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Original Research Article

The Possible Protective Effect of Vitamin E and Melatonin on Cyclophosphamide-Induced Ovarian Failure in Adult Female C57BL/6 Mice: Histological and Immunohistochemical Study

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ABSTRACT

Background: Cyclophosphamide has a toxic effect on the ovaries and may induce ovarian failure**.** Premature ovarian failure (POF) is a common condition that stands for cessation of the ovarian functions in women before the age of 40 years. Vitamin E shows antioxidant features that can protect ovary from toxicity of cyclophosphamide. Melatonin is known for having antioxidant features that help ovary to restore its normal structure.

Purpose: Study the potential protective role of co-administration of vitamin E and melatonin on the ovarian structure in cyclophosphamide induced ovarian failure.

Materials and Methods: The mice were divided into 5 groups (**group I:** control group that received orally distilled water and olive oil. **group II:** cyclophosphamide group (75 mg/kg/day, intraperitoneally), **group III:** cyclophosphamide+ vitamin E group (200 mg/kg/day, by orogastric tube), **group IV:** cyclophosphamide+ melatonin group (50 mg/kg/day, by orogastric tube), **group V:** cyclophosphamide+ vitamin E+ melatonin group. The ovarian sections were examined histologically and immunohistochemically.

Results: Ovaries of cyclophosphamide+ vitamin E group and cyclophosphamide+ melatonin group had partial improvement in the ovarian structure with reappearance of follicles at different stages of development, partial regression in the extent of cortical fibrosis and moderate decrease in immunohistochemical reaction against caspase-3 compared to cyclophosphamide group. Ovaries of cyclophosphamide+ vitamin E+ melatonin group showed restoration of nearly normal structure with normal appearance of follicles, zona pellucida and minimal cortical collagen deposition. Morphometric studies of the cyclophosphamide+ vitamin E+ melatonin group revealed a marked increase in follicle count, diameter and ovarian diameter with a decrease in collagen fibers deposition.

Conclusion: A Combination of vitamin E and melatonin with cyclophosphamide helps to preserve the ovary and restore normal ovarian structure in comparison to the use of vitamin E or melatonin alone.

KEY WORDS: C57BL/6 mice - caspase reaction - cyclophosphamide - follicles -melatonin - morphometric - premature ovarian failure – vitamin E.

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BACKGROUND

Cyclophosphamide is a widely used drug. It directly damages ovarian follicles by inducing apoptosis as a result of DNA damage. Additionally, oxidative stress and inflammation play role in cyclophosphamide induced ovarian failure [1,2]. It causes rapid reduction in the oocytes reserve and disappearance of the resting primordial follicles and other growing follicles [3]. It is linked to secondary premature ovarian failure (POF) that is characterized by amenorrhea and elevation of gonadotrophin hormones levels [4,5].

Different modalities are used in the management of cyclophosphamide induced POF including hormone replacement therapy (HRT) which is the most common method used. However, it can induce hormonal dependent cancer [6]. Second, the invasive method as in-vitro activation of dormant ovarian follicles which is useful for treatment of POF [7]. Third, mesenchymal stem cells (MSCs) are considered a possible modality in the management of POF. MSCs can restore the ovarian function and improve the structure of the damaged ovary but this method is very expensive and stem cells may turn into malignant cells [8].

Naturally occurring supplements show beneficial effects with minimal side effects as ginger and olive oil that were frequently used for POF treatment. To our knowledge no ideal therapy is found to protect ovaries from cyclophosphamide induced ovarian failure and to preserve fertility and the ovarian function [9].

Vitamin E is a lipid soluble vitamin that is found in many sources as green vegetables, vegetable oils and fruits and has antioxidant features [10].

Melatonin is produced mainly by the pineal gland and other extrapineal sites like retina, cerebellum, and ovary. Melatonin has anti-inflammatory and antioxidant properties [11]. It activates tumor suppressor genes so it exerts anti-tumor action [12].

The work aims to compare the potential protective roles of vitamin E or melatonin alone versus their concomitant administration on ovarian structure in ovarian failure induced by cyclophosphamide in adult female black mice, using histological and immunohistochemical studies.

MATERIALS AND METHODS

Animals: The present study was carried out on thirty-six adult female black mice aged about 8 weeks and weighed from 23 g to 29 g, obtained from the Animal House, Anatomy department, Faculty of Medicine, Alexandria University after the approval of Ethics Committee, Faculty of Medicine, Alexandria University [13].

All study groups are housed in standard housing conditions. Diet was administered in accordance with the Egyptian Institute of Nutrition (EIN). Throughout the experimental period, mice were given food and fresh water *ad libitum*. Diet was purchased from Tanta Oil and Soap Company, Egypt. Diet components: Bran – cotton seed meal - yellow corn - molasses - limestone powder - table salt. Mice were housed two per cage in a room temperature maintained at twenty degrees (20 ^{0}%C) on a 12:12 hour light: dark cycle [13].

The mice were divided into 5 groups 6 mice each except the control group that was 12 mice divided in two subgroups.

Group I: (control group) subdivided into

Subgroup Ia: 6 mice received distilled water which was used as vehicle for cyclophosphamide (5 ml/ Kg) intraperitoneally (IP), in the fifth, twelfth and nineteenth days of study.

Subgroup Ib: 6 mice received olive oil which was used as vehicle for vitamin E and melatonin (10 ml/Kg) by orogastric tube for 19 consecutive days from the first day of the experiment.

The olive oil was the solvent used for vitamin E and Melatonin as vitamin E is a fat-soluble vitamin and Melatonin is lipophilic. Distilled water is the solvent used for Cyclophosphamide.

Group II: (cyclophosphamide group) (Cyc G) included 6 mice received cyclophosphamide (75 mg/kg/day, intraperitoneally (IP), in the fifth, twelfth and nineteenth days of study [14].

The dose of cyclophosphamide was adjusted to induce ovarian failure in the experimental mice without resulting in their death. The frequency of administration was chosen based on the fact that cyclophosphamide inhibits its own activation within seven days [14].

Group III: (cyclophosphamide+ vitamin E group) (Cyc + Vit E G) included 6 mice received

vitamin E (200 mg/kg/day, by orogastric tube), for 19 days starting from day one of the study [15]. Then after half an hour from vitamin E administration the mice received cyclophosphamide (75 mg/kg/day, intraperitoneally (IP), in the fifth, twelfth and nineteenth days of study [14].

Group IV: (cyclophosphamide+ melatonin group) (Cyc +M G) included 6 mice received melatonin (50 mg/kg/day, by orogastric tube) for 19 days starting from day one of the study [16]. Then after half an hour from melatonin administration the mice received cyclophosphamide (75 mg/kg/day, intraperitoneally (IP), in the fifth, twelfth and nineteenth days of study [14].

Group V: (cyclophosphamide+ vitamin E+ melatonin group) (Cyc +Vit E +M) included 6 mice received vitamin E (200 mg/kg/day) and melatonin (50 mg/kg/day) both by orogastric tube for 19 days starting from day one of the study. Then the mice received cyclophosphamide (75 mg/kg/day, intraperitoneally (IP), in the fifth, twelfth and nineteenth days of study [14-16].

At the twenty sixth day of the experiment, the mice were sacrificed by neck dislocation adhering to the standards for use and care of animals that have been authorized by Ethics Committee, Faculty of Medicine, Alexandria University [13].

Drugs and materials:

Cyclophosphamide: It was purchased in the form of Endoxan (1 g/vial), (Baxter Oncology GmbH. Kantstrasse 2. D-33790 Halle. Germany). Vitamin E was purchased in the commercially available oral form 400mg/capsule, (Pharco Pharmaceuticals, Egypt). Melatonin (3mg/ capsule) was obtained from Organix Company, Egypt.

METHODS

I. Weight of mice: The mice were weighed on the first and twenty sixth days of the experiment using a conventional balance.

II. Weight of ovaries: The two ovaries were extracted then weighed using a sensitive scale.

III. Histological study: Both the right and left ovaries were utilized [17]. The ovaries were immediately fixed for 72 hours in 10% formol saline and paraffin blocks were formed. The automated microtome was used to section the paraffin blocks in serial cuts. Then sections were divided into four groups for different histological methods in this study and examined by light microscope (Leica DM750 Dual Viewing Pathology Microscope) in pathology laboratory, Pathology Department, Faculty of Medicine, Alexandria University. It was used to obtain light microscopic images.

A. H&E stain: Sections were stained by hematoxylin and eosin (H&E) to assess the structure of the ovaries.

B. Trichrome Stain: Sections from the paraffin blocks were stained by different dyes with varying molecular weights in a sequential manner to assess the amount of collagen fibers [18,19].

C. Periodic-Acid Schiff 's (PAS) Stain: Sections from the paraffin blocks were stained using periodic acid (1%) in distilled water, schiff's reagent and sulphurous acid reagents [19].

D. Immunohistochemical study: Sections from paraffin blocks were stained immuno-histochemically utilizing the streptavidin-biotin immune-enzymatic antigen detection method [20]. Anti caspase-3 kit was the monoclonal antibody used for the identification of apoptotic cells in the ovarian tissue utilizing imaging analysis system.

E. Morphometric studies: The number of follicles in the ovaries was counted. The diameter of the follicles and diameter of primary oocytes were determined. The percentage of surface area occupied by collagen fibers was measured. The density of anti-caspase 3 positive immune reaction was measured. All morphometric data was measured by imaging analysis system IMAGEJ (Rasband 1997–2019) in randomly selected 12 fields per each group, at Center of Excellence for Research in Regenerative Medicine and its Applications (CERRMA), Faculty of Medicine.

IV. Statistical analysis: The data were analyzed by SPSS software package version 20.0 (Armonk, NY: IBM Corp). The Shapiro-Wilk test was used to verify the normality of distribution. F-test (ANOVA): was used to compare between more than two groups, while Post Hoc test (Tukey) was used for pairwise comparisons, the level of significance was $P \leq 0.05$ [21].

RESULTS

I. Mice weight: There was no significant difference in the weight of all groups of mice at day 1

of the experiment. Mice of group II (cyclophosphamide group) showed significant decrease in the weight at day 26 compared to control group($p<0.001$). The other groups (group III, group IV and group V) and the control group did not significantly differ as regard weight ($P > 0.05$). **[table 1]**

II. Weight of ovaries: When compared to the control group, mice in group II (cyclophosphamide group) had significant decrease in the weight of the right ($p = 0.003$) and left ($p= 0.002$) ovaries. The other groups (group III, group IV and group V) and the control group showed no significant difference in the weight of the ovaries ($P > 0.05$). **[table 1]**

III. Histological study

A. H&E stain: In control group**,** ovary had a regular outline and it was divided into: outer cortex and inner medulla. The cortex was occupied by multiple follicles at different stages of maturation. Ovary showed a cover of germinal epithelium formed of single layer of low cuboidal cells **(Figure 1 a).** Primary follicles were formed of primary oocytes surrounded by a single layer of cuboidal follicular cells **(Figure 1 b)**, while secondary follicles showed multilayers of granulosa cells and theca cells surrounding the primary oocyte with multiple small antral cavities between granulosa cells. Corpus luteum was formed of large sized pale granulosa lutin cells surrounded by small dark eosinophilic theca lutin cells **(Figure 1 c)**. Graffian follicle was formed of primary oocyte surrounded by corona radiata projected in a single large antral cavity. Cumulus oophorus is formed of granulosa cells. Theca interna was formed of round shaped eosinophilic cells and theca externa was formed of collagen bundle and spindle shaped cells **(Figure 1 d)**. The medulla showed large blood vessels and spindle shaped stromal cells **(Figure 1 e)**.

In cyclophosphamide treated group**,** the ovarian structure was distorted with loss of the cortical follicles. Multiple atretic follicles and degenerated corpus lutea were seen **(Figure 2 a)**. Degenerated follicle appeared with irregular outline showing widely separated deeply stained granulosa cells some appeared detached from underlying basal lamina. Spindle shaped cells most probably fibroblasts with flat nuclei appeared in cortex. Remnants of theca cells were seen peripherally with dark nuclei. Zone of focal loss of theca cells with inflammatory cells infiltration were seen. Few primary follicles appeared in the cortex **(Figure 2 b)**. Hyaline exudate appeared in the cortex between follicles remnants. Thickening in the germinal epithelium was noticed. Eosinophilic material was found within degenerated cortical follicles. Atretic follicles were found in cortex with extravasated blood within follicle **(Figure 2 c, d)**. Medulla showed large number of spindle shaped cells most probably fibroblasts and inflammatory cells infiltration **(Figure 2 e)**.

In combined cyclophosphamide and vitamin E treated group**,** few follicles at different stages of development were found in the cortex **(Figure 3 a)**. Cortex showed primary follicles that had vacuolated follicular cells. Some follicles showed primary oocyte with vesicular nuclei but with irregular outline, while other follicles showed severely vacuolated cytoplasm and fragmented nucleus in association with vacuolated granulosa cells. Dense nuclear fragments and cytoplasmic dark bodies represent most likely apoptotic figures. Eosinophilic homogenous material was found **(Figure 3 b)**. Secondary follicles with primary oocyte revealed irregular zona pellucida and vacuolated granulosa cells with dark peripheral nuclei. Flat cells with flat nuclei surround primary oocyte were most probably pregranulosa cells which were transitional cells between primary and secondary follicles. Theca cells were found around follicle. Some secondary follicles showed degenerated primary oocytes. Moderate increase in thickness of germinal epithelium was noticed **(Figure 3 c, d)**. Atretic follicles presented in cortex. Some showed deeply stained degenerated granulosa cells, while others showed vacuolated pale-stained cells. Corpus luteum showed eosinophilic cells. Medulla showed stromal cellular infiltration and areas of hyaline exudate **(Figure 3 d)**.

In combined cyclophosphamide and melatonin treated group, few growing follicles at different stages of development were found in the cortex **(Figure 4 a)**. Primordial follicles showed primary oocytes with shrunken dark cytoplasm and

condensed nuclei. Primary follicles were found in the cortex. They showed primary oocyte with single layer of cuboidal follicular cells, some follicular cells showed minimal vacuolation with dark shrunken nucleus. Inflammatory cellular infiltration was found in the cortex and medulla. Congested blood vessels were found in the medulla **(Figure 4 b, c)**.

Secondary follicle showed primary oocyte with irregular outline that was peripherally placed and vacuolated granulosa cells **(Figure 4 d)**. Corpus luteum showed vacuolated granulosa lutein cells with loss of normal eosinophilia with dark shrunken nuclei **(Figure 4 e)**.

In combined cyclophosphamide, melatonin and vitamin E treated group, relative increase in the number of follicles in the cortex was noticed. Medulla showed minimal vascular congestion **(Figure 5 a, b)**. Germinal epithelium showed moderate increase in thickness. Primary oocyte had a regular contour and was surrounded by zona pellucida and granulosa cells. Theca cells appeared around basement membrane of follicle. Minimal congested capillaries were found in the cortex **(Figure 5 b, c, d)**. Secondary follicle had restored its normal architecture. It had primary oocyte surrounded by granulosa cells, theca externa and theca interna with multiple small antral cavities between granulosa cells **(Figure 5 d, e)**.

Graafian follicle was seen composed of primary oocyte surrounded by corona radiata projected in large antral cavity. Cumulus oophorus was formed of granulosa cells. The follicle was surrounded by theca externa and theca interna with normal structure **(Figure 5 d)**. Corpus luteum showed vacuolated cytoplasm of granulosa lutin cells surrounded by small dark eosinophilic theca lutin cells. Apoptotic bodies showed condensed eosinophilic fragmented bodies with dense nuclear fragments **(Figure 5 e)**.

B. Trichrome Stain: Control group showed scanty amount of collagen deposited in the ovarian cortex around primary follicles **(Figure 6 a)**, while group II showed extensive collagen deposition in ovarian cortex and subepithelial region **(Figure 6 b)**. Group III and group IV showed deposition of moderate amount of collagen in the cortex between ovarian follicles **(Figure 6 c, d)**. Group V showed minimal collagen deposition in the cortex **(Figure 6 e)**.

C. Periodic-Acid Schiff 's (PAS) Stain: Control group showed that zona pellucida had regular outline and rounded contour around primary oocyte **(Figure 7 a)**, while group II showed that zona pellucida was eroded and destroyed **(Figure 7 b)**. Group III and group IV showed irregular shape of the zona pellucida **(Figure 7 c, d)**. Group V showed that zona pellucida had regular outline and rounded contour around primary oocyte **(Figure 7 e)**.

D. Immunohistochemical study: Control group showed minimally positive anti caspase -3 immune reaction **(Figure 8 a)**, while group II showed highly positive anti caspase -3 immune reaction **(Figure 8 b)**. Group III and group IV showed moderately positive anti caspase -3 immune reaction **(Figure 8 c, d)**. Group V showed minimally positive anti caspase -3 immune reaction like control group **(Figure 8 e)**. [**table 2]**

E. Morphometric studies: According to number, diameter of follicles and diameter of primary oocyte.

All morphometric parameters in group II (cyclophosphamide group) except number of atretic follicles were significantly reduced compared to group I (control group) ($P < 0.001$). Morphometric analysis of group III (cyclophosphamide + vitamin E group) and group IV (cyclophosphamide+ melatonin group) showed significant improvement in morphometric parameters in comparison to group II ($P < 0.001$). Group IV showed significant improvement in the morphometric parameters in comparison to group III. Morphometric analysis of group V (cyclophosphamide+ vitamin E+ melatonin group) showed that the morphometric parameters were nearly like control group with no significant difference ($P > 0.05$). Group V revealed significant improvement in the morphometric parameters in comparison to group II, group III and group IV ($P < 0.001$)**. [table 2, 3,4]**

According to the percentage of surface area occupied by collagen fibers in sections stained by trichrome stain, the area% of collagen was significantly increased in group II compared to control group ($P < 0.001$). The area% of collagen in

group III and group IV was significantly decreased compared to group II (both P values <0.001). The area% of collagen in group IV was significantly decreased in comparison to area% of collagen in group III (P <0.001). The area% of collagen in group V was more significantly decreased than area% of collagen in group II, group III and group IV (P<0.001)**. [table 2]**

According to density (percentage of positive cells) in sections stained by anti-caspase 3, percentage of positive cells significantly increased in group II (P<0.001), group III (P<0.001) and group IV(P<0.001) compared to control group (group I). No significant difference in the percentage of positive cells was noticed between group V and control group (group I) (P = 0.397). **[table 2]**

Table 1: Comparison between the different studied groups according to weight of mice and ovaries.

	Group I (n = 12)		Group II	Group III	Group IV	Group V	F	p
	Subgroup Ia (n = 6)	Subgroup Ib (n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)		
Weight of mice (gram)								
Day 1								
Min. – Max.	24.0 – 26.0	23.0 – 26.0	23.0 – 28.0	25.0 – 27.0	25.0 – 28.0	25.0 – 29.0	2.643	0.052
Mean ± SD.	25.0 ± 0.89	24.60 ± 1.36	26.0 ± 2.10	26.0 ± 0.89	26.40 ± 1.02	26.60 ± 1.36		
Day 26								
Min. – Max.	24.0 – 27.0	23.0 – 27.0	20.0 – 22.0	23.0 – 29.0	22.0 – 30.0	23.0 – 29.0	9.510*	<0.001*
Mean ± SD.	25.40 ± 1.20	25.0 ± 1.41	21.0 ± 0.89	26.20 ± 1.94	26.40 ± 2.58	25.60 ± 2.15		
p_1			<0.001*	0.789	0.658	0.991		
p_2				<0.001*	<0.001*	0.001*		
p_3					1	0.976		
p_4						0.933		
Weight of ovaries (gram)								
Right								
Min. – Max.	0.036 – 0.040	0.032 – 0.037	0.027 – 0.037	0.031 – 0.037	0.030 – 0.036	0.031 – 0.039	4.671*	0.005*
Mean ± SD.	0.038 ± 0.002	0.035 ± 0.002	0.031 ± 0.004	0.034 ± 0.002	0.033 ± 0.002	0.035 ± 0.003		
p_1			0.003*	0.558	0.109	0.9		
p_2				0.209	0.684	0.07		
p_3					0.903	0.981		
p_4						0.616		
Left								
Min. – Max.	0.035 – 0.040	0.033 – 0.039	0.028 – 0.037	0.032 – 0.038	0.032 – 0.039	0.031 – 0.040	4.542*	0.005*
Mean ± SD.	0.038 ± 0.002	0.036 ± 0.002	0.032 ± 0.003	0.035 ± 0.002	0.035 ± 0.002	0.036 ± 0.003		
p_1			0.002*	0.65	0.569	0.8		
p_2				0.119	0.149	0.074		
p_3					1	0.999		
p_4						0.997		

SD: **Standard deviation**
F: **F for One way ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**
p: p value for comparing between the five studied groups (Total group I, II, III, IV and V)
p_1: p value for comparing between **Group I** and each other groups
p_2: p value for comparing between **Group II** and each other groups
p_3: p value for comparing between **Group III** and each other groups
p_4: p value for comparing between **Group IV** and each other groups
*: Statistically significant at $p \leq 0.05$

Table 2: Comparison between the different studied groups according to density (percentage of positive cells) in sections stained by anticaspase-3, diameter of primary oocyte and percentage of surface area occupied by collagen fibers (percentage of fibrosis) in sections stained by trichrome stain.

	Group I (n = 24)		Group II	Group III	Group IV	Group V	F	p
	Subgroup Ia (n = 12)	Subgroup Ib (n = 12)	(n = 12)	(n = 12)	(n = 12)	(n = 12)		
Density (percentage of positive cells%)								
Min. – Max.	2.00 – 3.50	2.00 – 3.10	80.00 – 85.00	6.00 – 7.50	6.00 – 9.00	2.00 – 4.00	21008.59*	<0.001*
Mean ± SD.	2.84 ± 0.56	2.58 ± 0.41	83.00 ± 1.54	6.84 ± 0.56	7.83 ± 0.94	3.25 ± 0.62		
p_1			<0.001*	<0.001*	<0.001*	0.397		
p_2				<0.001*	<0.001*	<0.001*		
p_3					0.046*	<0.001*		
p_4						<0.001*		
Diameter of primary oocyte (µm)								
Min. – Max.	71.66 – 86.30	72.10 – 82.10	14.85 – 17.50	39.50 – 58.85	49.75 – 68.30	53.60 – 87.00	272.48*	<0.001*
Mean ± SD.	78.98 ± 3.88	76.85 ± 3.19	15.72 ± 0.74	53.90 ± 5.33	59.79 ± 6.82	70.74 ± 9.02		
p_1			<0.001*	<0.001*	<0.001*	0.004*		
p_2				<0.001*	<0.001*	<0.001*		
p_3					0.078	<0.001*		
p_4						<0.001*		
percentage of surface area occupied by collagen fiber (Percentage of fibrosis %)								
Min. – Max.	12.03 – 13.44	15.63 – 16.90	32.70 – 48.18	29.87 – 32.13	18.28 – 32.74	9.70 – 23.40	182.841*	<0.001*
Mean ± SD.	12.69 ± 0.43	16.00 ± 0.35	40.03 ± 4.34	30.83 ± 0.60	24.69 ± 4.25	13.82 ± 3.90		
p_1			<0.001*	<0.001*	<0.001*	0.989		
p_2				<0.001*	<0.001*	<0.001*		
p_3					<0.001*	<0.001*		
p_4						<0.001*		

SD: **Standard deviation**
F: **F for One way ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**
p: p value for comparing between the five studied groups (Total group I, II, III, IV and V)
p_1: p value for comparing between **Group I** and each other groups
p_2: p value for comparing between **Group II** and each other groups
p_3: p value for comparing between **Group III** and each other groups
p_4: p value for comparing between **Group IV** and each other groups
*: Statistically significant at $p \leq 0.05$

Table 3: Comparison between the different studied groups as regard diameter of follicles.

Diameter of follicles (μm)	Group I (n = 24) Subgroup Ia (n = 12)	Group I (n = 24) Subgroup Ib (n = 12)	Group II (n = 12)	Group III (n = 12)	Group IV (n = 12)	Group V (n = 12)	Test of Sig.	p
Primary								
Min. – Max.	41.55 – 68.60	50.50 – 56.60	17.00 – 21.95	24.95 – 53.70	20.70 – 49.00	37.50 – 66.85	F=	<0.001*
Mean ± SD.	48.78 ± 7.36	53.68 ± 2.13	18.84 ± 1.49	35.15 ± 7.66	36.57 ± 9.58	52.88 ± 11.47	45.937*	
p_1			<0.001*	<0.001*	<0.001*	0.972		
p_2				<0.001*	<0.001*	<0.001*		
p_3					0.991	<0.001*		
p_4						<0.001*		
Secondary								
Min. – Max.	144.4 – 213.7	168.5 – 181.8	–	41.95 – 197.1	106.5 – 144.4	149.0 – 299.0	F=	<0.001*
Mean ± SD.	179.7 ± 17.92	173.6 ± 4.26	–	122.3 ± 44.08	123.1 ± 10.36	193.6 ± 39.74	22.744*	
p_1			–	<0.001*	<0.001*	0.328		
p_2				–	–	–		
p_3					1	<0.001*		
p_4						<0.001*		
Graffian								
Min. – Max.	419.4 – 442.3	429.2 – 449.3	–	–	–	413.2 – 440.5	t=	0.010*
Mean ± SD.	427.6 ± 6.06	439.0 ± 6.60	–	–	–	425.2 ± 7.93	2.726*	

SD: **Standard deviation t: Student t-test**
F: **F for One way ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**
p: p value for comparing between the five studied groups (Total group I, II, III, IV and V)
p_1: p value for comparing between **Group I** and each other groups
p_2: p value for comparing between **Group II** and each other groups
p_3: p value for comparing between **Group III** and each other groups
p_4: p value for comparing between **Group IV** and each other groups
*: Statistically significant at $p \leq 0.05$

Table 4: Comparison between the different studied groups as regard number of follicles.

Number of follicles	Group I (n = 24) Subgroup Ia (n = 12)	Group I (n = 24) Subgroup Ib (n = 12)	Group II (n = 12)	Group III (n = 12)	Group IV (n = 12)	Group V (n = 12)	F	p
Primary								
Min. – Max.	30.0 – 34.0	31.0 – 33.0	11.0 – 14.0	19.0 – 22.0	21.0 – 30.0	22.0 – 36.0	220.662*	<0.001*
Mean ± SD.	32.0 ± 1.04	32.0 ± 0.65	12.17 ± 0.85	20.83 ± 0.85	25.83 ± 2.60	31.17 ± 4.06		
p_1			<0.001*	<0.001*	<0.001*	0.786		
p_2				<0.001*	<0.001*	<0.001*		
p_3					<0.001*	<0.001*		
p_4						<0.001*		
Secondary								
Min. – Max.	17.0 – 20.0	15.0 – 18.0	0.0 – 0.0	5.0 – 8.0	10.0 – 15.0	10.0 – 25.0	219.881*	<0.001*
Mean ± SD.	18.18 ± 0.86	16.34 ± 0.99	0.0 ± 0.0	6.33 ± 0.88	13.0 ± 1.49	18.33 ± 4.01		
p_1			<0.001*	<0.001*	<0.001*	0.521		
p_2				<0.001*	<0.001*	<0.001*		
p_3					<0.001*	<0.001*		
p_4						<0.001*		
Atretic								
Min. – Max.	4.0 – 5.0	5.0 – 6.0	4.0 – 9.0	4.20 – 8.20	4.0 – 7.0	4.0 – 7.0	7.694*	<0.001*
Mean ± SD.	4.50 ± 0.40	5.66 ± 0.38	7.0 ± 1.46	6.03 ± 1.07	5.50 ± 1.15	5.67 ± 0.75		
p_1			<0.001*	0.068	0.763	0.475		
p_2				0.143	0.005*	0.015*		
p_3					0.695	0.899		
p_4						0.994		

SD: **Standard deviation**
F: **F for One way ANOVA test**, Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Tukey)**
p: p value for comparing between the five studied groups (Total group I, II, III, IV and V)
p_1: p value for comparing between **Group I** and each other groups
p_2: p value for comparing between **Group II** and each other groups
p_3: p value for comparing between **Group III** and each other groups
p_4: p value for comparing between **Group IV** and each other groups
*: Statistically significant at $p \leq 0.05$

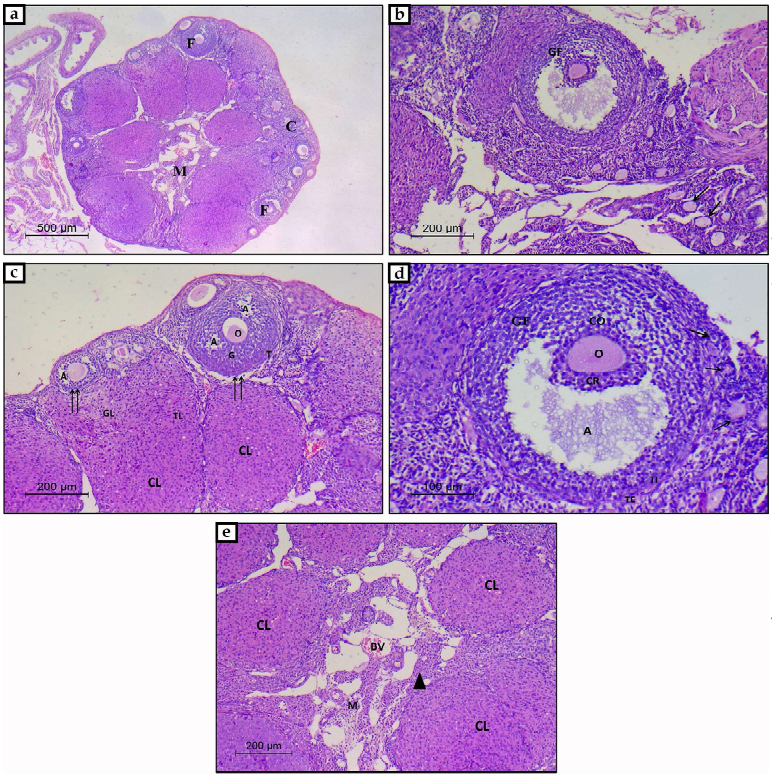

Figure 1(a- e): Microscopic sections of mouse's ovary of the control group (group I) showing ovary is divided into: cortex (C) with multiple follicles (F) and medulla (M) (1 a). Primary follicles (arrow) are formed of primary oocytes surrounded by a single layer of cuboidal follicular cells (1b). Secondary follicles (double arrow) show multilayers of granulosa cells (G) and theca cells (T) surrounding the primary oocyte (O) with small antral cavities (A) between granulosa cells. Germinal epithelium is formed of single layer of low cuboidal cells (arrow). Corpus luteum (CL) are seen showing granulosa lutin cells (GL) surrounded by theca lutin cells (TL) (1c). Graffian follicle (GF) is formed of primary oocyte (O) surrounded by corona radiata (CR) projected in large antral cavity (A). Cumulus oophorus (CO) is formed of granulosa cells. Theca externa (TE) is formed of collagen bundle and spindle shaped cells and theca interna (TI) is formed of round shaped eosinophilic cells. Normal appearance of the medulla (M) is seen with large blood vessels (BV) and spindle shaped stromal cells (arrow head). Multiple corpus lutea (CL) are seen (2e). (H&E. (a)x 40, (b, c, e) x 100, (d)x 200).

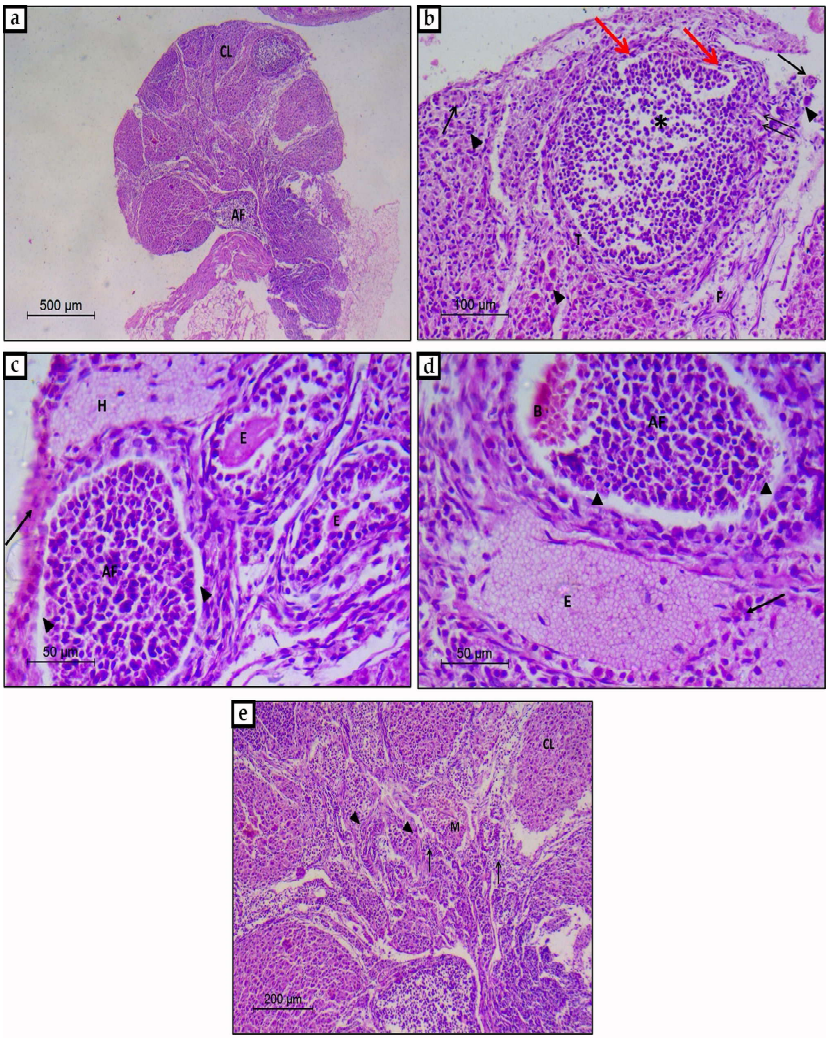

Figure 2(a-e): Microscopic sections of mouse's ovary of cyclophosphamide treated group (group II) showing that the ovarian structure is distorted with loss of the cortical follicles. Multiple atretic follicles (AF) and degenerated corpus lutea (CL) are seen (2a). Degenerated follicle (asterisk) showing deeply stained granulosa cells. Remnants of theca cells (T), zone of focal loss of theca cells with inflammatory cells infiltration (double arrow) are seen. Spindle shaped cells most probably fibroblasts with flat nuclei (F) are seen. Note numerous inflammatory cells with segmented nuclei (arrowhead) are found. Few primary follicles (arrow) appear in the cortex (2b). Eosinophilic material (H) is found in between degenerated follicles. The germinal epithelium shows marked thickening (arrow). Atretic follicle (AF) has degenerated dark granulosa cells. These cells appear detached from the underlying basal lamina (arrowhead) and the surrounding cells. Extravasated blood (B) is found within atretic follicle (2c, d). Inflammatory cells infiltration (arrow) is seen in the medulla (M). Spindle shaped cells (arrowhead). Corpus lutea (CL)(2e). (H&E. (a) X40, (b) X200, (c-d) X400, (e) X100)

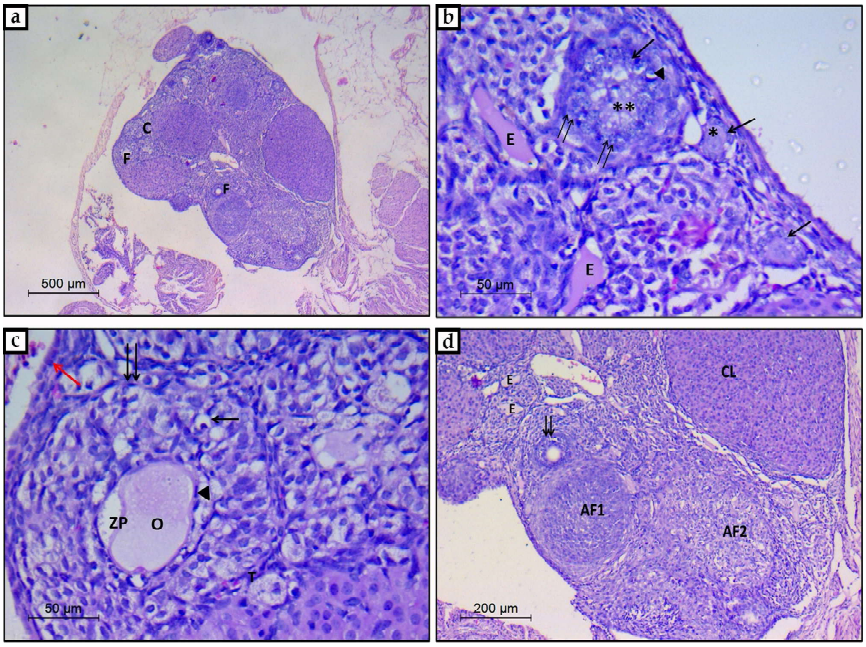

Figure 3(a-d): Microscopic sections of mouse's ovary of combined cyclophosphamide and vitamin E treated group (group III) showing few follicles (F) at different stages of development in the cortex (C) (3a). Three primary follicles (arrow) are found with vacuolated follicular cells. Some follicles show primary oocyte with vesicular nuclei but with irregular outline (star), while other shows severely vacuolated cytoplasm and fragmented nucleus (double star) in association with vacuolated granulosa cells (arrowhead). Some granulosa cells depict dense nuclear fragments and cytoplasmic dark bodies (double arrow) are found. Note eosinophilic homogenous material is found (E) (3b). Secondary follicle (double arrow) has primary oocyte (O) revealing irregular zona pellucida (ZP) and vacuolated granulosa cells with dark peripheral nuclei (arrow). Flat cells (arrowhead) with flat nuclei surround primary oocyte most probably pre-granulosa cells are seen. Theca cells (T) are found around follicle. Moderate increase in thickness of germinal epithelium (red arrow) is seen (3c). Multiple atretic follicles: first one (AF1) has deeply stained degenerated granulosa cells, while second one (AF2) shows vacuolated pale-stained cells. Secondary follicle (double arrow) with degenerated primary oocyte is noticed. Corpus luteum (CL) shows eosinophilic cells. Stromal cellular infiltration and areas of hyaline exudate (E) are seen in medulla (3d). (H&E. (a) X40, (b, c) x 400, (d)x 100)

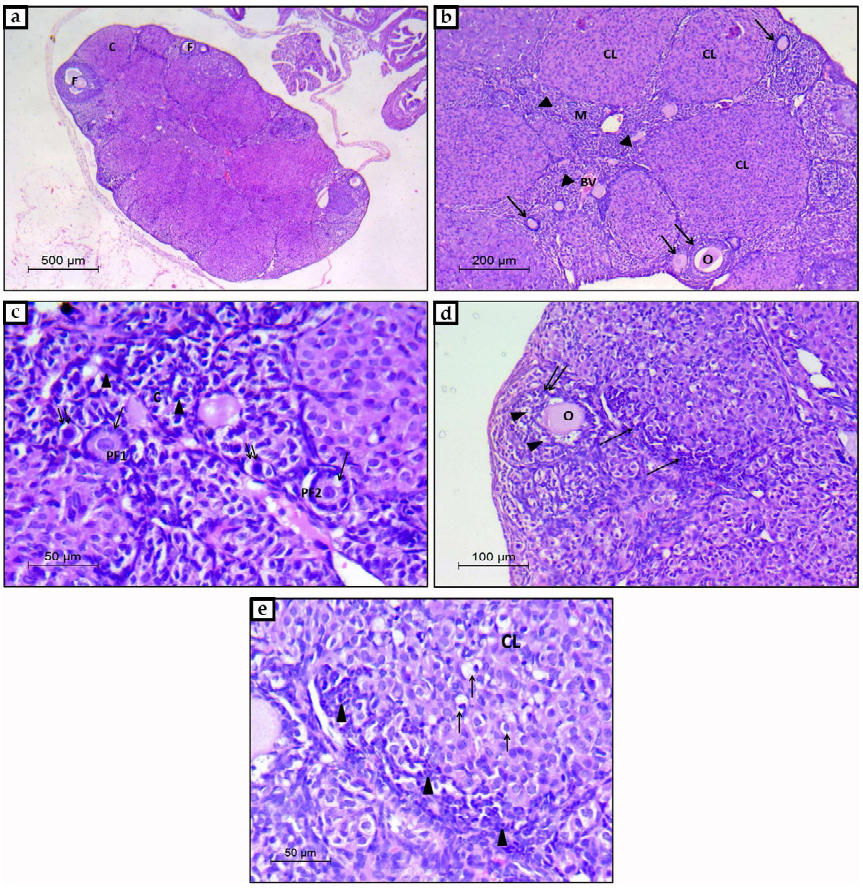

Figure 4(a-e): Microscopic sections of mouse's ovary of combined cyclophosphamide and melatonin treated group (group IV) showing few growing follicles (F) at different stages of development in the cortex (C) (4a). Primary follicles (arrow) are found in cortex. Corpus luteum (CL) shows eosinophilic cells. Cellular infiltration (arrowhead) is seen in the cortex. Medulla (M) shows inflammatory cells infiltration (arrowhead) and congested blood vessels (BV). Primordial follicles (double arrow) show primary oocytes with shrunken dark cytoplasm and condensed nuclei. Primary follicle (PF1) shows primary oocyte with single layer of cuboidal follicular cells, while (PF2) shows minimal vacuolation in the follicular cells with dark shrunken nucleus. (4b,c). Secondary follicle (double arrow) has irregular outline oocyte (O) and vacuolated granulosa cells (arrow heads). Note cellular infiltration (arrow) is seen (4d). Corpus luteum (CL) has vacuolated granulosa lutein cells (arrowhead) with loss of normal eosinophilia with dark shrunken nuclei. Inflammatory cellular infiltration is noticed (arrow) (4e). (H&E. (a) X40, (c, e)x 400, (b)x 100, (d)x 200)

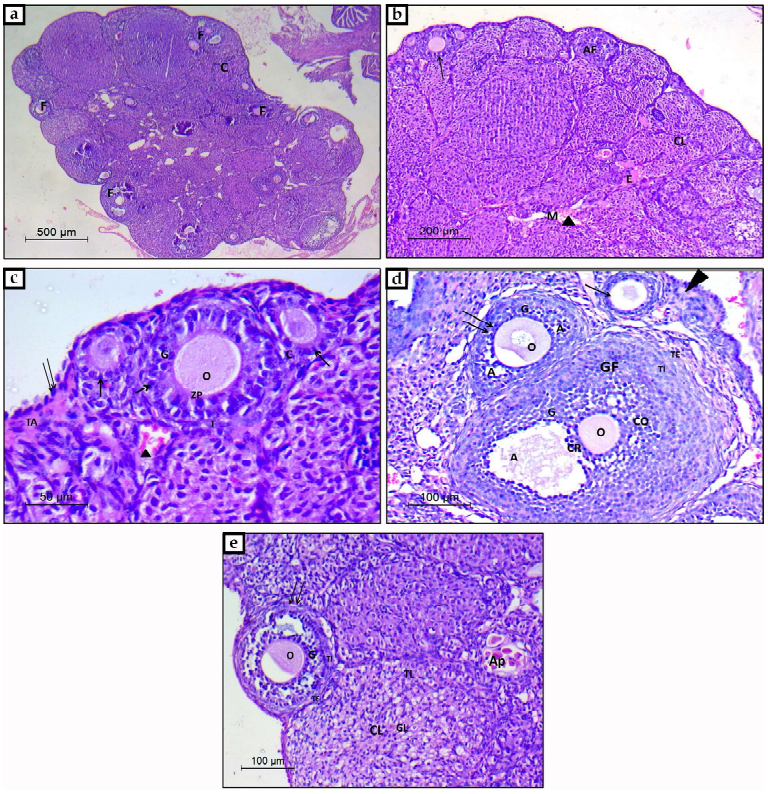

Figure 5(a- e): Microscopic sections of mouse's ovary of combined cyclophosphamide, vitamin E and melatonin treated group (group V) showing relative increase in the number of follicles (F) in the cortex (C) (5a). Primary follicles (arrow) show control like pattern. Medulla (M) shows minimal vascular congestion (arrowhead) and eosinophilic homogenous substance (E). Atretic follicle (AF) and corpus luteum (CL) are seen (5b). Primary follicle (arrow) has regular contour, well developed granulosa cells. Primary oocyte (O) is surrounded by zona pellucida (zp) and granulosa cells (G). Theca cells (T) appear around basement membrane of follicle. Minimal congested capillaries (arrowhead) appear in the cortex (C). Germinal epithelium (double arrow) shows moderate increase in thickness. Tunica albuginea (TA) lies underlying this epithelium (5c). Secondary follicle (double arrow) restored its normal architecture with multiple small antral cavities (A) between granulosa cells (G) that surround primary oocyte. Graffian follicle (GF) is seen composed of primary oocyte (O) surrounded by corona radiata (CR) projected in large antral cavity (A). Cumulus oophorus (CO) is formed of granulosa cells. The follicle is surrounded by theca interna (TI) and theca externa (TE) with normal structure. Thickened germinal epithelium is noticed. (arrowhead) (5d). Secondary follicle (double arrow) has primary oocyte (O). The follicle is surrounded by granulosa cells (G), theca externa (TE) and theca interna (TI). Corpus luteum (CL) shows vacuolated cytoplasm of granulosa lutin cells (GL) surrounded by small dark eosinophilic theca lutin (TL) cells. Apoptotic bodies (AP) show condensed eosinophilic fragmented bodies with dense nuclear fragments (5e). (H&E. (a) X40, (c)X 400, (b)X 100, (d, e)X 200)

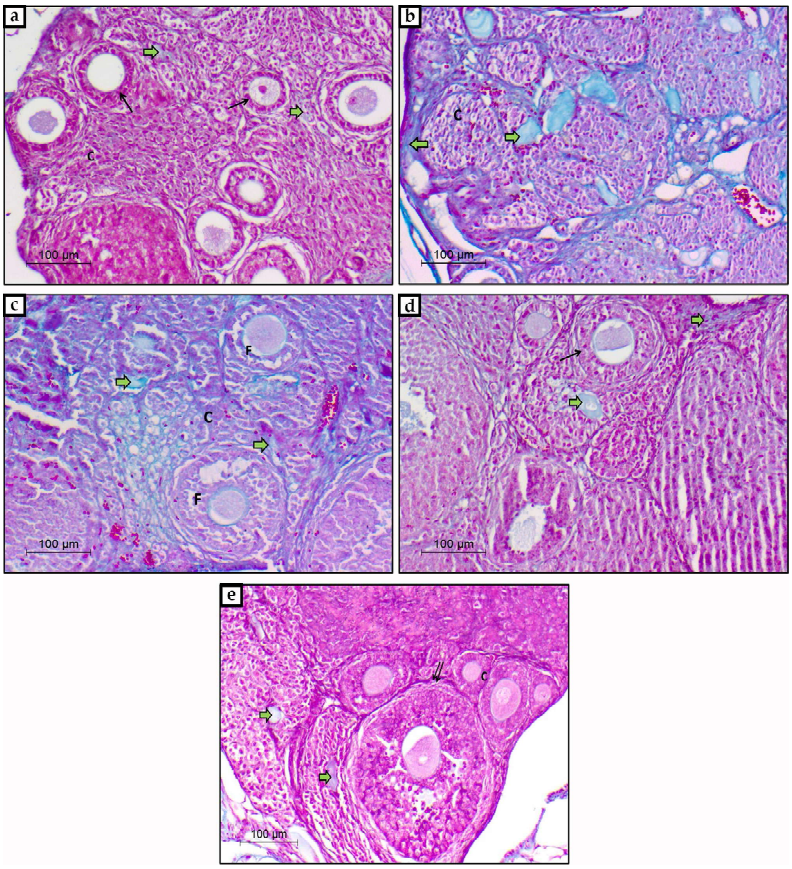

Figure 6(a- e): Microscopic sections of mouse's ovary of the control group (group I) showing scanty amount of collagen (green arrow) in the ovarian cortex (C) around primary follicles (arrow) (6a). Extensive collagen deposition (green arrow) is found in ovarian cortex (C) and subepithelial region in group II (6b). Deposition of moderate amount of collagen (green arrow) is found in the cortex (C) between ovarian follicles (F) in group III (6c). Moderate collagen deposition (green arrow) is found in the cortex (C) especially around primary follicle (arrow) and in subepithelial region in group IV (6d). Minimal collagen deposition (green arrow) is seen in the cortex (C) around secondary follicle (double arrow) in group V (6e). (Trichrome. x 200).

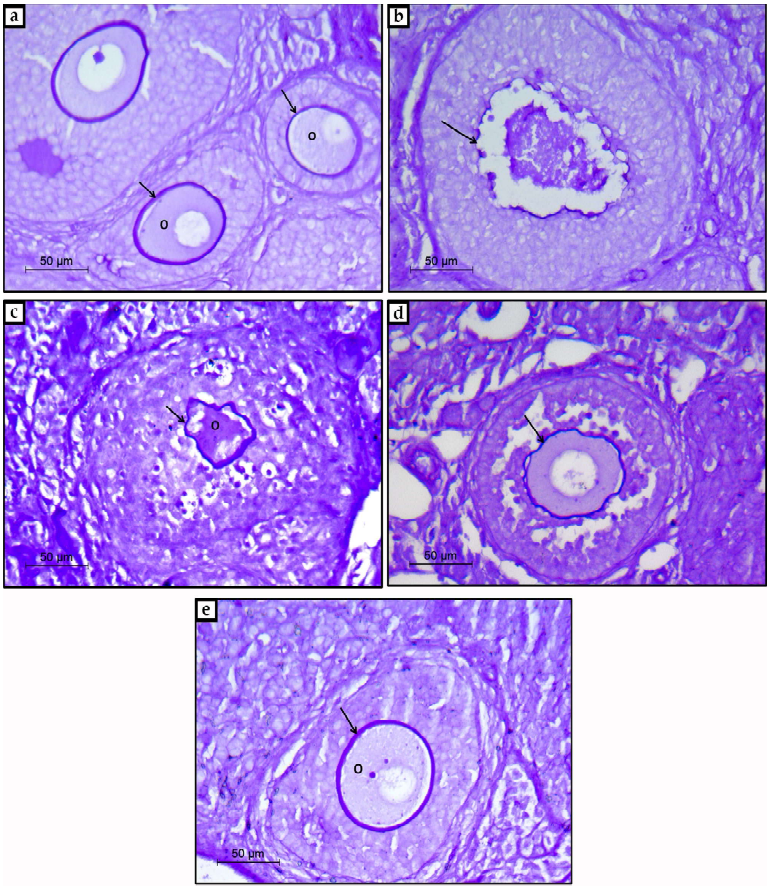

Figure 7(a- e): Microscopic section of mouse's ovary of the control group (group I) showing the zona pellucida (arrow) has regular outline and rounded contour around primary oocyte (O) (7a). The zona pellucida (arrow) is eroded and destroyed in group II (7b). Irregular shape of the zona pellucida (arrow) is noticed that surrounds degenerated primary oocyte (O) in group III (7c). Irregular shape of the zona pellucida (arrow) is found in group IV (7D). The zona pellucida (arrow) restores its normal shape. It has regular outline and rounded contour around primary oocyte (O) in group V (7e). (PAS. X400)

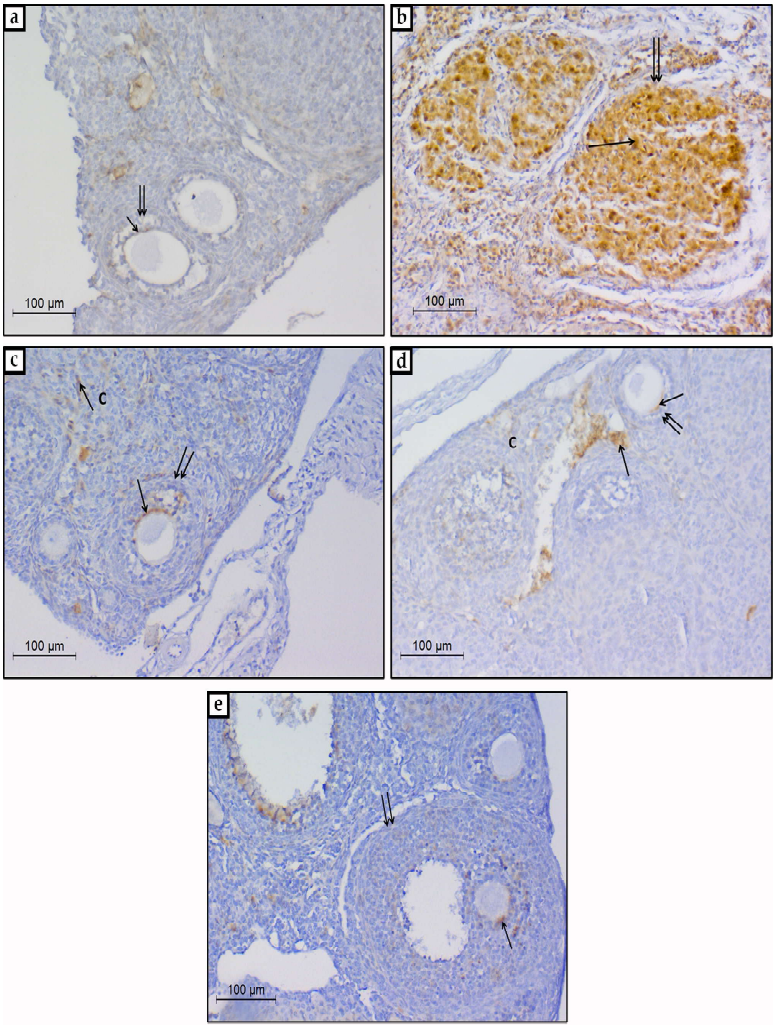

Figure 8(a- e): Microscopic section of mouse's ovary of control group (group I) showing minimal positive anti caspase 3 immune reaction in the apoptotic cells (arrow) in secondary follicle (double arrow) (8a). Group II shows high positive anti caspase 3 immune reaction in the apoptotic cells (arrow) in the atretic follicle (double arrow) (8b). Group III shows moderate positive anti caspase 3 immune reaction in the apoptotic cells (arrow) in secondary follicle (double arrow) and in the cortex (C) (8c). Group IV shows moderate positive anti caspase 3 immune reaction in the apoptotic cells (arrow) in the cortex (C) around and in secondary follicle (double arrow) (8d). Group V shows minimal positive anti caspase 3 immune reaction in the apoptotic cells (arrow) in secondary follicle (double arrow) (8e). (Immunoperoxidase. X 200)

DISCUSSION

In our study, cyclophosphamide caused severe ovarian affection, which was proved by histological and immunohistochemical methods. Vitamin E and melatonin exerted partial protection through their therapeutic potential.

Cyclophosphamide is used to treat many diseases, especially rheumatic diseases. However, gonadal toxicity and ovarian failure induced by cyclophosphamide remain a serious problem facing its therapeutic administration. Numerous studies have been conducted to reduce the impact of cyclophosphamide on fertility [2].

The dose of cyclophosphamide was adjusted to induce ovarian failure in the experimental mice without resulting in their death. This dose was adopted from previous studies using mice to assess the efficacy of platelet-rich plasma and melatonin in mitigating cyclophosphamide-induced ovarian failure [14]. The frequency of administration was chosen based on the fact that cyclophosphamide inhibits its own activation within seven days so the period between successive doses was seven days [22].

According to Mansour-Gueddes SB and Saidana-Naija D [10], vitamin E has been reported to be effective in protecting the ovary against the toxic effect of cyclophosphamide. Previous studies used the same dose of vit E to control the oxidative damage resulting from sperm cryopreservation and to treat diazinon-induced ovarian toxicity through modulation of apoptosis [15, 23, 24].

A study done by Megha KB et al. [11], concluded that melatonin reduced the side effects of cyclophosphamide by removing the free radicals. Liu et al. [16] used the same melatonin dose as in our study to improve cardiac function in a model of heart failure.

To our best knowledge, this study is the first to use vitamin E and melatonin together to address cyclophosphamide-induced ovarian toxicity.

The cyclophosphamide group revealed a significant decrease in the weight of mice and the ovaries compared to the control group. The histological examination showed ovaries with a distorted structure. There was thickening of the germinal epithelium, degeneration of the cortical follicles, inflammatory cellular infiltration, and extravasated blood vessels. A significant decrease in the ovarian follicles was encountered along with abundant atretic follicles. Significant increase in collagen was noticed by Trichrome stain. Eroded zona pellucida was evident in PAS-stained sections. Immunohistochemically, high anti-caspase-3 positivity was observed in many cells.

The reduction in body weight in the group II mice could be explained by cyclophosphamide's systemic multiorgan toxic effects [25]. Additionally, cyclophosphamide exerted cytotoxic effects on taste buds, leading to appetite loss and reduced food intake [26, 27].

The reason behind the distortion of the structure of the ovary is that cyclophosphamide activates p53 which activates the intrinsic pathway of apoptosis. It inhibits the anti-apoptotic proteins Bcl-2 and Bcl-xL. Additionally, by enhancing mitogen-activated protein kinase, cyclophosphamide induces apoptosis. Cyclophosphamide, in turn, can induce apoptosis in ovarian follicular cells, especially granulosa cells, which exhibit a high rate of proliferation [28-30]. Another explanation of the ovarian distortion is that cyclophosphamide is an alkylating agent. The active metabolite of cyclophosphamide causes intra- and inter-strand DNA crosslinking, leading to inhibition of DNA replication and gene transcription and inducing apoptosis [31].

Vacuolation of granulosa cells may result from inhibition of steroid synthesis, leading to lipid accumulation within the cells. Other steroid-producing cells, like the adrenal gland, may be affected. Hyaline exudate or (eosinophilic homogeneous material) can be attributed to the interruption of circulation, as a disturbance of normal angiogenesis, or the formation of thrombus.[32]

Oocyte degeneration has been shown to occur after exposure to cytotoxic drugs, so atresia of follicles begins with degeneration of the oocyte with nuclear changes in the form of chromatin condensation and pyknosis, followed by granulosa cells' degeneration [32].

Cyclophosphamide induced an inflammatory response by activating nuclear factor kappa B, leading to the production of proinflammatory cytokines, such as tumour necrosis factor alpha

(TNF - α). These proinflammatory cytokines stimulated inflammatory cells to invade the ovarian stroma [33].

The study by Liu et al. [34] reported degenerative changes in ovarian structure, resulting in a decrease in the number of telocytes after treatment with cyclophosphamide. Telocytes in the ovarian tissues play an important role in preserving the ovarian microenvironment and repairing the injured tissue.

Alkylating agents like cyclophosphamide can induce ovarian follicle loss through decreased micro-vascularisation of follicles and induction of follicle degeneration [35]. These findings were supported by a previous study that assessed the prophylactic role of Nigella sativa in adult mice, which reported marked degeneration of ovarian follicles [36].

Cyclophosphamide caused damage to the blood vessels of the ovary. An end-artery system supplies the ovary, so focal fibrosis is the consequence of blood vessel obstruction, which blocks the blood flow to some areas of the ovary [37]. Another explanation is that cyclophosphamide directly destroyed the follicles, leading to focal fibrosis. [37, 38].

The observed degeneration of zona pellucida could be explained as cyclophosphamide induces oxidative stress. It causes damage and depletion of glycoprotein in the zona pellucida. Cyclophosphamide decreases expression of Zp1, 2 and 3 those are necessary for proper formation of zona pellucida [39].

Group III (Cyc + Vit E G) showed no change in the weight of mice nor the weight of the ovaries at the end of experiment. The histological examination showed that ovaries of group III had improvement in the ovarian structure in the form of few follicles at different stages of development in the cortex. The number of the follicles in the ovaries, the diameter of the follicles and diameter of primary oocytes showed that all parameters except number of atretic follicles were significantly increased in group III compared to group II. Moderate collagen deposition was seen by Trichrome stain. Irregular zona pellucida as was depicted in PAS- stained sections. Immunohistochemically, group III showed moderate caspase 3 positivity in many cells.

The protective role of vitamin E is attributed to the strong antioxidant with many cytoprotective capacities. So, vitamin E can protect the ovary when it is taken as adjuvant therapy with cyclophosphamide [9].

According to study done by Haliciu et al. [40], vitamin E accelerated the improvement of ovarian structure as it stimulated the growth of the ovarian follicles. Raeeszadeh et al. [41] referred the increase in the number of normal follicles in rats received vitamin E with cyclophosphamide to the activity of vitamin E in modulating the immune system, promoting the growth of damaged follicles, and supporting the oxidative balance.

Vitamin E has an anti-inflammatory effect and an antioxidant effect so it can reduce neutrophil and macrophage infiltration. It neutralizes reactive oxygen species resulted from inflammatory processes [42].

A study done by Du et al. [43] showed marked decrease in collagen deposition in the ovaries of rats treated by vitamin E in cisplatin induced fibrosis as lipid peroxides accumulation can induce fibrosis so using vitamin E reverses the progression of fibrosis through its antioxidant characteristics.

Vitamin E has a protecting role against lipid peroxidation. Vitamin E protects the zona pellucida components like lipid and protein against degradation induced by oxidative stress [44].

Vitamin E has anti-apoptotic properties. It can down regulate the apoptotic markers. Vitamin E can decrease free radicals which are responsible for the activation of apoptosis cascade. In this way, vitamin E can protect cells against damage and excessive apoptosis [45].

Group IV (Cyc +M G) showed no change in the weight of mice or weight of ovaries at the end of experiment. The histological examination showed that ovaries of group IV had improvement in the ovarian structure in the form of growing follicles in the cortex and decrease in the sign of inflammation and degeneration. The number of the follicles in the ovaries, the diameter of the follicles and diameter of primary oocytes showed that all parameters except number of atretic follicles were significantly

increased in group IV compared to group II. Moderate collagen deposition was seen by Trichrome stain. Irregular zona pellucida was evident in PAS- stained sections. Immuno-histochemically, group IV showed moderate caspase 3 positivity in many cells.

The protective role of melatonin is explained by mitigating the status of oxidative stress caused by cyclophosphamide. It can protect granulosa cells and primary oocytes from the harmful effect of cyclophosphamide [46]. Barberino et al. [47] reported the protective effect of melatonin on the ovary as melatonin promoted folliculogenesis and increased the number of normal follicles in the cortex. A study done by Feng et al. [48] reported that melatonin attenuated follicles loss induced by cyclophosphamide as melatonin is a free radical scavenger in the ovarian follicles, it protects granulosa cells from the apoptosis and promotes oocyte maturation.

Melatonin has anti-fibrotic properties because it can inhibit the cells injury in the initial phase of fibrosis as impaired cells induce fibrogenic response. Melatonin inhibits the release of inflammatory cytokines. It can decrease the deposition of collagen and the development of fibrosis. It helps the organs recovery after the development of pathological fibrosis [49].

A study done by Goktepe et al. [50] showed the major role of melatonin as anti-fibrotic as it can inhibit epithelial cell injury either via apoptotic or necrotic changes. Melatonin can also decrease expression of factors inducing fibrosis as cytokines and chemokines.

Normally, follicles of the ovary contain melatonin in their fluid. Melatonin can protect zona pellucida against oxidative stress by decreasing degradation of carbohydrate in oocytes and zona pellucida. Melatonin has an anti-apoptotic activity as it can inhibit caspase 3 leading to blockage of the apoptosis process either the intrinsic or the extrinsic pathways [51].

Group V (Cyc + Vit E + M G) showed no change in the weight of mice at the end of experiment or weight of ovaries. The histological examination showed that ovaries of group V had restored the normal ovarian structure in the form of appearance of nearly normal follicles in the cortex. all parameters were nearly like control group. Minimal collagen deposition was seen by Trichrome stain. The normal rounded shape of zona pellucida was evident in PAS- stained sections. Immunohistochemically, group V showed minimal caspase 3 positivity in many cells.

Mallamaci R et al. [52] in their study reported melatonin, vitamin E and Trolox were more effective in the reduction of the oxidative stress than using a single drug so the combination therapy can afford maximal protection against the harmful effects of drugs inducing oxidative stress.

Melatonin and vitamin E together prevent oxidative stress, inflammatory processes, and cardio-renal dysfunction, hence reducing drug toxicity on different body organs [53]. According to the results of Demir K et al. [54], the combination of melatonin, vitamins D, B12, E, and folic acid increase the fertility rate, that was referred to higher levels of antioxidants which may help infertile women to increase conception.

A study done by Aykutoglu G et al. [55], revealed that the co-administration of vitamin E and melatonin prevented homocysteine-induced apoptosis more effectively than using vitamin E or melatonin alone because melatonin with vitamin E reduce the levels of reactive oxygen species produced by homocysteine and downregulate the pro-apoptotic proteins. The antioxidant capacity increased and oxidative stress was effectively inhibited. Additionally, melatonin acts by amplifying the benefits of other antioxidants [56, 57].

It is concluded that, Vitamin E and melatonin have a great role in the protection the ovarian structure. Using both vitamin E and melatonin as an adjuvant therapy with cyclophosphamide can decrease the harmful effects of cyclophosphamide on the ovary. It is more effective than using vitamin E or melatonin alone.

CONCLUSION

Concomitant use of vitamin E and melatonin with cyclophosphamide helps to preserve ovary and restores normal ovarian structure in comparison to the use of vitamin E or melatonin alone.

Limitations of the study: small sample size**.**

DECLARATIONS

Ethical approval: The research was approved by Ethics Committee of Faculty of Medicine, Alexandria University (IRB No: 00012098- FWA No: 00018699). Serial number 0107513 and following the guidelines for care and use of animals in accordance and adherence with ARRIVE guidelines.

Consent to participate: not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study will be available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no conflicts of interest.

Authors' Contribution: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Melad N. Kelada, Reham Abelfatah Menesy, Inass Ibrahim Zaki, Heba G. Ibrahim, Mohamed Gamal Ayoub. The first draft of the manuscript was written by Melad N. Kelada, Elsayed Aly Mohamed Metwally and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

REFERENCES

[1]. Rosario R, Stewart HL, Spears N, Telfer EE, Anderson RA. Anti-Mullerian hormone attenuates both cyclophosphamide-induced damage and PI3K signalling activation, while rapamycin attenuates only PI3K signalling activation, in human ovarian cortex in vitro. Human Reproduction. 2024 Feb 1;39(2):382-92. https://doi.org/10.1093/humrep/dead255 PMid:38070496 PMCid:PMC10833070

[2]. Barberino RS, Silva RLS, Palheta Junior RC, Smitz JEJ, Matos MHT. Protective Effects of Antioxidants on Cyclophosphamide-Induced Ovarian Toxicity. Biopreserv Biobank. 2023 Apr;21(2):121-141. https://doi.org/10.1089/bio.2021.0159 PMid:35696235

[3]. Bhardwaj JK, Bikal P, Sachdeva SN. Chemotherapeutic drugs induced female reproductive toxicity and treatment strategies. Journal of Biochemical and Molecular Toxicology. 2023 Jul;37(7):e23371. https://doi.org/10.1002/jbt.23371 PMid:37098697

[4]. Busuioc RE, Paduraru DN, Alexandra Stanescu AM, Barbalata DC, Musat F, Albu AR, et al. Regenerative medicine options in treating premature ovarian failure. Romanian Medical Journal. 2023 Jan 1;70(1). https://doi.org/10.37897/RMJ.2023.1.9

[5]. Chon SJ, Umair Z, Yoon MS. Premature ovarian insufficiency: past, present, and future. Frontiers in cell and developmental biology. 2021 May 10; 9:672890. https://doi.org/10.3389/fcell.2021.672890 PMid:34041247 PMCid:PMC8141617

[6]. Rahman R, Panay N. Diagnosis and management of premature ovarian insufficiency. Best Practice & Research Clinical Endocrinology & Metabolism. 2021 Dec 1;35(6):101600. https://doi.org/10.1016/j.beem.2021.101600 PMid:34823999

[7]. Vo KC, Kawamura K. In vitro activation early follicles: from the basic science to the clinical perspectives. International Journal of Molecular Sciences. 2021 Apr 6;22(7):3785. https://doi.org/10.3390/ijms22073785 PMid:33917468 PMCid:PMC8038686

[8]. Li Z, Zhang M, Tian Y, Li Q, Huang X. Mesenchymal stem cells in premature ovarian insufficiency: Mechanisms and prospects. Front Cell Dev Biol 2021; 9:718192. https://doi.org/10.3389/fcell.2021.718192 PMid:34414193 PMCid:PMC8369507

[9]. Cemali Ö, Akdevelioğlu Y. Female infertility and the Mediterranean Diet. Journal of Gazi University Health Sciences Institute. 2022;4(2):96-106.

[10]. Mansour-Gueddes SB, Saidana-Naija D. Vitamin E: natural antioxidant in the Mediterranean diet. Vitam. E Health Dis.-Interact. Dis. Health Asp. 2021 Oct 6;10. https://doi.org/10.5772/intechopen.99705

[11]. Megha KB, Arathi A, Shikha S, Alka R, Ramya P, Mohanan PV. Significance of melatonin in the regulation of circadian rhythms and disease management. Molecular Neurobiology. 2024 Jan 11:1-31. https://doi.org/10.1007/s12035-024-03915-0 PMid:38206471

[12]. Savage RA, Zafar N, Yohannan S, Miller JMM. Melatonin. In: StatPearls. Treasure Island (FL): StatPearls Publishing, 2022.

[13]. Alexandria University. Regulatory framework in ethics of animal research. 2018. Available from: http://www.alexu.edu.eg/index.php/en/research/4401-regulatory-framework-in-ethics-of-animal-research. [Accessed in: Aug, 2023].

[14]. Safwat M, Elagwany AM, Abd El Mottelib LM, Zahran NM. Effect of platelet-rich plasma in cyclophosphamide-induced ovarian failure in albino rats: histological and anatomical study. Egypt J Histol 2022; 45:597-618.

[15]. Raeeszadeh M, Shokrollahi B, Khademi N, Akbari A. Superior effect of broccoli methanolic extract on control of oxidative damage of sperm cryopreservation and reproductive performance in rats: A comparison with vitamin C and E antioxidant. Theriogenology 2022; 181:50-8. https://doi.org/10.1016/j.theriogenology.2022.01.010 PMid:35063921

[16]. Liu Y, Li LN, Guo S, Zhao XY, Liu YZ, Liang C, et al. Melatonin improves cardiac function in a mouse model of heart failure with preserved ejection fraction. Redox Biol 2018; 18:211-21. https://doi.org/10.1016/j.redox.2018.07.007 PMid:30031269 PMCid:PMC6076208

[17]. Eggan K, Jurga S, Gosden R, Min IM, Wagers AJ. Ovulated oocytes in adult mice derive from non-circulating germ cells. Nature 2006; 29;441(7097):1109-14. https://doi.org/10.1038/nature04929 PMid:16799565

[18]. Gunasegaran JP. Textbook of Histology and A Practical guide, 4e-E-book. Elsevier Health Sciences; 2020 Mar 14.

[19]. Rasmussen R. Tissue acquisition and processing. InMethods in Inhalation Toxicology 2020 Apr 30 (pp. 101-123). CRC Press. https://doi.org/10.1201/9780429332852-7

[20]. Hasic E. Immunohistochemistry Fundamentals. Immunohistochemistry: A Technical Guide to Current Practices. 2022 Jul 7:1. https://doi.org/10.1017/9781009106924.002

[21]. Sprent P. Data driven statistical methods. Routledge; 2019 Dec 6. https://doi.org/10.1201/9781315140780

[22]. Abdoon AS, Al-Atrash AM, Soliman SS, El-Sanea AM, Gamal el Din AA, Fahmy HM. Lyophilized equine platelet-rich plasma (L-GFequina) antagonize the Reproductive toxicity and oxidative stress Induced by Cyclophosphamide in female rats. Journal of Ovarian Research. 2023 Apr 28;16(1):84. https://doi.org/10.1186/s13048-023-01161-x PMid:37118826 PMCid:PMC10141944

[23]. Zarei S, Molavi F, Abasnezhad FA, Majidi B, Mohammadihosseinabad S, Ranjbar FE, et al. The effects of vitamin E supplementation on sperm parameters, chromatin integrity, and gene expression before and after freezing in aged mice. Korean Journal of Fertility and Sterility. 2024 Jan 30. https://doi.org/10.5653/cerm.2023.06632 PMid:38853131 PMCid:PMC11372309

[24]. Sargazi Z, Nikravesh MR, Jalali M, Sadeghnia HR, Rahimi Anbarkeh F, Mohammadzadeh L. Diazinon-induced ovarian toxicity and protection by vitamins E. Iran J Toxicol 2014;8:1130-5.

[25]. Al-Salih HA, Al-Sharafi NM, Al-Qabi SS, Al-Darwesh AA. The Pathological Features of cyclophosphamide induced multi-organs toxicity in male wister rats. Systematic Reviews in Pharmacy. 2020 Jun 1;11(6):45-9. https://doi.org/10.31838/srp.2020.6.10

[26]. Adrian PH, Hunisand M, Lipovestky F. Nutrition in the Patient with Cancer Clinical Physiological and Therapeutic Aspects. Int J Cancer Res Ther, 6 (2): 49. 2021;61. https://doi.org/10.33140/IJCRT.06.02.11

[27]. Onaolapo AY, Ojo FO, Onaolapo OJ. Biflavonoid quercetin protects against cyclophosphamide-induced organ toxicities via modulation of inflammatory cytokines, brain neurotransmitters, and astrocyte immunoreactivity. Food and chemical toxicology. 2023 Aug 1; 178:113879. https://doi.org/10.1016/j.fct.2023.113879 PMid:37301500

[28]. Alshahrani S, Ali Thubab HM, Ali Zaeri AM, Anwer T, Ahmed RA, Jali AM, et al. The protective effects of sesamin against cyclophosphamide-induced nephrotoxicity through modulation of oxidative stress, inflammatory-cytokines and apoptosis in rats. International Journal of Molecular Sciences. 2022 Oct 1;23(19):11615. https://doi.org/10.3390/ijms231911615 PMid:36232918 PMCid:PMC9569534

[29]. Barberino RS, Lins TL, Monte AP, Silva RL, Andrade KO, Campinho DS, Junior RC, Smitz JE, Matos MH. Epigallocatechin-3-gallate attenuates cyclophosphamide-induced damage in mouse ovarian tissue via suppressing inflammation, apoptosis, and expression of phosphorylated Akt, FOXO3a and rpS6. Reproductive Toxicology. 2022 Oct 1; 113:42-51. https://doi.org/10.1016/j.reprotox.2022.08.010 PMid:35981663

[30]. Abogresha NM, Mohammed SS, Hosny MM, Abdallah HY, Gadallah AM, Greish SM. Diosmin mitigates cyclophosphamide induced premature ovarian insufficiency in rat model. Int J Mol Sci 2021; 22:3044. https://doi.org/10.3390/ijms22063044 PMid:33802633 PMCid:PMC8002508

[31]. Ýpek E, Hesapçýoðlu M, Karaboða M, Avcý H. Selenium protection from DNA damage and regulation of apoptosis signaling following cyclophosphamide induced cardiotoxicity in rats. Biotechnic & Histochemistry. 2023 Oct 3;98(7):534-42. https://doi.org/10.1080/10520295.2023.2253424 PMid:37695070

[32]. Yaeger MJ. Pathology of the Female Reproductive System. Atlas of Veterinary Surgical Pathology. 2023 Mar 17:178-98. https://doi.org/10.1002/9781119261254.ch5

[33]. Adeyemi DH, Hamed MA, Oluwole DT, Omole AI, Akhigbe RE. Acetate attenuates cyclophosphamide-induced cardiac injury via inhibition of NF-kB signaling and suppression of caspase 3-dependent apoptosis in Wistar rats. Biomedicine & Pharmacotherapy. 2024 Jan 1; 170:116019. https://doi.org/10.1016/j.biopha.2023.116019 PMid:38128178

[34]. Liu T, Wang S, Li Q, Huang Y, Chen C, Zheng J. Telocytes as potential targets in a cyclophosphamide-induced animal model of premature ovarian failure. Mol Med Rep 2016; 14:2415-22. https://doi.org/10.3892/mmr.2016.5540 PMid:27485835 PMCid:PMC4991733

[35]. Markowska A, Antoszczak M, Markowska J, Huczyñski A. Gynotoxic Effects of Chemotherapy and Potential Protective Mechanisms. Cancers. 2024 Jan;16(12):2288. https://doi.org/10.3390/cancers16122288 PMid:38927992 PMCid:PMC11202309

[36]. Amalia A, Hendarto H, Mustika A, Susanti I. Effects of Nigella Sativa on Female Infertility: A Systematic Review. InProceedings of the 6th International Conference on Medical and Health Informatics 2022 May 13 (pp. 234-237). https://doi.org/10.1145/3545729.3545776

[37]. Ermiþ M, Karaman E, Yalçýn B, Aygen EM. An evaluation of the effects on the ovaries of hyperbaric oxygen therapy in a rat model of premature ovarian failure created with cyclophosphamide. Turk J Obstet Gynecol. 2023; 20:46-52. https://doi.org/10.4274/tjod.galenos.2023.47817 PMid:36908093 PMCid:PMC10013088

[38]. Guo Y, Xue L, Tang W, Xiong J, Chen D, Dai Y, Wu C, Wei S, Dai J, Wu M, Wang S. Ovarian microenvironment: challenges and opportunities in protecting against chemotherapy-associated ovarian damage. Human Reproduction Update. 2024 Sep;30(5):614-47. https://doi.org/10.1093/humupd/dmae020 PMid:38942605 PMCid:PMC11369228

[39]. Kim J, You S. Extended adverse effects of cyclophosphamide on mouse ovarian function. BMC Pharmacol Toxicol 2021; 22:3. https://doi.org/10.1186/s40360-020-00468-5 PMid:33413693 PMCid:PMC7792169

[40]. Haliciu AM, Strat L, Amihaesei C, Stan CI, Zamfir AS, Folescu R, Zamfir CL. Antioxidant effect of vitamin e on cyclophosphamide-induced oxidative stress in female rats. Romanian Journal of Functional & Clinical, Macro & Microscopical Anatomy & of Anthropology/Revista Româna de Anatomie Functionala si Clinica, Macro si Microscopica si de Antropologie; 2016;15:161.

[41]. Raeeszadeh M, Saleh Hosseini SM, Amiri AA. Impact of co-administration of N-Acetylcysteine and vitamin E on cyclophosphamide-induced ovarian toxicity in female rats. J Toxicol 2022; 2022:9073405. https://doi.org/10.1155/2022/9073405 PMid:36051383 PMCid:PMC9427260

[42]. Jiang Q, Im S, Wagner JG, Hernandez ML, Peden DB. Gamma-tocopherol, a major form of vitamin E in diets: Insights into antioxidant and anti-inflammatory effects, mechanisms, and roles in disease management. Free Radical Biology and Medicine. 2022 Jan 1; 178:347-59. https://doi.org/10.1016/j.freeradbiomed.2021.12.012 PMid:34896589 PMCid:PMC8826491

[43]. Du R, Cheng X, Ji J, Lu Y, Xie Y, Wang W, et al. Mechanism of ferroptosis in a rat model of premature ovarian insufficiency induced by cisplatin. Sci Rep 2023; 13:4463. https://doi.org/10.1038/s41598-023-31712-7 PMid:36932163 PMCid:PMC10023701

[44]. Sezer Z, Yilmaz TE, Gungor ZB, Kalay F, Guzel E. Effects of vitamin E on nicotine-induced lipid peroxidation in rat granulosa cells: Folliculogenesis. Reproductive Biology. 2020 Mar 1;20(1):63-74. https://doi.org/10.1016/j.repbio.2019.12.004 PMid:31918980

[45]. Ebrahim NA, Abdalla HA, Yassin NAE, Maghrabia AE, Morsy AI. Effect of Sofosbuvir on rats' ovaries and the possible protective role of vitamin E: biochemical and immunohistochemical study. Anat Cell Biol 2023; 56:526-37. https://doi.org/10.5115/acb.23.079 PMid:37654017 PMCid:PMC10714087

[46]. Xu H, Bao X, Kong H, Yang J, Li Y, Sun Z. Melatonin Protects Against Cyclophosphamide-induced Premature Ovarian Failure in Rats. Hum Exp Toxicol. 2022 Jan-Dec; 41:9603271221127430. https://doi.org/10.1177/09603271221127430 PMid:36154502

[47]. Barberino RS, Lins TLBG, Monte APO, Gouveia BB, Campinho DSP, Palheta RC Jr, et al. Melatonin attenuates cyclophosphamide-induced primordial follicle loss by interaction with MT1 receptor and modulation of PTEN/Akt/FOXO3a proteins in the mouse ovary. Reprod Sci 2022;29:2505-14. https://doi.org/10.1007/s43032-021-00768-z PMid:34642909

[48]. Feng J, Ma WW, Li HX, Pei XY, Deng SL, Jia H, et al. Melatonin prevents cyclophosphamide-induced primordial follicle loss by inhibiting ovarian granulosa cell apoptosis and maintaining AMH expression. Front Endocrinol (Lausanne) 2022; 13:895095. https://doi.org/10.3389/fendo.2022.895095 PMid:35992124 PMCid:PMC9381702

[49]. Hosseinzadeh A, Pourhanifeh MH, Amiri S, Sheibani M, Irilouzadian R, Reiter RJ, Mehrzadi S. Therapeutic potential of melatonin in targeting molecular pathways of organ fibrosis. Pharmacological Reports. 2024 Feb;76(1):25-50. https://doi.org/10.1007/s43440-023-00554-5 PMid:37995089

[50]. Goktepe O, Balcioglu E, Baran M, Cengiz O, Ceyhan A, Suna PA, et al. Protective effects of melatonin on female rat ovary treated with nonylphenol. Biotech Histochem 2023; 98:13-9. https://doi.org/10.1080/10520295.2022.2075566 PMid:35611760

[51]. Al-Shahat A, Hulail MAE, Soliman NMM, Khamis T, Fericean LM, Arisha AH, et al. Melatonin mitigates cisplatin-induced ovarian dysfunction via altering steroidogenesis, inflammation, apoptosis, oxidative stress, and PTEN/PI3K/Akt/mTOR/AMPK signaling pathway in female rats. Pharmaceutics 2022; 14:2769. https://doi.org/10.3390/pharmaceutics14122769 PMid:36559263 PMCid:PMC9786155

[52]. Mallamaci R, Barbarossa A, Carrieri A, Meleleo D, Carocci A. Evaluation of the Potential Cytoprotective Effect of Melatonin in Comparison with Vitamin E and Trolox against Cd2+-Induced Toxicity in SH-SY5Y, HCT 116, and HepG2 Cell Lines. Int J Mol Sci. 2024 Jul 24;25(15):8055. https://doi.org/10.3390/ijms25158055 PMid:39125623 PMCid:PMC11312335

[53]. Oyagbemi AA, Ajibade TO, Esan OO, Adetona MO, Awoyomi OV, Omobowale TO, et al. Cardioprotective and renoprotective effects of melatonin and vitamin E on fluoride-induced hypertension and renal dysfunction in rats. Comparative Clinical Pathology. 2024 Feb;33(1):33-45. https://doi.org/10.1007/s00580-023-03519-5

[54]. Demir K, Caliskan ST, Celik S, Akdeniz M, Goc RY. The effect of Folic Acid, B12, D, and E Vitamins and Melatonin levels in the follicular fluid taken by the Intracytoplasmic Sperm Injection method on pregnancy. Pakistan Journal of Medical Sciences. 2024 Jan;40(3Part-II):433. https://doi.org/10.12669/pjms.40.3.7929 PMid:38356841 PMCid:PMC10862457

[55]. Aykutoglu G, Tartik M, Darendelioglu E, Ayna A, Baydas G. Melatonin and vitamin E alleviate homocysteine induced oxidative injury and apoptosis in endothelial cells. Molecular Biology Reports. 2020 Jul; 47:5285-93. https://doi.org/10.1007/s11033-020-05607-z PMid:32592115

[56]. Erdemli ME, Zayman E, Erdemli Z, Gul M, Gul S, Gozukara Bag H. Protective effects of melatonin and vitamin E in acetamiprid-induced nephrotoxicity. Environmental Science and Pollution Research. 2020 Mar; 27:9202-13. https://doi.org/10.1007/s11356-019-06754-y PMid:31916150

[57]. Rashad S, Ahmed S, El-Sayed M, Ahmed D. The toxic effect of bisphenol a on albino rat testicles and the possible protective value of vitamin e and melatonin. Egyptian Society of Clinical Toxicology Journal. 2021 Dec 1;9(2):1-2. https://doi.org/10.21608/esctj.2021.63294.1001

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