (fig. 20).

The oocytic organelles became scanty and haphazard in distribution. The mitochondria appeared electron-dense or showed destructed cristae.The fibril lamellae became scanty and widely separated. The piles of the Golgi apparatus became electron-dense, haphazard and dilated. Some cytoplasmic vacuoles were also present (fig. 21).

Two-and-half month old rat: In control animals, this age group showed mainly huge-sized follicles, the mature Graafian follicles (the pre-ovulatory follicles). These follicles were characterized by large amount of follicular fluid, the liquor folliculi which distended the antrum (fig. 22). Their oocyte with its surrounding well-formed corona radiata became eccentric in position. The oocyte and the corona were mound on a group of granulosa cells, the cumulus oophorus. The stratum granulosum formed continuous layers around the antral cavity. The theca folliculi was markedly thickened and became differentiated into theca interna which contained polyhedral cells, theca externa which contained fusiform cells (the fibroblasts) and collagenous fibers. The boundary between the two layers was somewhat indistinct (fig. 22).

Rehabilitated group: In this group the follicles were less affected. There was resumption of the normal number and order of granulosa cell layers and normal thickness of the theca layer. Apoptosis was apparent in some follicles and absent in others (fig. 23). Cysts were observed in some follicles with discontinuation of the theca, granulosa layers and vacuolation of oocytes (fig. 24). Discontinuity of the basal lamina with leakage of the liquor folliculi in the interstitial tissue was observed in some follicles (fig. 25).

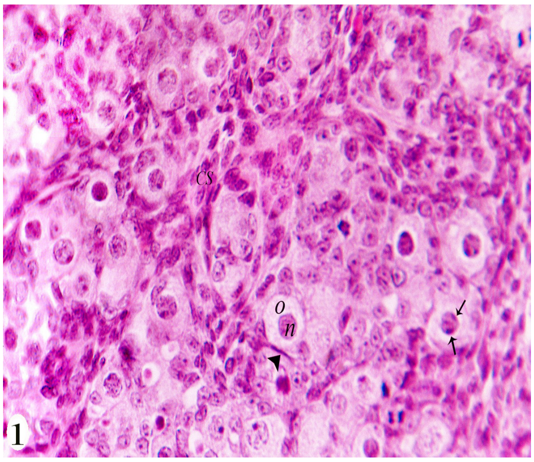

Fig. 1: A photomicrograph of a sagittal section in the ovary of newborn control rat showing primordial follicles each contain 1ry oocyte (o) with a large vesicular nucleus (n) slightly eccentric. Some show one or more prominent nucleoli (arrows). Notice a single layer of flattened follicular cells surrounding the oocytes (arrowhead) and the dense cortical stroma (Cs). Hx. & E.; X400

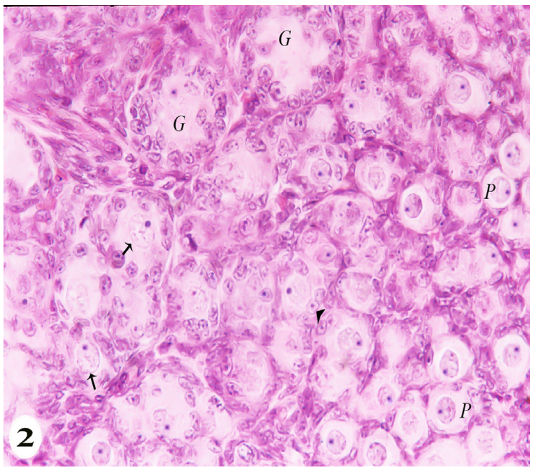

Fig. 2: A photomicrograph of a sagittal section in the ovary of one-week-old control rat showing primordial follicles (P) on the right and growing follicles (G) on the left where the oocytes and their nuclei increase in size (arrows). Notice cuboidal follicular cells (arrowhead). Hx. & E.; X400

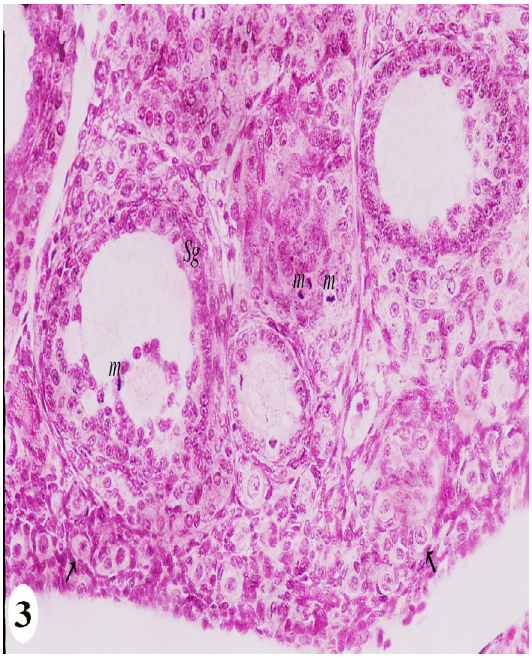

Fig. 3: A photomicrograph of a sagittal section in the ovary of two-week-old control rat showing growing follicles at variable stages of development. Some of them become multilaminar forming the stratum granulosum (Sg). Some granulosa cells show active mitosis (m). Note that the primordial follicles are now grouped at the periphery (arrows) underneath the germinal epithelium. Hx. & E.; X250

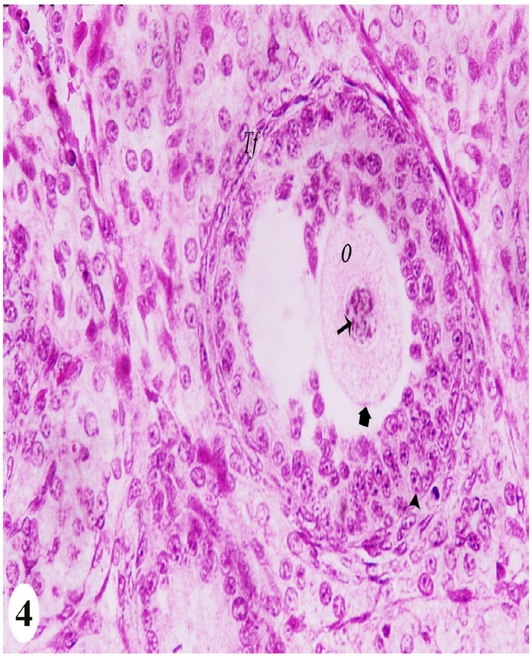

Fig. 4: A photomicrograph of a sagittal section in the ovary of two-week-old control rat showing a growing early 2ry follicle (pre-antral) consisting of enlarged ovum (o), large nucleus with dispersed chromatin (arrow). The epithelium of stratum granulosum becomes columnar (arrowhead). Notice stromal theca folliculi (Tf) and the thin vitelline membrane (thick arrow). Hx. & E.; X400

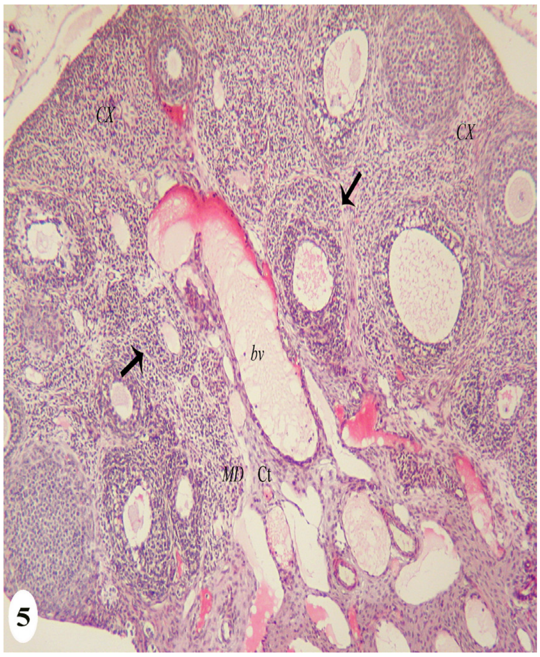

Fig. 5: A photomicrograph of a sagittal section in the ovary of one-and-half month old control rat showing two distinguished zones, the outer cortical zone (CX) formed of a compact stroma containing growing follicles regularly arranged and an inner medullary zone (MD) consisting of connective tissue (Ct) which contains dilated blood vessels (bv) that are surrounded by atretic follicles (arrows). Hx. & E.; X100

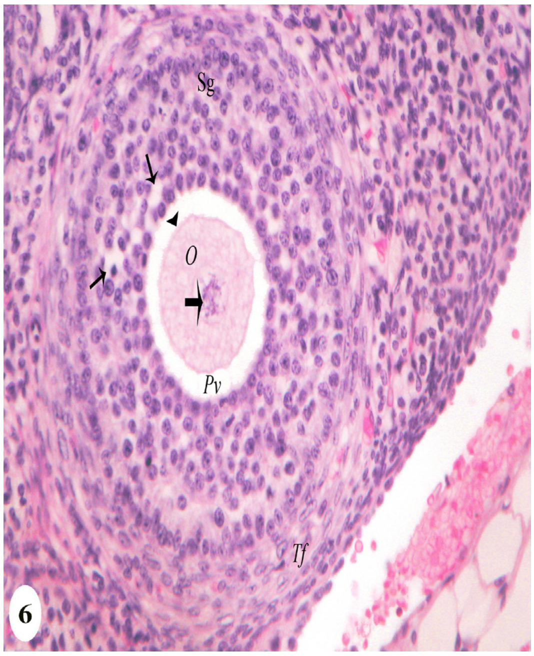

Fig. 6: A photomicrograph of a sagittal section in the ovary of one-and-half month old control rat showing 2ry follicle with large oocyte (o) containing large nucleus with dispersed chromatin (thick arrow) and surrounded by well developed vitelline membrane (arrowhead), perivitelline space (Pv) and the stratum granulosum (Sg). Notice the spaces that in-between the granulosa cells (thin arrows) and the thick theca folliculi (Tf). Hx. & E.; X400

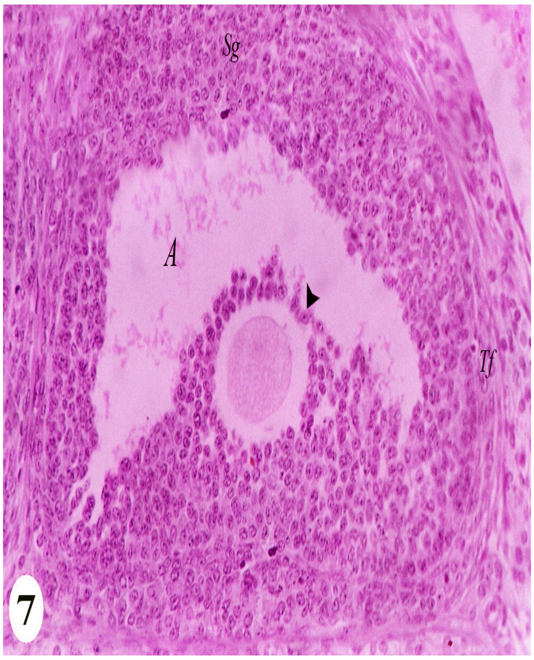

Fig. 7: A photomicrograph of a sagittal section in the ovary of one-and-half month old control rat showing large tertiary or antral follicle with single antral cavity (A) surrounded by the stratum granulosum (Sg) and the later is surrounded by well developed theca folliculi (Tf). Note that some cuboidal granulosa cells start to encircle the oocyte to form the future corona radiata (arrowhead). Hx. & E.; X400

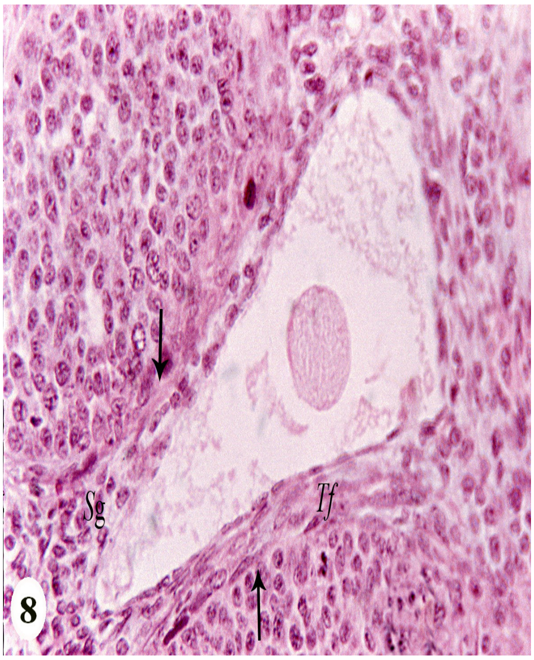

Fig. 8: A photomicrograph of a sagittal section in the ovary of one-and-half month old treated rat showing triangular-shaped atretic follicle with thickened theca folliculi (Tf)and remnants of the stratum granulosum (Sg). The follicle seems to be compressed by two neighboring follicles (arrows). Hx. & E.; X400

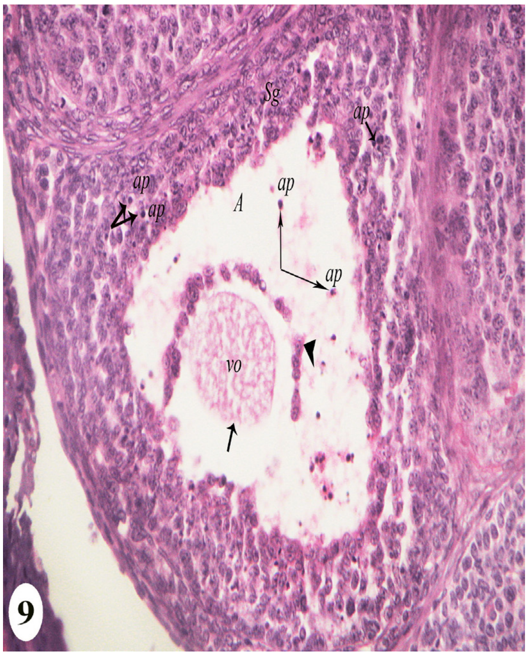

Fig. 9: A photomicrograph of a sagittal section in the ovary of one-and-half month old treated rat showing reduced thickness of stratum granulosum (Sg) with incomplete ring of granulosa cells (arrowhead) around a vacuolated oocyte (vo). Notice the partial disappearance of the vitelline membrane (arrow) and abundant apoptosis (ap) in between the granulosa cells in what was antrum (A). Hx. & E.; X400

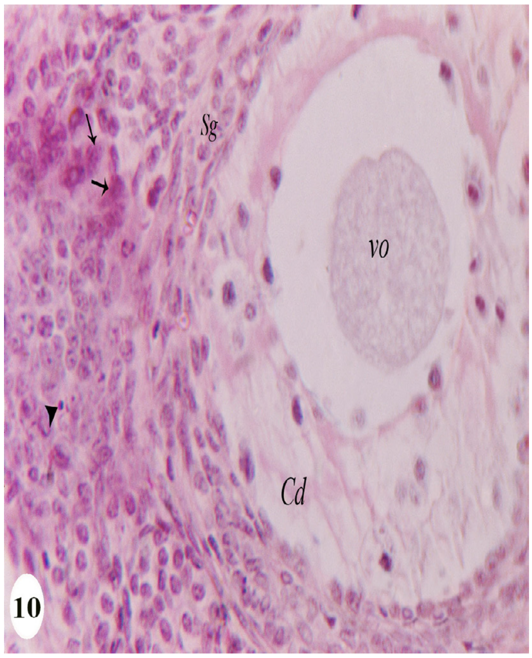

Fig. 10: A photomicrograph of a sagittal section in the ovary of one-and-half month old treated rat showing marked reduction and disorganization of the stratum granulosum (Sg), with cellular debris (Cd) and vacuolated oocyte (vo). Notice large size granulosa cells in comparison with normal (arrows) and apoptotic cells (arrowhead). Hx. & E.;X400

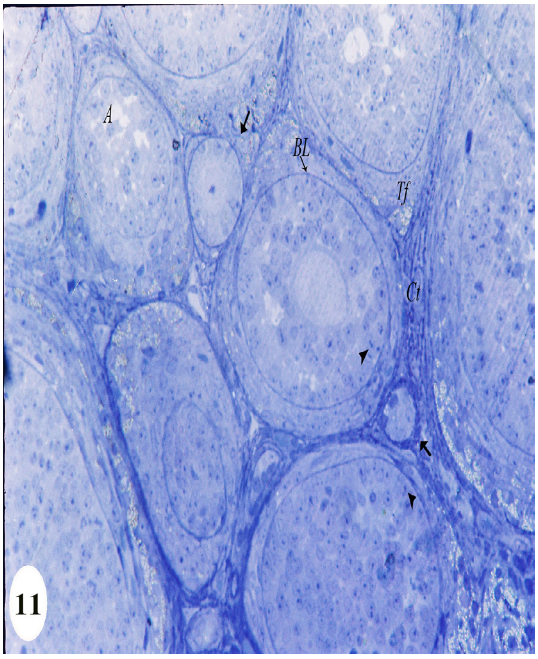

Fig. 11: A photomicrograph of semi-thin section in the ovary of one-and-half month old control rat showing growing follicles. Some show antral spaces (A). The granulosa cells are limited by basal lamina (Bl) and are active in mitosis (arrowheads). The later are encapsulated by the theca folliculi (Tf). The follicles are separated by connective tissue stroma (Ct). Note the atretic follicles that lie within the stroma (arrows). Toluidine blue; X 250

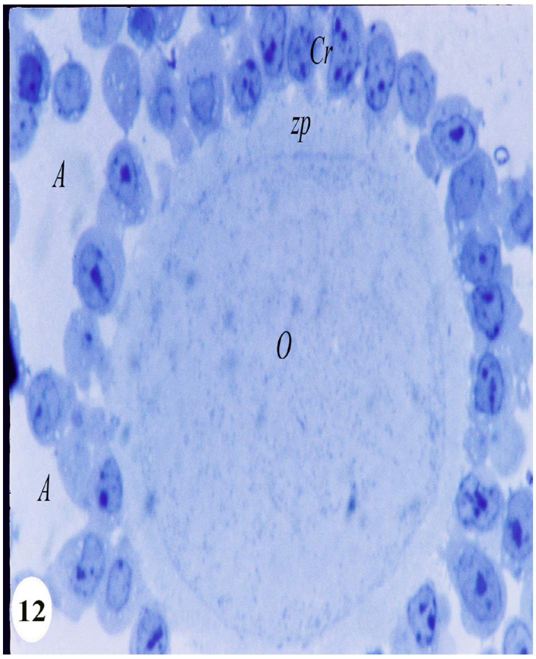

Fig. 12: A photomicrograph of semi-thin section in the ovary of one-and-half month old control rat showing an oocyte (o) surrounded by zona pellucida (zp) which encircled by corona radiata (Cr). Note the antral spaces (A) in-between the granulosa cells. Toluidine blue; X 1000

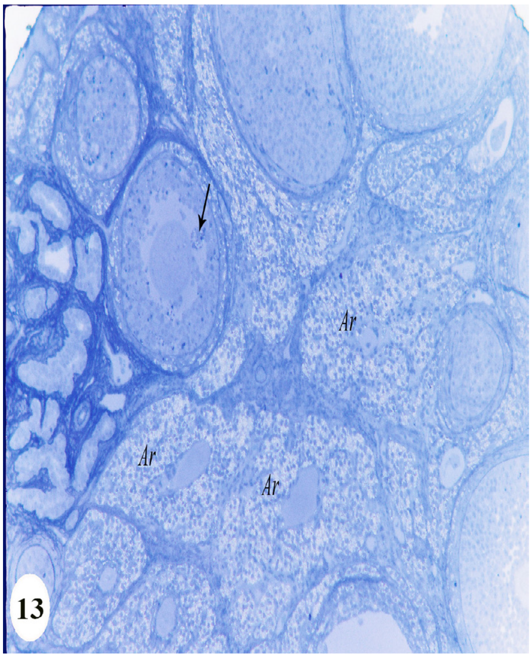

Fig. 13: A photomicrograph of semi-thin section in the ovary of one-and-half month old treated rat showing large number of atretic follicles (Ar) at the centre in their advanced stage of atresia and some apparently healthy growing follicles at the periphery containing antral masses of apoptotic cells engulfed by phagocytic cells (arrow). Toluidine blue; X100

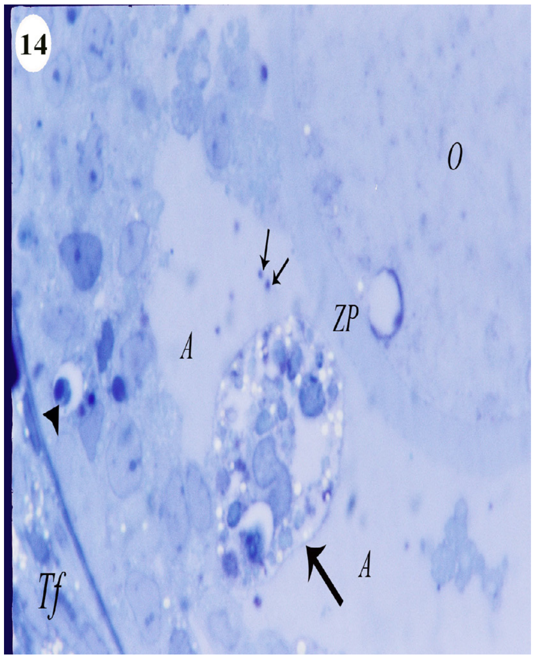

Fig. 14: A photomicrograph of semi-thin section in the ovary of one-and-half month old treated rat showing part of an oocyte (o) with its surrounding zona pellucida (zp), part of theca layer (Tf) and the antrum (A). Some apoptotic cells appeared partly to be phagocytosed by the nearby granulosa cells (arrowhead) and partly by the phagocytic cells forming a mass of cellular debris (big arrow). Note the apoptotic cells in the antrum (small arrows) and absent corona radiata. Toluidine blue; X1000

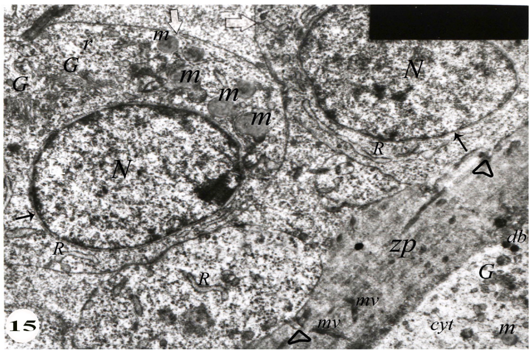

Fig. 15: An electron micrograph of the ovary of one-and-half month old control rat showing granulosa cells (white arrows) containing euchromatic large nuclei (N) with regular nuclear membrane (arrows). Their cytoplasm contains regularly arranged Golgi saccules (G) near the nucleus, mitochondria (m), rough endoplasmic reticulum (R) and free ribosomes (r). The zona pellucida contains microvilli (mv) and granulosa cell processes (arrowheads). Notice part of the oocyte's cytoplasm (cyt) with mitochondria (m), Golgi (G) and dense particles (dp). dX 5,000

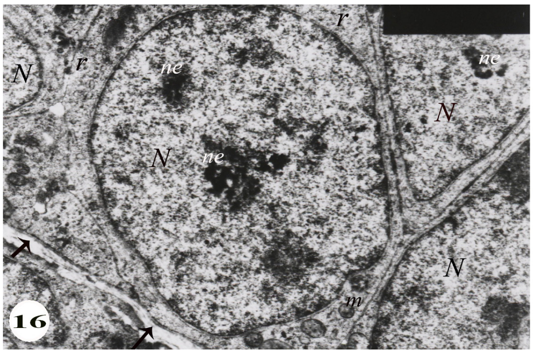

Fig. 16: An electron micrograph of the ovary of one-and-half month old control rat showing granulosa cells with large nuclei (N) surrounded by regular nuclear membrane containing one or more prominent nucleoli (ne) and rest on the basal lamina (arrows). The cytoplasm contains free ribosomes (r) and mitochondria (m). Note parts of adjacent nuclei and nucleoli. X 6,700

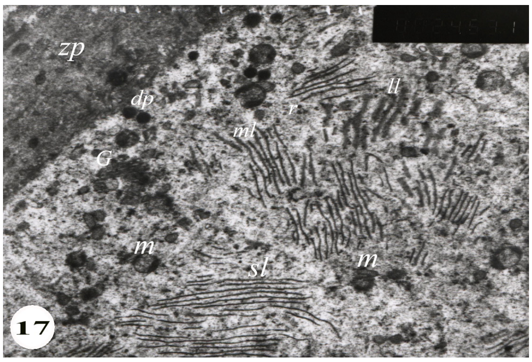

Fig. 17: An electron micrograph of the ovary of one-and-half month old control rat showing part of the zona pellucida (zp) and the cytoplasm of the oocyte. The later shows abundant well organized cell organelles like mitochondria (m), Golgi (G), ribosomes (r), variable sized fibril lamellae, small (sl), medium sized (ml) and large (ll) and some dense particles (dp). X 8,000

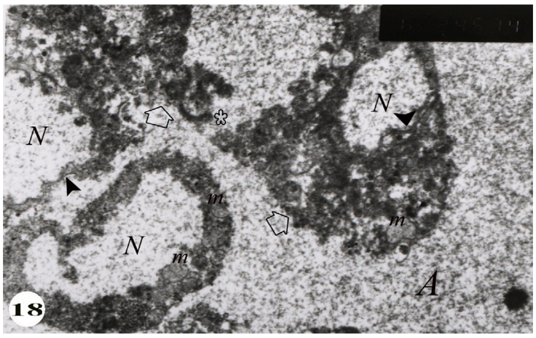

Fig. 18: An electron micrograph of the ovary of one-and-half month old treated rat showing totally destructed granulosa cells with loss of their architecture and leakage of the nuclear and cytoplasmic contents into the antrum (A), giving them clumped appearance (arrows). Some nuclei (N) are still limited by irregular nuclear membrane (arrowheads), while other membranes are absent (*). Note that some abnormal mitochondria (m) are present within the clumped materials. X 6,700

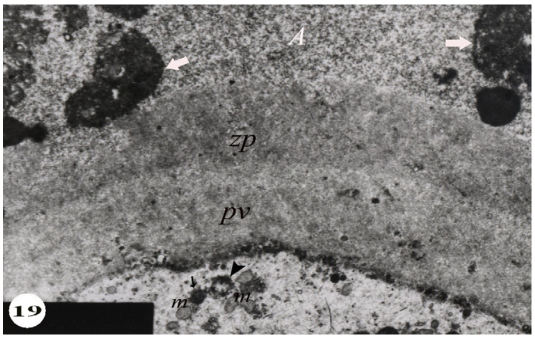

Fig. 19: An electron microghragph of the ovary of one-and-half month old treated rat showing part of the zona pleucida (zp) with no microvilli or granulosa cell processes, the perivitelline space (pv) which appears less dense than the zona, the oocytic cytoplasm shows some electro-dense mitochomdria (arrow), destructed mitochondria (arrowhead) and disfigured mitochondria (m). On the other side there are totally destructed pyknotic granulosa cells with leakage of their material (white arrows) in the antrum (A). X 5,000

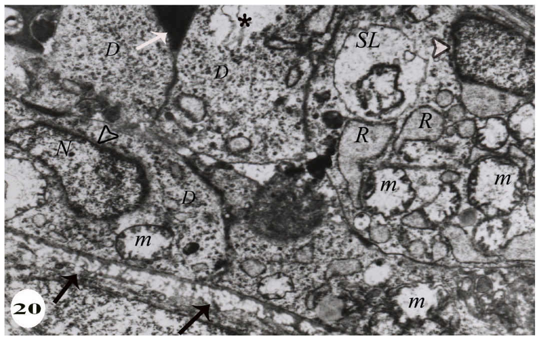

Fig. 20: An electron micrograph of the ovary of one-and-half month old treated rat showing disfigurement of granulosa cells (D), with shrunken and fragmented nuclei (N). The nuclear membranes are irregular and thickened (arrow heads). Notice The dilated and destructed mitochondria (m), dilated rough endoplasmic reticulum (R), secondary lysosomes (SL), part of apoptotic cell (white arrow), rarified cytoplasm (*) and thickened basement membrane (arrows). X 6,700

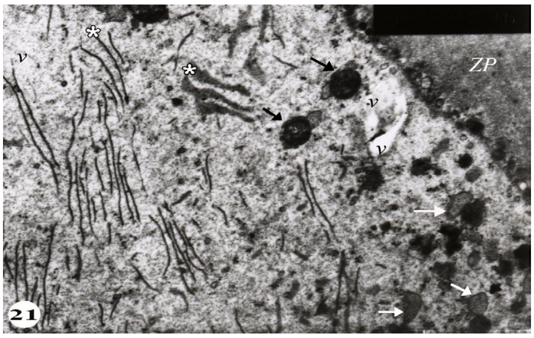

Fig. 21: An electron micrograph of the ovary of one-and-half month old treated rat showing part of the zona pellucida (zp) and the cytoplasm of the oocyte. The later shows scanty haphazard cell organelles. Note disfigured (white arrows) and electro-dense mitochondria (black arrows), the fibril lamellae are scanty and widely separated (*) and the cytoplasm is vacuolated and rarified (v). X 8,000

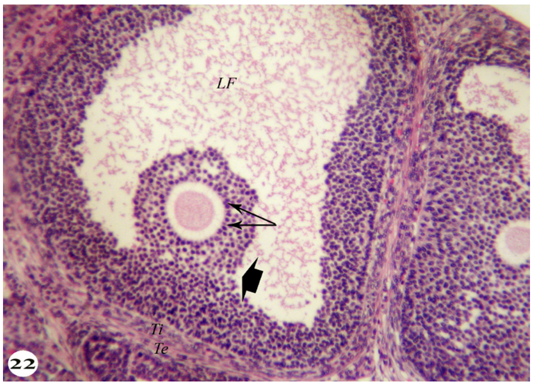

Fig. 22: A photomicrograph of a sagittal section in the ovary of two-and-half month old control rat showing a large preovulatory follicle (Graafian follicle), with eccentric oocyte and a large amount of liquor folliculi (LF). The oocyte is surrounded by well formed corona radiata (long arrows) and rests on the cumulus oophrous (thick arrow).The theca cells are differentiated into theca interna and externa (Ti & Te). Hx. &E.; X 200

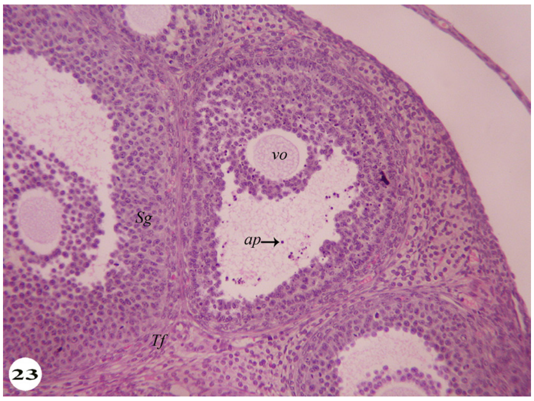

Fig. 23: A photomicrograph of a sagittal section in the ovary of two-and-half month old rehabilitated rat showing some regenerative features in the follicles. The left one show absent apoptosis, resumption of the normal thickness of the stratum granulosum (Sg) and the theca layer (Tf). The right one is still apoptotic (ap) with more vacuolated oocyte (vo) Hx. &E.; X 250

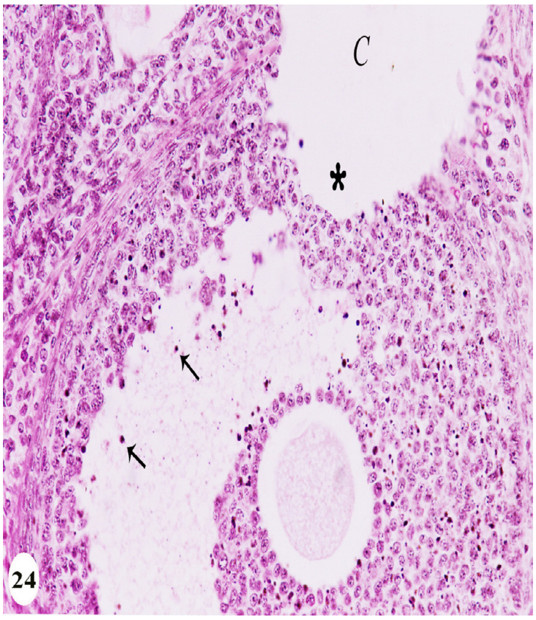

Fig. 24: A photomicrograph of a sagittal section in the ovary of two-and-half month old rehabilitated rat showing part of a follicle and an upper cyst (C). Despite of some regeneration in this follicle, it shows some degeneration in the form of discontinuity of the stratum granulosum and the theca folliculi (*) that it has been compressed by an upper cyst. The antrum shows apoptosis (arrows). Hx. & E.; X 400

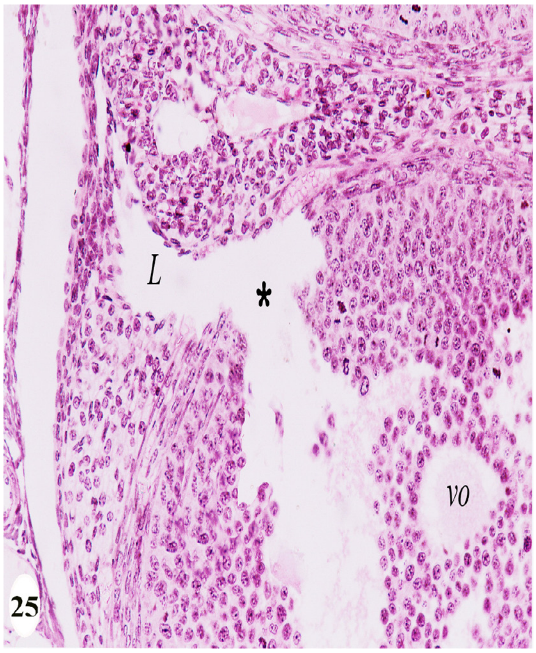

Fig. 25: A photomicrograph of a sagittal section in the ovary of two- and- half month old rehabilitated rat showing a follicle with absent apoptosis. The granulosal thickness is complete in some areas but still deficient in other areas (*) with leakage of the antral fluid (L) in the interstitial tissue. The oocyte shows vacuolation and degeneration (vo). Hx. & E.; X 400

Table: Morphometric measurements in the four groups of animals in an area of 315.9x234.7 µm.

	Control 1.5 m (*N*=4)	Treated 1.5 m (*N*=4)	Difference	p value	Control 2.5 m (*N*=4)	Rehabi-litated 2.5 m (*N*=4)	Difference	p value
Average number of healthy follicles	4.15± 1.93	1.30± 1.08	2.85	*** <0.001	2.55± 1.15	1.55± 0.94	1	* 0.0192
Average number of atretic follicles	13.55± 5.51	18.65± 6.75	-5.1	** 0.0097	20.80± 5.81	24.05± 5.50	-3.25	0.1109
Average follicular diameter (µm)	233.89± 55.11	146.06± 12.07	87.83	*** <0.001	405.10± 50.38	343.75± 97.69	61.35	* 0.0282
Average oocytic diameter (µm)	60.15± 4.33	45.49± 2.01	14.66	*** <0.001	79.12± 13.88	68.31± 5.76	10.81	** 0.0015
Average number of granulosa cell layers	8.18± 1.91	5.93± 1.31	2.25	0.0845	10.23± 2.53	6.10± 1.79	4.13	** 0.0062

N= Number ; *m*= month
Significance probability:
p<0.01 ⟶ (*) significant difference
P<0.005 ⟶ (**) high significant difference
P<0.001 (***) very high significant difference

MORPHOMETERY: Table

The morphometeric measurements were conducted into two age groups, the one-and-half and two-and-half months in an area of 315.9x234.7 µm.

DISCUSSION

There is close similarity between the human and the rat ovary in shape, histological structure, embryogenesis and physiology, but they differ in the size, the site and the onset of puberty.

Development of the rat ovary:

In the present work the ovary of the new-born rat showed only primordial follicles, each of which consisted of an immature ovum (the primary oocyte) and a single layer of flattened epithelial (follicular) cells. These findings are in line with those of *Mattison and Schulman (1980), Richards (1980), Roy and Greenwald (1985) and Hirshfield (1991).*

The ovary of the one-week-old rat showed the appearance of primary follicles where the oocytes and their nuclei became larger in size and their follicular epithelium became cuboidal. At the age of two weeks, the secondary or pre-antral follicles were evident where the follicles became more developed in size and structure. The follicular layer became multi-laminar. The oocytes increased in size and became surrounded by the vitelline membrane. *Boubekri et al. (2007)* described the same findings but they added that the zona pellucida appeared with the primary follicles and became well developed by the formation secondary follicles.

At one-and-half month aged rats, the ovary showed differentiation into outer cortical zone which consisted mainly of follicles at variable stages of development and inner medullary zone consisting mainly of connective tissue. The same findings were found by *Leeson et al. (1988)* who added that there was no sharp demarcation between the two zones and that the vascular connective tissue core consisted of numerous blood vessels, lymphatics and nerves. The present work showed the development of the pre-antral follicles at this age through formation of more granulosa cell layers, the increased size of oocytes that became surrounded by a well developed vitelline membrane and perivitelline space and separated from the granulosa layer by zona pellucida. Intercellular spaces had started to appear in some follicles and to become confluent to form the antrum. These results confirm previous studies done by *Richards (1980), Hirshfied (1991) and Boubekr et al. (2007)*.

At the age of two-and-half month- old albino rat, mature Graffian follicles (the pre-ovulatory follicles) were characterized by huge size and large amount of follicular fluid. The theca folliculi were markedly thickened and differentiated into theca interna and theca externa. These results are in accordance with that of *Boubekri et al. (2007)* who also observed that the large healthy antral follicles developed into maturing follicles to form pre-ovulatory follicles.

Effect of cyclophosphamide:

Cyclophosphamide is a commonly used cytotoxic drug *(Calabresi & Parks, 1980)*. Its usage is not limited to malignant conditions, but has been extended towards some non-malignant conditions *(Waxman, 1983; Chabner et al., 2006)*. In the current study, cyclophosphamide was administered at a dose of 100 mg/kg once weekly for four successive weeks because sometimes it is clinically administered in such high dose to achieve maximal therapeutic effect *(Chabner et al., 2006)*. Also, this dose was based on other studies *(Jarrell, et al., 1987; Ataya et al., 1989)*. Immature female rats (2 weeks) were used in this study, as the start of drug therapy, because it is the age of formation and development of the growing follicles and the formation of granulosa cells that are mainly targeted by the drug *(Burkl & Schiechl, 1978; Shiromizu et al., 1984; Plowchalk & Mattison, 1991, 1992; Davis & Heindel, 1998)*.

Histological and morphometeric studies of one-and-half month-old treated rats

Ovarian follicles and granulosa cells:

In the present work there was reduction in the size and number of healthy antral follicles, their mean diameters and their number in comparison to those of the control group. These results are in agreement with those of *Shiromizu et al.* (1984), *Goebelsmann (1986), Ataya et al. (1989) and Ataya et al. (1990)* who observed that the effect of cyclophosphamide on the ovary was mainly through the rapid pooling of the follicles to the growing stage. This could be explained on the basis that cyclophosphamide destroys the developing follicles by attacking the dividing granulosa cells thus reducing estradiol secretion and inhibin production from the ovary. This leads to an increase in pituitary gonadotrophin release. All of this enhanced the further recruitment of follicles into the pool of maturing follicles that made them more vulnerable to cytotoxic effects of cyclophosphamide than resting small follicles. This resulted in vicious cycle of accelerated atresia and premature depletion of ovarian follicles. This result is supported by the fact that the number of germ cells was limited, fixed since fetal life, and gradually declined with age *(Meirow et al., 2001)*.

The present work suggested that cyclophosphamide targeted specifically the granulosa cells causing their enlargement due to increase in their nuclear size in comparison to the control. This coincides with the results of the study performed by *Meyn and Murray (1986)* that attributed this increase in the nuclear size to the accumulation of DNA that might be due to cross linkage of DNA by the alkyl group of the drug.

In this study it was found that the drug affected the healthy antral follicles rather than the atretic follicles as seen by very high significant reduction in the average number of the healthy antral follicle. Moreover, there was a high significant increase in the number of the atretic antral follicles after treatment in comparison to control. In addition, there was reduction in the size of antral follicles as noticed by reduction in their average diameter in comparison to the control. *Jarrell et al. (1987)* also noted that the main effect of cyclophosphamide was on the number and the average diameters of healthy antral follicles and no significant effect was noticed on the number and average diameters of primordial and pre-antral follicles. *Burkl and Schiechl (1978)* observed that secondary and antral follicles specifically disappeared after short period of treatment with cyclophosphamide.

In this study, the number of granulosa cell layers decreased and became markedly disorganized probably due to destruction caused by apoptosis with leakage of the cellular material in the antrum. These results are concomitant with Burkl and *Schiechl (1978)*. *Lopez and Luderer (2004)* investigated the mechanism of destruction of granulosa cells by cyclophosphamide in rats and they concluded that it was due to the induction of apoptosis. *Tsai-Turton et al. (2007)* provided an explanation to the mechanism of apoptosis and attributed it to the oxidative stress induced by glutathione depletion.

Oocytes:

The current study showed vacuolated and degenerated oocytes. In some follicles the oocytes were completely absent which was a sign of advanced atresia. *Lopez and Luderer (2004)* as well as *Desmeules and Devine (2006)* found that cyclophosphamide appeared to target the oocytes for apoptotic destruction in primordial and small primary follicles, whereas in larger follicles, it induced granulosa cell apoptosis followed by death of the oocyte. *Gougeon (1996) and Abir et al. (2001)* observed that when follicles were damaged by direct cytotoxicity of cyclophosphamide to the granulosa cell layers, the oocyte could not remain viable which lead to follicular loss.

Semi-thin sections of the treated group in the present study showed that there were large numbers of atretic follicles with darkly stained shrunken pyknotic granulosa cells indicating apoptosis. Their oocytes became shrunken indicating an advanced stage of atresia. The interstitial tissue appeared thicker in comparison to the control. Some follicles contained in the antrum some apoptotic cells which were phagocytosed by the phagocytic cells. Other follicles showed absence of phagocytic cells and the apoptotic granulosa cells appeared to be phagocytosed by neighboring healthier ones. *Gaytan et al. (1998)* showed that the presence of advanced atretic follicles lead to the appearance of secondary interstitial tissue from the day 25-30 of life, thus it became thicker.

Electron microscopic results:

Electron microscopic study of treated ovaries showed pyknotic, shrunken and electron-dense granulosa cell. Some showed fragmentation of nuclear and cytoplasmic contents and their leakage through the destructed membranes. These findings suggested signs of granulosa cells atresia and are in accordance with those observed by *Devine et al. (2000). Wyllie et al. (1980), Payne et al. (1995), Hsueh et al. (1996) and Tilly (1998)* explained that the underlying mechanism of ovarian atresia was apoptosis.

Tilly (1996) noticed shrinkage of the granulosa cells and condensation of the chromatin into dense masses which were signs for degeneration. *Moore (1991)* put an explanation to the cause of the cell death, that cyclophosphamide underwent hepatic transformation to form an active metabolite, phospharamide mustard. This agent irreversibly bound to a nucleophilic site, especially to DNA. The DNA strands then broke and could not be further synthesized, leading to cell death.

The present study revealed that both granulosa cell processes and the oocytic microvilli appeared to be retracted and became fewer or lost from the zona pellucida in comparison to the control. This could be an early sign of degeneration as this was expected to occur physiologically at an older age (at the time of the initiation of the formation of the first polar body within the ovary) according to *Sotelo and Porter (1959)*. *Devine et al. (2000)* noticed the same results and they explained them on the basis that oocytes were removed by physiological cell death rather than by apoptosis. Thus, their affection was slow and delayed in comparison to that of the granulosa cells.

The present work showed remnants of some cytoplasmic organelles of the granulosa cells with dilated and destructed rough endoplasmic reticulum, swollen mitochondria with destruction of their cristae. The suggested causes of mitochondrial apoptotic pathway were initiated either by damaging stimuli such as oxidative stress *(Berrigan et al., 1987; Venkatesan & Chandrakasan, 1995; Murata et al., 2004)* or DNA damage by DNA alkylation *(Gamcsik et al., 1999; Coppola & Ghibelli, 2000)*. While the oocytic organelles became scanty and haphazard in distribution, the mitochondria were either showing destructed cristae or appeared electron-dense (late sign of atretic oocyte). The Golgi apparatus became also, electron-dense and abnormal. Moreover, the basement membrane appeared thicker than that of the control group. These results are in accordance with those of *Devine et al. (2000)*. They also, reported that granulosa organelles suffering from the above changes were in their early stages of atresia. In advanced stage, the cytoplasmic organelles of granulosa cells were difficult to identify and the cell became pyknotic, darkly stained and then completely lost. They added an explanation that apoptosis is an active process thought to protect the rest of the healthy dividing cells from an aberrant cells.

At the same time, *Familiari et al. (1993) and Abir et al. (2001)* observed an increase in the vacuoles and lipid droplets in occytes and granulosa cells in addition to abnormally thick basement membrane. They suggested that this was due to deterioration in follicular health. *However, Marcello et al. (1990)* attributed the thickening of the basement membrane to physiological transition from the primary to secondary follicles and not due to the effect of the drug.

REFERENCES

Abir, R., Fisch, B., Nitke, S., et al. 2001. Morphological study of fully and partially isolated early human follicles. Fertility and Sterility 75 (1):141-146.

Ataya, K., Pydyn, E., Young, J. and Struck, R. 1990. The uptake and metabolism of cyclophosphamide by the ovary. Selective Cancer Therapeutics 6 (2): 83-92.

Ataya, K. M., Valeriote, F. A. and Ramahi Ataya, A. J. 1989. Effect of cyclophosphamide on the immature rat ovary. Cancer Research 49 (7): 1660-1664.

Berrigan, M. J., Struck, R. F., and Gurtoo, H. L. 1987. Lipid peroxidation induced by cyclophosphamide. Cancer Biochemistry Biophysics 9 (3): 265-270.

Bines, J., Oleske, D. M. and Cobleigh, M. A. 1996. Ovarian function in premenopausal women treated with adjuvant chemotherapy for breast cancer. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology 14 (5): 1718-1729.

Blumenfeld, Z. 2002. Preservation of fertility and ovarian function and minimalization of chemotherapy associated gonadotoxicity and premature ovarian failure: The role of inhibin-A and -B as markers. Molecular and Cellular Endocrinology 187 (1-2): 93-105.

Boubekri, A., Gernigon-Spychalowicz, T., Khammar, F. and Exbrayat, J. M. 2007. Histological and immunohistological aspects of the ovarian cycle of the algerian wild sand rat, Psammomys obesus Cretzschmar, 1828. Folia Histochemica Et Cytobiologica 45 (Suppl. 1): 41-49.

Brock, N., Hilgard, P., Peukert, M., et al. 1988. Basis and new developments in the field of oxazaphosphorines. Cancer Investigation 6(5): 513-532.

Burkl, W. and Schiechl, H. 1978. The growth of follicles in the rat ovary under the influence of busulphan and endoxan. Cell and Tissue Research 186 (2): 351-359.

Calabresi, P. and Parks, R. E., Jr. 1980. Antiproliferative agents and drugs used for immunosuppression: in the pharmacological basis of therapeutics. Edited by L. S. Goodman and A. Gilman, Macmillan, New York, 1263.

Chabner, B. A., Amerein, P. C., Druker, B. et al. 2006. Antineoplastic agents: in Goodman and Gilman's the pharmacological basis of therapeutics. Edited by J Brunton LL, Lazo JS and Parker KL., McGraw Hill, NewYork, 1322-1328, 1694.

Chabner, B. A., Ryan, D. P., Paz-Ares, L. and et al. 2001. Antineoplastic agents: in goodman and Gilman's the pharmacological basis of therapeutics. Edited by Hardman JG, Limbird LE, Gilman A., McGraw-Hill, NewYork, 1389-1459.

Colvin, M., Brundrett, R. B., Kan, M. N., et al. 1976. Alkylating properties of phosphoramide mustard. Cancer Research 36 (3): 1121-1126.

Coppola, S. and Ghibelli, L. 2000. GSH extrusion and the mitochondrial pathway of apoptotic signalling. Biochemical Society Transactions 28 (2): 56-61.

Davis, B. J. and Heindel, J. J. 1998. Ovarian toxicants: Multiple mechanisms of action: in reproductive and developmental toxicology. Edited by K. S. Korach., Marcel Dekker, New York, 373-395.

Demirer, T., Buckner, C. D., Appelbaum, F. R., et al. 1996. Busulfan, cyclophosphamide and fractionated total body irradiation for allogeneic marrow transplantation in advanced acute and chronic myelogenous leukemia: Phase I dose escalation of busulfan based on targeted plasma levels. Bone Marrow Transplantation 17 (3): 341-346.

Desmeules, P. and Devine, P. J. 2006. Characterizing the ovotoxicity of cyclophosphamide metabolites on cultured mouse ovaries. Toxicological Sciences 90 (2): 500-509.

Devine, P. J., Payne, C. M., McCuskey, M. K. and Hoyer, P. B. 2000. Ultrastructural evaluation of oocytes during atresia in rat ovarian follicles. Biology of Reproduction 63 (5): 1245-1252.

Drury, R. A. B. and Wallington, E. A. 1980. Carleton's histological technique. 5th ed., Oxford, Oxford University Press, New York.

Familiari, G., Caggiati, A., Nottola, S. A., et al. 1993. Ultrastructure of human ovarian primordial follicles after combination chemotherapy for Hodgkin's disease. Human Reproduction (Oxford, England) 8 (12): 2080-2087.

Gamcsik, M. P., Dolan, M. E., Andersson, B. S. and Murray, D. 1999. Mechanisms of resistance to the toxicity of cyclophosphamide. Current Pharmaceutical Design 5 (8): 587-605.

Gaytan, F., Morales, C., Bellido, C., et al. 1998. Ovarian follicle macrophages: Is follicular atresia in the immature rat a macrophage-mediated event? Biology of Reproduction 58 (1): 52-59.

Gobelsmann, U. 1986. The menstrual cycle. In Infertility, contraception and reproductive endocrinology. Edited by D. R. Jr Mishell and V. Davajan, Oradell, New Jersey: Medical Economics Books, 69-90.

Gougeon, A. 1996. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocrine Reviews 17 (2): 121-155.

Hirshfield, A. N. 1991. Development of follicles in the mammalian ovary. International Review of Cytology 124: 43-101.

Hsueh, A. J., Eisenhauer, K., Chun, S. Y., et al. 1996. Gonadal cell apoptosis. Recent Progress in Hormone Research discussion, 51:433-456.

Jarrell, J., Lai, E. V., Barr, R., et al. 1987. Ovarian toxicity of cyclophosphamide alone and in combination with ovarian irradiation in the rat. Cancer Research 47 (9): 2340-2343.

Leeson, T. S., Leeson, C. R. and Paparo, A. A. 1988. Text/atlas of histology. W.B. Saunders, Philadelphia.

Lopez, S. G. and Luderer, U. 2004. Effects of cyclophosphamide and buthionine sulfoximine on ovarian glutathione and apoptosis. Free Radical Biology and Medicine 36 (11): 1366-1377.

M***arcello, M. F., Nuciforo, G., Romeo, R., et al. 1990.*** Structural and ultrastructural study of the ovary in childhood leukemia after successful treatment. Cancer 66(10):2099-2104.

Mattison, D. R. 1980. How xenobiotic compounds can destroy oocytes. Contemporary Obstetrics and Gynecology 15:157-169.

Meirow, D., Epstein, M., Lewis, H., et al. 2001. Administration of cyclophosphamide at different stages of follicular maturation in mice: Effects on reproductive performance and fetal malformations. Human Reproduction 16 (4): 632-637.

Meyn, R. E. and Murray, D. 1986. Cell cycle effects of alkylating agents. In Cell cycle effects of drugs. Edited by L. A. Dethlefsen, Pergamon Press, New York, 170-188.

Moore, M. J. 1991. Clinical pharmacokinetics of cyclophosphamide. Clinical Pharmacokinetics 20 (3): 194-208.

Murata, M., Suzuki, T., Midorikawa, K., et al. 2004. Oxidative DNA damage induced by a hydroperoxide derivative of cyclophosphamide. Free Radical Biology and Medicine 37 (6): 793-802.

Payne, C. M., Bernstein, C. and Bernstein, H. 1995. Apoptosis overview emphasizing the role of oxidative stress, DNA damage and signal-transduction pathways. Leukemia and Lymphoma 19 (1-2): 43-93.

Plowchalk, D. R. and Mattison, D. R. 1991. Phosphoramide mustard is responsible for the ovarian toxicity of cyclophosphamide. Toxicology and Applied Pharmacology 107 (3): 472-481.

Plowchalk, D. R. and Mattison, D. R. 1992. Reproductive toxicity of cyclophosphamide in the C57BL/6N mouse: 1. Effects on ovarian structure and function. Reproductive Toxicology (Elmsford, N.Y.) 6 (5): 411-421.

Rao, R., Shammo, J. M., Enschede, S. H., et al. 2005. The combination of fludarabine, cyclophosphamide and granulocyte-macrophage colony-stimulating factor in the treatment of patients with relapsed chronic lymphocytic leukemia and low-grade non-Hodgkin's lymphoma. Clinical Lymphoma 6 (1): 26-30.

Ray, B. and Potu, B. K. 2010. Ovarian folliculogenesis: Detrimental effect of prenatal exposure to cyclophosphamide: a preliminary study. Bratislava Medical Journal 111 (7): 369-372.

Richards, J. S. 1980. Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiological Reviews 60 (1): 51-89.

Rooseboom, M., Commandeur, J. N. and Vermeulen, N. P. 2004. Enzyme-catalyzed activation of anticancer prodrugs. Pharmacological Reviews 56 (1): 53-102.

Ross, W. C. J. 1962. Alkylating agents, in Biological alkylating agents. Butterworth, London. 232.

Roy, S. K. and Greenwald, G. S. 1985. An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects. Biology of Reproduction 32 (1): 203-215.

Shiromizu, K., Thorgeirsson, S. S. and Mattison, D. R. 1984. Effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. Pediatric Pharmacology (New York, N.Y.) 4 (4): 213-221.

Sotelo, J. R. and Porter, K. R. 1959. An electron microscope study of the rat ovum. Journal of Biophysical and Biochemical Cytology 5 (2): 327-342.

Tew, K. D., Colvin, M. and Chabner, B. A. 1996. Cancer chemotherapy and biotherapy. Lippincott-Raven, Philadelphia.

Tilly, J. L. 1996. Apoptosis and ovarian function. Reviews of Reproduction 1 (3): 162-172.

Tilly, J. L. 1998. Molecular and genetic basis of normal and toxicant-induced apoptosis in female germ cells. Toxicology Letters 102-103: 497-501.

Tsai Turton, M., Luong, B. T., Tan, Y. and Luderer, U. 2007. Cyclophosphamide-induced apoptosis in COV434 human granulosa cells involves oxidative stress and glutathione depletion. Toxicological Sciences 98 (1): 216-230.

Velez de la Calle, J. F., de Queiroz, F., Garnier, D. H., et al. 1989. Reproductive effects of the anticancer drug cyclophosphamide in male rats at different ages. Archives of Andrology 22 (3): 251-263.

Venkatesan, N. and Chandrakasan, G. 1995. Modulation of cyclophosphamide-induced early lung injury by curcumin, an anti-inflammatory antioxidant. Molecular and Cellular Biochemistry 142 (1): 79-87.

Waxman, J. 1983. Chemotherapy and the adult gonad: A review. Journal of the Royal Society of Medicine 76 (2): 144-148.

Wyllie, A. H., Kerr, J. F. and Currie, A. R. 1980. Cell death: the significance of apoptosis. International Review of Cytology 68: 251-306.

تأثير السيكلوفوسفاميد علي مبيض الفأر الأبيض في مرحلة ما بعد الولادة

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ملخص البحث

يعتبر عقار السيكلوفوسفاميد من الأدوية شائعة الاستخدام في علاج الكثير من الأورام السرطانية كسرطان الرئة والرحم والثدي والدم والغدد الليمفاوية، وكذلك يستخدم في تثبيط جهاز المناعة في الكثير من الحالات المرضية المرتبطة بزيادة نشاط الجهاز المناعي.

يعمل عقار السيكلوفوسفاميد على إحداث تغييرات بالحامض الأمينى فى نواة الخلايا وخاصة الخلايا التناسلية والخلايا سريعة النمو. ولذلك فإن له أعراضا جانبية على الخصوبة فى الجنسين. كما وجد أن السيكلوفوسفاميد يؤدى إلى تأخر نمو البويضات ويؤدى إلى تقلص فى عدد الحويصلات المبيضية.

الغرض من هذا البحث دراسة التأثيرات التركيبية والإستيريولوجية للسيكلوفوسفاميد علي مبيض الفأر الأبيض قبل مرحلة البلوغ.وقد استُخدم فى هذا البحث عدد ستة وثلاثون من إناث الفئران البيضاء. أثنى عشرة منها تم إعطائها عقار سيكلوفوسفاميد بجرعة ١٠٠ ملجم/ كجم مرة واحدة أسبوعيا لمدة أربعة أسابيع، أما باقى الفئران فقد تم إعطاؤها محلول دكستروز ٥٪ (المجموعة الضابطة). ثم استخراج المبيض و فحصها بالميكروسكوب الضوئى والالكتروني.

بالفحص المجهري الضوئي لعينات فئران المجموعة الضابطة وجد أن ابتداءاً من عمر حديثي الولادة حتى عمر ما قبل البلوغ مباشرةً يزداد نمو الحويصلات ومحتواها من خلايا حويصلية ويزداد نمو البويضات وأنويتها. أما المجموعة التي تمت معالجتها بهذا العقار فقد وجد اختلال فى توزيع وحجم الحويصلات داخل المبيض مما أدى إلى ضمورها. وكذلك حدث اختلال في عدد طبقات الخلايا الحبيبية و حجم هذه الخلايا فمعظمها حدث به ضمور، ولكن البعض الأخر حدث به زيادة بالحجم. وعند إيقاف استخدام العقار لمدة أربعة أسابيع حدث تجدد للخلايا الحبيبية في بعض الحويصلات. و قد تم عمل القياسات الإستيريولوجية عن طريق استخدام جهاز كمبيوتر معد لهذه القياسات والتى أوضحت تأثر عدد وأبعاد الحويصلات المبيضية في الحيوانات التى تم إعطاؤها هذا العقار.

اوبفحص مبيض الفئران التي تمت معالجتها باستخدام المجهر الإلكتروني تبين وجود تغيرات بشكل النواة في الخلايا الحبيبية أو حدوث تحلل كامل بالنواة. وكذلك تبين وجود تحلل بأجزاء الخلايا الحبيبية. أما الخلايا البويضية فقد وجد بها تحلل بأجزاء الخلية وفراغات بالسيتوبلازم، كما لوحظ زيادة فى سمك الغشاء القاعدي.