Effect of Platelet-Rich Plasma in Cyclophosphamide-Induced Ovarian Failure in Albino Rats: Histological and Anatomical Study

Original Article

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ABSTRACT

Backgroud: Amenorrhea and ovarian disorders are a common concern of women under the age of 40, a condition medically known as premature ovarian failure (POF). The incidence of POF among Egyptian women before the age of 40, is about 8% that exceeds the global incidence which is about 1%. Chemotherapeutic agents were linked to toxicity of ovarian tissues and disturbance of ovarian functions. Provision of a suitable protective agent with high efficacy, low side effects and cost is a major challenge in the field of oncofertility.

Aim of the Study: Is to evaluate the mechanism of PRP treatment in induced ovarian failure due to cyclophosphamide.

Materials and Methods: The present study was carried out on twenty-nine albino rats. Twenty-four adult female albino rats were used as the experimental group. Five age-matched healthy male albino rats were used to get the PRP. The twenty-four adult female albino rats were randomly assigned into three groups (control, group II: received cyclophosphamide, group III: received cyclophosphamide and PRP). Plasma estradiol and progesterone levels were measured on day 22 after cyclophosphamide treatment before experimental rats euthanasia. The rats were euthanized and ovaries were anatomically examined using dissecting stereomicroscope. Sections of the ovaries were prepared and examined histologically, immunohistochemicaly and using transmission electron microscope.

Results: Cyclophosphamide treated groups showed signs of degeneration of follicles and oocyte and deterioration of ovarian functions. PRP treated group showed improved appearance of ovarian follicles and improved ovarian functions.

Conclusion: PRP seems to have protective effects on ovarian tissues and functions and its concomitant use with cyclophosphamide helps to restore the ovarian tissues.

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Key Words: Congestion; cyclophosphamide; immunohistochemistry; ovarian failure; platelet rich plasma.

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INTRODUCTION

Malignancy, idiopathic diseases and autoimmune diseases are eminently rising health issues throughout the past five years, as affecting females at fertility period. They are usually represented by amenorrhea and infertility, a condition medically known as premature ovarian failure (POF)[1,2]. The incidence of POF among Egyptian women before the age of 40, is about 8%[3] that exceeds the global incidence which is about 1%[4]. While malignancy is a common etiology of POF, chemotherapeutic agents and concomitant radiotherapy add to the incidence among those patients[5].

Chemotherapeutic agents are divided into five types: alkylating agents, antimetabolites, antitumor antibiotics, taxanes and platinum-based drugs[6]. At the beginning of the treatment, chemotherapeutic agents induce apoptosis of growing follicle[7]. On long term administration, they affect primordial follicles either by inducing DNA damage or over recruitment of these follicles[8].

Recent research works to find solutions that would help protecting ovarian functions during chemotherapy preventing subsequent induced POF[9-12]. In *vitro* preservation of follicles and oocytes is a solution that could help women with cancer induced infertility and POF to achieve pregnancy just after treatment[13]. Other solutions as different fetoprotective agents[14-16] which act by inhibiting apoptosis of primordial follicles. Others[17] can prevent chemotherapy adverse effects by inhibition of nuclear activation. These techniques are difficult to execute and have high cost, thus improving the currently available approaches and designing new fertility maintenance techniques are major challenges in the field of oncofertility[5].

Platelet rich plasma (PRP) is considered as a source of biological active components such as growth factors and cytokines[18-21]. It is used as autologous biological treatment which stimulate normal healing cascade and tissue regeneration[22,23]. It is used in the treatment of musculoskeletal disorders[24,25], hair loss[26] with promising results. It was shown that plasma growth factors may have

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a role in follicular development, growth and preservation of ovarian tissue[27,28].

The aim of this study was to assess the potential effect of PRP on preservation of ovarian follicular structure and function after cyclophosphamide induced ovarian failure using anatomical, histological, immunohistochemical and biochemical studies.

MATERIALS AND METHODS

Animals

The present study was carried out on twenty-nine adult albino rats obtained from the Animal House Center in Faculty of Agriculture, Alexandria University after approval of Ethics Committee, Faculty of Medicine, Alexandria University[29].

Twenty-four adult female albino rats aged about 8 weeks and weighed about 220 ± 20 g were used as the experimental group. They were randomly assigned to three groups:

Group I (Control groups): The group was divided into two subgroups:

- Subgroup a: Negative control group, included six adult female albino rats received sodium chloride 0.9% (1mg/kg/dose) by intraperitoneal injection on the first, eighth and fifteenth days of the study as a placebo.
- Subgroup b: Positive control group, included six adult female albino rats received PRP.

Group II: (Cyclophosphamide treated group) Included six adult female albino rats received cyclophosphamide.

Group III: (Combined cyclophosphamide and PRP treated group) Included six female rats received Cyclophosphamide, half an hour later the rats received PRP.

Five age-matched healthy adult male albino rats aged about 8 weeks and weighed about 300 ± 50 g were used to get the PRP.

All animals were kept under standard housing conditions; in a room temperature (24◦C) and 12:12 hour light: dark cycle. Standard diet and water were given ad labitum[29].

Drugs and materials

Cyclophosphamide

The drug was obtained from the pharmaceutical market (1 g vial/ injection form). Trade name is Endoxan (Baxter Oncology GmbH. Kantstrasse 2. D-33790 Halle. Germany). The dose of cyclophosphamide was 75 mg/kg/dose[27,30] given by intraperitoneal injection in the first, eighth and fifteenth days[31,32] of the study.

Platelet-rich plasma[27,33]

The preparation process was done under strict sterile condition at Biochemistry Department, Faculty of Medicine, Alexandria University.

The whole blood of each rat was withdrawn through cardiac puncture and transferred into test tubes with 3.2% sodium citrate. The blood was centrifuged at 400 g for 10 minutes and then the supernatant was transferred to another sterile tube, centrifuged again at 800 g for another 10 minutes. The upper 2/3, which consists of platelet-poor plasma (PPP) was removed and discarded. The remaining lower 1/3 was considered as PRP and preserved in sterile ebindurf. PRP was stored and frozen at -20ºC for further use. PRP was given as 200 µl/dose by intraperitoneal injection on the first, eighth and fifteenth days[28,32] of the study.

Platelets count

An automated cell counter was used to detect the PRP platelets count at clinical pathology department, Faculty of Medicine, Alexandria University. The average was 2380×103 platelets/µL. which was 5 times the whole blood levels.

I. Biochemical study

The blood samples (2ml for each sample) were collected from retro orbital venous plexus in a dry clean non-heparinized test tubes for assessing the serum level of estradiol (E2) and progesterone.

Biochemical parameters of serum estradiol (E2) and progesterone were estimated using estradiol (rat) ELISA kit and progesterone (rat) ELISA kit, Abbot, Austria. at Clinical Pathology department, Faculty of Medicine, Alexandria University. Steps were done following manufacturer instructions. Results were statistically analyzed.

II. Anatomical study

The experimental rats were euthenized following the Regulatory framework of Ethics of animal research, Faculty of Medicine, Alexandria University[29].

The rats were dissected and the right and left ovaries were extracted, weighed using sensitive scale and the volume of each ovary was estimated by displacement method.

Gross features were examined using Olympus SZ Dissecting Stereo Microscope at Experimental Embryology Laboratory, Anatomy and Embryology Department, Faculty of Medicine, Alexandria University.

III. Histological study

Vaginal smears[34,35] were collected daily for 5 days before the beginning of the experiment to detect the estrous cycle to make sure that all experimental rats at the same stage of estrous cycle (the estrus phase, 2nd day of the estrous cycle) and examined using light microscopy at Experimental Embryology Laboratory, Anatomy and Embryology Department, Faculty of Medicine, Alexandria University.

Both right and left ovaries were used in histological studies based on the theory adopted by Linares R et.al.[36] which stated that no difference in the number of oocyte released either from the right or the left ovary of rats each cycle.

A. H&E stain and morphometric studies

The right ovary was immediately fixed in 10% formol saline for an additional 72 hours and paraffin blocks were made.

The paraffin blocks were sectioned in serial cuts using automated microtome and sections stained by hematoxylin and eosin[37].

H & E sections were studied with a light microscopy (Olympus CX41 Binocular LED – Sample Microscope) in Center of Excellence for Reasearch in Regenerative Medicine and its Applications (CERRMA), Faculty of Medicine, Alexandria University.

3-5 serial sections were taken for image analysis. The number and diameter of ovarian follicle and diameter of oocytes were measured with determination of the type of follicles using imaging analysis system IMAGEJ (Rasband 1997–2019).

B. Immunohistochemical study

Sections from paraffin blocks were immunohistochemically studied using streptavidin-biotin immunoenzymatic antigen detection system[38]. Tissues were treated with anti caspase-3 kit (mouse monoclonal antibody), purchased from Biocare Medical, USA. The density of immunohistochemical stain was measured using Leica Application Suite (Version 4.12.0 (Build: 86). Leica Microsystems CMS GmbH) at Pathology Department, Faculty of Medicine, Alexandria University.

C. Ultrastructure study by electron microscopy

The left ovary of each rat was immediately fixed in 4% formaldehyde and 1% gluteraldehyde (4F1G)[39]. Sections were prepared[40] and examined using Transmission Electron Microscope (JEM 1400 plus-Made in Japan) at Faculty of Science, Alexandria University.

Statistical analysis

Morphometric and histological data was presented as mean ± SD and differences between groups and were analyzed with SPSS software package version 20.0. "t" test was used during comparison between the means of different sample groups and ANOVA was performed for comparison between more than two groups[41].

RESULTS

I. Biochemical study

Plasma estradiol and progesterone levels: (Table I, II, Figures 1, 2)

The comparison between the different studied groups revealed the following

Plasma estradiol and progesterone levels were significantly lower in cyclophosphamide treated group as compared to control groups. Plasma estradiol and progesterone levels were significantly higher in combined cyclophosphamide and PRP treated group as compared to those in cyclophosphamide treated group but still lower than control groups.

II. Anatomical study

i. The comparison between the different studied groups regarding volume and weight of the ovaries: (Table III, IV, Figures 3,4)

There was significant decrease in the volume and weight of the ovaries of cyclophosphamide treated group as compared to those of control groups. The volume and weights of ovaries of combined cyclophosphamide and PRP treated group were significantly higher than cyclophosphamide treated group with values approaching those of control groups.

ii. Examination of the ovaries using dissecting stereomicroscope revealed the following: (Figure 5 a-d)

In negative control group, the ovaries were pinkish in color with nodular glistening surface. In positive control group, they were moderately congested with nodular glistening surface. In cyclophosphamide treated group, the ovaries were smaller in size as compared to those of all other groups, slightly congested with nodular surface. Congestion of the ovary was prominent in combined cyclophosphamide and PRP treated group. The surface was nodular and glistening.

III. Histological examination of the ovaries revealed the following

A. Light microscopic results

Control groups showed (Figure 6 a-e)

Low magnification revealed the classical structure of rat's right ovary with irregular outline. The cortex was occupied by multiple primary, secondary and Graffian follicle with classical structure. (Figure 6 a)

The secondary follicle formed of a central primary oocyte surrounded by multiple layers of granulosa cells separated by multiple antral spaces. Theca interna appeared with vaculated acidophilic cytoplasm. (Figure 6 b,c)

Cortical mature Graffian follicles (Figure 6 d) showing characteristic appearance of oocyte surrounded by granulosa cells and theca interna and externa.

Corpus luteum were clearly identified by their classical central vacuolated granulosa lutein cells and peripherally located smaller and darker theca lutein cells. (Figure 6 e)

Sections of ovarian rat medulla (Figure 7 a,b) showed blood vessels surrounded by smooth muscles (Figure 7 a).While positive control subgroup showed mild vascular congestion with perivascular cellular infiltrates (Figure 7 b).

Cyclophosphamide treated group showed (Figure 8 a-d)

Low magnification revealed some degenerative changes with defective number of ovarian follicles (Figure 8a). Ovarian follicles with vacuolated granulosa cells with dense nuclei. Oocyte appeared small, ill-defined zona pellucida and surrounded by granulosa cells with rarified cytoplasm. (Figure 8b)

Corpus luteum appeared with acidophilic granulosa lutein cells with dense central nuclei while theca lutein showed densely acidophilic cytoplasm. (Figure 8c). Multiple atretic follicles and corpus albicans encouted inbetween thick walled blood vessels of the medulla. (Figure 8d)

Combined cyclophosphamide and PRP treated group showed (Figure 9 a-f)

Low magnification revealed improved appearance of ovarian follicle in comparison to cyclophosphamide treated group. Multiple ovarian follicles and corpora lutea occupied the cortex. (Figure 9 a,b)

Mature Graffian follicle appeared with detached oocyte, zona pellucida and cumulus oophrous. Detatched granulosa cells appeared in their lumen.Congested blood vessels were detected at theca interna and externa. (Figure 9 c,d).

Extravasation of RBCs was detected inbetween cells of corpus luteum (Figure 9e).

Ovarian medulla was illustrated with congested thickened blood vessesls (Figure 9f).

Histological data and morphometric studies

Number, diameter of follicles and diameter of oocyte (Table V-VII, Figures 10-12) calculated using "t" test during comparison between the means of different sample groups and ANOVA was performed for comparison between more than two groups.

Number and diameter of primary, secondary and mature Graffian follicles and diameter of oocyte reduced in cyclophosphamide treated group compared to control groups. Number and diameter of primary, secondary and mature Graffian follicles and diameter of oocyte increased in combined cyclophosphamide and PRP treated group compared to cyclophosphamide treated group.

B. Immunohistochemical findings

The immunohistochemical staining appeared as intracytoplasmic +/- nuclear brown coloration[42] of the ovarian follicles, in some stromal cells as well as in endothelial lining of medullary vessels of the ovary.

Comparison of the studied groups (Table VIII, Figure 16) showed the following

Regarding control groups the ovarian tissue showed low caspase 3 positivity in apoptotic cells among the whole ovarian tissue when compared to other studied groups, with mild intensity. (Figure 13 a-e)

In cyclophosphamide treated group, ovarian tissue showed the highest degree of capase 3 positivity in apoptotic cells when compared to other studied groups with severe intensity. (Figure 14 a-e)

Combined cyclophosphamide and PRP treated group showed a considerable recovery of the ovary with a low caspase 3 positivity in apoptotic cells in comparison to cyclophosphamide treated group with mild intensity. (Figure 15 a-d)

C. Ultrastructure results

Sections of control groups' ovaries showed normal architecture of oocyte, ooplasm filled with rough endoplasmic reticulum and mitochondria. The oocyte surrounded by glycoprotein of zona pellucida traversed by microvilli between oocyte and adjacent granulosa cells. (Figure 17 a,b). Primordial follicle lined by flat follicular cells and condensed stromal cells. (Figure 17c)

Sections of cyclophosphamide treated group ovaries showed irregular outline between oocyte and granulosa cells with apparent ooplasmic vacuoles (Figure 18a). Apoptotic bodies were frequently seen in between granulosa cells (Figure 18b). Some of granulosa cells nuclei were with irregular marginated chromatin, other cells with rarified cytoplasm and loss of organelles (Figure 18c). Theca lutein cells of corpus luteum showed dilated rough endoplasmic reticulum filled with retained secretion. Dense small nuclei of surrounding cells were noted (Figure 18d). Theca interna cells with multiple variable size empty vacuoles (Figure 18e). Theca interna showed also multiple vacuoles filled with secretion surrounded by flat nuclei of fibroblast from theca externa (Figure 18f).

Sections of combined cyclophosphamide and PRP treated group ovaries showed rarified ooplasm of oocyte surrounded by zona pellucida and granulosa cells. Granulosa cells with fragmented dense nuclei and rarified cytoplasm (Figure 19 a-c). Primordial follicle with flat follicular cells surrounded by stromal cells (Figure 19d). Secondary follicle with multiple layer of granulosa cells separated by antral spaces. The follicle was surrounded by theca cells (Figure 19e). Increased activity of fibroblast cells with deposition of collagen was noted (Figure 19f). Corpus luteum with vacuolated theca lutein cells, some vacuoles appeared with dense retained secretion. Plasma cells also appeared (Figure 19g). Congested blood vessels with RBCs, vacuoles with heterogenous densities were noted at hilum of the ovary. (Figure 19h).

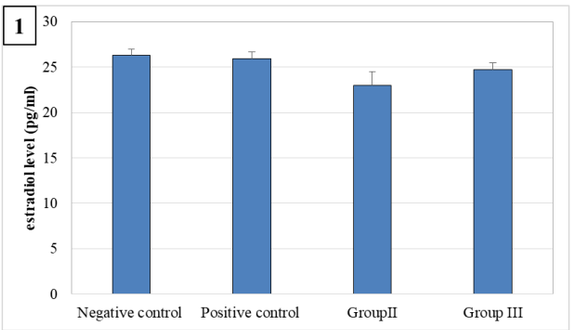

Fig. 1: Comparison between the different studied groups regarding estradiol level.

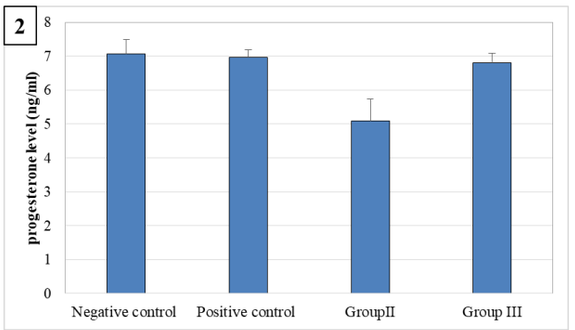

Fig. 2: Comparison between the different studied groups regarding progesterone level.

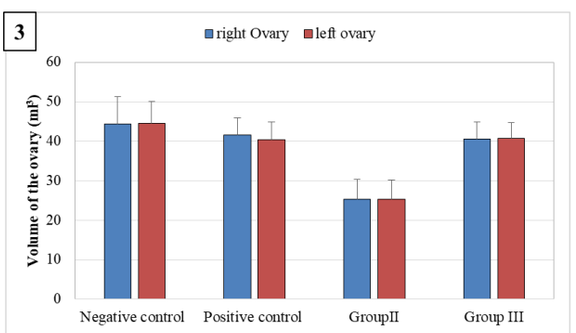

Fig. 3: Comparison between different studied groups regarding the volume of right and left ovary.

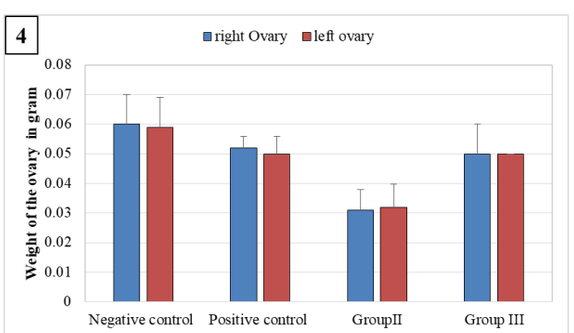

Fig. 4: Comparison between different studied groups regarding the weight of right and left ovary.

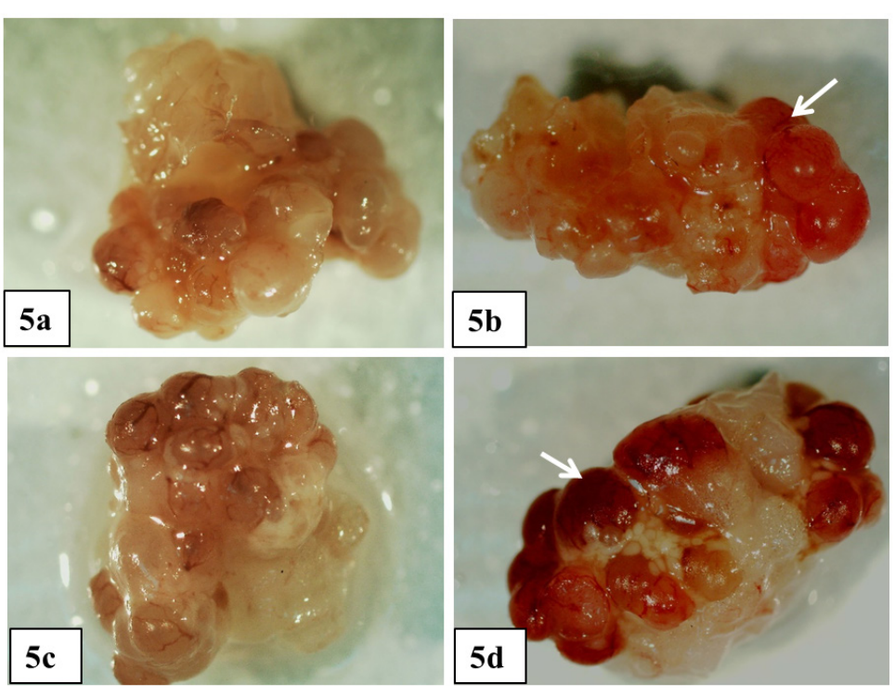

Fig. 5 (a-d): Photographs of rat's ovary from different studied groups. 5 a) Rat's ovary from negative control group showing the ovary had a pinkish color with nodular glistening surface (x8). 5 b) Rat's ovary from positive control group showing the ovary moderately congested (↑) with nodular glistening surface (x8) 5 c) Rat's ovary from cyclophosphamide treated group showing that the ovary was smaller than those of control groups, slightly congested with nodular surface (x8). 5 d) Rat's ovary from combined cyclophosphamide and PRP treated group showing that the ovary was highly congested with nodular surface. Congestion (↑) (x8).

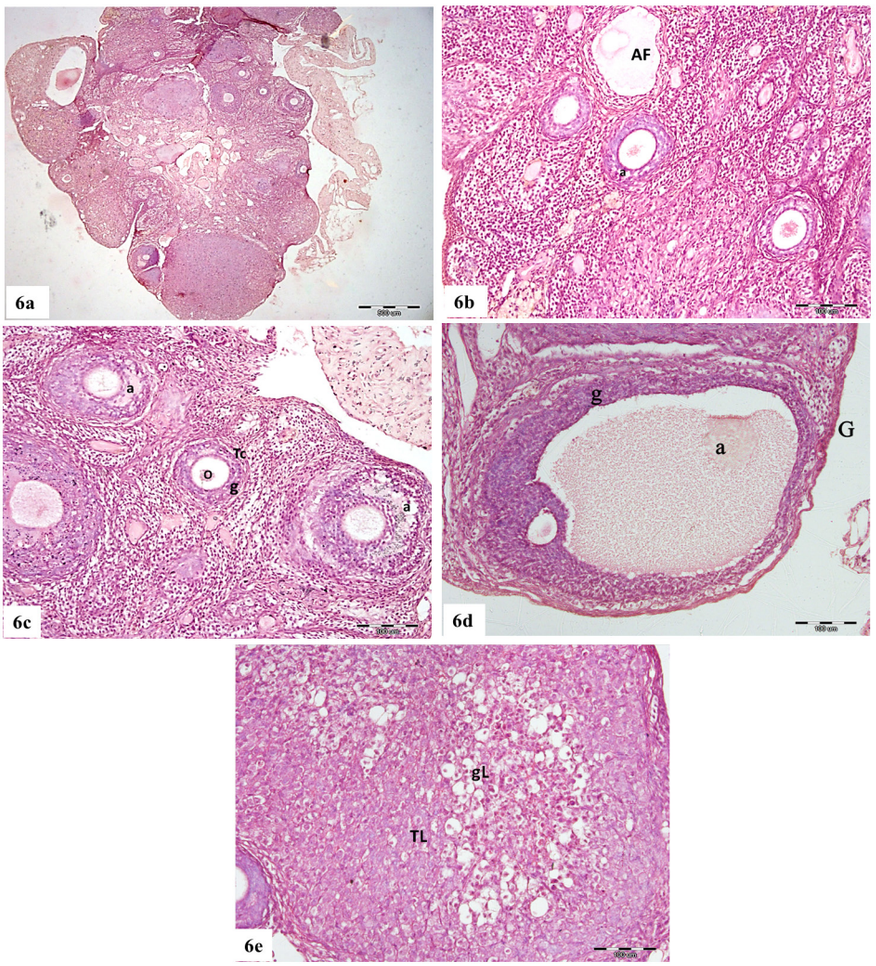

Fig. 6 (a-e): Light photomicrographs of control groups rat's ovarian sections showing irregular ovary outline with cortical follicles (6 a). (6 b & c) revealing primary and secondary ovarian follicles, oocyte (o), granulosa cells (g) vacuolated theca interna appears at the periphery (Tc) with prescence of antral spaces (a) filled with secretion. Notice the presence of atretic follicle (AF). (6 d) Mature Graffian follicle (G) with single antrum (a) surrounded by granulosa cells (g), theca interna and theca externa. Corpus luteum in (6 e) appears with large vacuolated granulosa lutein cells (gL) surrounded by small dense acidophilic theca lutein cells (TL). (H&E Mic Mag (a) x 40, (b-e) x200)

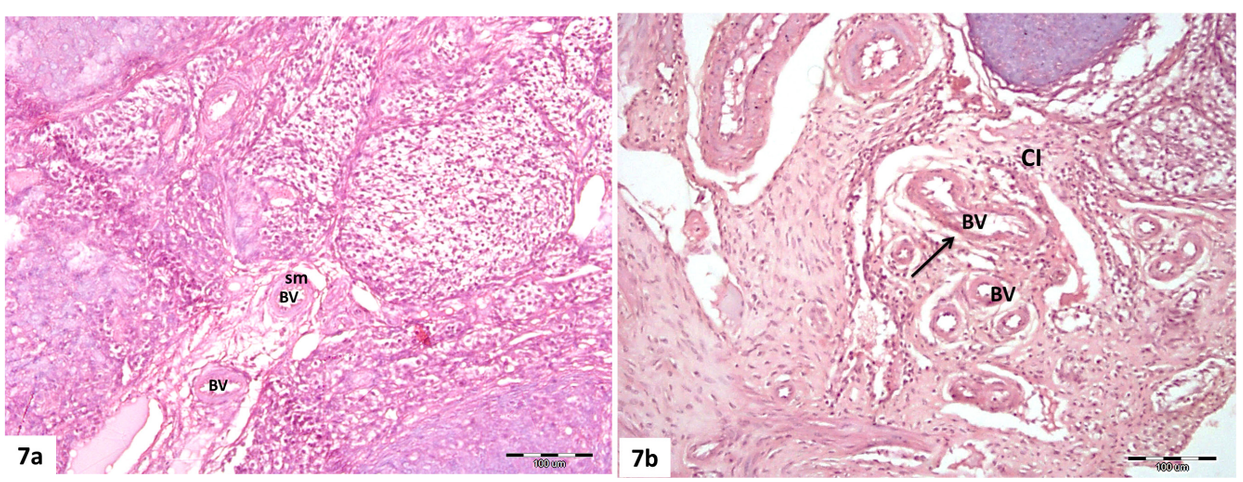

Fig.7 (a-b): Light photomicrographs of control groups rat's ovarian sections showing the medulla. 7 a) Negative control subgroup showing blood vessels (BV) surrounded by smooth muscles (sm). 7 b) Positive control subgroup showing mild vascular congestion (↑) and perivascular cellular infiltrates (CI). (H&E Mic Mag a-b x 200).

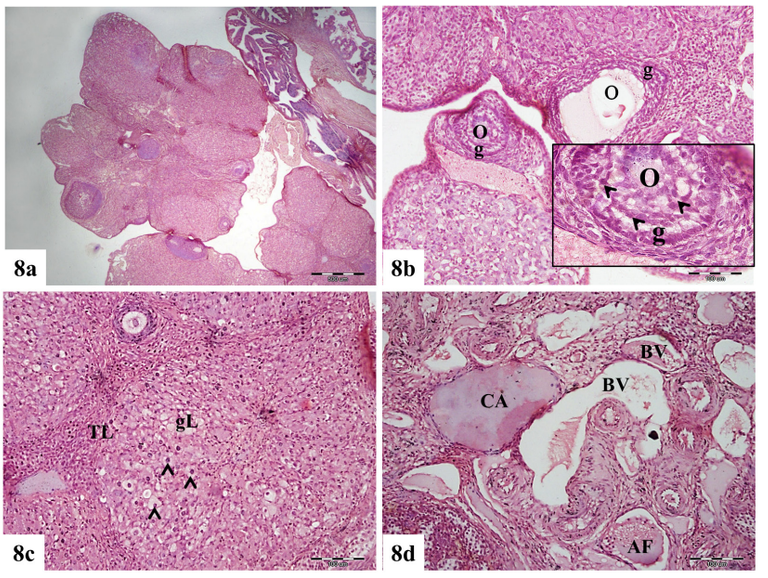

Fig. 8 (a-d): Light photomicrographs of cyclophosphamide treated group rat's ovarian sections revealing some degenerative changes with defective number of ovarian follicles in Figure (8 a). (8 b) Showing ovarian follicles with vacuolated granulosa cells (g) with dense nuclei (▲). Oocyte (o) appeared small, surrounded by granulosa cells (g) with rarified cytoplasm.
(8 c) Corpus luteum appeared with acidophilic granulosa lutein cells (gL) with dense central nuclei (▲) while theca lutein (TL) showing densely acidophilic cytoplasm. (8 d) Multiple atretic follicles (AF) and corpus albicans (CA) encountered inbetween thick walled blood vessels (BV) of the medulla. (H & E Mic Mag (a) x 40, (b-d) x 200, inset x 400).

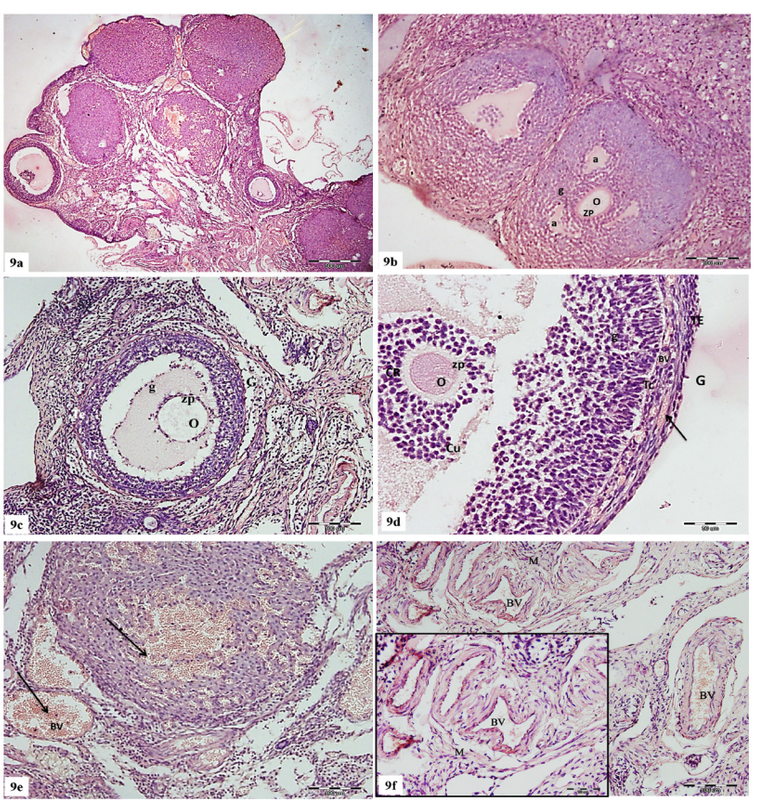

Fig. 9 (a-f): Light photomicrographs of combined cyclophosphamide and PRP treated group rat's ovarian sections (9 a) showing ovarian follicles and corpora lutea located in the cortex. (9 b): antral follicle, multiple antral cavities(a) between granulosa cells (g), oocyte (o) surrounded by zona pellucida (zp). (9 c, d): Mature Graffian follicle (G) with detached granulosa cells (g), cumulus oophrous (cu), corona radiata (CR), oocyte (o), zona pellucida (zp), and congested blood vessels (↑) were detected at theca interna (Tc) and externa (Te). (9 e-f) showing thickening and congestion (↑) of medullary blood vessels (BV). (H&E Mic Mag (a)x 40, (b-f)x 200, inset x 400).

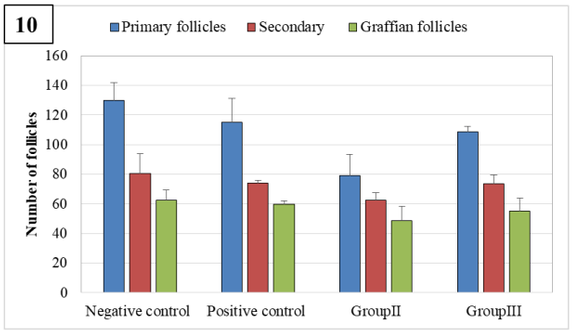

Fig. 10: Comparison between different studied groups regarding number of follicles.

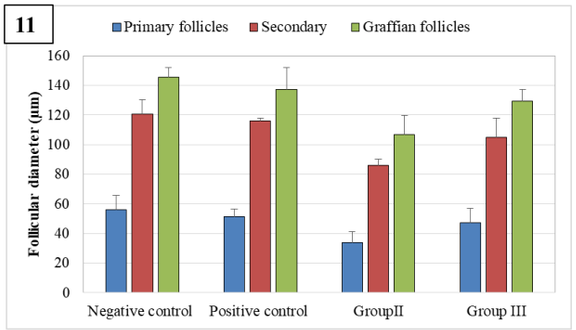

Fig. 11: Comparison between different studied groups regarding the follicles diameters.

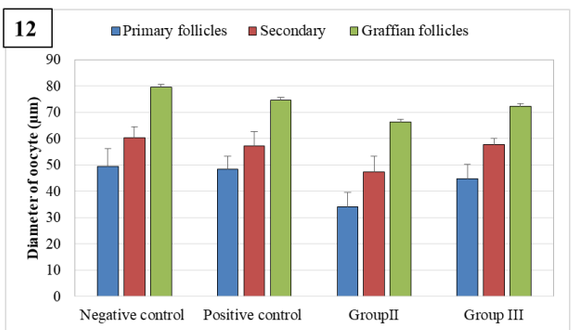

Fig. 12: Comparison between different studied groups regarding the diameter of oocyte.

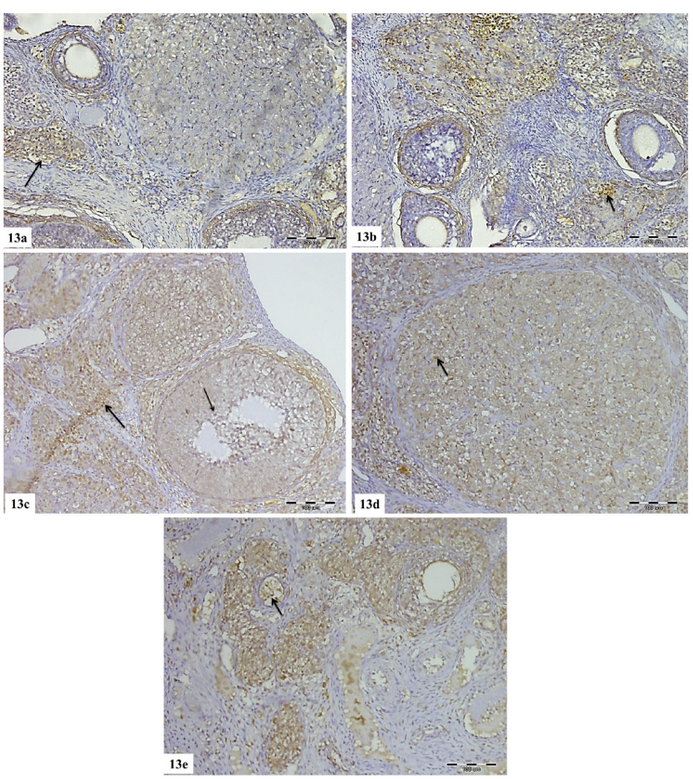

Fig. 13 (a-e): Ovarian tissues of rats from control groups featuring low caspase 3 positivity in apoptotic cells within ovarian tissue 13 a) low caspase 3 positivity in apoptotic cells within ovarian cortex. 13 b) low caspase 3 positivity in apoptotic cells within the medulla of negative control subgroup. 13 c) low caspase 3 positivity in apoptotic cells within secondary follicle. 13 d) low caspase 3 positivity in apoptotic cells within corpus luteum. 13 e) low caspase 3 positivity in apoptotic cells within the medulla of positive control subgroup. (Caspase 3 immunohistochemical staining, x 200) ↑: caspase 3 positive apoptotic cells.

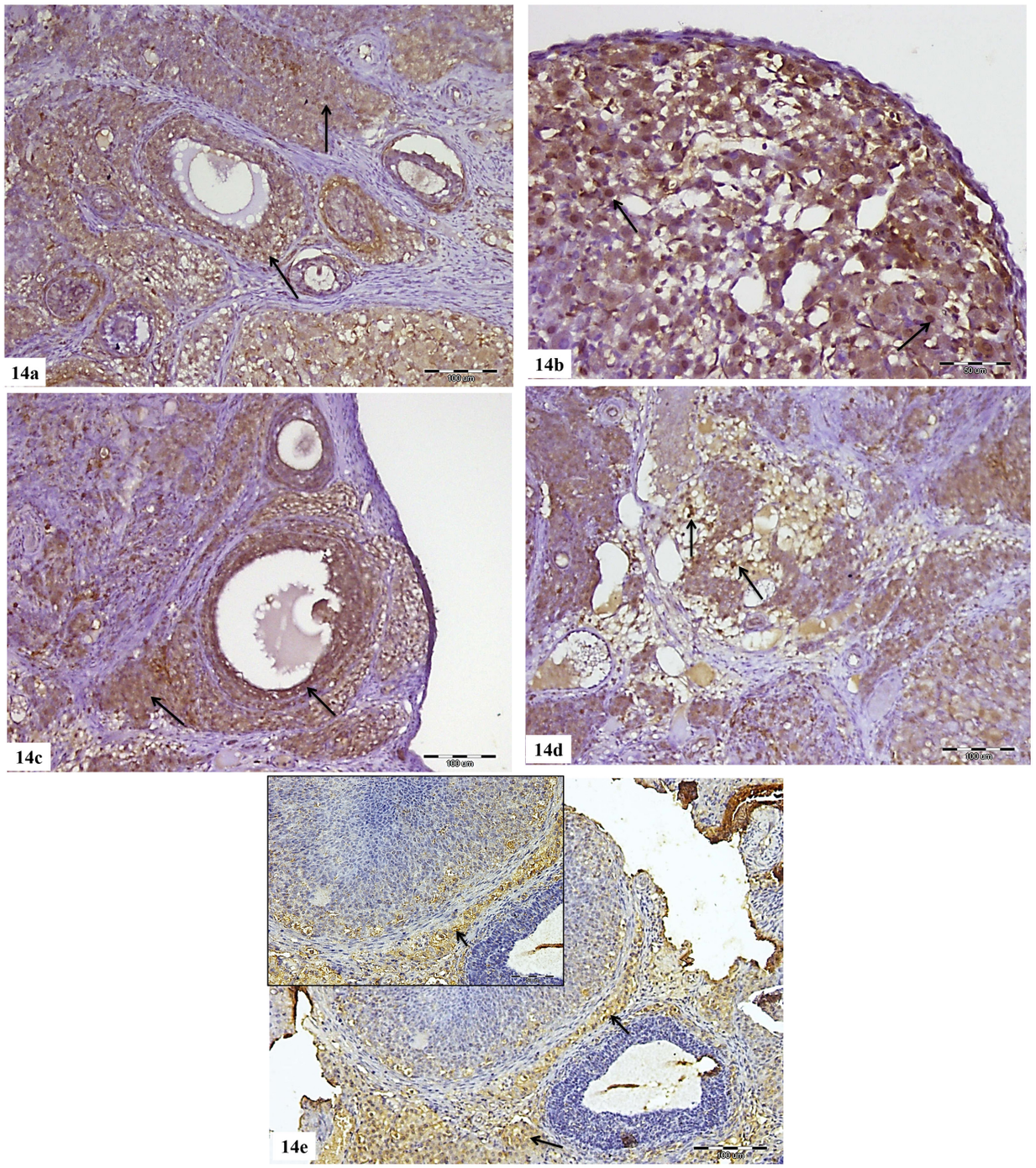

Fig. 14 (a-e): Ovarian tissues of rats from cyclophosphamide treated group featuring high caspase 3 positivity in apoptotic cells within ovarian tissue. 14 a, c) high caspase 3 positivity in apoptotic cells within mature Graffian follicle. 14 b, e) high caspase 3 positivity in apoptotic cells within corpus luteum. 14 d) high caspase 3 positivity in apoptotic cells among the medulla. (Caspase 3 immunohistochemical staining, (a, c, d, e) x 200, (b) x 400, inset x 400) ↑: caspase 3 positive apoptotic cells.

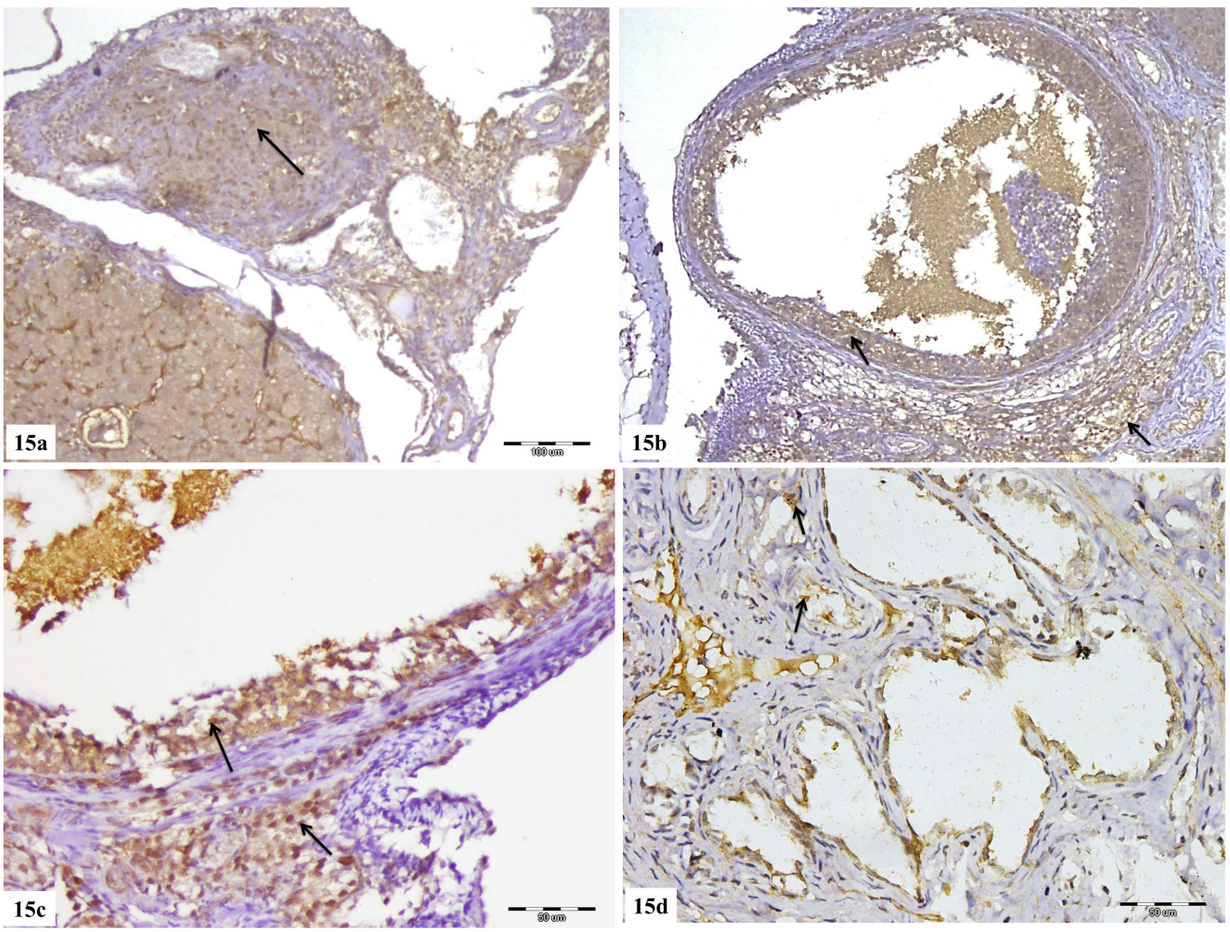

Fig. 15 (a-d): Ovarian tissues of rats from combined cyclophosphamide and PRP treated group featuring low caspase 3 positivity in apoptotic cells within ovarian tissue. 15 a, d) low caspase 3 positivity in apoptotic cells among the medulla. 15 b, c) low caspase 3 positivity in apoptotic cells within mature Graffian follicle. (Caspase 3 immunohistochemical staining, (a, b) x 200, (c, d) x 400). ↑: caspase 3 positive apoptotic cells.

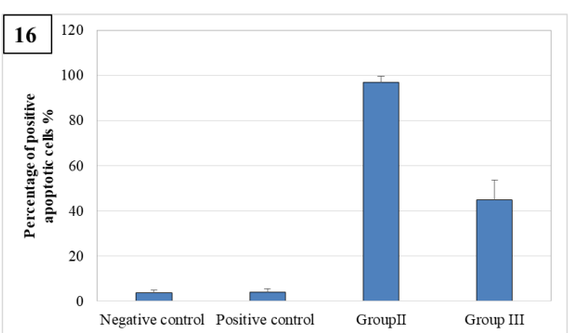

Fig. 16: Comparison between different studied groups regarding the percentage of positive apoptotic cells.

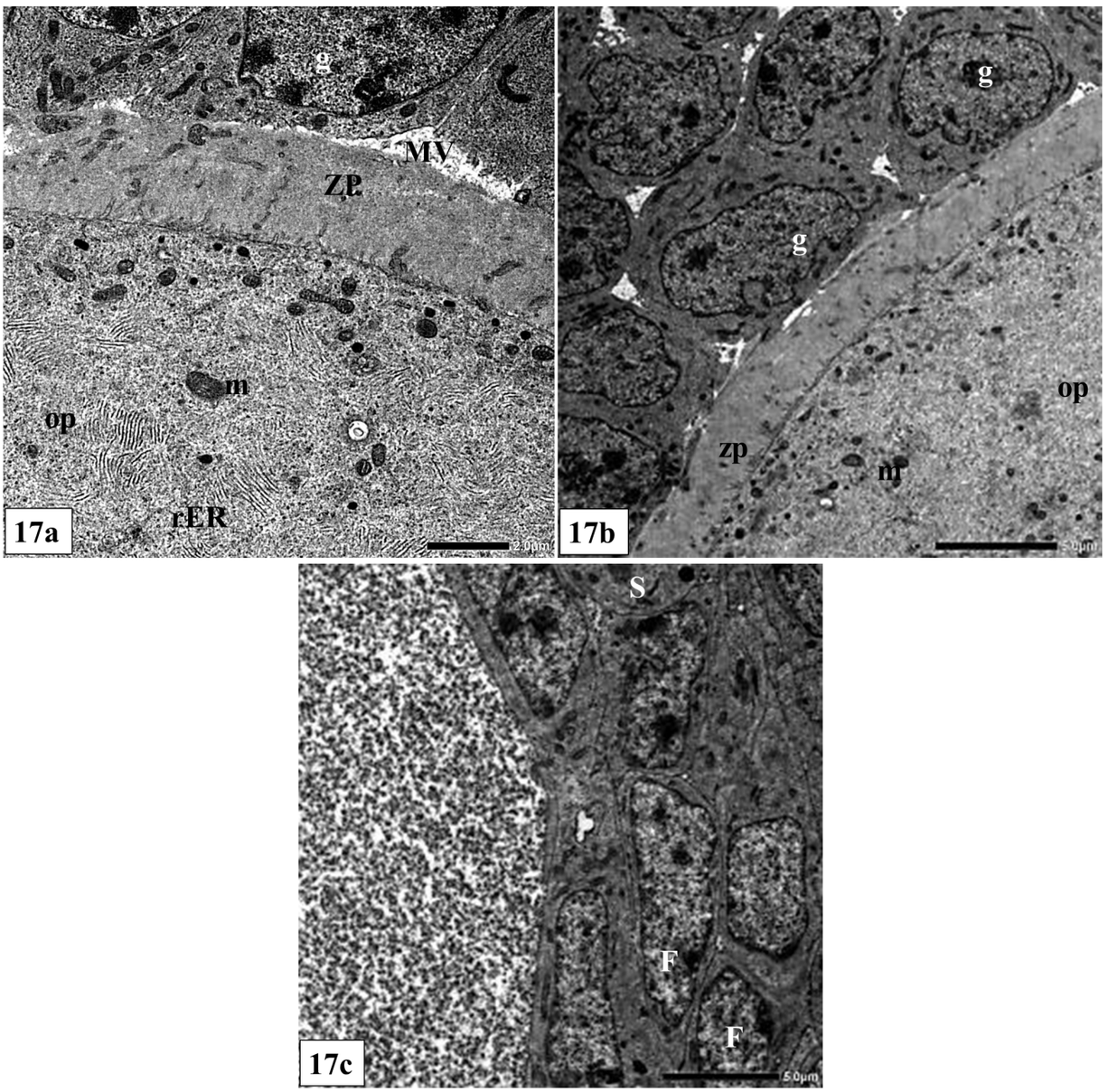

Fig. 17 (a-c): Electron photomicrographs of sections of control groups ovaries showing normal architecture of oocyte, ooplasm (op) filled with rough endoplasmic reticulum (rER) and mitochondria (m). The oocyte surrounded by glycoprotein of zona pellucida (zp) traversed by microvilli (MV) between oocyte and adjacent granulosa cells (g) (17 a, b). (17 c) Part of primordial follicle lined by flat follicular cells (F) and condensed stromal cells (S). (Mic. Mag. (a) x 2500, (b-c) x 1500)

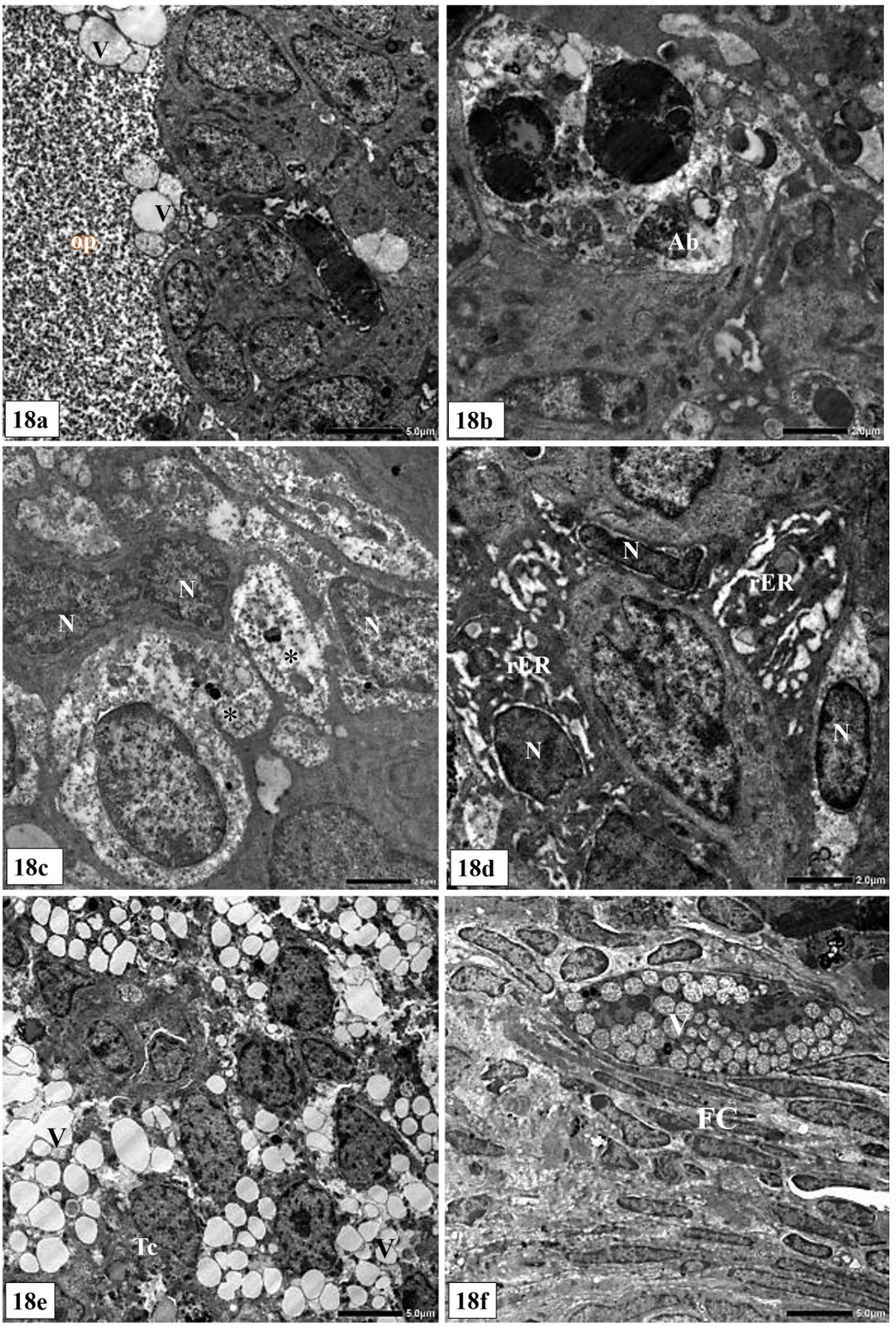

Fig. 18 (a- f): Electron photomicrographs of sections of cyclophosphamide treated group ovaries showing irregular outline between oocyte and granulosa cells with apparent ooplasmic vacuoles (V) within ooplasm (op) (18 a). (18 b) Apoptotic bodies (Ab) frequently seen in between granulosa cells. (18 c) Higher magnification of granulosa cells, some nuclei (N) with irregular marginated chromatin, other cells with rarified cytoplasm (*) and loss of organelles. (18 d) Theca lutein cells of corpus luteum showing dilated rough endoplasmic reticulum (rER) filled with retained secretion. Notice dense small nuclei (N) of surrounding cells. (18 e) Theca interna cells (Tc) with multiple variable size empty vacuoles (V). (18 f) Theca interna showing multiple vacuoles (V) filled with secretion surrounded by flat nuclei of fibroblast (FC) from theca externa. (Mic.Mag (a)x 1200, (b-d)x 2500,(e-f) x 1000)

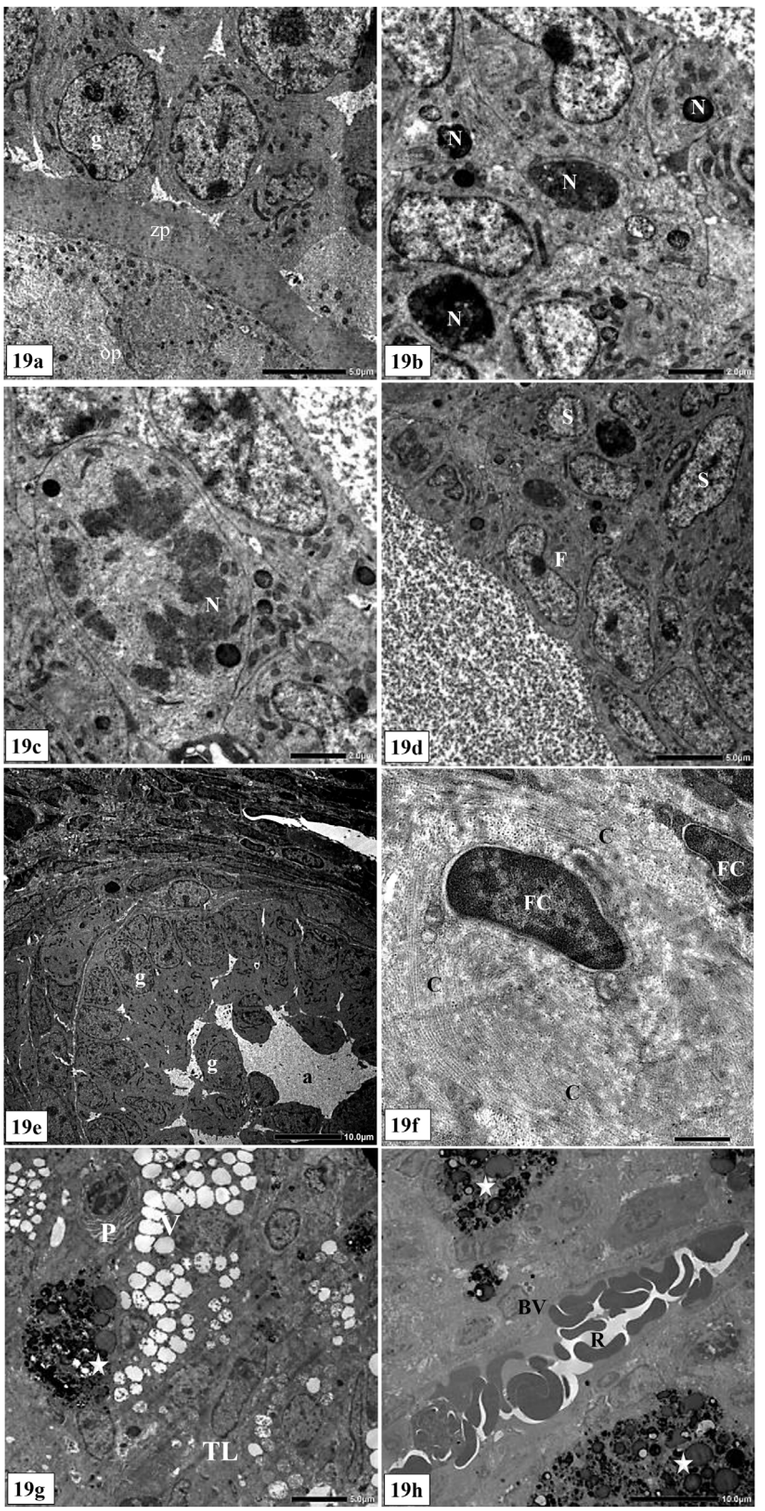

Fig. 19 (a-h): Electron photomicrographs of sections of combined cyclophosphamide and PRP treated group ovaries showing rarified ooplasm (op) of oocyte surrounded by zona pellucida (zp) and granulosa cells (g). Notice some cells with dense shrunken nuclei (N) (19 a- b). (19 c) Granulosa cells with fragmented dense nuclei (N) and rarified cytoplasm. (19 d) Primordial follicle with flat follicular cells (F) surrounded by stromal cells (S). (19 e) Secondary follicle appeared with multiple layer of granulosa cells (g) separated by antral spaces (a). The follicle was surrounded by theca cells. (19 f) Higher magnification of fibroblast cells (FC) with deposition of collagen (C). (19 g) Part of corpus luteum with vacuolated theca lutein cells (TL), some vacuoles appeared with dense retained secretion (*). Plasma cell (P) was noted. (19 h) Congested blood vessels (BV) with RBCs (R). Notice presence of vacuoles with heterogenous densities (*) (Mic. Mag. (a)x 1500, (b-c) x 2500, (d) x 1200, (e) x 600, (f) x 5000, (g) x 1000, (h) x 800)

Table I: Comparison between the different studied groups regarding estradiol level

Estradiol (pg/ml)	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
Range	25.2-27.1	24.5-25.6	21.1-25.3	24.1-26.1
Mean	26.30	25.89	22.97	24.70
SD	0.66	0.79	1.53	0.78
ANOVA		17.33		
P value		0.001*		
P1		0.072	0.001*	0.002*
P2			0.003*	0.312
P3				0.017*

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

Table II: Comparison between the different studied groups regarding progesterone level

Progesterone (ng/ml)	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
Range	6.3-7.5	6.58-7.13	4.5-6.1	6.5-7.3
Mean	7.07	6.97	5.08	6.80
SD	0.41	0.21	0.66	0.28
ANOVA		14.33		
P value		0.0001*		
P1		0.307	0.001*	0.111
P2			0.001*	0.134
P3				0.001*

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

Table III: Comparison between different studied groups regarding the volume of right and left ovary

Volume (ml^3)	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
right Ovary				
Range	35-55	35-50	18.2-30	35-48
Mean	44.30	41.65	25.27	40.50
SD	6.99	4.25	5.05	4.37
ANOVA		20.11		
P value		0.001*		
P1				0.143
P2		0.0856	0.005*	0.0001*
P3			0.001*	0.011*
left ovary				
Range	36.5-51.48	31.45-45.6	18.746-30.9	36.75-47.04
Mean	44.56	40.31	25.38	40.72
SD	5.50	4.65	4.86	4.05
ANOVA		19.85		
P value		0.001*		
P1				0.099
P2		0.085	0.002*	0.416
P3			0.003*	0.001*

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

Table IV: Comparison between different studied groups regarding the weight of right and left ovary

Weight (gram)	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
right Ovary				
Range	0.043-0.066	0.044-0.052	0.02-0.042	0.045-0.065
Mean	0.06	0.052	0.031	0.05
SD	0.01	0.004	0.007	0.01
ANOVA		10.85		
P value		0.008*		
P1		0.0785	0.050*	0.4731
P2			0.0002*	0.108
P3				0.001*
left ovary				
Range	0.045-0.065	0.043-0.055	0.020-0.044	0.045-0.058
Mean	0.059	0.050	0.0320	0.050
SD	0.01	0.006	0.0077	0.00
ANOVA		12.85		
P value		0.001*		
P1		0.079	0.005*	0.0156*
P2			0.0006*	0.243
P3				0.003*

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

Table V: Comparison between different studied groups regarding number of follicles

Number of follicles	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
Primary follicles				
Range	120.0-154.0	92.0-134.0	63.0-102.0	103.0-114.0
Mean	129.67	115.17	79.0	108.67
SD	12.26	16.02	14.25	3.56
ANOVA		16.85		
P value		0.001*		
P1		0.054	0.0001*	0.001*
P2			0.001*	0.177
P3				0.0001*
Secondary				
Range	71.0-107.0	72.0-76.0	55.0-69.0	65.0-82.0
Mean	80.50	74.00	62.33	73.67
SD	13.38	1.90	5.47	5.75
ANOVA		20.6		
P value		0.001*		
P1		0.133	0.006*	0.139
P2			0.002*	0.448
P3				0.003*
Graffian follicles				
Range	50.0-68.0	53.0-64.0	36.0-64.0	42.0-62.0
Mean	62.50	59.65	48.67	55.33
SD	6.69	2.42	9.79	8.62
ANOVA		2.65		
P value		0.236		
P1		0.265	0.009*	0.069
P2			0.032*	0.312
P3				0.12

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

Table VI: Comparison between different studied groups regarding the follicles diameters

Follicles diameters (µm)	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
Primary follicles				
Range	42.0-72.0	44.0-59.0	25.0-42.0	36.0-64.0
Mean	56.17	51.33	33.83	47.17
SD	9.6	5.05	7.52	9.91
ANOVA		11.69		
P value		0.012*		
P1		0.15	0.001*	0.071
P2			0.001*	0.19
P3				0.12*
Secondary				
Range	108.0-132.0	108.0-120.0	80.0-92.0	91.0-122.0
Mean	120.83	116.1	85.83	105.0
SD	9.3	1.79	4.4	13.05
ANOVA		15.85		
P value		0.006*		
P1		0.072	0.0001*	0.018*
P2			0.001*	0.172
P3				0.003*
Graffian follicles				
Range	139.0-157.0	122.0-162.0	92.0-123.0	120.0-144.0
Mean	145.5	137.17	107.0	129.33
SD	6.41	14.76	12.87	8.09
ANOVA		26.52		
P value		0.001*		
P1		0.117	0.0001*	0.002*
P2			0.002*	0.14
P3				0.002*

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

Table VII: Comparison between different studied groups regarding the diameter of oocyte

Diameter of oocyte (µm)	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
Primary follicles				
Range	39.0-59.0	44.0-54.0	30.0-45.0	41.0-55.0
Mean	49.5	48.32	34.0	44.83
SD	6.8	4.9	5.62	5.34
ANOVA		17.11		
P value		0.001*		
P1		0.069	0.001*	0.108
P2			0.001*	0.136
P3				0.003*
Secondary				
Range	56.0-67.0	52.0-62.0	40.0-55.0	55.0-62.0
Mean	60.33	57.17	47.33	57.67
SD	4.13	5.38	5.92	2.42
ANOVA		20.1		
P value		0.001*		
P1		0.140	0.001*	0.101
P2			0.007*	0.420
P3				0.001*
Graffian follicles				
Range	71.0-91.0	60.0-85.0	62.0-75.0	67.0-78.0
Mean	79.67	74.67	66.33	72.33
SD	7.31	8.89	4.5	4.59
ANOVA		19.05		
P value		0.001*		
P1		0.156	0.002*	0.032*
P2			0.034*	0.29
P3				0.023*

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

Table VIII: Comparison between different studied groups regarding percentage of positive apoptotic cells (Density of immunohistochemical stain)

Density (Percentage of positive cells)	Group I (negative control)	Group I (positive control)	Group II (CX treated)	Group III (CX + PRP)
Range	2-5	2-6	92.0-100.0	38.0-56.0
Mean	3.67	4.0	97.0	45.0
SD	1.21	1.55	2.65	8.58
ANOVA		102.1		
P value		0.001*		
P1		0.291	0.0001*	0.001*
P2			0.0001*	0.001*
P3				0.001

P1 comparison between group I (negative control) and other groups.
P2 comparison between group I (positive control) and other groups.
P 3 comparison between group II and group III
* Significant at level 0.05

DISCUSSION

Chemotherapy treatment has drawbacks on fertility affecting females of different age groups up to menopause[13] Chemotheraputic agents are used in the treatment of malignant diseases such as lymphomas, melanomas, central nervous system tumers in young age while in older age it is used in treatment of breast cancer, cancer cervix and lung cancer[43]. Cancer patients usually treated with combination of alkylating as cyclophosphamide and alkylating-like drugs which linked to toxicity of ovarian tissues and disturbance of ovarian functions[44].

Several studies were carried to decrease the impact of chemotherapeutic drugs on fertility[45-47]. The available studies are not enough with respect to provision of a suitable protective agent with high efficacy, low side effects and cost.

Aim of the present work was to assess the degenerative changes on rat ovarian tissue after cyclophosphamide treatment and to evaluate the protective effect of PRP by using light microscopy and electron microscope to estimate the ultrastructure changes, and through immunohistochemical methods for apoptosis detection.

The dose of cyclophosphamide used in the present work were chosen according to our pilot study to mimic chemotherapeutic therapy sessions in human and in the same time induce ovarian tissue damage without causing death among the experimental rats. The studies done by Vural B *et al*.[30] Dehghani F *et al*.[27] supported the use of the same dose of cyclophosohamide in rats as in the present work while Tang H *et al*.[48] and Zheng Q *et al*.[49] used different doses of cyclophosphamide to induce POF in rats.

Cyclophosphamide inhibits its own activation after seven days in rats according to the study done Angley M *et al*.[32] so the interval between successive doeses was seven days.

In the present study, Cyclophosphamide treated group showed small, slightly congested ovaries with significant decrease in weight and volume. Histologically, the ovarian structure was distorted with reduction of all morphometric parameters. There was a higher degree of capase 3 positivity in apoptotic cells. This was confirmed by Electron microscope which showed apoptotic bodies, dilated rough endoplasmic reticulum. Combined cyclophosphamide and PRP treated group showed rounded irregular vacuoles appeared in the ooplasm. These results were accompanied by decrease levels of estrogen and progesterone.

The mechanism of action of cyclophosphamide could explain these findings, as it alkylates DNA and inhibits protein synthesis by forming DNA and RNA crosslinking[50].

Moreover, cyclophosphamide irreversibly induces reduction of microvascularization of ovarian follicles as indicated by study done by Ezoe K *et al*.[51].

Luan Y *et al*.[52] in their study on inhibitors of apoptosis in a trial to protect the ovary after cyclophosphamide therapy concluded that cyclophosphamide induced apoptosis of granulosa cells and oocyte which is parallel to the findings of the present work.

Giusti I *et al*.[53] studied the ovarian tissue using electron microscope and considered that alternated structure of zona pellucida and retained secretory vacuoles in theca interna layer as ultrastructural signs of apoptosis.

In the present work, PRP was used as a blood product rich of growth factors in a trial to enhance ovarian tissue and function after administration of cyclophosphamide. It was prepared from the blood of five adult male rats. Male not female, rats were chosen based on the theory adopted by Weil-Fugazza J *et al*.[54] who stated that the platelet count and growth factors in PRP increases during ageing in male rats and slightly decreases in female rats.

The ovaries of combined cyclophosphamide and PRP treated group were very congested with significantly higher weight and volume than cyclophosphamide treated group. Histologically there was improvement of morphometric parameters. The ovaries showed low caspase 3 positivity in apoptotic cells. There were abundant fibroblast cells detected by electron microscope and dilated blood vessels in the hilum of the ovary. Abundant secretory vacuoles appeared in theca interna. This was confirmed biochemically by elevated plasma estradiol and progesterone level which were significantly higher than those of cyclophosphamide treated group.

These findings could be explained by a theory adopted by El-Sharkawy H *et al*.[55] who thought that PRP with its rich source of growth factors may inhibit cytokine release, decrease inflammation, and as a result enhance tissue regeneration.

Cheng H *et al*.[56] suggested that PRP induces angiogenesis via its content of growth factors especially vascular endothelial growth factor (VEGF) that would agree with and explain the increased congestion concomitant with the use of PRP observed in positive control group and combined cyclophosphamide and PRP treated group.

Agreeing with the current work results, Van der Bijl I *et al*.[57] stated that PRP stimulates fibroblast proliferation, migration through its content of tissue growth factor β1 (TGF-β1) and platelet derived growth factor (PDGF) which may explain the presence of abundant fibroblast detected by electron microscope.

Hsu C *et al*.[58] reported a successful in *vitro* fertilization in woman with POF received combined PRP and gonadotropins. They concluded that PRP helped to restore antral follicles and improved ovarian functions but they warned from unknown possible side effects such as developing ovarian cancer.

A study done by Abdullah TH *et al*.[59] about the possible role of PRP in restoration of ovarian tissues concluded that PRP injection had good amelioration effects on ovaries in women with infertility.

Melo P *et al*.[60] in their study compared the use of PRP versus no intervention in women with infertility revealed that PRP injection into ovaries was safe and improved the ovarian reserve as measured by morphometric and biochemical studies. However, they could not evaluate the effect of PRP on pregnancy outcome.

However, disadvantages of PRP where mentioned by Yuan X *et al*.[61] in their book about regenerative medicine. They claimed that the lack of standard method for preparation of PRP, standard dose of PRP, variations among patients and donors regarding age, gender and chronic diseases limit the use of PRP as regenerative treatment.

From the above findings, it is obvious that PRP seems to have protective effects on ovarian tissues and functions and its concomitant use with cyclophosphamide helps to restore the ovarian tissues near normal.

Further studies are needed to confirm the safety of use of PRP and to investigate the appropriate concentration of PRP, technique and ways of delivery targeting ovarian follicles on different experimental models.

ABBREVIATIONS

- **CERRMA:** Center of Excellence for Reasearch in Regenerative Medicine and its Applications.
- **PDGF:** platelet derived growth factor.
- **POF:** premature ovarian failure.
- **PPP:** platelet-poor plasma.
- **PRP:** platelet- rich plasma.
- **TGF-β1:** tissue growth factor β1.
- **VEGF:** vascular endothelial growth factor.
- **4F1G:** 4% formaldehyde and 1% gluteraldehyde.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تأثير البلازما الغنية بالصفائح الدموية في حالات فشل المبيض المستحدث بواسطة عقار السيكلوفوسفاميد في الجرذان البيضاء: دراسة هستولوجية و تشريحية

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المقدمة: يعرف فشل وظائف المبيض المبكر بأنه استنفاذ مخزون المبيض بشكل غير طبيعي مما يؤدئ إلي انقطاع الطمث. و يبلغ معدل حدوثه بين السيدات المصريات قبل سن الأربعين حوالي ٨٪ بينما معدل حدوثه عالميا حوالي ١٪.
الهدف: بحث التأثير الواقي المحتمل للبلازما الغنية بالصفائح الدموية علي أنسجة المبيض في حالات فشل المبيض المستحدث باستخدام عقار السيكلوفوسفاميد.
مواد وطرق البحث: تم إجراء هذه الدراسة علي تسعة و عشرين جرذا أبيض. أربعة و عشرون جرذا من إناث الجرذان استخدموا كمجموعة تجريبية. خمسة جرذان ذكور استخدموا للحصول علي البلازما الغنية بالصفائح الدموية. المجموعة التجريبية تم تصنيفهم عشوائيا إلي خمس مجموعات:(المجموعة الضابطة، المجموعة الثانية: تم إعطاؤها عقار السيكلوفوسفاميد، المجموعة الثالثة: تم إعطاؤها عقار السيكلوفوسفاميد بالإضافة إلي البلازما الغنية بالصفائح الدموية). قياس مستوي هرمون الاستراديول و البروجسيتيرون تم في اليوم الثاني و العشرين من التجربة قبل ذبح جرذان المجموعة التجريبية، و بعد ذبحها تم فحص المبايض بإستخدام الميكروسكوب التشريحي ثم تحضير شرائح من المبيض لفحصها هستولوجيا و هيستوكيميائيا و للفحص بإستخدام الميكروسكوب الإلكتروني.
النتائج: أظهرت المجموعات التي تلقت عقار السيكلوفوسفاميد ارتفاع معدل الموت المبرمج لحويصلات المبيض و البويضات بينما أظهرت المجموعات التي تلقت البلازما الغنية بالصفائح الدموية تحسنا ملحوظا في مظهر حويصلات المبيض.
الإستنتاج: البلازما الغنية بالصفائح الدموية تحمي أنسجة المبيض و تساعد علي استعادة وظائفه عند استخدامها بصحبة عقار السيكلوفوسفاميد.