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An investigation of the protective effects of Dehydroepiandrosterone (DHEA) in chemotherapatic Cyclophosphamide (CP) induced ovarian damage on rats

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Abstract

Objective: In this study, we aimed to investigate the preventive effect of Dehydroepiandrosterone (DHEA) on Cyclophosphamide (CP) induced damage on rat ovarium.

Material and Method: Wistar Albino Rats have been used for the study and three groups have been created. Group 1 (the control Group): no treatment was administered. Intact ovarian tissue was removed and blood samples were taken for anti-mullerian hormone (AMH) test. Group 2 (the CP Group): Rats received CP intraperitoneally at a single dose of 150 mg / kg. Group 3 (the CP + DHEA Group): Rats received CP intraperitoneally at a single dose of 150 mg / kg at baseline and DHEA has been administrated subcutaneously for 10 days at a dose of 60 mg / kg daily. Rats in groups 2 and 3 were sacrificed at the end of 10 days, ovarian tissues were removed and blood samples have been collected for AMH test.

Results: While normal ovarian tissue damage scores were zero, CP showed significant damage and histopathological changes on ovarian tissue in all CP administrated rats. CP group had higher vascular congestion ($p=0.004$) and total damage scores ($p=0.010$) than normal ovarian group. CP + DHEA group had higher edema ($p<0.001$), vascular congestion ($p<0.001$) and total damage scores ($p<0.001$). CP group had a decrease in primordial ($p = 0.001$), primary ($p = 0.043$) and preantral follicles ($p = 0.006$). CP + DHEA group showed a decrease in primordial ($p = 0.001$) and antral follicles ($p = 0.018$). AMH levels did not decrease in both groups.

Conclusions: It was found that the use of DHEA to prevent CP-induced ovarian damage in rats did not produce significant changes in antral follicle counts, ovarian volume, and AMH levels, which were important for clinical practice.

Keywords: CP; dehydroepiandrosterone; anti-mullerian hormone; ovary; rat

Introduction

It is well known that chemotherapy, which is used to treat cancers commonly observed in girls and women, can deplete ovarian reserve and cause infertility or early menopause (1). Cancer treatments increase survival in both pediatric and young patients. However, the fertility problems associated with the anti-tumor treatments administered to this patient group have not yet been resolved. New treatments and new strategies are needed to prevent ovarian damage and to restore ovarian functions (1,2).

The most ovotoxic chemotherapy agents are the nitrogen mustard-derived alkylators (e.g. CP, cisplatin, etc.) (1). Today, approximately 5% of malignant neoplasms occur in women younger than 35 years of age (3). Women who have survived severe treatment protocols are likely to suffer from ovarian infertility problems (4).

CP is widely used in the treatment of many diseases, since it is a low-cost and effective therapeutic agent (5).

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These include many diseases such as cancers (breast cancer, ovarian cancer, hematological cancers, etc.), hematological diseases, nervous system diseases, immune diseases, rheumatoid arthritis and nephrotic syndrome (6,7). CP belongs to the oxazaphosphorine family of mustard-alkylating agents (8) and has been used for more than 50 years in the clinical practice (9).

It is highly ovotoxic, and its ovarian toxicity and relationship with infertility have been proven (9). It has been reported that it is the agent with the highest impact on women's fertility(10, 11) and is associated with a high risk of ovarian insufficiency (4). Early ovarian insufficiency, premature menopause and impaired reproductive potential after chemotherapy can significantly affect quality of life in this young age group (12,13). Despite efforts to understand the details of ovarian damage and insufficiency caused by CP, the mechanisms remain unclear (14).

Dehydroepiandrosterone (DHEA) is an endogenous steroid produced by the zona reticularis of the adrenal cortex and ovarian theca cells (15). It promotes follicular development and granulosa cell proliferation by increasing intraovarian androgen concentrations in the ovaries (16). The responses given in a worldwide survey conducted in 196 in vitro fertilization (IVF) units in 45 countries, representing a total of 124,700 IVF cycles, revealed that DHEA was included in more than a quarter of treatment protocols concerning patients with reduced ovarian reserve (17). Several reports have shown that DHEA supplementation in patients with low ovarian reserve helps to improve ovarian reserve parameters (18,19), increase ovarian response (20), increase pregnancy rates and reduce age-related aneuploidy (21).

Our aim in this experimental study was to investigate whether DHEA has protective effects on ovarian damage caused by CP.

Materials and Methods

This study was conducted at the Animal Testing Laboratory of the University of Üsküdar in July 2019, after the approval of the Ethics Committee.

Laboratory animals and the care of animals in research: Ten-twelve weeks old, female Wistar Albino (Rattus Norvegicus species) rats weighing 175 to 210 grams were used in this study. Rats were exposed to light for 12 hours a day (from 08:00 to 20:00) and had access to food (standard rodent pellet) and drinking water (tap water) without restriction and were kept at room temperature of 21 to 23ºC and a humidity of 40 to 50%. 4 or 5 rats were placed in every cage. The number of rats was determined in line with the previous studies. Rats were randomly assigned to four groups of 8. Considering bowel transit time, rats were not fed within 6 hours before laparotomy to empty the gut and allow surgery but they had access to drinking water.

Study groups: Group 1 (the control group): These rats underwent a laparotomy procedure at baseline and their ovaries were removed. Blood was drawn from the inferior vena cava for anti-mullerian hormone (AMH) testing.

Group 2 (the CP group): These rats received CP intraperitoneally at a dose of 150 mg/kg at baseline (22) and underwent an oophorectomy procedure at the end of the 10th day of the study. After the rats were sacrificed, at least 2-3 ml of blood was collected for AMH testing. Then laparotomy was performed and both ovaries were excised for histopathological examination.

Group 3 (the CP + DHEA Group): Rats received CP intraperitoneally at a dose of 150 mg/kg at baseline. In addition they received DHEA (Cayman Chemical, Michigan, USA, CAS registry no: 53-43-0, item no:15728) subcutaneously for 10 days at a dose of 60 mg/kg daily as dissolved in 0.1 ml of sesame oil. (23, 24)After the rats were sacrificed, at least 2-3 ml of blood was collected for AMH testing. Then laparotomy was performed and both ovaries were excised for histopathological examination.

CP dose and preparation: CP was administered intraperitoneally only at baseline at a dose of 150 mg/kg. While preparing the drug,we used the central drug preparation unit of our hospital (with Robotic Chemotherapy Drug Preparation System) in a closed environment where microbiological contamination and employee exposure risks were eliminated under conditions that comply with national and international standards.

This Drug Preparation System uses a negative pressure indoor air environment complying with ISO 5, Class 100 and GMP Class A, double HEPA filter air cleaning system, safe waste management system, high capacity laminator current and dose sensitivity information (gravimetric and volumetric) measurement and uses the barcode system during drug preparation process.

Surgical procedures: Laparotomy was performed after decapitation of the animals. Sterile, powder-free, latex gloves were used during all surgical procedures. The procedure was performed while rats were lying in a supine position. Abdominal area was shaved before the procedure and the surgical site was prepped using 10% Povidone-iodine solution (Batticon; Adeka Laboratories, Istanbul, Turkey).

A 5 cm median (on the line between the xiphoid process and pubis) incision was made to enter into the abdominal cavity and each surgical procedure lasted 5 to 10 minutes to protect the drying effect of the room air (Figure 1). After the removal of ovaries for histological examination, animals were disposed of in red waste containers.

Histopathological examinations: Surgically excised ovaries were fixed in 10% formalin. Paraffin blocks were prepared 24 hours after the oophorectomy procedure. Tissue sections of 5 micrometers were taken and follicular activity was assessed in 5 randomly selected samples from each ovary. Slides were stained with hematoxylin eosin and examined under the light microscope. The paraffin blocks were sectioned using a microtome blade (Leica, Nussloch, Germany). Every slide was blindly assessed by the same pathologist. A light microscope (Olympus Clinical Microscope, Tokyo, Japan) was used to analyze the sections. Edema, vascular congestion, inflammation, cellular degeneration and hemorrhage were examined as

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histopathological injury scores. The scores were evaluated as described by Celik et al. Pathological findings were rated. Grade 0 indicated normal alterations, no abnormal findings; Grade 1 indicated mild edema, mild vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 2 indicated moderate edema, moderate vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 3 indicated severe edema, severe vascular occlusion, minimal hemorrhage and minimal leukocyte infiltration, Grade 4 indicated severe edema, severe vascular occlusion, hemorrhage and leukocyte infiltration. (Figure 2)

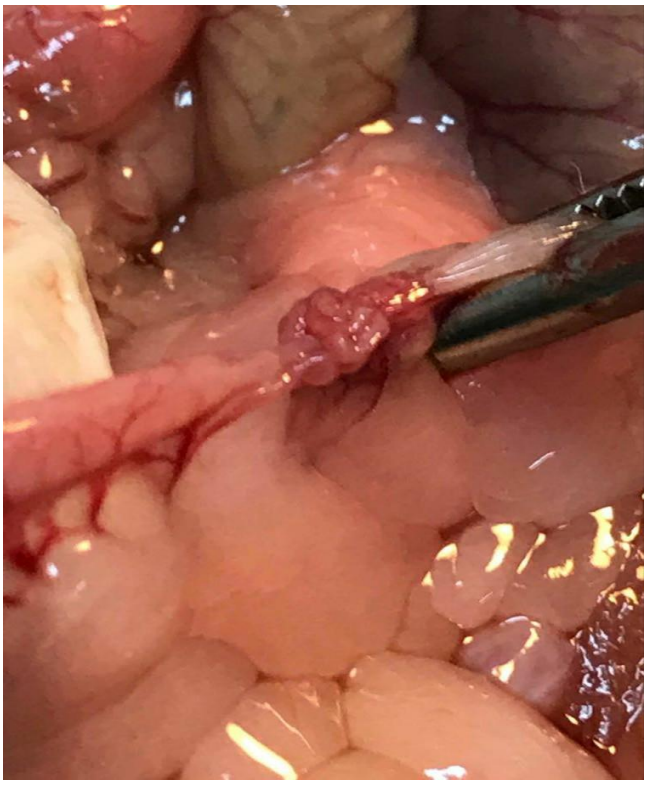

Figure 1. Excision of the ovary

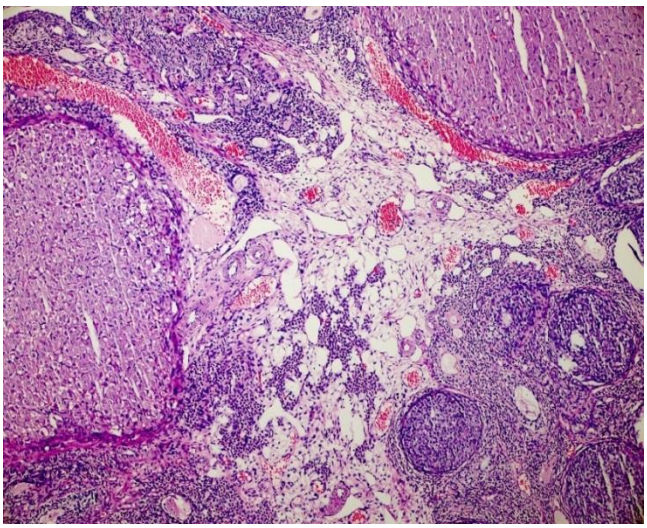

Figure 2. Edema in the medullar region - dilated vessels x200 hematoxylin eosin

All follicles were counted to assess ovarian reserve. Primordial, primary, secondary (pre-antral), tertiary (antral) and atretic follicles were counted (figures 3). Follicles were evaluated as described by Parlakgumus et al. (25). Primordial, primary, secondary (pre-antral) and tertiary (antral) follicles were counted. Primordial follicle is described as an oosit, surrounded with only one layer of epithelial cell layer, primer follicle is surrounded with one or more layers of cuboidal granulosa cells. Secondary/ pre-antral follicle is surrounded with more than two cell layers and consists of antrum folliculi and zona pellucida. The follicle which has an antrum and stratum granulosum and is surrounded with cumulus oophorus is defined as a tertiary follicle. Atretic follicle, the basement that separated the oocyte from granulosa cells often thickens to become the glassy membrane. Fibrous material replaces the granulosa cells and loss of cohesion may also occur in granulosa cells.

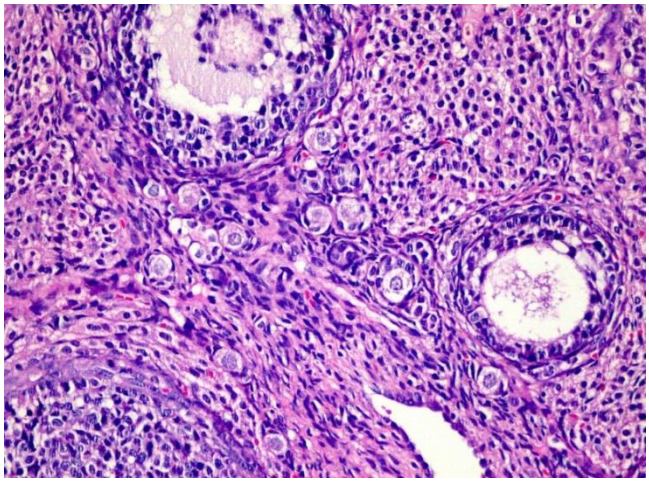

Figure 3. Multiple primary and primordial follicles x400 hematoxylin eosin

AMH assays: Blood samples were collected into tubes containing lithium heparin (BD Vacutainer Plasma tubes, Manchester, England). The concentration of the Lithium Heparin additive in these tubes was 17 international units of heparin/ml of blood. Blood samples were centrifuged within 30 minutes of sampling. After 15 minutes of centrifugation at 1000xg, serum was removed and the remaining plasma was transferred into an Eppendorf tube and stored frozen at -20ºC until the time of analysis. AMH concentrations were measured in “ng/ml” plasma using ELISA method. The rat AMH kit used in study had a sensitivity of 0.10 g/mL, a detection range of 0.16 to 10 ng/mL and a coefficient variation of less than 10% (Elabscience, Rat AMH kit; Houston, Texas, ABD). The laboratory technician of the laboratory of the university hospital was blinded to the study groups and unaware of which samples belonged to which rat. All samples were analyzed in the same assay.

Statistical analysis: Statistical analysis was performed with SPSS version 17.0 program. The suitability of the variables to normal distribution was examined by histogram graphs and Kolmogorov-Smirnov test. Mean, standard deviation, median and IQR values were used to present descriptive analyzes.

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Non-parametric variables were evaluated between the two groups and Mann Whitney U Test was used. Spearman Correlation Test was used to analyze the measured data of each group in relation to each other. The cases where the P-value was less than 0.05 were evaluated as statistically significant results.

Results

Histopathological damage scores were compared between the groups. Vascular congestion and total damage scores were higher in the CP group than in the normal group. CP + DHEA group had higher edema, vascular congestion and total score values than the normal group (Table 1).

Follicle numbers and AMH values were compared according to the groups. In the CP group, the number of Primordial, Primary, Secondary follicles and ovarian volume were lower than the normal group. In the CP + DHEA group, the number of primordial and tertiary follicles and ovarian volume were lower than in the normal group. AMH values do not differ between the groups (Table 2).

The correlation between AMH and rat weight, ovarian volume, total damage score, number of atretic follicles, secondary and tertiary follicles were evaluated between the groups. Accordingly, there was a strong positive correlation between AMH and ovarian volume in the normal group (Table 3).

Table 1. Comparison of histopathological damage scores of normal ovary vs CP, CP + DHEA

	Normal ovary	CP	P*	CP +DHEA	P**
Edema					
Mean SD Median- IQR	0,00±0,00 0,00(0,00-0,00)	0,25±0,46 0,00(0,00-0,50)	0,143	2,50±0,53 2,50(2,00-3,00)	**<0,001**
Vascular congestion					
Mean SD Median- IQR	0,00±0,00 0,00(0,00-0,00)	1,13±0,83 1,00(0,50-2,00)	**0,004**	1,50±0,53 1,50(1,00-2,00)	**<0,001**
Inflammation					
Mean SD Median- IQR	0,00±0,00 0,00(0,00-0,00)	0,00±0,00 0,00(0,00-0,00)	1,000	0,00±0,00 0,00(0,00-0,00)	1,000
Cellular degeneration					
Mean SD Median- IQR	0,00±0,00 0,00(0,00-0,00)	0,25±0,71 0,00(0,00-0,00)	0,317	0,00±0,00 0,00(0,00-0,00)	1,000
Hemorrhage					
Mean SD Median- IQR	0,13±0,35 0,00(0,00-0,00)	0,00±0,00 0,00(0,00-0,00)	0,317	0,00±0,00 0,00(0,00-0,00)	0,317
Total score					
Mean SD Median- IQR	0,13±0,35 0,00(0,00-0,00)	1,62±1,30 1,50(0,50-3,00)	**0,010**	4,00±0,93 4,00(3,00-5,00)	**<0,001**

Mann Whitney U Test

Table 2. Comparison of normal ovary vs CP, CP + DHEA groups in terms of follicle count and AMH values

	Normal ovary	CP	P*	CP +DHEA	P**
Primordialfollicle					
Mean SD Median- IQR	12,75±1,91 12,50(11,50-14,00)	2,88±1,36 2,50(2,00-4,00)	**0,001**	4,63±1,77 4,50(3,00-5,50)	**0,001**
Primerfollicle					
Mean SD Median- IQR	10,50±2,33 11,00(8,50-12,00)	6,13±4,45 3,50(3,00-9,00)	**0,043**	9,50±4,57 10,50(7,50-11,50)	0,671
Secondary (pre-antral) follicle					
Mean SD Median- IQR	12,25±1,83 12,50(10,50-13,50)	8,63±2,39 9,50(6,50-10,00)	**0,006**	12,13±4,05 11,50(8,50-15,50)	0,833
Tertiary (antral) follicle					
Mean SD Median- IQR	21,50±3,21 22,00(19,50-23,50)	17,75±5,97 17,00(12,50-22,00)	0,140	17,00±2,83 16,50(14,50-19,00)	**0,018**
Atreticfollicle					
Mean SD Median- IQR	0,25±0,46 0,00(0,00-0,50)	0,25±0,71 0,00(0,00-0,00)	0,643	0,00±0,00 0,00(0,00-0,00)	0,143
AMH (ng/mL)					
Mean SD Median- IQR	3,42±0,79 3,37(2,64-4,06)	2,96±0,57 3,03(2,57-3,29)	0,189	3,71±0,75 3,64(3,18-4,05)	0,528
Ovaryvolume (mm3)					
Mean SD Median- IQR	55,49±9,14 54,12(50,19-55,53)	23,27±5,04 24,07(19,15-27,68)	**0,001**	38,70±12,16 36,22(27,80-50,19)	**0,017**

Mann Whitney U Test

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Table 3. Correlations between rat weights, ovary volume, total damage score, number of atretic follicles and AMH levels

	Normal rat AMH	CP AMH	CP+DHEA AMH
Rat weight (grams)	0,443	-0,160	-0,562
Ovary volume (mm3)	**0,778***	0,476	-0,168
Total damage score	0,082	0,222	0,095
Atretic follicle count	0,000	-0,412	0,000
Pre-antral+antral follicle count	-0,072	-0,325	0,611

Spearman Correlation Test *p<0,050

Discussion

Many previous studies have investigated the effects of CP on reproductive function in women and have found that it is associated with the highest risk of iatrogenic infertility following the treatment of common cancers frequently observed in women of reproductive age and children (26). CP induces its effects by mitotically diminishing the stock of active cells (4). It was found that ovarian tissues had prominent atrophy, fibrosis, prominent follicular atresia and did not have normal follicular stages (27-29). In the pathological examinations of the ovary, it was generally seen that there was decreased primordial follicles, ovarian blood vessel damage, and ovarian atrophy (30,31). To investigate these effects associated with CP, we conducted histopathological examinations on ovarian tissue and performed volume evaluations. We examined the correlation of AMH with rat weight, ovarian volume, total damage score, atrial follicle count, and secondary and tertiary follicle counts. As such, we determined that there was a strong positive correlation between AMH and ovarian volume in the normal group. However, ovarian volume decreased significantly compared with normal rats in all rats administered with CP. Although the DHEA supplementation reduced this decrease numerically, it could not prevent a statistical decrease. In addition, although ovarian volume decreased in both groups, AMH levels were not affected.

CP causes progressive and irreversible damage due to the destruction of oocytes, follicular depletion and severe vascular damage in the ovary (32,33). Severe ovarian damage develops as a result of ovarian atrophy, destruction of growing follicles, and hence a decrease in follicle counts (34). We evaluated the histopathological damage scores in order to examine ovarian damage. We found that there was a significant increase in vascular congestion and total damage score compared with normal ovarian tissue. We determined that the addition of DHEA to CP application did not lead to a difference in any of these damage scores.

Alkylating drugs such as CP are the most effective agents in inducing ovarian failure(35). In studies, it is usually used as a single dose of 200 mg/kg(36,37).It has been reported that in the treatment of mice with CP, a process related to the p53-upregulated modulator of apoptosis (PUMA), a pro-apoptotic protein, results in a massive net follicle loss within five days(38).

Primordial follicles can also become the target of direct chemotherapy, since cytotoxic agents with a non-specific cell cycle, such as CP, can also damage resting cells(39).

It has been shown that the alkylating agent CP induces primordial follicle loss in mice by resting follicle activation and burn-out (40).

In our study, we examined the effects of CP on ovarian follicles. We determined that compared with normal ovarian tissue, there was a significant decrease in primordial, primary and preantral follicles in the group treated with CP alone. We also found that the addition of DHEA to chemotherapy prevented a decrease in primordial and primary follicles, although preantral follicles still remained at a low level. There was no significant change in terms of atretic follicles in both groups treated with CP.

The anti-mullerian hormone levels are associated with follicular reserves, in particular with preantral follicle reserves (4). The effects of anti-tumoral therapies on the ovaries can be evaluated with many different markers. Serum early phase FSH, estradiol, AMH levels and antral follicle count can be used for these evaluations (41).Although there are many ovarian reserve tests with varying predictive capabilities, antral follicle count (AFC) and AMH have been found to have the best diagnostic accuracy for consistently predicting poor ovarian reserve (42,43).

In rats treated with CP, it was observed that ovarian damage increased and that total vascular congestion and total damage scores increased significantly compared with normal rats. There was a decrease in ovarian volume, primordial, primary and preantral follicle numbers. It was determined that in the group that received DHEA in addition to CP, there was no decrease in primary and preantral follicles. It was also foundthat there were changes in all other parameters, and that there were no significant differences in terms of tissue damage, follicle counts and AMH values, which are important for clinical practice.

Conclusion

It was determined that the use of DHEA to prevent CP-related ovarian damage in rats did not produce significant changes in antral follicle counts, ovarian volume and AMH levels, which are important in clinical practice.

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Ethical issues: Author declare, originality and ethical approval of research. The study was conducted under defined rules by the Local Ethics Commission guidelines and audits.

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