Original Article

Dose-Duration Dependent Changes Induced by Atrazine in the Ovary of Adult Albino Rat and the Possible Protective Role of L-Carnitine: Histological and Immunohistochemical Study

Reham H. Abdel-Kareem, Mohey Elsayed Hulail, Eman M. Kamal El-Din El-Sherbiny, and Ola Ali Abdelwahab

Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt

ABSTRACT

Introduction: Atrazine (ATR) is the most common herbicide used in the world. Several studies showed that ATR has many reproductive adverse effects. L-Carnitine (LC) is a flavonoid antioxidant.

Aim of the work: The goal of this research was to elucidate the structural and biochemical alterations induced by ATR in the ovary of the adult albino rats and to clarify the possible protective role of LC.

Material and Methods: 70 adult female albino rats were separated into 6 groups; Control, LC (given 100 mg LC/kg/day for 30 days), ATR sub-chronic treated (given 6.5 mg ATR/kg/day for 30 days), ATR sub-acute treated (given 65 mg/kg/day for 14 days), Sub-chronic protected (given 6.5 mg ATR/kg/day +LC 100 mg/kg/day for 30 days), and Sub-acute protected (given 65 mg ATR/kg/day +LC 100 mg/kg/day for 14 days). All doses were administrated orally. Blood and tissue samples were obtained and processed for biochemical, histological, and immunohistochemical study. Statistical analysis was also done.

Results: Serum luteinizing hormone (LH) and estradiol (E2), ovarian superoxide dismutase (SOD) and catalase (CAT) significantly decreased while the ovarian malondialdehyde (MDA) and nitric oxide (NO) significantly increased in ATR treated groups as compared to control group. Ovarian tissue of ATR treated groups showed significant increase in number of atretic follicles and loss of zona pellucida continuity in PAS stain as compared to control group. Atresia was confirmed by significant increase in immuohistochemical study of P53 in ATR treated groups. Also, there was significant decrease in mitotic division in ATR treated groups confirmed by immuohistochemical study of Ki-67 as compared to control group. Moreover, there was significant difference between the two ATR treated groups in both biochemical and structural findings. While, these changes were less obvious in protected groups after administration of LC.

Conclusion: Supplementation of LC could have a beneficial effect against ATR-induced ovarian damage.

Received: 28 August 2021, **Accepted:** 22 October 2021

Keywords: Atrazine, biochemical, histological, L-carnitine, ovary.

Corresponding Author: Eman M. Kamal El-Din El-Sherbiny, MSc, Department of Human Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt, **Tel.**: +201098087628, **E-mail:** emanelshrbiny7@gmail.com

ISSN: 1110-0559, Vol. 46, No. 1, March 2023

INTRODUCTION

The reproductive system of women is extremely vulnerable to a variety of adverse environmental stresses[1]. Exposure to these stressors like air pollution and chemicals in the environment has been linked to numerous health problems, including malignancies, neurological diseases (such as Parkinson's disease) and problems of reproduction (such as increase the risk of miscarriage and decrease male and female fertility)[2,3]. Environmental chemicals which impair endogenous hormone signaling pathways are known as endocrine disrupting chemicals (EDCs)[4,5].

Atrazine (ATR) consists of a ring structure, called the triazine ring, along with five nitrogen atoms and a chlorine atom. Pure form is an odorless white powder[6]. It belongs to the group of chlorotriazine herbicides[7] and is the most widely used herbicide on the globe because of its low price, great efficacy and flexibility of its application[8]. Agriculture is a field in which it is used to manage weeds in sugarcane, corn, pineapple, soybean, and sorghum by inhibiting photosynthesis[9]. It is intensively used in agricultural production with consumption of 70-90 thousand-tones annually[10]. It is also used in industrial and entertaining areas to control weeds[7].

Exposure to pesticides and herbicides may be generally classed into three chief categories, namely occupational, non-occupational and intentional (accidental/suicidal) exposures[11]. Contaminated water is the main route of atrazine exposure in humans[12-14]. In addition, it is possible to be exposed to it through skin contact and inhalation from occupational sources[15].

Carnitines are special groups of nutrients called 'quasi-vitamins' or 'conditionally essential' nutrients[16]. Levo-carnitine (L-Carnitine) (LC) is a water-soluble, biologically active antioxidant molecule[17,18]. It is found in human serum and tissues and plays an important function in fatty acid metabolism[19]. It has a protective effect on lipid peroxidation by the reduction in hydrogen peroxide production, and it has the ability to remove the superoxide radical[20].

 DOI: 10.21608/ejh.2021.92935.1551

Now, researchers are considering application of L-Carnitine as a treatment for female infertility[18,21,22]. Both L-Carnitine and Acetyl L-Carnitine supplementation produces improvement in many disorders as polycystic ovary syndrome[22], endometriosis and amenorrhea[23].

MATERIALS AND METHODS

2.1. Chemicals:

Atrazine (ATR): was purchased from Kafr El Zayat Pesticides and Chemicals CO. as white odorless powder; purity of 97%. Powder was weighed according to weight of rats and dissolved in distilled water.

L-Carnitine (LC): was supplied as capsules of 350 mg/capsule, in a box of 20 capsules, from MEPACO-MEDIFOOD Company for Pharmaceuticals and Medicinal plants, Sharkeya, Egypt. Capsules were opened and dissolved in distilled water.

2.2. Animals:

Seventy adult female albino rats (Sprague Dawley) weighing 230-250 gm were used in this study. They were obtained from Zagazig University's Faculty of Medicine's animal house. All of the animals were kept in sanitary conditions. Standard food and tap water were given to the participants. They were kept in ventilated polypropylene cages with stainless steel covers and bedding made of wood shavings. The temperature was kept at 22°C. They were given 15 days to adjust to the laboratory settings before being tested. Estrous phases were determined two weeks before the start of the treatment for three cycles, demonstrating its regularity. Vaginal smears in the morning (08:00–10:00) were used to assess the estrous cycle phases of rats on a daily basis for three days[24]. In each rat, estral cyclicity was monitored to ensure that it was in the proestrous phase at the start of the experiment[25]. All rats were handled in accordance with Zagazig University's Institutional Animal Care and Use Committee's standards and regulations (ZU-IACUC committee), number of approval ZU-IACUC/3/F/186/2019.

2.3. Chemical preparation for treatment:

Solutions were made to get 2 distinct dosages of atrazine and only one dose of L-Carnitine. First, 16.25 mg of ATR was dissolved in 10 cm of distilled water. One cm was given for each rat (about 1.625 mg). Secondly, 162.5 mg of ATR was dissolved in 10 cm distilled water. One cm was given for each rat (16.25 mg). Finally, each capsule of LC (350 mg) was opened and dissolved in 14 cm of distilled water. One cm was given for each rat (about 25 mg).

2.4. Experimental design:

The animals were separated into six groups randomly as follow: Group I: (Control group); subdivided into Control A; they did not receive any supplementation for basic parameters to be measured and Control B; they were given 1 cm distilled water by oral gavage daily for 30 days. Group II: (LC group); they were given LC at a dose of 100 mg/kg body weight (B.W)[26-28] by oral gavage for 30 days[29]. Group III: (ATR sub-chronic treated group); they were given ATR at dose of 6.5 mg/kg B.W which is 1/100 of lethal dose (LD50) for rat (672 mg/kg)[30] and according to the safety data sheet[31] by oral gavage daily for 30 days[25]. Group IV: (ATR sub-acute treated group); they were given ATR at a dose of 65 mg/kg B.W which is 1/10 of lethal dose (LD50) for rat (672 mg/kg) by oral gavage daily for 14 days[25]. Group V: (Sub-chronic protected group); they were given ATR at a dose of 6.5 mg/kg B.W plus LC at a dose of 100 mg/kg by oral gavage daily for 30 days. Group VI: (Sub-acute protected group); they were given ATR at a dose of 65 mg/kg B.W plus LC at a dose of 100 mg/kg by oral gavage daily for 14 days.

Rats were sacrificed on the first diestrous stage of the estrous cycle following 14 and 30 days treatment[32]. Animals were anesthetized by intra-peritoneal injection of thiopental 50 mg/kg[33] then blood samples were collected from each group under complete aseptic conditions, for estimation of serum luteinizing hormone (LH) and estradiol (E2). Laparotomy was performed to expose the ovary (Figure 1: B) and ovarian specimens were taken for histopathological and biochemical studies.

2.5. Hormonal assay:

After allowing blood to coagulate at room temperature, samples were centrifuged for 20 minutes at 4000 rpm using a cooling centrifuge (Sigma 3-30 k, USA). Biomarkers were isolated from the clear serum layer and kept at -80°C. Serum LH and E2 estimation has been carried out using enzyme-linked immune-sorbent assay (ELISA)[34].

2.6. Biochemical analysis:

Ovarian tissues were washed out in ice-cold saline then dried and stored at -80°C for subsequent homogenization. They were homogenized in cold 50 mM sodium phosphate buffer (pH 7.0) which has 0.1 mM EDTA. After blending, the mixture was centrifuged at 5000-10000 rpm for 10 minutes. The supernatant was immediately taken and examined. Estimation of lipid peroxidation, oxidative stress and antioxidant defense system was detected using biodiagnostic kits to measure levels of malondialdehyde (MDA) according to[35], nitric oxide (NO) according to[36], superoxide dismutase enzyme (SOD) according to[37] and catalase (CAT) according to[38].

2.7. Hematoxylin and Eosin (H&E) and periodic acid–Schiff (PAS) staining:

H&E and PAS staining techniques were done according to[39]. Follicles at various stages of development were counted in 10 distinct sections of H&E in diestrous rats per group at magnification power X40. Moreover, if the oocyte was lost or degenerated and the granulosa cells had pyknotic nuclei or cytoplasmic shrinkage, the follicle was considered atretic[40].

2.8. Immunohistochemistry staining of P53 and Ki-67and image analysis:

On positive slides, prepared 5 μm slices were placed and immunostained with an avidin-biotin method. To suppress endogenous peroxidase activity, sections were deparaffinized, rehydrated, and prepared with 0.01 percent hydrogen peroxide (H_2O_2). To mask the antigenic location, slices were incubated in a 0.01 M citrate buffer at ph 6 for 10 minutes and then in ethanol for 10 minutes. After that, 20 minutes of microwave-assisted antigen retrieval was performed. Ki-67 and p53 monoclonal mouse antibodies were used to incubate sections overnight at 4°C with diluted primary antibodies at dilutions of 1/500 and 1/100. After 60 minutes of incubation with the avidin-biotin complex (ABC) reagent, the sections were placed in a peroxidase solution for 6–10 minutes. Finally, a counter stain of Mayer's hematoxylin was utilized. For both antigens, immunoreactivity was seen in the nucleus. The particular 1ry antibody was substituted with phosphate buffer saline for the negative control slide[41,42]. Positive results for P53 and Ki-67 antibodies were indicated by brown coloration occupying the nucleus of the cells[43].

ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) was used to calculate the area percent of immunological expression for every stain in high-power fields (X 400) in every slide[44].

Using an electric light microscope (Leica DM500) (German), all sections were examined and photographed at image analysis of Anatomy and Embryology department, Faculty of Medicine, Zagazig University.

2.9. Statistical analysis:

SPSS was used to analyze the data. Data for continuous variables were summarized using the mean and standard deviation if normally distributed, or the median and interquartile ranges if not normally distributed. The analysis of variance was used to examine for differences in mean values between experimental groups (ANOVA). Tukey's multiple comparison test was used as a post hoc ANOVA test. The threshold of significance is fixed at 5% level (*P value*). $p<0.05$ value was considered statistically significant and $p>0.05$ value was considered non-statistically significant.

RESULTS

3.1. Phases of estrous cycle:

The following estrous cycle phases were monitored and detected using a vaginal swab: Proestrous phase: consisted of rounded epithelial cells (Figure 1: A1), estrous phase: characterized by predominance of cornified cells (Figure 1: A2), metestrous phase: has different cell types with predominance of leucocytes (Figure 1: A3) and diestrous phase: consisted of predominance of leucocytes (Figure 1: A4).

3.2. Hormonal assays and biochemical results:

There was no significant difference between control and LC groups in all variables.

Serum levels of LH & E2 significantly decreased in ATR treated groups in comparison to the control group. Moreover, ATR sub-acute treated group showed significant decrease as compared to ATR sub-chronic treated group. Results enhanced after LC administration in protected groups, as LH & E2 levels were significantly increased as compared to the corresponding treated groups. In comparing to control group; there was no significant difference with sub-chronic protected group, but there was still a significant difference with sub-acute protected group. The sub-chronic protected group revealed significant increase as compared to the sub-acute protected group (Table 1; Figure 2: A and B).

According to ovarian MDA & NO contents; they were significantly increased in ATR treated groups as compared with the control group. Furthermore, ATR sub-acute treated group showed significant increase as compared to ATR sub-chronic treated group. On the other hand, there was improvement in protected groups; with a significant decrease in MDA & NO levels as compared to corresponding treated groups. There was no significant difference between sub-chronic protected and control group. While, results showed a significant difference between sub-acute protected and control group. The sub-chronic protected group revealed significant decrease as compared to the sub-acute protected group (Table 1; Figure 2: C and D).

According to ovarian SOD & CAT contents; they were significantly decreased in ATR treated groups in comparison to control group. Moreover, ATR sub-acute treated group showed significant decrease as compared to ATR sub-chronic treated group. After LC administration in protected groups; there was a significant increase in SOD & CAT levels as compared to their corresponding treated groups. There was no significant difference between ovarian SOD & CAT contents of sub-chronic protected and control group. In comparison to control group, the sub-acute protected group showed a significant difference in ovarian SOD and CAT contents. The sub-chronic protected group revealed significant increase as compared to the sub-acute protected group (Table1; Figure 2: E and F).

3.3. H &E Histopathological and morphometric results:

Control group (Figure 3: A, B & C) and LC group (Figure 3: D, E & F) showed nearly the same histological results of normal ovarian structure. Ovary was consisted of outer cortex and central medulla. Ovarian cortex demonstrated all phases of follicular development including; primordial follicles, primary follicles, preantral follicles, antral follicles, mature Graffian follicle and corpora lutea (old and recent).

On the other hand, ovary of ATR sub-chronic treated group was made up of multiple atretic follicles with increased interstitial cells in between. These atretic follicles had degenerated oocytes with cytoplasmic vacuolations and disorganized vacuolated granulosa cells with pyknotic dark stained nuclei. Also, there were old corpus luteum, multiple areas of hemorrhage, Graffian follicle with ill differentiation between layers of theca cells and vacuolated degenerated preantral follicle (Figure 4: A, B & C). While, ovary of ATR sub-acute treated group showed multiple cystic follicles with retention of follicular fluid and there was persistence of many old corpora lutea with absence of recent ones. Also, there were areas of hemorrhage, congested blood vessels and atretic follicles with complete loss of oocyte or with degenerated vacuolated oocyte, micronuclei formation and disarranged vacuolated granulosa cells with pyknotic nuclei (Figure 4: D, E & F). There was apparent decrease of primordial follicles in both treated groups.

In contrast, ovary of sub-chronic protected group showed presence of primordial follicles, multiple growing follicles, recent corpus luteum and decreased number of atretic follicles when compared with ATR sub-chronic treated group (Figure 5: A, B & C). The ovary of sub-acute protected group revealed decrease in number of ovarian cysts, number of atretic follicles and vascular changes (blood congestion and hemorrhage) with still absence of primordial follicles as compared to ATR sub-acute treated group (Figure 5: D, E & F).

According to number of primordial, primary, and preantral follicles; it was significantly decreased in ATR treated groups as compared to control group. There was no significant difference between ATR sub-chronic and sub-acute treated groups. There was a significant increase in sub-chronic protected group as compared to ATR sub-chronic treated group and control group. However, there was no significant increase in sub-acute protected group in comparison to ATR sub-acute treated group and there was still a significant decrease in sub-acute protected group in comparison to control group. There was a significant increase in sub-chronic protected group in comparison to sub-acute protected group (Table 2; Figure 6 A).

According to number of antral follicles; it was significantly decreased in ATR treated groups as compared to control group. There was no significant difference in ATR sub-acute treated group as compared to ATR sub-chronic treated group. There was a significant increase in sub-chronic protected group as compared to corresponding treated group. There was non-significant difference in sub-chronic protected group as compared to control group. There was still a significant decrease in sub-acute protected group in comparison to control group, as there was no significant difference between sub-acute protected group and corresponding treated one. There was a significant increase in sub-chronic protected group in comparison to sub-acute protected group (Table 2; Figure 6 B).

According to number of atretic follicles; it was significantly increased in both ATR treated groups as compared to control group. There was no significant difference between the two ATR treated groups. There was a significant decrease in sub-chronic protected and sub-acute protected groups as compared to corresponding treated groups. There was no significant difference in sub-chronic protected group as compared to control group, while there was still a significant difference in sub-acute protected group in comparison to control group. There was a significant decrease in sub-chronic protected group in comparison to sub-acute protected group (Table 2; Figure 6 C).

According to number of corpora lutea; there was a significant decrease in ATR treated groups in comparison to control group. Moreover, ATR sub-acute treated group showed significant decrease as compared to ATR sub-chronic treated group. There was a significant increase in sub-chronic protected and sub-acute protected groups as compared to corresponding ATR treated groups. There was no significant difference in sub-chronic protected group as compared to control group, but there was still a significant difference in sub-acute protected group in comparison to control group. There was a significant increase in sub-chronic protected group in comparison to sub-acute protected group (Table 2; Figure 6 D).

3.4. PAS staining results:

PAS stained ovarian sections of control and LC groups showed continuous zona pellucida (Figure 7: A and B). However, there was loss of continuity of zona pellucida in PAS stained ovarian sections of ATR sub-chronic treated group (Figure 7: C) and ATR sub-acute treated group (Figure 7: D). Restoration of continuity of zona pellucida in sub-chronic protected group (Figure 7: E) and sub-acute protected group (Figure 7: F) was demonstrated.

3.5. Immunohistochemistry and morphometry:

Immunostained ovarian sections for P53 and Ki-67 showed normal expression in nuclei of granulosa cells in both control and LC groups (Figure 8: A&B, Figure 9: A&B). ATR sub-chronic treated group presented positively stained nuclei of most of the granulosa cells in P53 (Figure 8: C) while most of the granulosa cells presented negative stained nuclei in Ki-67 (Figure 9: C). ATR sub-acute treated group's granulosa cells showed extensively positive stained nuclei in P53 (Figure 8: D) while extensively negative stained nuclei of the granulosa cells in Ki-67 (Figure 9: D). Sub-chronic protected group presented negative stained nuclei of most of the granulosa cells in P53 (Figure 8: E) and positive stained nuclei of the majority of the granulosa cells in Ki-67 (Figure 9: E). Sub-acute protected rats showed negative stained nuclei of a little number of the granulosa cells and positive stained nuclei of majority of them in P53 (Figure 8: F) and negative stained nuclei of a little number of granulosa cells and positive stained nuclei of the majority of them in Ki-67 (Figure 9: F).

According to area percent of positivity of P53-immunostained sections; it was significantly increased in both ATR treated rats as compared with control group. ATR sub-acute treated group showed significant increase as compared to ATR sub-chronic treated group. There was a significant decrease in both sub-chronic and sub-acute protected groups in comparison with corresponding treated groups, but still there was a significant difference with control group. The sub-chronic protected group revealed significant decrease in area percentage of positivity of P53 as compared to the sub-acute protected group. On the other hand, area percentage of positivity of Ki-67-immunostained sections significantly decreased in both ATR treated rats in comparison with control group. ATR sub-acute treated group showed significant decrease in area percentage of positivity of Ki-67 as compared to ATR sub-chronic treated group. There was a significant increase in both sub-chronic and sub-acute protected groups in comparison with corresponding treated groups, while there was still a significant difference with control group. The sub-chronic protected group revealed significant increase in area percentage of positivity of Ki-67 as compared to the sub-acute protected group (Table 2; Figure 6: E & F).

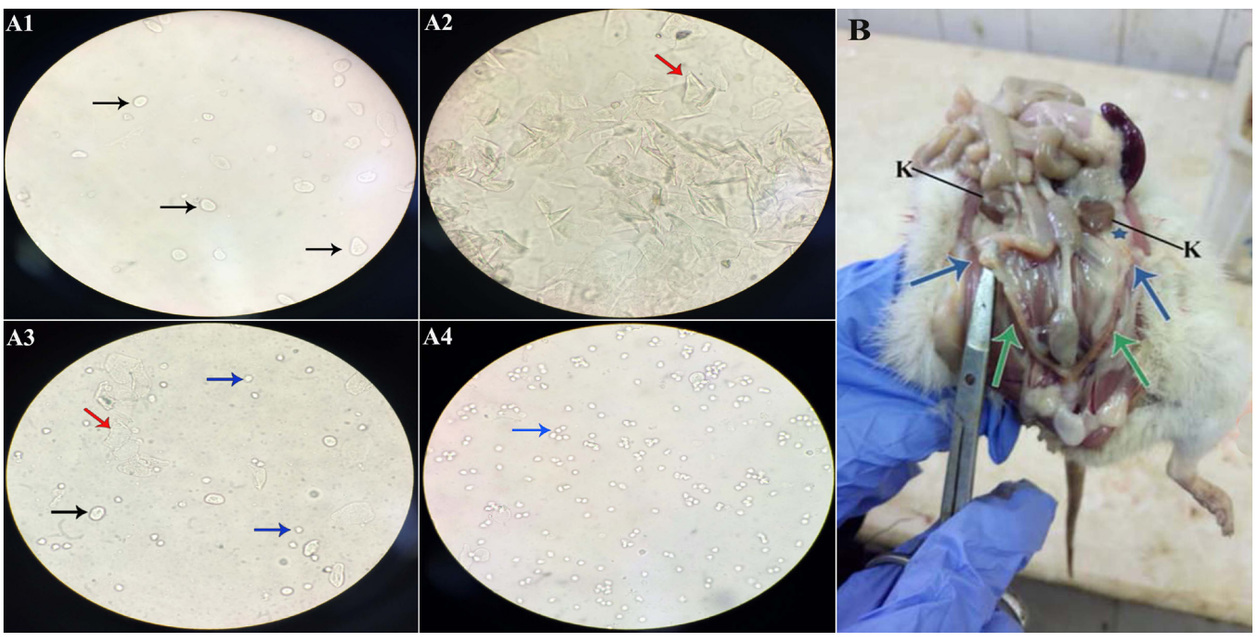

Fig. 1: (A): Unstained vaginal smear of different stages of estrous cycle: proestrous phase (A1) showing; oval epithelial cells (black arrows). Estrous phase (A2) consisting of; cornified epithelial cells (red arrow). Metestrous phase (A3) containing; different cell types; oval epithelium (black arrow), cornified epithelium (red arrow) and predominance of leucocytes (blue arrows). Diestrous phase (A4) showing predominance of leucocytes (blue arrow). (B): a photo showing paired ovaries (blue arrows) are situated at caudal poles of kidneys (K) and embedded in fat (blue star). Uterus is formed of 2 horns (green arrows).

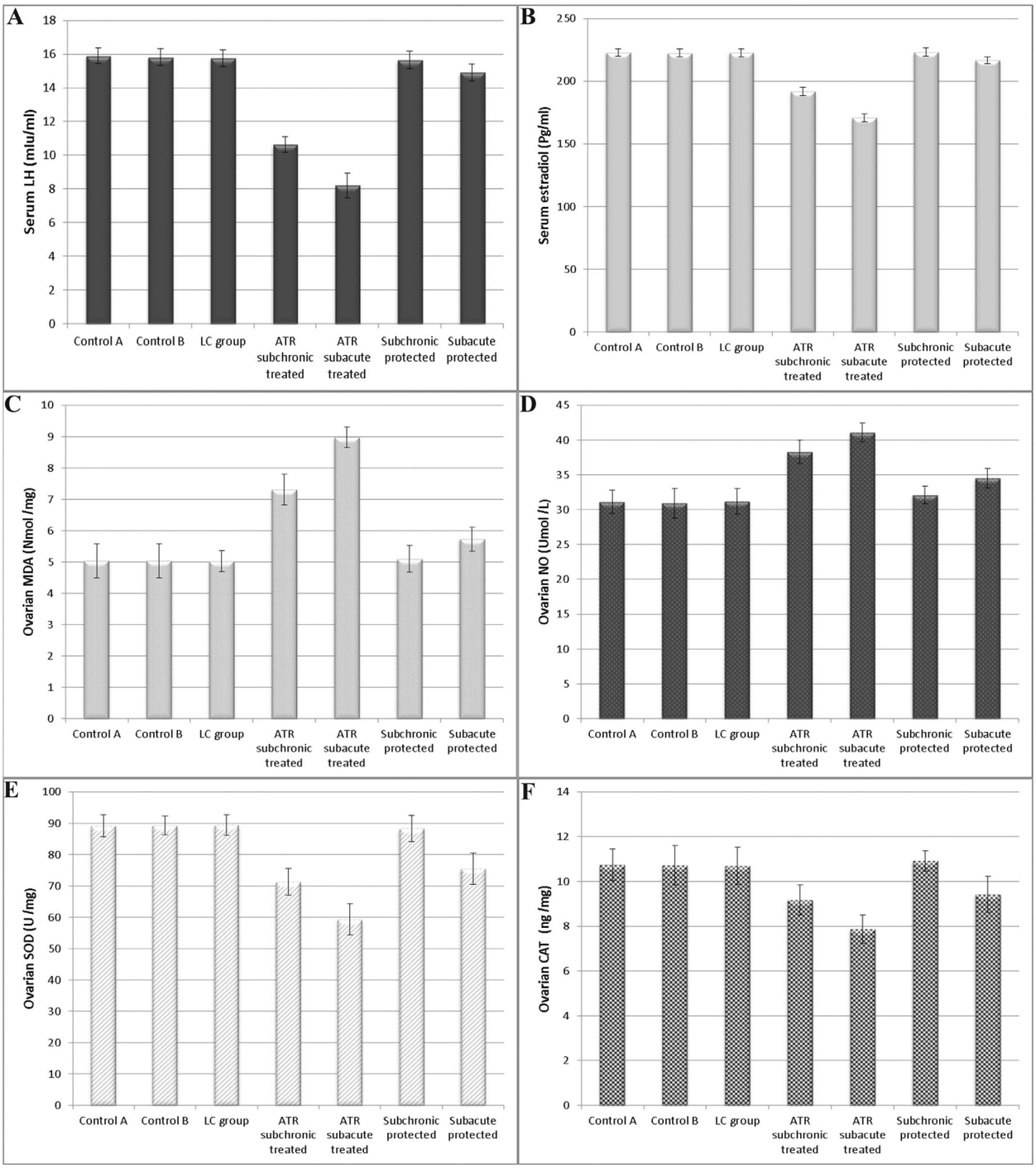

Fig. 2: Bar charts showing hormonal levels & ovarian contents of oxidant and antioxidant enzymes in different studied groups. A: serum LH, B: serum estradiol, C: MDA, D: NO, E: SOD, and F: CAT. Results are represented as mean ± SD.

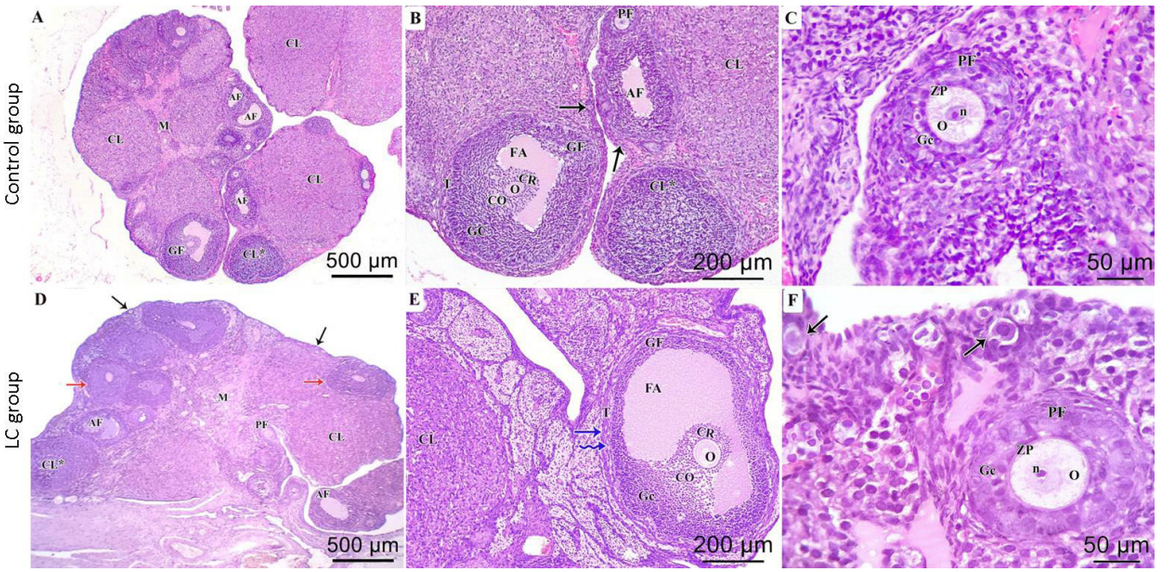

Fig. 3: H&E stained sections of the ovarian tissue: control group (A, B & C) is showing normal ovarian structure formed of outer cortex containing different stages of follicular development and central medulla (M). Primordial follicles (black arrows), primary follicle (PF), multiple antral follicles (AF) and mature Graffian Follicle (GF) which is formed of outer lining of theca cells (T), multiple layers of granulosa cells (Gc), follicular antrum (FA), corona radiata (CR) and cumulus oophorus (CO) that connects the oocyte (O) with the rest of the granulosa cells. Recent corpora lutea (CL*) and old corpora lutea (CL) are also seen. The highest magnification is showing primary follicle (PF) formed of an oocyte (O) having clear nucleus (n) and surrounded by zona pellucida (ZP) and few layers of granulosa cells (Gc).
LC group (D, E & F) is showing normal ovarian structure formed of outer cortex and central medulla (M). Primordial follicles (black arrows), primary follicle (PF), preantral follicles (red arrows) and multiple antral follicles (AF) are seen. The mature Graffian Follicle (GF) is formed of outer lining of theca cells (T) which are divisible into theca interna (blue wavy arrow) and theca externa (blue straight arrow), multiple layers of granulosa cells (Gc), follicular antrum (FA), corona radiata (CR) and cumulus oophorus (CO) that connects the oocyte (O) with the rest of the granulosa cells. Recent corpora lutea (CL*) and old corpora lutea (CL) are also seen. The highest magnification is showing primordial follicles (black arrows) formed of oocyte surrounded by a single layer of squamous cells and primary follicle (PF) formed of an oocyte (O) having clear nucleus (n) and surrounded by zona pellucida (ZP) and few layers of granulosa cells (Gc). (Bar: A&D: 500 µm X40- B&E 200 µm X100 - C&F: 50 µm X400).

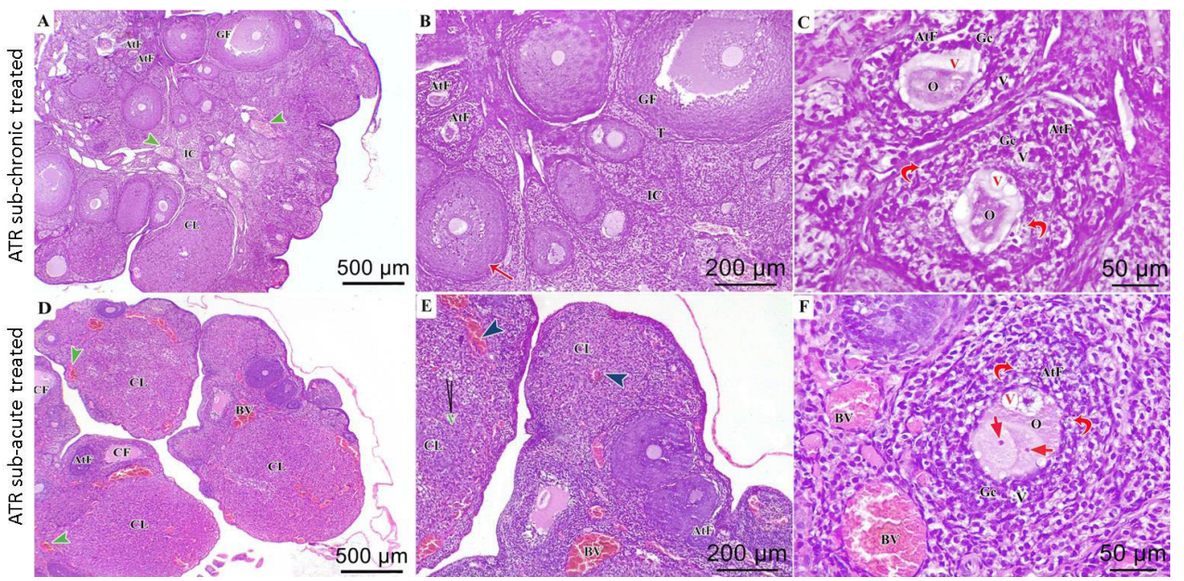

Fig. 4: H&E stained sections of the ovarian tissue: ATR sub-chronic treated group (A, B & C) is showing multiple atretic follicles (AtF) with apparent increase in interstitial cells (IC) in between, old corpus luteum (CL), multiple areas of hemorrhage (green arrow heads), Graffian follicle (GF) with ill differentiation between layers of theca cells (T) and vacuolated degenerated preantral follicle (red arrow). The highest magnification is showing atretic follicles (Atf) having vacuolated (black V) and disorganized granulosa cells (Gc) with pyknotic nuclei (red curved arrows) and degenerated vacuolated (red V) oocyte (O).
ATR sub-acute treated group (D, E & F) is showing multiple cystic follicles (CF), atretic follicles (AtF) with complete loss of oocyte, many old corpora lutea (CL) with vascular changes (blue arrow heads) and vacuolations (green V), areas of hemorrhage (green arrow heads) and congested blood vessels (BV). The highest magnification is showing atretic follicle (AtF) with degenerated vacuolated (red V) oocyte (O), micronuclei formation (red short arrows) and disarranged vacuolated (black V) granulosa cells (Gc) with pyknotic nuclei (red curved arrows). (Bar: A&D: 500 µm X40- B&E 200 µm X100 - C&F: 50 µm X400).

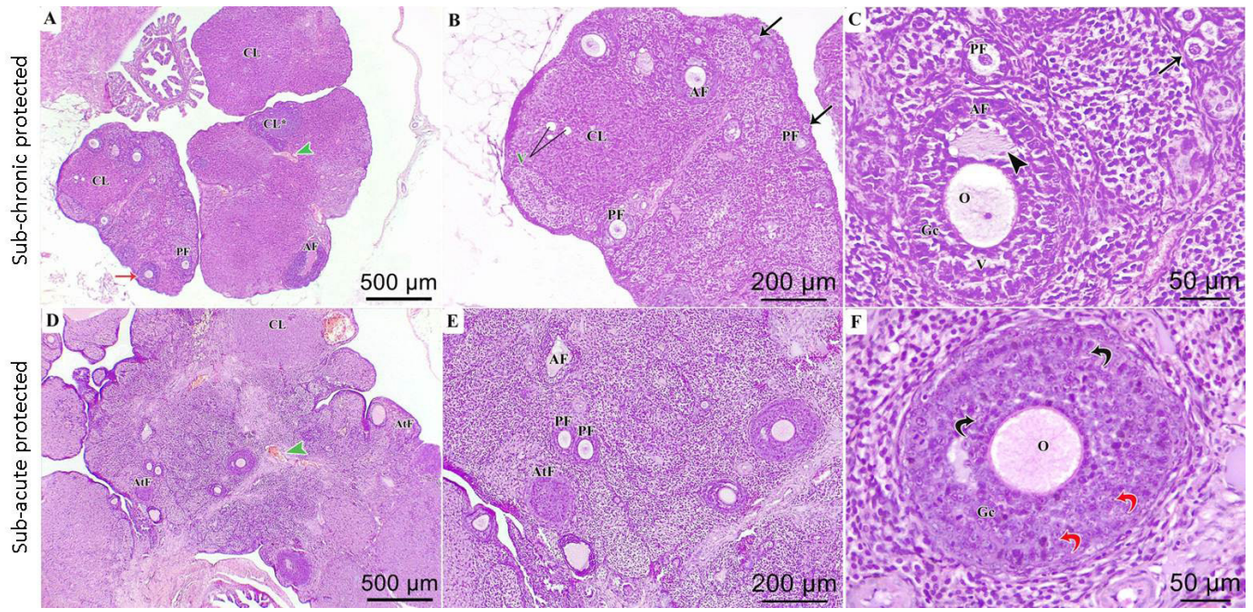

Fig. 5: H&E stained sections of the ovarian tissue: Sub-chronic protected group (A, B & C) is showing primordial follicles (black arrows) and recent corpus luteum (CL*). Also, there are primary follicles (PF), preantral follicle (red arrow), antral follicle (AF), old corpora lutea (CL) with little vacuolation (green V) and small area of hemorrhage (green arrow head). The highest magnification is showing primordial follicle (black arrow), primary follicle (PF) and early antral follicle (AF) having multiple layers of granulosa cells (Gc) with some vacuolations (V), a small cavity (black arrow head) and oocyte (O).

Sub-acute protected group (D, E & F) is showing primary follicles (PF), antral follicle (AF), old corpus luteum (CL), atretic follicles (AtF) and area of hemorrhage (green arrow head). The highest magnification is showing early antral follicle with oocyte (O) surrounded by granulosa cells (Gc) having pyknotic (red curved arrows) or karyorrhetic (black curved arrows) nuclei. Notice absence of primordial follicles. (Bar: A&D: 500 µm X40- B&E 200 µm X100 - C&F: 50 µm X400).

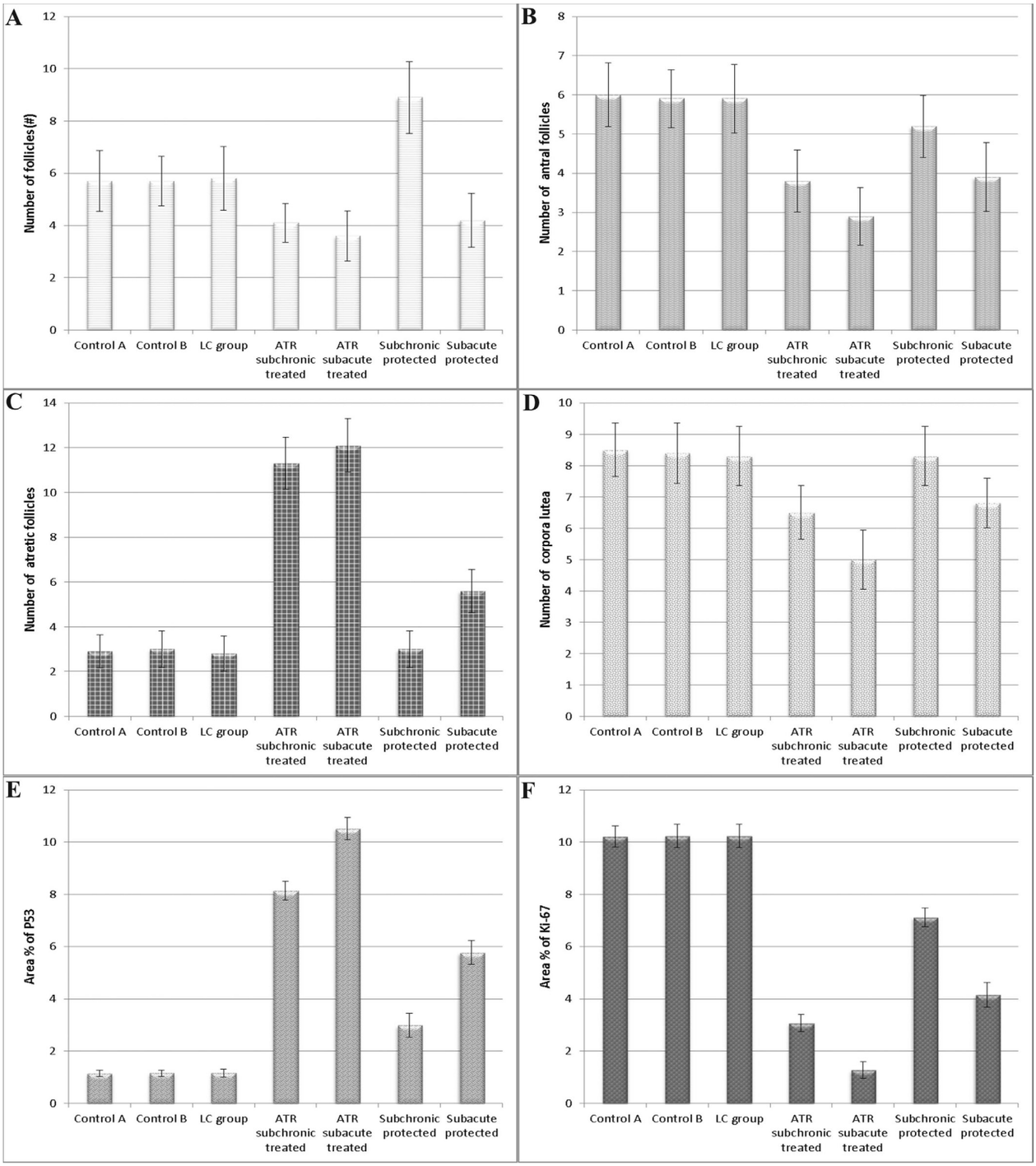

Fig. 6: Bar charts showing number of different follicles and area percent of immunoexpression in different studied groups. A: number of follicles (primordial, primary, and preantral), B: number of antral follicles, C: number of atretic follicles, D: number of corpora lutea, E: area % of P53 and F: area % of Ki-67. Results are represented as mean ± SD.

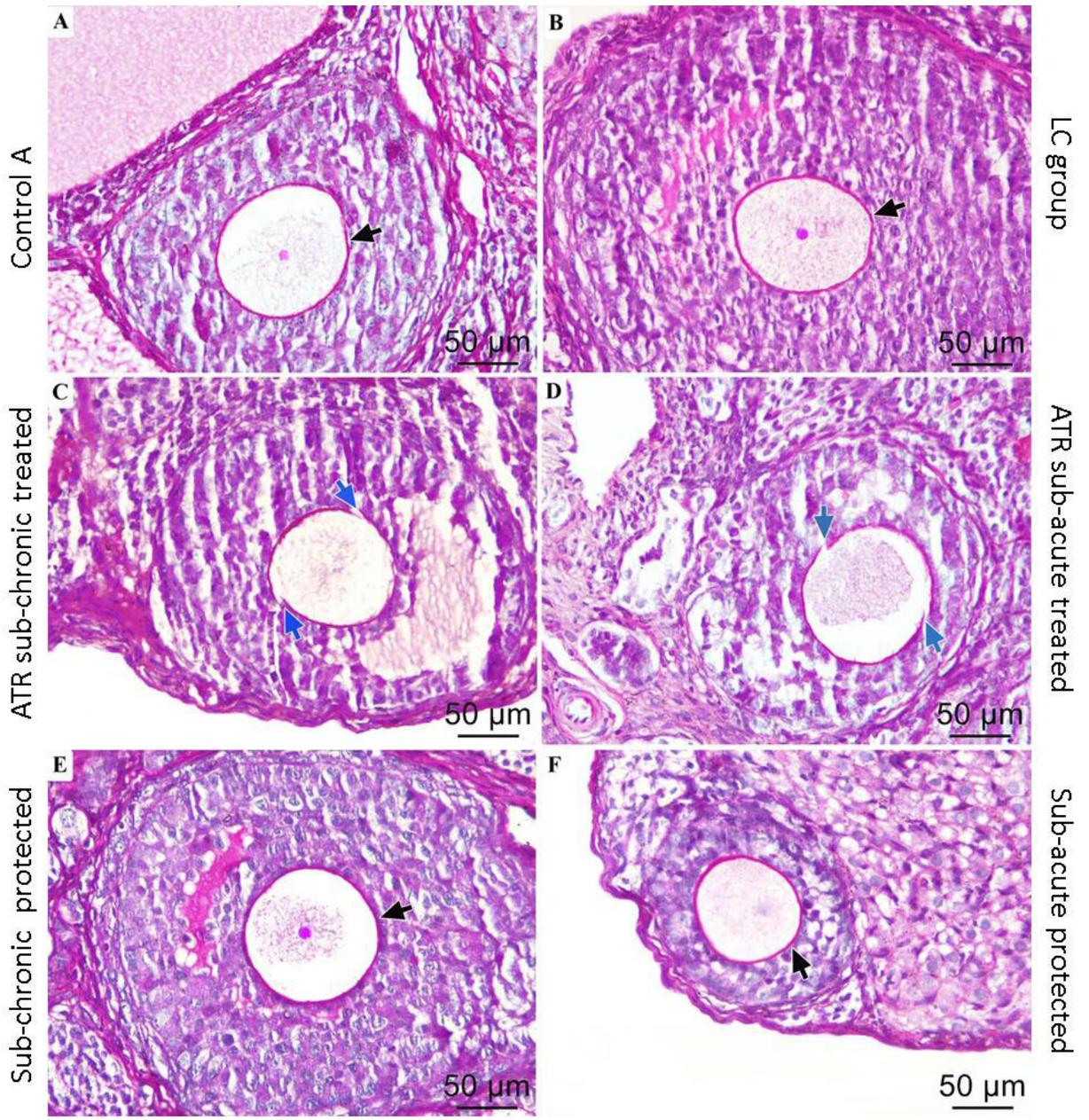

Fig. 7: PAS stained ovarian sections in different studied groups: control (A) and LC (B) groups are showing continuous zona pellucida (black short arrow). While, ATR sub-chronic treated (C) and ATR sub-acute treated (D) groups are showing loss of continuity of zona pellucida (blue short arrows). Restoration of of zona pellucida's continuity in sub-chronic protected group (E) and sub-acute protected group (F) was demonstrated (black short arrow). (Bar: 50 µm X400).

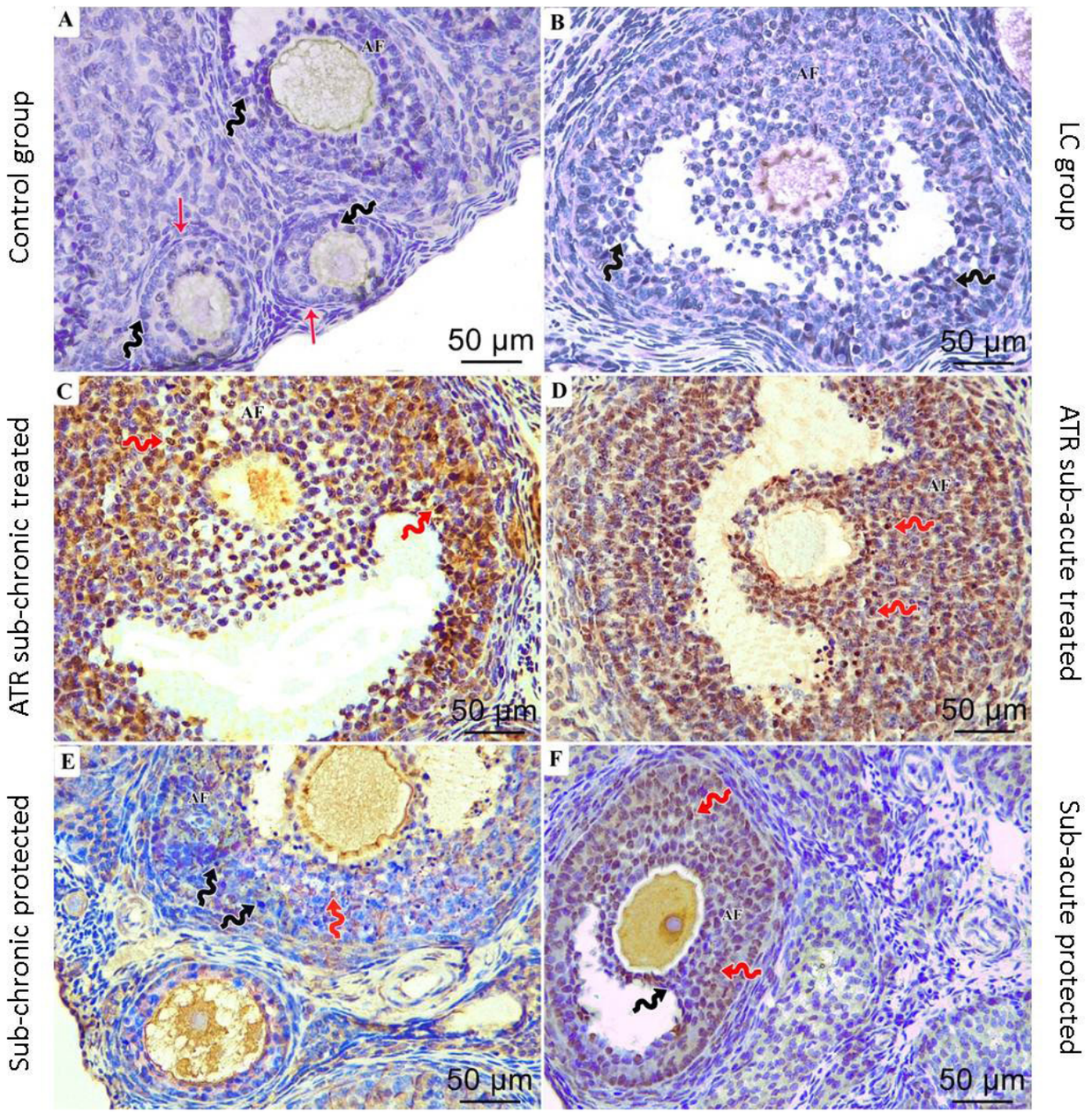

Fig. 8: P53 immunostained ovarian sections of the different studied groups: preantral follicles (red straight arrows), antral follicle (AF), negative stained nuclei of granulosa cells are taking blue color (black zigzag arrows), and positive stained nuclei of granulosa cells are taking brown color (red zigzag arrows). A: control group, B: LC group, C: ATR sub-chronic treated group, D: ATR sub-acute treated group, E: sub-chronic protected group and F: sub-acute protected group. (Bar: 50 µm X400).

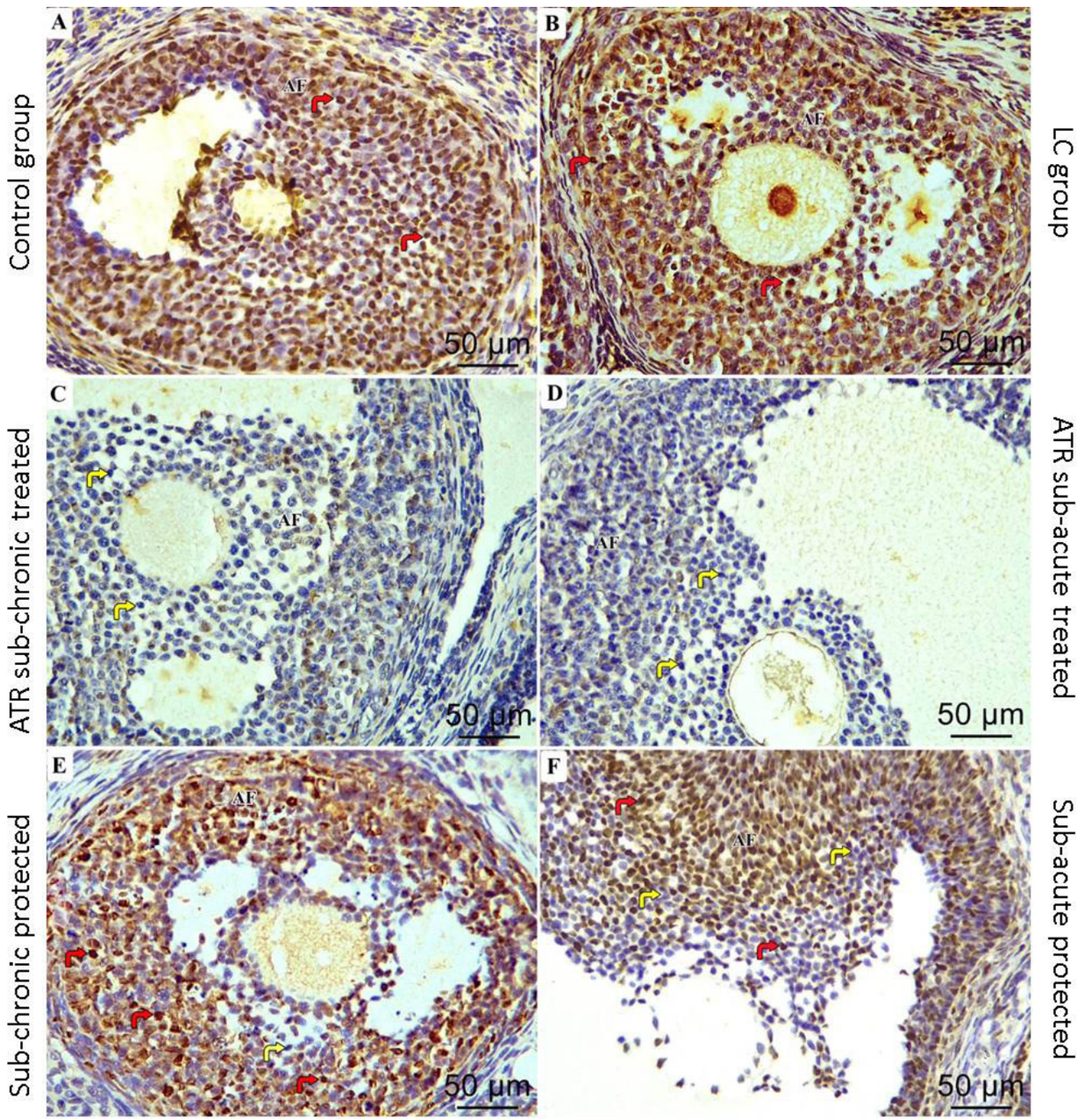

Fig. 9: Ki-67 immunostained ovarian sections of the different studied groups: antral follicle (AF), positive stained nuclei of granulosa cells are taking brown color (red right angled arrows), negative stained nuclei of granulosa cells are taking blue color (yellow right angled arrows). A: control group, B: LC group, C: ATR sub-chronic treated group, D: ATR sub-acute treated group, E: sub-chronic protected group and F: sub-acute protected group. (Bar: 50 µm X400).

Table 1: Statistical comparison of mean values of serum LH and estradiol and ovarian MDA, NO, SOD and CAT by ANOVA & Tukey HSD post hoc test among different groups:

Group	Control group		LC group	ATR subchronic treated	ATR subacute treated	Subchronic protected	Subacute protected	F	*P-value*
Variable	A	B							
Serum LH (uIm/ml)	15.898 ± 0.471	15.817 ± 0.495	15.761 ± 0.494	10.6330 ± 0.452^{a*}	8.215 ± 0.72^{a*,c*}	15.666 ± 0.523^{b1*}	14.907 ± 0.51^{a*,b2*,d*}	344.718	*<0.001*
Serum estradiol (Pg/ml)	222.70 ± 3.057	222.5 ± 3.206	222.60 ± 3.273	191.80 ± 3.490^{a*}	170.90 ± 3.247^{a*,c*}	223.30 ± 3.466^{b1*}	216.70 ± 2.83^{a*,b2*,d*}	408.393	*<0.001*
MDA (nmol/mg)	5.037 ± 0.55	5.0370 ± 0.541	5.024 ± 0.343	7.3100 ± 0.491^{a*}	8.977 ± 0.324^{a*,c*}	5.10 ± 0.424^{b1*}	5.729 ± 0.385^{a*,b2*,d*}	120.070	*<0.001*
NO (umol/L)	31.10 ± 1.663	30.90 ± 2.132	31.20 ± 1.814	38.30 ± 1.703^{a*}	41.10 ± 1.370^{a*,c*}	32.10 ± 1.287^{b1*}	34.50 ± 1.354^{a*,b2*,d*}	60.857	*<0.001*
SOD (U/mg)	89.10 ± 3.542	89.30 ± 2.983	89.40 ± 3.204	71.30 ± 4.296^{a*}	59.30 ± 4.968^{a*,c*}	88.30 ± 4.111^{b1*}	75.50 ± 4.972^{a*,b2*,d*}	85.175	*<0.001*
CAT (ng/mg)	10.73 ± 0.717	10.725 ± 0.882	10.687 ± 0.827	9.179 ± 0.674^{a*}	7.86 ± 0.647^{a*,c*}	10.911 ± 0.451^{b1*}	9.434 ± 0.802^{a*,b2*,d*}	24.962	*<0.001*

N= 10 for each subgroup. **LH:** Luteinizing hormone. **MDA:** malondialdehyde. **NO:** nitric oxide. **SOD:** super oxide dismutase. **CAT:** catalase.
***:** significant difference (*P <0.05*)
a: Comparison in relation to control A group.
b: Comparison in relation to treated groups.
b1: sub-chronic group.
b2: sub-acute group.
c: Comparison between sub-acute and sub-chronic treated groups
d: Comparison between sub-acute and sub-chronic protected groups

Table 2: Statistical comparison of number of different follicles and area % of P53 & Ki-67 by ANOVA & Tukey HSD post hoc test among different groups:

Group	Control group		LC group	ATR subchronic treated	ATR subacute treated	Subchronic protected	Subacute protected	F	*P-value*
Variable	A	B							
Follicles (#)	5.70 ± 1.16	5.70 ± 0.949	5.80 ± 1.229	4.10 ± 0.738^{a*}	3.60 ± 0.966^{a*}	8.90 ± 1.370^{a*,b1*}	4.20 ± 1.033^{a*,d*}	27.041	*<0.001*
Antral follicles	6.00 ± 0.816	5.90 ± 0.738	5.90 ± 0.876	3.80 ± 0.789^{a*}	2.90 ± 0.738^{a*}	5.20 ± 0.789^{b1*}	3.90 ± 0.876^{a*,d*}	24.294	*<0.001*
Atretic follicles	2.90 ± 0.738	3.00 ± 0.816	2.80 ± 0.789	11.30 ± 1.160^{a*}	12.10 ± 1.197^{a*}	3.00 ± 0.816^{b1*}	5.60 ± 0.966^{a*,b2*,d*}	193.599	*<0.001*
CL	8.50 ± 0.85	8.40 ± 0.966	8.30 ± 0.949	6.50 ± 0.850^{a*}	5 .00 ± 0.943^{a*,c*}	8.30 ± 0.949^{b1*}	6.80 ± 0.789^{a*,b2*,d*}	22.066	*<0.001*
P53 (Area %)	1.1530 ± 0.119	1.154 ± 0.12	1.153 ± 0.158	8.148 ± 0.356^{a*}	10.517 ± 0.424^{a*,c*}	2.986 ± 0.467^{a*,b1*}	5.769 ± 0.459^{a*,b2*,d*}	1286.412	*<0.001*
Ki-67 (Area %)	10.217 ± 0.408	10.233 ± 0.453	10.235 ± 0.443	3.072 ± 0.323^{a*}	1.272 ± 0.313^{a*,c*}	7.126 ± 0.36^{a*,b1*}	4.148 ± 0.467^{a*,b2*,d*}	904.330	*<0.001*

N= 10 for each subgroup. **Follicles (#):** primordial, primary and preantral follicles. **CL:** corpora lutea.
***:** significant difference (*P <0.05*)
a: Comparison in relation to control A group.
b: Comparison in relation to treated groups.
b1: sub-chronic group.
b2: sub-acute group.
c: Comparison between sub-acute and sub-chronic treated groups
d: Comparison between sub-acute and sub-chronic protected groups.

DISCUSSION

The current study looked at the sub-chronic and sub-acute effects of ATR on the adult rat ovary, as well as the predicted protective impact of LC. Recently, there has been an increasing interest to detect the toxicological effects of environmental chemicals on reproductive system of human and mammals. Numerous reports have suggested that ATR has adverse effects on the reproductive function[45].

In the present study, serum LH level in ATR sub-chronic and sub-acute treated groups showed significant decrease, in comparison to that of the control group. These findings were consistent with some investigators[32] who reported significant decrease in serum LH level in atrazine treated group for 2 weeks. As atrazine and its metabolites are lipid soluble, can pass the blood brain barrier and interact with gonadotropin releasing hormone (GnRH) neurons[46, 47]. ATR alteration of GnRH signaling to the pituitary causes disturbed downstream secretion of LH and inhibition of subsequent ovulation[48].

In the current study, serum E2 levels in the ATR sub-chronic and sub-acute treated groups showed significant decrease, as compared to the control group. These results were in agreement with the study done by[49]. As one of the main targets of ATR in reproductive system is disturbed steroidogenesis (biosynthesis of estradiol and progesterone) in the ovarian granulosa cells[50-53].

Androstenedione is transformed to estradiol by aromatase enzyme[54]. Some researchers[55] revealed that ATR inhibits activity of aromatase enzyme and stimulates E2 metabolism, so it has antiestrogenic effect.

On the other hand, a research by[56] found no changes in serum E2 after exposing Long-Evans rats to 100 mg/kg daily of ATR. This is may be due to variable affection of ATR on steroidogenesis according to age, dose and experimental model[57].

The current study revealed that serum LH and E2 levels significantly increased in the protected groups as compared with ATR treated groups. These findings were supported by some researchers[28] who gave 100 mg/kg/day LC with sodium fluoride in tap water for 30 days and found significant increase in serum LH and estrogen.

In this study, ATR treated groups showed significant increase in MDA and NO ovarian contents and significant decrease in SOD and CAT ovarian contents. This finding was in the same line with[58] who found that oxidative stress occurred in ovarian tissue. This was explained as ATR causes oxidative stress by forming reactive oxygen species[59], lipid peroxidation and antioxidant depletion[60].

In this study, MDA and NO ovarian contents significantly decreased while SOD and CAT ovarian levels significantly increased in sub-chronic and sub-acute protected groups in comparison with corresponding treated groups. In the same line, some researchers[61] found that LC administration in diabetic rats for 21 days decreased MDA and increased SOD and CAT ovarian levels compared with the diabetic rats. As LC possesses antioxidant properties and inhibits protein breakdown[62] by decreasing free radicals formation and mitochondrial dysfunction[63]. Also, the protective roles of LC may include its capacity to decrease the harmful fatty acids accumulation around mitochondria and/or its capacity to increase source of energy necessary for survival of cell under situations of oxidative stress[64].

In the present study, ATR sub-chronic treated group revealed presence of multiple atretic follicles at different stages of development with apparent increase in interstitial cells (due to increased atresia) and presence of old corpus luteum. These observations were consistent with[58] who observed atretic follicles in ovarian tissue of rats treated by 5mg/kg/day for 28 days. Also, some investigators[49] showed presence of atretic follicles with vacuolated granulosa cells in ovarian sections of rats exposed to a low dose of ATR (150 mg atrazine/kg) for 30 days. This is explained as the action of low-dose endocrine disruptive substances like atrazine have evolved to behave as strong amplifiers, so significant alterations in cell function may occur in response to extremely low concentrations[65].

In the current study, ATR sub-acute treated group showed multiple cystic follicles and persistence of many old corpora lutea with absence of recent one. This means stoppage of follicular growth due to strong gonadotropin inhibition by high doses and elongation of luteal phase. Also, there were atretic follicles with complete loss of oocyte or with micronuclei formation and disarranged vacuolated granulosa cells with pyknotic nuclei. These findings were in agreement with some researchers[49]. Micronuclei formation in the nucleus of oocyte is an indicator for DNA damage as explained by[66]. These histopathological findings are due to LH suppression caused by ATR resulting in anovulation[67] and increased number of atretic follicles[68].

In our study, there was a significant decrease in number of primordial and primary follicles in ATR treated groups as compared to control group. As exposure to EDCs as ATR may cause premature ovarian failure (POF)[69], a disorder which is associated with follicular depletion and death of granulosa cells in process of follicular atresia[70]. Moreover in POF, there is an increase in the primordial follicle activation rate, so depletion of ovarian reserve occurs[71]. Also, a study done by[72] showed that exposure to EDCs led to POF due to alteration of growth and development of ovarian follicles, depletion of primordial and primary follicles and affection of steroidogenesis.

In the present study, sub-chronic protected group revealed appearance of primordial follicles which is a good sign with presence of recent corpus lutum. Also, sub-acute protected group revealed decrease in number of ovarian cysts and atretic follicles, but there was absence of primordial follicles. These observations were consistent with some scientists[28].

In the current study, number of primordial follicles was significantly increased in sub-chronic protected group, while there was no significant increase in sub-acute protected group which means reserve depletion. This may be explained as in sub-chronic protected group there was sufficient time to restore the reservoir of primordial follicles. While number of atretic follicles significantly decreased in both protected groups. This was in line with some investigators[61] who stated that LC administration to diabetic rats increased the number of primordial, primary and secondary follicles. It also decreased atretic follicles number.

In our study, sub-acute protected group showed decrease in number of cystic follicles. Some scientists[73] revealed that impairment of folliculogenesis in a polycystic ovary syndrome (PCOS) mouse model was partially prevented by LC administration. Because LC is a ROS scavenger and an energy production promoter, it may have beneficial effects on follicular survival and ovarian function[74-76].

Numerous reports have highlighted that women with PCOS had low serum levels of carnitines[22,77,78]. Moreover, LC supplementation has been effectively employed in PCOS patients, as it revealed improvements in hormonal and metabolic parameters[21,79,80].

Zona pellucida is a glycoprotein coat which separates the granulosa cells from oocyte. It is penetrated by thin granulosa cells cytoplasmic processes which contact with microvilli projecting from basement membrane of oocyte. This cell-cell communication mechanism directs the growth of primary oocyte in addition to progression of its cell cycle. This is called granulosa cell-oocyte bidirectional pathway which is important for folliculogenesis. The interruption of this pathway leads to impairment of folliculogenesis as occurs in PCOS and leads to infertility[81].

In the present study, PAS stained sections of ATR treated groups revealed discontinuation of zona pellucida. However, a research by[25] revealed intact zona pellucida in Xylidine Ponceau stained sections of ATR sub-chronic treated rats and discontinuation in ATR sub-acute treated group. While, PAS stained ovarian sections of protected groups showed restoration of continuity of zona pellucida. So, ATR impairs folliculogenesis due to destruction of zona pellucida which agrees with some investigators[82] who showed that formation of ZP is essential for normal folliculogenesis and fertility; particularly in mice and generally in mammals.

Many studies reported that ATR causes oxidative stress, cytotoxicity and apoptosis[15,83,84]. Atresia was confirmed by immunohistochemical study of P53. In our study, the area % of P53 positivity was significantly increased in ATR treated groups when compared with the control group. The increase of apoptosis was significant in ATR sub-acute treated group as compared to ATR sub-chronic treated group. This was in agreement with some investigators[58], [85] who found increased apoptotic nuclei in the ovarian tissues of rats exposed to ATR.

In the current study, the area % of P53 positivity was significantly decreased in protected groups as compared to their corresponding treated groups. This decrease in apoptosis was statistically significant in sub-chronic protected group as compared to sub-acute protected group. LC possesses anti-apoptotic properties[86] through inhibiting the production of pro-apoptotic proteins[87]. Some researchers[74] discovered that LC prevents apoptosis of follicle and improves the frozen-thawed ovaries function in mice. Also, other researchers[88] showed that LC stimulates growth of cells, decreases ROS levels, inhibits lipid peroxidation and reduces apoptosis in grasp carp ovary cells.

Effect of ATR on decreased mitotic division is confirmed by immunohistochemical study of Ki-67. Antigen Ki-67 is a nuclear protein which is associated with and may be essential for synthesis of ribosomal RNA and cell proliferation[89]. In the present study, ATR sub-chronic and sub-acute treated rats presented significant reduction of area % of immune-expression of Ki-67 suggesting inhibited cellular proliferation. This was in line with some investigators[90] who found that cell proliferation was decreased in the epithelium of ventral prostate of atrazine treated rats. Oxidative stress role in inhibition of cellular proliferation is controversial. Whereas several researches revealed that cell proliferation may be reduced by oxidative stress[91,92], another studies revealed that cell proliferation may be increased by oxidative stress[93,94].

This study revealed significant increase in area % of immune-expression of Ki-67 in ovaries of protected groups compared to corresponding treated groups. This increase of proliferation was statistically significant in sub-chronic protected group as compared to sub-acute protected group. This was in line with some scientists[95] who revealed that the positive reaction of Ki-67 immunostaining of testicular sections was improved after treatment with 150 mg/kg of LC for 35 days simultaneously with low and high doses of monosodium glutamate.

So in our study, ATR has been revealed to affect two vital ovarian processes; folliculogenesis and steroidogenesis, by altering oocyte development, depletion of primordial follicles, and inducing atresia in follicles during several stages of development. LC had an important role in improvement of steroidogenesis and restoration of primordial follicles. This improvement was more prominent in sub-chronic than sub-acute protected groups, as time factor was essential to restore the primordial reservoir.

CONCLUSION

As regards effect of LC which was used as an antidote to atrazine in this study, there was a significant decrease of atrazine toxicity in female rats but it didn't eliminate the effect of atrazine completely. Yet with prolonged administration of LC, the antidote effect became more prominent and atrazine toxicity became less obvious.

RECOMMENDATION

We recommend performing more researches about usage of antioxidants as L-Carnitine by different doses in cases of infertility due to ovarian disorders.

ACKNOWLEDGEMENTS

Special thanks to Human Anatomy Department, Faculty of Medicine, Zagazig University. Many thanks to the team of Zagazig scientific and medical research center (ZSMRC).

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Ali AA, El-Seify GH, El Haroun HM, Soliman MAEMM: Effect of monosodium glutamate on the ovaries of adult female albino rats and the possible protective role of green tea. Menoufia Medical Journal. (2014) 27(4):793.
2. Van Der Mark M, Brouwer M, Kromhout H, Nijssen P, Huss A, Vermeulen R: Is pesticide use related to Parkinson disease? Some clues to heterogeneity in study results. Environmental health perspectives. (2012) 120(3):340-7.
3. Mostafalou S, Abdollahi M: Pesticides and human chronic diseases: evidences, mechanisms, and perspectives. Toxicology and applied pharmacology. (2013) 268(2):157-77.
4. Uzumcu M, Zama AM, Oruc E: Epigenetic mechanisms in the actions of endocrine-disrupting chemicals: gonadal effects and role in female reproduction. Reproduction in domestic animals. (2012) 47:338-47.
5. Alavian-Ghavanini A, Rüegg J: Understanding epigenetic effects of endocrine disrupting chemicals: from mechanisms to novel test methods. Basic & clinical pharmacology & toxicology. (2018) 122(1):38-45.
6. Pathak RK, Dikshit AK: Atrazine and human health. International Journal of Ecosystem. (2011) 1(1):14-23.
7. Konstantinou IK, Hela DG, Albanis TA: The status of pesticide pollution in surface waters (rivers and lakes) of Greece. Part I. Review on occurrence and levels. Environmental Pollution. (2006) 141(3):555-70.
8. Udiković-Kolić N, Scott C, Martin-Laurent F: Evolution of atrazine-degrading capabilities in the environment. Applied microbiology and biotechnology. (2012) 96(5):1175-89.
9. Barr DB, Panuwet P, Nguyen JV, Udunka S, Needham LL: Assessing exposure to atrazine and its metabolites using biomonitoring. Environmental health perspectives. (2007) 115(10):1474-8.
10. Baxter L, Brain RA, Hosmer AJ, Nema M, Müller KM, Solomon KR, *et al*.: Effects of atrazine on egg masses of the yellow-spotted salamander (Ambystoma maculatum) and its endosymbiotic alga (Oophila amblystomatis). Environmental Pollution. (2015) 206:324-31.
11. Sabarwal A, Kumar K, Singh RP: Hazardous effects of chemical pesticides on human health–Cancer and other associated disorders. Environmental toxicology and pharmacology. (2018) 63:103-14.
12. Bray LD, Szarka AZ, Heard NE, Hackett DS, Kahrs RA: Dietary exposure assessment of the triazine herbicides. The Triazine Herbicides. (2008) 413.
13. Bohn T, Cocco E, Gourdol L, Guignard C, Hoffmann L: Determination of atrazine and degradation products in Luxembourgish drinking water: origin and fate of potential endocrine-disrupting pesticides. Food Additives & Contaminants: Part A. (2011) 28(8):1041-54.
14. Komsky-Elbaz A, Roth Z: Effect of the herbicide atrazine and its metabolite DACT on bovine sperm quality. Reproductive Toxicology. (2017) 67:15-25.
15. Abarikwu SO, Duru QC, Njoku R-CC, Amadi BA, Tamunoibuomie A, Keboh E: Effects of co-exposure to atrazine and ethanol on the oxidative damage of kidney and liver in Wistar rats. Renal failure. (2017) 39(1):588-96.
16. Vassiliadis S, Athanassakis I: A "conditionally essential" nutrient, L-carnitine, as a primary suspect in endometriosis. Fertility and sterility. (2011) 95(8):2759-60.
17. Brass EP: Supplemental carnitine and exercise. The American journal of clinical nutrition. (2000) 72(2):618S-23S.
18. Dunning KR, Robker RL: Promoting lipid utilization with l-carnitine to improve oocyte quality. Animal reproduction science. (2012) 134(1-2):69-75.
19. Zarbakhsh S, Safari R, Sameni HR, Yousefi B, Safari M, Khanmohammadi N, *et al*.: Effects of co-administration of bone marrow stromal cells and L-carnitine on the recovery of damaged ovaries by performing chemotherapy model in rat. International journal of fertility & sterility. (2019) 13(3):196.
20. Hamza RZ, Al-Eisa RA, Mehana AE, El-Shenawy NS: Effect of l-carnitine on aspartame-induced oxidative stress, histopathological changes, and genotoxicity in liver of male rats. Journal of basic and clinical physiology and pharmacology. (2019) 30(2):219-32.
21. Ismail AM, Hamed AH, Saso S, Thabet HH: Adding L-carnitine to clomiphene resistant PCOS women improves the quality of ovulation and the pregnancy rate. A randomized clinical trial. European Journal of Obstetrics & Gynecology and Reproductive Biology. (2014) 180:148-52.
22. Samimi M, Jamilian M, Ebrahimi FA, Rahimi M, Tajbakhsh B, Asemi Z: Oral carnitine supplementation

reduces body weight and insulin resistance in women with polycystic ovary syndrome: a randomized, double-blind, placebo-controlled trial. Clinical endocrinology. (2016) 84(6):851-7.

23. Dionyssopoulou E, Vassiliadis S, Evangeliou A, Koumantakis E, Athanassakis I: Constitutive or induced elevated levels of L-carnitine correlate with the cytokine and cellular profile of endometriosis. Journal of reproductive immunology. (2005) 65(2):159-70.
24. Hubscher C, Brooks D, Johnson J: A quantitative method for assessing stages of the rat estrous cycle. Biotechnic & Histochemistry. (2005) 80(2):79-87.
25. Juliani C, Silva-Zacarin E, Santos D, Boer P: Effects of atrazine on female Wistar rats: morphological alterations in ovarian follicles and immunocytochemical labeling of 90 kDa heat shock protein. Micron. (2008) 39(5):607-16.
26. Bodea F, Bocea A, Decea N: L-carnitine decreases oxidative stress induced by experimental hypobaric hypoxia. Pediatric Endocrinology, Diabetes and Metabolism. (2010) 16(2):78-81.
27. Galal G, Abou Zed T, Kahilo K, Shukry M: Effect of L-carnitine on Lead-induced Infertility in Female Rats. (2016) reactions.11:16.
28. Al_Shammari ZM, Alsaidi ZZ: Assessment the Estrogenic Efficacy of L-carnitine on Female Rats treated with Sodium Fluoride. Systematic Reviews in Pharmacy. (2020) 11(3).
29. 29. Abd-Elrazek A, Ahmed-Farid O: Protective effect of L-carnitine and L-arginine against busulfan-induced oligospermia in adult rat. Andrologia. (2018) 50(1):e12806.
30. Wujcik EK, Londoño NJ, Duirk SE, Monty CN, Masel RI: An acetylcholinesterase-inspired biomimetic toxicity sensor. Chemosphere. (2013) 91(8):1176-82.
31. (https://www.caymanchem.com/msdss/13375m.pdf).
32. Quignot N, Arnaud M, Robidel F, Lecomte A, Tournier M, Cren-Olivé C, *et al*.: Characterization of endocrine-disrupting chemicals based on hormonal balance disruption in male and female adult rats. Reproductive Toxicology. (2012) 33(3):339-52.
33. Altun MA, Ozaydin A, Arkan H, Demiryas S, Akbas F, Bahtiyar N, *et al*.: Anesthesia may alter mRNA expression of certain wound healing-associated genes in dermal wound environment of the rats. Molecular biology reports. (2019) 46(3):2819-27.
34. Obinna VC, Kagbo HD: Endocrine effect of beta cypermethrin on female albino rats. Journal of Advances in Medicine and Medical Research. (2017) :1-7.
35. Del Rio D, Stewart AJ, Pellegrini N: A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutrition, metabolism and cardiovascular diseases. (2005) 15(4):316-28.
36. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. (1976) 72(1-2):248-54.
37. Sun Y, Oberley LW, Li Y: A simple method for clinical assay of superoxide dismutase. Clinical chemistry. (1988) 34(3):497-500.
38. Aebi H: [13] Catalase in vitro. Methods in enzymology. (1984) 105:121-6.
39. Suvarna KS, Layton C, Bancroft JD: Bancroft's theory and practice of histological techniques E-Book: Elsevier Health Sciences. (2018)
40. Amini L, Tehranian N, Movahedin M, Ramezani Tehrani F, Soltanghoraee H: Polycystic ovary morphology (PCOM) in estradiol valerate treated mouse model. Int J Women's Health Reprod Sci. (2016) 4:13-7.
41. Goncalves R, DeSchryver K, Ma C, Tao Y, Hoog J, Cheang M, *et al*.: Development of a Ki-67-based clinical trial assay for neoadjuvant endocrine therapy response monitoring in breast cancer. Breast cancer research and treatment. (2017) 165(2):355-64.
42. Suzuki H, Kawaguchi T, Hasegawa T, Yonechi A, Ohsugi J, Higuchi M, *et al*.: Prognostic impact of p53 protein overexpression in patients with node-negative lung adenocarcinoma. Cancer letters. (2006) 237(2):242-7.
43. Khalaf HA, Elmorsy E, Mahmoud E-HM, Aggour AM, Amer SA: The role of oxidative stress in ovarian toxicity induced by haloperidol and clozapine—a histological and biochemical study in albino rats. Cell and tissue research. (2019) 378(2):371-83.
44. Sabha Jr M, Emirandetti A, Cullheim S, De Oliveira ALR: MHC I expression and synaptic plasticity in different mice strains after axotomy. Synapse. (2008) 62(2):137-48.
45. Yang S, Jia ZC, Chen JY, Hu JX, Zhang LS: Toxic effects of atrazine on reproductive system of male rats. Biomedical and Environmental Sciences. (2014) 27(4):281-8.
46. Gojmerac T, Kniewald J: Atrazine biodegradation in rats--a model for mammalian metabolism. Bulletin of environmental contamination and toxicology. (1989)
47. Wirbisky SE, Freeman JL: Atrazine exposure and reproductive dysfunction through the hypothalamus-pituitary-gonadal (HPG) axis. Toxics. (2015) 3(4):414-50.
48. Cooper RL, Laws SC, Das PC, Narotsky MG, Goldman JM, Lee Tyrey E, *et al*.: Atrazine and reproductive

function: mode and mechanism of action studies. Birth Defects Research Part B: Developmental and Reproductive Toxicology. (2007) 80(2):98-112.

49. Salem MM, Nabeeh A: Therapeutic Effects of Ascorbic Acid on Hormonal and Histological Alteration Produced in The Reproductive System of Albino Rats Intoxicated by Herbicide Atrazine. Egyptian Academic Journal of Biological Sciences, B Zoology. (2021) 13(1):1-16.
50. Tinfo NS, Hotchkiss MG, Buckalew AR, Zorrilla LM, Cooper RL, Laws SC: Understanding the effects of atrazine on steroidogenesis in rat granulosa and H295R adrenal cortical carcinoma cells. Reproductive Toxicology. (2011) 31(2):184-93.
51. Fa S, Pogrmic-Majkic K, Samardzija D, Glisic B, Kaisarevic S, Kovacevic R, *et al*.: Involvement of ERK1/2 signaling pathway in atrazine action on FSH-stimulated LHR and CYP19A1 expression in rat granulosa cells. Toxicology and applied pharmacology. (2013) 270(1):1-8.
52. Pogrmic-Majkic K, Samardzija D, Fa S, Hrubik J, Glisic B, Kaisarevic S, *et al*.: Atrazine enhances progesterone production through activation of multiple signaling pathways in FSH-stimulated rat granulosa cells: evidence for premature luteinization. Biology of reproduction. (2014) 91(5):124, 1-10.
53. Samardzija D, Pogrmic-Majkic K, Fa S, Glisic B, Stanic B, Andric N: Atrazine blocks ovulation via suppression of Lhr and Cyp19a1 mRNA and estradiol secretion in immature gonadotropin-treated rats. Reproductive Toxicology. (2016) 61:10-8.
54. Anderson SA, Barry JA, Hardiman PJ: Risk of coronary heart disease and risk of stroke in women with polycystic ovary syndrome: a systematic review and meta-analysis. International journal of cardiology. (2014) 176(2):486-7.
55. Oh SM, Shim SH, Chung KH: Antiestrogenic action of atrazine and its major metabolites in vitro. Journal of health science. (2003) 49(1):65-71.
56. Goldman JM, Davis LK, Murr AS, Cooper RL: Atrazine-induced elevation or attenuation of the LH surge in the ovariectomized, estrogen-primed female rat: role of adrenal progesterone. Reproduction. (2013) 146(4):305-14.
57. Piazza MJ, Urbanetz AA: Environmental toxins and the impact of other endocrine disrupting chemicals in women's reproductive health. JBRA assisted reproduction. (2019) 23(2):154.
58. Zhao F, Li K, Zhao L, Liu J, Suo Q, Zhao J, *et al*.: Effect of Nrf2 on rat ovarian tissues against atrazine-induced anti-oxidative response. International journal of clinical and experimental pathology. (2014) 7(6):2780.
59. Gely-Pernot A, Hao C, Becker E, Stuparevic I, Kervarrec C, Chalmel F, *et al*.: The epigenetic processes of meiosis in male mice are broadly affected by the widely used herbicide atrazine. BMC genomics. (2015) 16(1):1-22.
60. Abass MA, Elkhateeb SA, Abd EL-Baset SA, Kattaia AA, Mohamed EM, Atteia HH: Lycopene ameliorates atrazine-induced oxidative damage in adrenal cortex of male rats by activation of the Nrf2/HO-1 pathway. Environmental Science and Pollution Research. (2016) 23(15):15262-74.
61. Majidi FZ, Rezaei N, Zare Z, Dashti A, Shafaroudi MM, Abediankenari S: The Protective Effects of l-Carnitine and Zinc Oxide Nanoparticles Against Diabetic Injury on Sex Steroid Hormones Levels, Oxidative Stress, and Ovarian Histopathological Changes in Rat. Reproductive Sciences. (2021) 28(3):888-96.
62. Samir SM, Abbas AM, Safwat SM, Elserougy HG: Effect of L-carnitine on diabetes-induced changes of skeletal muscles in rats. Journal of basic and clinical physiology and pharmacology. (2018) 29(1):47-59.
63. Hino K, Nishikawa M, Sato E, Inoue M: L-carnitine inhibits hypoglycemia-induced brain damage in the rat. Brain research. (2005) 1053(1-2):77-87.
64. Morimoto N, Hashimoto S, Yamanaka M, Satoh M, Nakaoka Y, Fukui A, *et al*.: Treatment with Laevo (l)-carnitine reverses the mitochondrial function of human embryos. Journal of assisted reproduction and genetics. (2021) 38(1):71-8.
65. Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS: Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environmental health perspectives. (2003) 111(8):994-1006.
66. Alchalabi AS, Rahim H, Aklilu E, Al-Sultan II, Malek MF, Ronald SH, *et al*.: Histopathological changes associated with oxidative stress induced by electromagnetic waves in rats' ovarian and uterine tissues. Asian Pacific Journal of Reproduction. (2016) 5(4):301-10.
67. Shibayama H, Kotera T, Shinoda Y, Hanada T, Kajihara T, Ueda M, *et al*.: Collaborative work on evaluation of ovarian toxicity 14) Two-or four-week repeated-dose studies and fertility study of atrazine in female rats. The Journal of toxicological sciences. (2009) 34(Special):SP147-SP55.
68. Khayal E, Atta W, Elmesallamy G, Omar M: ENDOCRINAL DISRUPTION EFFECTS OF ATRAZINE ON THE PUBERTY OF JUVENILE ALBINO RATS: A SUB-CHRONIC STUDY. Egyptian Society of Clinical Toxicology Journal. (2013) 1(1):1-16.

69. Vabre P, Gatimel N, Moreau J, Gayrard V, Picard-Hagen N, Parinaud J, *et al*.: Environmental pollutants, a possible etiology for premature ovarian insufficiency: a narrative review of animal and human data. Environmental Health. (2017) 16(1):1-18.

70. Akogullari D, Uluer ET, Vatansever HS, editors: Investigation of the relation between follicular atresia and granulosa cells in terms of cell death mechanisms in premature ovarian failure model. Multidisciplinary Digital Publishing Institute Proceedings. (2018)

71. Nelson LM: Primary ovarian insufficiency. New England Journal of Medicine. (2009) 360(6):606-14.

72. Tran DN, Jung E-M, Yoo Y-M, Ahn C, Kang HY, Choi K-C, *et al*.: Depletion of follicles accelerated by combined exposure to phthalates and 4-vinylcyclohexene diepoxide, leading to premature ovarian failure in rats. Reproductive Toxicology. (2018) 80:60-7.

73. Kalhori Z, Mehranjani MS, Azadbakht M, Shariatzadeh MA: L-Carnitine improves endocrine function and folliculogenesis by reducing inflammation, oxidative stress and apoptosis in mice following induction of polycystic ovary syndrome. Reproduction, Fertility and Development. (2019) 31(2):282-93.

74. Zhang Q, Wang S-M, Yao P-B, Zhang L, Zhang Y-J, Chen R-X, *et al*.: Effects of L-carnitine on follicular survival and graft function following autotransplantation of cryopreserved-thawed ovarian tissues. Cryobiology. (2015) 71(1):135-40.

75. Khanmohammadi N, Movahedin M, Safari M, Sameni HR, Yousefi B, Jafari B, *et al*.: Effect of L-carnitine on in vitro developmental rate, the zona pellucida and hatching of blastocysts and their cell numbers in mouse embryos. International Journal of Reproductive BioMedicine. (2016) 14(10):649.

76. Lin Z, Liu F, Shi P, Song A, Huang Z, Zou D, *et al*.: Fatty acid oxidation promotes reprogramming by enhancing oxidative phosphorylation and inhibiting protein kinase C. Stem cell research & therapy. (2018) 9(1):1-14.

77. Celik F, Kose M, Yilmazer M, Köken GN, Arioz DT, Kanat Pektas M: Plasma L-carnitine levels of obese and non-obese polycystic ovary syndrome patients. Journal of Obstetrics and Gynaecology. (2017) 37(4):476-9.

78. Salehpour S, Nazari L, Hoseini S, Moghaddam PB, Gachkar L: Effects of L-carnitine on polycystic ovary syndrome. JBRA assisted reproduction. (2019) 23(4):392.

79. Fenkci V, Fenkci S, Yilmazer M, Serteser M: Decreased total antioxidant status and increased oxidative stress in women with polycystic ovary syndrome may contribute to the risk of cardiovascular disease. Fertility and sterility. (2003) 80(1):123-7.

80. Jamilian M, Foroozanfard F, Kavossian E, Kia M, Aghadavod E, Amirani E, *et al*.: Effects of chromium and carnitine co-supplementation on body weight and metabolic profiles in overweight and obese women with polycystic ovary syndrome: a randomized, double-blind, placebo-controlled trial. Biological trace element research. (2020) 193(2):334-41.

81. Kierszenbaum AL, Tres L: Histology and cell biology: an introduction to pathology E-book: Elsevier Health Sciences. (2015)

82. Wassarman PM, Litscher ES: Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility. International Journal of Developmental Biology. (2013) 56(10-11-12):833-9.

83. Adesiyan AC, Oyejola TO, Abarikwu SO, Oyeyemi MO, Farombi EO: Selenium provides protection to the liver but not the reproductive organs in an atrazine-model of experimental toxicity. Experimental and toxicologic pathology. (2011) 63(3):201-7.

84. Farombi E, Abarikwu S, Adesiyan A, Oyejola T: Quercetin exacerbates the effects of subacute treatment of atrazine on reproductive tissue antioxidant defence system, lipid peroxidation and sperm quality in rats. Andrologia. (2013) 45(4):256-65.

85. Sargazi Z, Nikravesh MR, Jalali M, Sadeghnia HR, Rahimi Anbarkeh F: Apoptotic effect of organophosphorus insecticide diazinon on rat ovary and protective effect of vitamin E. Iranian Journal of Toxicology. (2016) 10(2):37-44.

86. Ishii T, Shimpo Y, Matsuoka Y, Kinoshita K: Anti-apoptotic effect of acetyl-L-carnitine and L-carnitine in primary cultured neurons. The Japanese Journal of Pharmacology. (2000) 83(2):119-24.

87. Chao H-H, Liu J-C, Hong H-J, Lin J-w, Chen C-H, Cheng T-H: L-carnitine reduces doxorubicin-induced apoptosis through a prostacyclin-mediated pathway in neonatal rat cardiomyocytes. International journal of cardiology. (2011) 146(2):145-52.

88. Wang Q, Ju X, Chen Y, Dong X, Luo S, Liu H, *et al*.: Effects of L-carnitine against H 2 O 2-induced oxidative stress in grass carp ovary cells (Ctenopharyngodon idellus). Fish physiology and biochemistry. (2016) 42(3):845-57.

89. Bullwinkel J, Baron-Lühr B, Lüdemann A, Wohlenberg C, Gerdes J, Scholzen T: Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. Journal of cellular physiology. (2006) 206(3):624-35.

90. Martins-Santos E, Pimenta CG, Campos PRN, Oliveira AG, Mahecha GAB, Oliveira CA: Atrazine affects the morphophysiology, tissue homeostasis and aromatase expression in the efferent ductules of adult rats with mild alterations in the ventral prostate. Chemosphere. (2018) 193:958-67.

91. Miethling-Graff R, Rumpker R, Richter M, Verano-Braga T, Kjeldsen F, Brewer J, *et al*.: Exposure to silver nanoparticles induces size-and dose-dependent oxidative stress and cytotoxicity in human colon carcinoma cells. Toxicology in vitro. (2014) 28(7):1280-9.

92. Carrasco-Torres G, Baltiérrez-Hoyos R, Andrade-Jorge E, Villa-Treviño S, Trujillo-Ferrara JG, Vásquez-Garzón VR: Cytotoxicity, oxidative stress, cell cycle arrest, and mitochondrial apoptosis after combined treatment of hepatocarcinoma cells with maleic anhydride derivatives and quercetin. Oxidative medicine and cellular longevity. (2017)

93. Burdon RH, Gill V, Rice-Evans C: Oxidative stress and tumour cell proliferation. Free radical research communications. (1990) 11(1-3):65-76.

94. Galli A, Svegliati-Baroni G, Ceni E, Milani S, Ridolfi F, Salzano R, *et al*.: Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. Hepatology. (2005) 41(5):1074-84.

95. Abdellateif AM, Twfeq WS: Testicular Ameliorative Effect of L-Carnitine on Monosodium Glutamate-Induced Testicular Structure Alterations in Male Mice. Egyptian Academic Journal of Biological Sciences, D Histology & Histochemistry. (2021) 13(1):13-30.

الملخص العربى

التغييرات الناجمة عن الأترازين - اعتماداً على الجرعة والمدة - في مبيض الجرذ الأبيض البالغ و الدور الوقائي المحتمل لـ إل- كارنيتين : دراسة هستولوجية وهستوكيميائية مناعية

ريهام حلمى عبدالكريم، محيي السيد هليل، إيمان محمد كمال الدين الشربيني، علا علي عبد الوهاب

قسم التشريح الآدمي والأجنة، كلية الطب البشرى، جامعة الزقازيق، مصر

مقدمة: الأترازين هو أكثر مبيدات الأعشاب شيوعًا في العالم. وقد أظهرت العديد من الدراسات أن له آثاراً سلبية كثيرة على الإنجاب. إل- كارنيتين هو أحد مضادات الأكسدة الفلافونويدية.

الهدف من الدراسة: كان الهدف من هذا البحث هو توضيح التغييرات التركيبية والكيميائية التي يسببها الأترازين في مبيض الجرذان البيضاء البالغة ولتوضيح الدور الوقائي المحتمل لـ إل- كارنيتين.

المواد والطرق المستخدمة: تم تقسيم ٧٠ أنثى من الجرذان البالغة إلى ٦ مجموعات:

المجموعة الضابطة ، مجموعة إل- كارنيتين (تم إعطاؤهم ١٠٠ مجم إل- كارنتين / كجم / يوم لمدة ٣٠ يوم) ، المجموعة المعالجة بالأترازين شبه المزمنة (تم إعطاؤهم ٦,٥ مجم أترازين/ كجم/ يوم لمدة ٣٠ يوم) ، المجموعة المعالجة بالأترازين شبه الحادة (تم إعطاؤهم ٦٥ مجم أترازين/ كجم/ يوم لمدة ١٤ يوم) ، المجموعة المحمية شبه المزمنة (تم إعطائهم ٦,٥ مجم أترازين/ كجم/ يوم بالإضافة الى ١٠٠ مجم إل- كارنيتين / كجم/ يوم لمدة ٣٠ يوم) والمجموعة المحمية شبه الحادة (تم إعطائهم ٦٥ مجم أترازين/ كجم/ يوم بالإضافة الي١٠٠ مجم إل- كارنيتين / كجم / يوم لمدة ١٤ يوم). تم إعطاء كل الأدوية عن طريق الفم. و تم الحصول على عينات الدم والأنسجة ومعالجتها من أجل التحليلات البيوكيميائية ، والدراسات النسيجية و صبغها بالتصبغ المناعي الكيميائي. كما تم إجراء التحليل الإحصائي.

النتائج: انخفض بشكل ملحوظ مستوي هرمون LH والإستراديول ومحتوي المبيض من SOD و CAT بينما زاد بشكل ملحوظ محتوى المبيض من MDA & NO في المجموعات المعالجة بـالأترازين ATR مقارنة بالمجموعة الضابطة. وأظهرت أنسجة المبيض للمجموعات المعالجة بـالأترازين زيادة ملحوظة فى عدد الحويصلات الضامرة، وفقدان استمرارية الغشاء المحاط بالنواة في صبغة PAS مقارنة بالمجموعة الضابطة.تم التأكد من الضمورمن خلال زيادة ملحوظة في الدراسة المناعية الكيميائية لـ P53 في المجموعات المعالجة بـالأترازين. كما كان هناك انخفاضاً ملحوظاً في الانقسام الميتوزي في المجموعات المعالجة بـالأترازين مقارنة بالمجموعة الضابطة وتم تأكيده عن طريق الدراسة المناعية الكيميائية لـ Ki-67. علاوة على ذلك، اختلفت المجموعتان المعالجتان بـالأترازين اختلافًا ملحوظاً في كل من النتائج البيوكيميائية والنسيجية ، بينما كانت هذه التغييرات أقل وضوحًا في المجموعات المحمية بعد إعطاء إل- كارنيتين.

الخلاصة: المكملات الغذائية لـ إل- كارنيتين لها تأثير مفيد ضد تدمير الأترازين للمبيض.