

The protective effects of hormonal suppression by a gonadotropin-releasing hormone agonist or an oral contraceptive on the decreased ovarian reserve in female rats exposed to isotretinoin

S. BAS¹, N. CETINKAYA², E. OZGU³, E. KORKMAZ⁴, M. OZ⁵, M. ISIKALAN⁶, M. CAYDERE⁷, S. HUCUMENOGLU⁷, T. GUNGOR⁸, U. BUYUKKAGNICI⁹

¹Department of Gynecology and Obstetrics, City Education and Research Hospital, Adana, Turkey

²Department of Gynecology and Obstetrics, Basaksehir Cam and Sakura City Hospital, Istanbul Turkey

³Department of Gynecology and Obstetrics, Acibadem Hospital, Ankara, Turkey

⁴Department of Gynecology and Obstetrics, Umraniye Education and Research Hospital, Istanbul, Turkey

⁵Department of Gynecology and Obstetrics, Memorial Hospital, Ankara, Turkey

⁶Department of Gynecology and Obstetrics, Mehmet Isikalan, Education and Research Hospital, Adiyaman, Turkey

⁷Department of Pathology, Ankara Education and Research Hospital, Ankara, Turkey

⁸Department of Gynecology and Obstetrics, Zekai Tahir Burak Women's Health Education and Research Hospital, Ankara, Turkey

⁹Department of Biochemistry, 19 Mayıs Hospital, Ankara, Turkey

Abstract. – OBJECTIVE: The objective of our study was to evaluate whether ovarian suppression by two different hormonal methods may spare the ovary the cytotoxic effects of isotretinoin in a rat model.

MATERIALS AND METHODS: Four groups (n=8 Sprague-Dawley albino rats per group) were studied: control (Group I), 7.5 mg/kg/day isotretinoin (Group II), isotretinoin plus the combination of 0.030 mg ethinyl estradiol/0.15 mg levonorgestrel (combined oral contraceptive, COC), and isotretinoin plus 100 µg (microgram) leuprolide acetate (GnRHa) (Group III and IV, respectively). Four rats from each group were decapitated on the 30th day of treatment, and the remaining rats were decapitated on the 30th day of untreated follow-up. Serum anti-Müllerian hormone (AMH) concentrations, healthy and atretic follicle numbers, and apoptotic activity of follicles in oophorectomy specimens were compared between the groups.

RESULTS: There were no significant differences in AMH levels among the study groups before, immediately after (first month), and one month after their last medication (second month) ($p=0.08$, 0.47 , and 0.08 , respectively). At the end of the first month, the control group had a higher median count of healthy primordial follicles compared to the study groups: 13.5 ($8-22$), 5.5 ($3-11$), 6 ($2-13$), and 1 ($0-1$) in control, isotretinoin, isotretinoin+COC, and isotretinoin+GnRHa groups, respectively ($p=0.02$). However, there was no statistically significant difference in the number of

healthy primordial follicles between the groups one month after the last medication ($p=0.33$). The median atretic antral follicle counts in the first month were 2 ($1-4$), 3.5 ($1-4$), 0 ($0-2$), and 0 ($0-0$) in the control, isotretinoin, isotretinoin+COC, and isotretinoin+GnRHa groups, respectively ($p=0.02$). Otherwise, there were no significant differences in other types of follicles among the control and treated groups ($p>0.05$). There was also no statistical difference between the groups regarding immunostaining intensity for active caspase-3 evaluated in the first or second month of treatment ($p=0.8$ and 0.2 , respectively).

CONCLUSIONS: Our results show that GnRH agonists or COC have no protective effects on ovarian reserve when co-administered with isotretinoin in the rat model.

Key Words:

Isotretinoin, Ovarian reserve, Leuprolide, Combined oral contraceptive.

Introduction

Acne vulgaris is an inflammatory disease of the pilosebaceous duct, commonly found in adolescents. As part of normal pubertal physiology, the prevalence has been reported¹ to be up to 87% in teenagers, and half of them experience the disease during adulthood.

Isotretinoin (13-cis retinoic acid) is a non-aromatic retinoid approved by the Food and Drug Administration (FDA) as an oral capsule formulation for the treatment of severe recalcitrant acne. It has sebo-suppressive, comedolytic, anti-inflammatory, and possibly immunological effects². However, it also has many devastating side effects, especially on mucocutaneous tissues. It is a category X drug associated with fetal craniofacial, cardiovascular, neurological, and thymic malformations if exposed during pregnancy³. Thus, contraception is necessary during treatment.

Additionally, studies^{4,6} have found that isotretinoin can negatively impact ovarian functions, both in clinical and experimental settings. Abali et al⁴ presented the detrimental effects of isotretinoin treatment on the rat ovaries by providing evidence of decreased serum anti-Mullerian hormone (AMH) concentrations and increased number of ovarian follicles containing apoptotic cells with advanced atretic features⁴. Similarly, Sikar Akturk et al⁵ demonstrated a decline in serum AMH concentrations, and Ozturk et al⁶ showed decreased levels of mean follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2) without significant difference in the antral follicle counts and ovarian volume in women treated with isotretinoin.

The fact that acne is more common in adolescence and young adulthood raises concerns that isotretinoin used in this age group may adversely affect fertility. Besides, early deterioration in ovarian functions puts women at risk of psychosocial dysfunction, osteoporosis, and ischemic heart diseases⁷.

Hormonal interventions using GnRHa or COC have been suggested^{8,9} as a viable option for temporarily suppressing ovarian function while undergoing gonadotoxic drugs. The effectiveness of such interventions has been studied^{8,9} in patients receiving chemotherapy for breast cancer, hematological malignancy, and autoimmune disease to preserve ovarian functions. However, there is still a debate in literature on the potential effectiveness of these interventions in cancer survivors, and their specific mechanism has not been completely understood.

To date, there is no study in the literature dealing with the effects of oral contraceptives or GnRHa on isotretinoin-induced ovarian damage. The aim of our investigation is (i) to determine whether the detrimental effects of isotretinoin are reversible; (ii) to study if ovarian suppression by two different hormonal methods may spare the ovary the cytotoxic effects of isotretinoin using a rat model.

This paper constitutes a continuation of our prior research, which we designated as Part I¹⁰. In that earlier work, we evaluated the potentially reversible impact of isotretinoin on ovarian function and reported on the relevant biochemical findings. The present study aims to complement those findings by providing a detailed analysis of the histopathological data and additional information on the protective effects of employing simultaneous administration of GnRHa and oral contraceptives, as was previously noted in the Part I of our research.

Materials and Methods

Experimental Design

12-week-old reproductive-aged female non-pregnant, nulligravid Sprague-Dawley albino rats, weighing 250-300 grams, bred at Yeditepe University Experimental Research Center (YUDE-TAM), were used in this study after approval by the Ethics Committee on Animal Experimentation of the Yeditepe University Medical Faculty (Reference Number: 10/2014-422). 32 rats were randomly divided into four groups, 8 in each, depending on the treatment protocol. Group I was selected as a control group and received no active medication except 1.0 ml of soybean oil through a feeding needle for 30 days. Group II was selected as an isotretinoin treatment group and was treated with a 7.5 mg/kg/day isotretinoin suspended in soybean oil in a volume of 1.0 ml through a feeding needle for 30 days. Next, we tested whether pretreatment with a COC or a GnRHa could reduce the predicted ovarian toxic effect due to isotretinoin. Group III and IV were selected as other investigation groups and treated with 0.030 mg ethinyl estradiol (EE) plus 0.15 mg levonorgestrel (LNG) or 100 µg leuprolide acetate for 30 days through a feeding needle concurrent with isotretinoin administration, respectively. Hormonal suppression by combined EE & LNG and GnRHa were started six days before the beginning of the concurrent isotretinoin treatment, after the confirmation of the diestrus cycle by vaginal smears, to ensure the inhibition of endogenous gonadotropin production, which is expected to occur on the 5th day of the cycle¹¹.

At the beginning of the study, approximately 1.0 ml of blood was withdrawn from the right jugular vein of the rats under isoflurane inhalation anesthesia to measure the baseline and mean AMH in each rat and group, respectively. Then, the blood samples were centrifuged with 4,000

RPM at 4°C for 5 minutes, and the supernatants were stored in the freezer at -80°C till the end of the study for biochemical analysis.

After the 30th day of the drug treatments, four rats were randomly selected from each group to investigate the treatment results. The selected rats were decapitated by a guillotine under inhalation anesthesia with isoflurane, and 1.0 ml of blood samples were collected from neck vessels to evaluate the serum anti-Mullerian hormone levels in each group. In addition, the abdominal cavity was entered by a midline abdominal incision, and bilateral oophorectomy was performed in these selected rats. The blood samples were centrifuged, and the supernatants were stored in the freezer at -80°C, as previously performed. The ovarian tissues were then put into a 10% neutral buffered formaldehyde solution and stored for further histopathologic and immunohistochemical evaluations. The other four rats in each group were anesthetized by isoflurane, and about 1.0 ml of blood was withdrawn from the right jugular vein for AMH investigations. Serum samples were created and stored in the same way as previously stated. After the blood samples were taken, all the treatments were stopped in the remaining rats in each group.

After the 60th day of the study and the 30th day of the cage activity without any treatment, the remaining rats in each group were decapitated, and blood and tissue samples were retrieved as previously performed.

Drug Preparations and Administration

Rats in the combined oral contraceptive group (Group III) were orally treated with a combination of 0.030 mg EE and 0.15 mg LNG at 9 a.m. of the first diestrus for six days before 7.5 mg/kg/day isotretinoin administration and then continued concurrently with isotretinoin for 30 days. For this purpose, tablets of a combined oral contraceptive containing 0.030 mg EE and 0.15 mg LNG (Microgynon; Bayer, NSW, Australia) were crushed in a mortar, and the product was dissolved in 0.4% aqueous solution of sodium carboxymethylcellulose (prepared *ex tempore*) to produce a stock solution in the form of suspension after thoroughly mixing by electromagnetic shaper (RHbasic; IKA Labortechnik, Staufen, Germany). The whole solution was administered to 8 rats of Group III in a volume of 1.0 ml, equal to 1 tablet combined oral contraceptive per rat/day, through a feeding needle for 36 days. The admixture was well-shaped by a vortex mixer

(MS1 Minishaker; IKA Labortechnik) for 2 minutes before each administration. In the GnRH agonist group (Group IV), each rat was given 100 µg (0.02 ml) leuprolide acetate subcutaneously (Lucrin Daily 5 mg/ml, 2.8 ml; Abbott, Famar L'Aigle, Saint Rémy Sur Avre, France) at 10 a.m. of the first diestrus for 6 days prior to 7.5 mg/kg/day isotretinoin treatment and then continued for 30 days with the isotretinoin therapy.

Rats in Group II, III, and IV were treated with 7.5 mg/kg/day isotretinoin to produce serum isotretinoin concentrations comparable to those of humans treated with 1 mg/kg/day isotretinoin and to have a detrimental effect on rat ovaries (decreased ovarian reserve) based on some previous studies^{8,12}. For this purpose, capsules containing 20 mg of isotretinoin (Roaccutane; Roche, R.P. Scherer GmbH, Germany) were opened and transferred to class A volumetric flasks and diluted with soybean oil (cat. no. TR34K014949; KRK nutrition) and mixed by electromagnetic shaper to obtain a suspension at the desired concentration. Isotretinoin suspension was prepared in a darkened room, and the product was stored in amber bottles on a refrigerator shelf. All rats were administered 7.5 mg/kg/day isotretinoin in a volume of 1.0 ml suspension through a feeding needle for 30 days at 3 p.m. to avoid the possible drug interactions between the isotretinoin and hormonal treatment. The isotretinoin suspension was also well-shaped by a vortex mixer for 2 minutes before each administration. Control rats in Group I received 1.0 ml of soybean oil used as a vehicle.

AMH Concentrations

Throughout the study, the serum samples were coded with serial numbers from 1 to 80 and stored at -80°C in the freezer until the end of the study. The measurement of AMH concentrations was performed by the biochemist who participated in the research but was blinded to the treatment groups (UB).

AMH concentrations were measured in rat serum by "Rat Mullerian Inhibiting Substance/Anti-Mullerian Hormone (MIS/AMH) Enzyme-linked immunosorbent assay (ELISA) Kit" (Cat. no: CK-E30083; Hangzhou East Biopharm Co., Ltd., China). This kit is used to assay MIS/AMH based on the Biotin double antibody sandwich technology with a sensitivity of 0.051 ng/ml, assay range 0.1-40 ng/ml and intra-assay/inter-assay coefficients of variation (CV) <10%, and <12%, respectively.

All the subsequent antibodies, enzymes, substrates, and solutions were included in the kit and were prepared according to the manufacturer's instructions. A microplate was coated with Mullerian inhibiting substance/anti-Mullerian hormone (MIS/AMH) monoclonal antibody as a capture antibody; a sample was added, and any antigen that was presently bound to capture antibody; anti-MIS/AMH antibodies labeled with biotin was added as a detecting antibody, and attached to AMH; streptavidin conjugated to Horse Radish Peroxidase (HRP) was added, and bound to detecting antibody during incubation at 37°C for 60 minutes; substrate (chromogen solution A and B) was added, and converted by the enzyme to detectable form as color development in proportion to the amount of AMH during incubation for 10 minutes. The stop solution was added to stop the reaction, and the absorbance (optical density; OD) was measured under 450 nm wavelength within 10 minutes after adding the stop solution. According to the standards' concentrations and the corresponding OD values, the linear regression equation of the standard curve was calculated. Then, according to the OD value of the samples, the concentration of the corresponding sample was calculated.

Histopathological Investigation

Oophorectomy specimens were fixed in a 10% neutral buffered formaldehyde solution. After the dehydration procedure, all pieces were embedded in paraffin. Three (3) µm-thick sections were made by a microtome, and after deparaffinization, samples were stained with Hematoxyline & Eosin (HE). Slides were inspected under a light microscope (Olympus BX53; Olympus). They were examined by two pathologists (MC and SH) who participated in the study and were blinded to the treatment groups.

H&E-stained slides, including the largest cross-sectional ovarian tissue, in 10 different areas were examined to count the total amount of atretic and normal follicles with primordial, primary, pre-antral, and antral differentiation.

Small follicles located close to the outer edge of the cortex and surrounded by a single layer of flattened ovarian follicular epithelial cells were determined as primordial. Primary follicles, instead, had at least two layers of follicular cells. They differentiated from the pre-antral follicles by the absence of small accumulations of follicular fluid in the intracellular spaces and the differentiation of the surrounding theca.

Antral follicles were larger than the pre-antral ones and recognized with a single space called the antrum, surrounded by the follicular cells and the cumulus oophorus structure. Also, a basement membrane between the granulosa cells and the theca interna was easily identified. Atretic follicles were specified in each stage of follicular differentiation and observed with progressive cellular degeneration and the presence of pyknotic cells or apoptotic bodies. The disintegration of the granulosa layer was also detected in the later stage of atresia.

Immunohistochemistry

We examined the expression of active caspase-3 in rat ovarian tissue to determine apoptosis. Immunohistochemistry (IHC) was performed on 3-micron paraffin sections on 3-aminopropyltriethoxysilane-coated slides, using prediluted antibodies (1:1000 dilution; anti-caspase-3 [EPR18297] (ab184787), ABCAM, Cambridge, UK), with known positive controls by polymer horseradish peroxidase IHC detection system, following the manufacturer's instructions (Leica Biosystems, Mount Waverley, Vic., Australia). The slides were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen, counterstained with hematoxylin, and mounted.

Immunohistochemical staining was scored in a semi-quantitative manner to determine the differences between the control group and the experimental groups. The numbers of the positive staining were recorded as absence [0.1 (±)], few [2 (++)], medium [3 (+++)], and high [4 (++++)]. This analysis was performed in serial sections from each animal at 400 magnifications.

Statistical Analysis

Data were analyzed with SPSS version 23 (IBM Corp., Armonk, NY, USA). Conformity to the normal distribution was evaluated using the Shapiro-Wilk test. One-way analysis of variance was used to compare the normally distributed data according to the groups, and multiple comparisons were analyzed by the Duncan test. The Kruskal-Wallis' test was used to compare the data that were not normally distributed according to the groups. An Independent two-sample *t*-test was used to compare normally distributed data according to paired groups, and the Mann-Whitney U test was used for non-normally distributed data. Repeated analysis of variance was used to compare normally distributed AMH values within groups according to time. The Chi-square

test was used to compare categorical variables according to groups. Analysis results mean \pm standard deviation (SD) for quantitative data. Categorical data as deviation and median were presented as frequency and percentage. $p < 0.05$ was considered statistically significant.

Results

Two rats died in the isotretinoin+GnRHa group within two weeks from the beginning of the study, and none in the remaining groups. Therefore, the statistical analysis was performed on a sample size of six rats for Group IV and 8 for the rest of the groups.

We first examined the gonadotoxic effects of isotretinoin on the ovaries and whether it was reversible. We found that AMH values tended to increase one month after the cessation of treatment in rats which were administered isotretinoin and published our findings in our Part I study¹⁰. This study investigated the effectiveness of using GnRHa or COC and isotretinoin to reduce the gonadotoxic effect and presented with histopathological data.

Serum AMH Concentrations

Table I and II present the AMH values in each group before the treatment, immediately after (first month), and one month after the last medication (second month). Before treatment, there were no statistically significant differences in AMH levels among the groups, indicating that the groups were well standardized and ensuring reliable results ($p = 0.81$). Additionally, there were no significant differences in AMH values between the groups in post-treatment (first and second months) measurements ($p = 0.47$, $p = 0.08$), as shown in Table I. While the first-month measures were evaluated in the data analysis, the non-parametric test was used because the normal distribution condition was not met, and the values were given in the table as median (minimum-maximum) in addition to mean \pm SD.

The mean AMH levels were significantly decreased in the isotretinoin group in the first month compared to levels taken before the treatment had begun, then increased slightly during the second month, which is statistically significant ($p = 0.02$). Otherwise, no significant differences were found in the mean AMH levels in

Table I. The comparison of AMH values between groups before, at the end of the 1st month (immediately after), and at the end of the 2nd month (1 month after the last medication).

Group	AMH values, ng/ml		
	Before medication (mean \pm SD)	1 st month (mean \pm SD) (median = min-max.)	2 nd month (mean \pm SD)
Control group [†]	8.16 \pm 1.48	6.96 \pm 1.87/6.61 (4.6-9.7)	6.28 \pm 0.72
Isotretinoin group [†]	8.20 \pm 1.48	6.56 \pm 1.45/6.37 (4.9-8.8)	7.07 \pm 0.96
Isotretinoin+COC group	7.57 \pm 1.37	6.69 \pm 1.71/6.08 (5.2-10.4)	5.35 \pm 1.72
Isotretinoin+GnRHa group	8.0 \pm 1.65	5.86 \pm 1.25/5.71 (4.6-8.1)	4.90 \pm 1.07
<i>p</i> -value*	0.81*	0.47**	0.08*

[†], ^{*}These values were presented in our Part I study mentioned in the article. ^{*}One-way analysis of variance, values are expressed as means \pm SD. ^{**}Kruskal-Wallis' test; values are expressed as means \pm SD and median minimum maximum.

Table II. The AMH values in four groups before, at the end of the 1st month (immediately after), and at the end of the 2nd month (1 month after the last medication).

Group	AMH values, ng/ml (mean \pm SD)			
	Before medication	1 st month	2 nd month	<i>p</i> -value
Control group [†]	8.06 \pm 1.95	6.50 \pm 1.82	6.28 \pm 0.72	0.35
Isotretinoin group [†]	9.21 \pm 1.37 ^a	6.98 \pm 1.79 ^b	7.07 \pm 0.96 ^a	0.02
Isotretinoin+COC group	7.25 \pm 2.01	6.57 \pm 1.15	5.35 \pm 1.72	0.47
Isotretinoin+GnRHa group	7.54 \pm 1.62	5.11 \pm 0.58	4.90 \pm 1.07	0.08

[†], ^{*}These values were presented in our Part I study mentioned in the article. ^{*}Repeated measures analysis of variance. The letters (^{a,b}) indicate the mean AMH values, which lead to the statistical difference.

the isotretinoin+COC and isotretinoin+GnRHa groups before, immediately after, and one month after their last medication (with *p*-values of 0.47 and 0.08, respectively) (Table II).

Follicle Counts

The number of healthy and atretic follicles at each stage of folliculogenesis was calculated separately at the end of the first and second months. Sixty ovarian tissues from 30 rats (six rats for Group IV and eight rats for each other group) were examined at 400 × in the largest section in each sample. A total of 1,061 ovarian follicles (396 primordial, 203 primary, 299 preantral, and 163 antral) were evaluated.

Isotretinoin had no significant effects on the number of healthy primary, preantral, and antral follicles in the ovary, but there was a considerable decrease in the number of primordial follicles compared to the control group in the first month. The co-administration of GnRHa or COC with isotretinoin did not prevent the loss of primordial follicles. The median healthy primordial follicle counts in the first month were 13.5 (8-22), 5.5 (3-11), 6 (2-13), and 1 (0-1) in control, isotretinoin, isotretinoin+COC, and isotretinoin+GnRHa groups, respectively (*p*=0.02). The mean number of primordial follicles in all three study groups was lower than in the control group in the second month, but this difference was not statistically significant (*p*=0.33).

The total atretic follicles were slightly lower in the two groups that received GnRHa or COC with isotretinoin compared to the isotretinoin group, but the difference was not statistically significant. In the first month, the mean total atretic follicles were 7.25±2.8, 12.50±6.5, 8.00±5.9, and 9.33±5.1 in the control, isotretinoin, isotretinoin +COC, and isotretinoin +GnRHa groups, respectively (*p*=0.54). In the second month, the median total atretic follicles were 12 (8-15), 11 (8-17), 9 (9-11), and 8 (4-10) in the same groups (*p*=0.19). No significant differences were found in the mean total

atretic follicle counts in the control and study groups before, immediately after, and one month after their last medication. (*p*>0.05) (Table III).

Upon examining the various stages of follicle development, we found a difference only in the number of atretic antral follicles in the first month. The median atretic antral follicle counts in the first month were 2 (1-4), 3.5 (1-4), 0 (0-2), and 0 (0-0) in the control, isotretinoin, isotretinoin+COC, and isotretinoin+GnRHa groups respectively (*p*=0.02). Otherwise, there was no statistically significant number of atretic primordial, preantral, and antral follicles among the control and study groups. **Supplementary Table I** contains statistical comparisons of the numbers of healthy and atretic follicles at various developmental stages between the groups.

Apoptosis

We examined histological sections of the ovary, whether isotretinoin causes apoptotic death of oocytes and surrounding granulosa cells in the ovary, and the protective effect of hormonal ovarian suppression. There was no statistical difference between the groups regarding immunostaining intensity for active caspase-3 evaluated in the first or second month of treatment (Table IV, Figure 1).

Discussion

In this experimental study, we evaluated the effectiveness of co-administered GnRHa or COC to prevent the gonadotoxic effect of isotretinoin. Our findings indicate that the implementation of these hormonal interventions did not suppress the depletion of the ovarian primordial follicle pool or the decreased AMH values induced by isotretinoin in rats.

Retinoic acids (RAs) are vitamin A-derivatives and potent substances for regulating the expression of many different genes within the body. Isotretinoin (13-cis RA) is a synthetic retinoid

Table III. The total atretic follicles.

Group	1 st month mean±SD	2 nd month mean±SD/median (min-max)	<i>p</i> -value
Control group	7.25±2.87	11.75±2.99/12 (8-15)	0.07***
Isotretinoin	12.50±6.56	12.40±3.58/11 (8-17)	0.97***
Isotretinoin+COC	8.00±5.94	9.67±1.15/9 (9-11)	0.62****
Isotretinoin+GnRHa	9.33±5.13	7.33±3.06/8 (4-10)	0.59***
<i>p</i> -value	0.54*	0.19**	

*One-way analysis of variance, values are expressed as means ± SD. **Kruskal-Wallis test, values are expressed as means ± SD and median (minimum-maximum). ****Independent two-sample *t*-test, ****Mann-Whitney U test; values are expressed as means ± SD.

Table IV. The immunohistochemical staining intensity of caspase-3.

Groups					
	Control	Isotretinoin	Isotretinoin+OCS	Isotretinoin+GnRHa	<i>p</i> -value*
1st month					
Absence	2 (50)	2 (50)	3 (75)	2 (66.7)	0.85
Few	2 (50)	2 (50)	1 (25)	1 (33.3)	
2nd month					
Absence	3 (75)	0 (0)	2 (66.7)	2 (66.7)	0.21
Few	1 (25)	3 (60)	1 (33.3)	1 (33.3)	
Medium	0 (0)	2 (40)	0 (0)	0 (0)	

*Chi-square test.

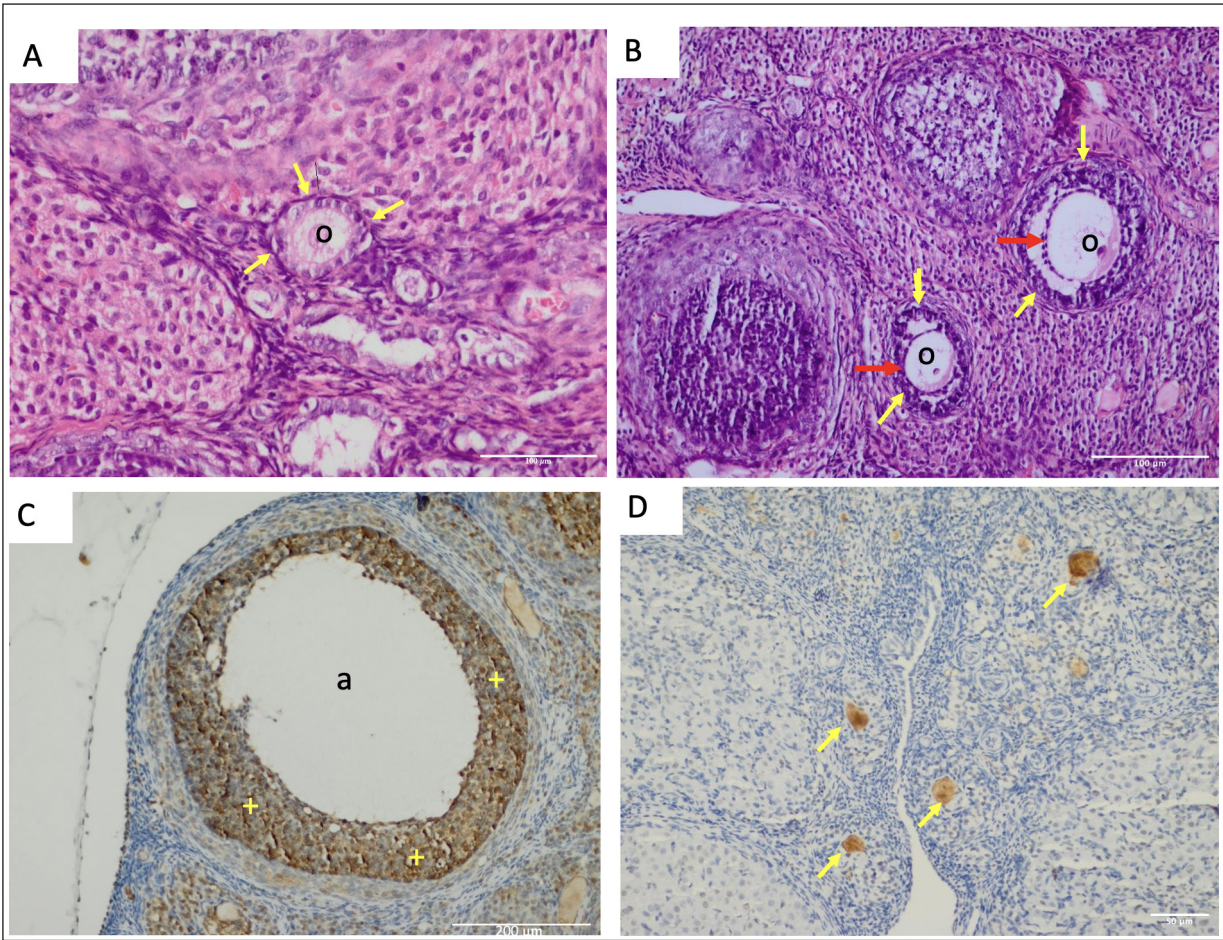


Figure 1. **A**, Normal histological appearance of the primary follicle indicated by arrows (o; oocyte, H&E \times 100). **B**, Degenerate (atretic) secondary follicles indicated by yellow arrows (red arrows; zona pellucida, o; oocyte, H&E \times 100). **C**, Immune-stained for caspase-3 exhibited brown cytoplasmic reaction in granulosa cells of an antral follicle (+; granulosa cells, a; antrum, intensity score; +++, H&E \times 200). **D**, Immunostaining of the active caspase-3 in granulosa cells of primordial follicles indicated by arrows (intensity score; +++, H&E \times 100).

considered to be the most effective pharmacologic agent for treating severe acne vulgaris. It acts as a pro-drug. After isomerization to more transcriptionally active all-trans-retinoic

acid (ATRA), 9-*cis* retinoic acid (9-*cis* RA), and other possible metabolites, it affects cellular differentiation, cell cycle progression, cell survival and apoptosis variety of cell types¹².

Retinoids are essential for normal fetal development as they guide the formation of many organs, including the ovaries, brain, and heart. It is known that they are necessary for normal sperm development in males during adulthood, but the impact of retinoids on follicle development in females has yet to be extensively studied^{13,14}. Animal studies¹³ have shown that exposure to RAs at levels of 2-5 μM (micromole) can have a positive impact on the growth, maturity, and quality of ovarian follicles, but higher concentrations may lead to negative effects such as oocyte degeneration.

Abali et al⁴ investigated the detrimental impacts of isotretinoin treatment in a dose-dependent manner in rats (7.5 vs. 15 mg/kg/day). The study found that the groups receiving either dose had lower AMH concentrations than the control group. However, there was no significant difference in AMH concentrations between the two groups receiving different doses of isotretinoin. The study also found that increasing doses of isotretinoin led to a considerable increase in degenerated follicles at all stages. These findings suggest that the adverse effects of isotretinoin on the ovaries depend on the dosage used. In our Part I study¹⁰, we followed the rat's serum AMH levels to investigate whether the effect of isotretinoin treatment on ovarian reserve is reversible for a longer period than the study by Abali et al⁴. In a more extended follow-up, we showed that the AMH levels increased in the second month compared to the first-month measurements and suggested that the negative impact may be reversible. Likewise, Cinar et al¹⁵ evaluated the effect of isotretinoin exposure on ovarian functions in 82 young women. They found that the total ovarian volume (TOV), total antral follicle number (TAFC) and AMH values increased close to the pretreatment values after 18 months of systemic isotretinoin treatment (following a 12-month treatment-free period), and they interpreted these results as the disappearance of the toxic ovarian effect of the drug. Consistent with this research, Öztürk et al⁶ found no difference in the number of antral follicles and ovarian volume evaluated by ultrasonography in women treated with oral isotretinoin before and immediately after 5- to 8-month treatment. Our study corroborates these results and suggests that further clinical studies are required to determine the long-term effects of isotretinoin on ovarian functions in women. It is vital to reveal fertility and ovarian damage complications, especially considering the age group in which the drug is frequently used.

Efforts to protect the ovaries against gonadotoxic medications are essential for fertility preservation in women of reproductive age, especially with limited ovarian reserves. Studies¹⁶⁻¹⁸ in cancer survivors have shown conflicting results regarding using GnRH agonists with chemotherapy to suppress ovarian function temporarily. While some trials have shown a protective effect, others have not¹⁶⁻¹⁸. Although it is unclear how the GnRH agonists protect the ovaries from the gonadotoxic agents, some potential mechanisms have been proposed. Theoretically, GnRHa induces a hypogonadotropic hypoestrogenic milieu via the persistent occupation of GnRH receptors in the pituitary gland due to the downregulation of the own receptors. These effects provide suppression of primordial follicle development. Also, the hypoestrogenic state decreases utero-ovarian perfusion and reduces drug delivery to the ovary. Moreover, GnRH agonists up-regulate intragonadal anti-apoptotic molecules of the ovaries independently from gonadotropins. A similar role has also been proposed¹⁹ for oral contraceptives that may produce a hypogonadotropic milieu and protect the ovary from chemotherapy-induced follicular destruction.

In this study, we constructed a rat model to evaluate the potential protective effect of combining GnRHa or COC with isotretinoin on gonadal function. The assessment consisted of analyzing both histopathological and biochemical evidence. Considering the AMH values, we found that using GnRHa or COC did not have a protective effect on ovarian reserve. Our histopathological examination also revealed that isotretinoin has resulted in a significant decline in primordial follicle counts, regardless of whether it was used in conjunction with GnRH agonist or COC. Otherwise, our study groups had no significant difference in the number of other healthy or apoptotic follicles. These results contradict the advantages of hormonal suppression.

After applying a GnRH agonist, the gonadotropic activity increases until the GnRH receptors down-regulate, a phenomenon known as "the flare-up effect". The ovary is exposed to a greater cytotoxic effect if gonadotoxic medication is administered during this period. Patients should receive GnRHa medication at least two weeks before their first gonadotoxic treatment in humans to prevent an increase in gonadotrophin production. Whereas in rats, it takes only four days of GnRHa administration, which is in accordance with their much shorter reproductive cycle (5 days)²⁰.

Therefore, we started GnRHa administration five days before isotretinoin treatment in Group III. Similarly, the rats were given COC treatment for four consecutive days and a placebo on the fifth day based on the 5-day estrous cycle of the rat to resemble COC administration in women. The COC regimen repeated for 30 days, allowing for roughly five to six cycles to elapse.

Publications are using different doses of GnRHa from 20 µg/kg/day to 200 µg/kg/day to provide adequate suppression of gonadotropin levels for various indications in rats^{21,22}. Through our research, we have effectively suppressed ovarian function by optimizing the dosage to align with our current laboratory capabilities. The significant decrease in the number of healthy follicles and AMH values observed at the end of the first month in Group III could be attributed to the doses of leuprolide acetate that were administered. We found that these variables tended to increase in the second month compared to the first values following the termination of drug administration. However, the number of primordial follicles in the second month is still the lowest in Group III compared to the other three groups. These findings suggest that using lower doses of GnRHa is sufficient for ovarian suppression in rats.

There is currently no proven molecular mechanism of action for ovarian protection with ovarian quiescence provided by hormonal suppression in the literature. In an *in vivo* study by Hasky et al²³, the administration of GnRHa with doxorubicin (Dxr) delayed the recovery in AMH level and decreased the level of VEGF; thus, it altered the ovarian recovery process in response to Dxr-induced vascular toxicity. On the other hand, co-administration of GnRHa with cyclophosphamide (Cyc) resulted in less follicular apoptosis, preserved AMH levels, and prevented the accelerated depletion of the ovarian reserve²⁴⁻²⁶. Their results showed that the gonadotoxic effects of these two chemotherapeutic drugs occurred through different mechanisms; while VEGF did not significantly mediate Cyc-induced ovarian gonadotoxicity, it did have a role in Dxr-induced toxicity. In translational research conducted by Bildik et al²⁷, GnRHa co-administered with various chemotherapy agents neither activated the expression of anti-apoptotic genes (Bcl-2, Bcl-xL, Bcl-2L2, Mcl-1, BIRC2 and XIAP) nor prevented follicle loss, DNA damage and apoptosis induced by these drugs in human ovarian samples. However, in clinical practice, GnRHa is a treatment option for ovarian protection in patients using gonadotoxic

drugs. American College of Obstetricians and Gynecologists Committee on Adolescent Health Care recommends that “GnRH agonist should be considered and discussed with premenopausal patients who will be treated with chemotherapeutic agents since GnRHa was associated with trends toward more favorable outcomes in maintaining ovarian functions in clinic studies^{28,29}”.

It is essential to note that the studies conducted so far in literature have only focused on specific chemotherapeutic agents, and each drug has unique pharmacological effects that cannot be generalized. So far, this is the first report about the use of both retinoic acid and hormonal interventions simultaneously. This highlights the need for more comprehensive studies to evaluate the effectiveness of ovarian suppression using varying dosages and combinations.

The primary mechanism of action of isotretinoin treatment is inducing apoptosis and cell cycle arrest in the human sebaceous gland. Studies²⁴ conducted on animals have confirmed that isotretinoin administration during pregnancy can increase the apoptosis of neural crest cells and the appearance of malformations. Apoptosis plays a crucial role in maintaining ovarian function. During adult life, a few primordial follicles start growing during each menstrual cycle. Usually, only one follicle will ovulate, and the fate of the rest of the follicles is atresia through apoptotic cell death. In addition, apoptosis is responsible for luteal regression. Caspases, a group of proteolytic enzymes, are crucial in inducing ovarian apoptosis. Caspases involved in apoptosis have been subclassified according to their mechanism of action and divided into initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7). Once activated, a single executioner caspase can cleave and activate other executioner caspases, leading to an accelerated feedback loop of caspase activation²⁴. In granulosa cells, caspases are activated through two distinct pathways: the extrinsic pathway, mediated by cell surface death receptors, and the intrinsic pathway, involving members of the Bcl-2 protein family. Caspase-3 is present in the luteal and thecal cells of a healthy corpus luteum and the granulosa cells of atretic follicles. However, it is not detectable in the granulosa cells of healthy follicles²⁶.

We evaluated immunohistochemical detection of caspase-3 activity in ovarian follicles to investigate apoptotic cell death. We found no evidence of increased apoptosis through IHC in the isotretinoin, isotretinoin+GnRHa, or isotretinoin+COC

groups compared to the control group. Although these data are compatible with comparing total atretic follicle counts and AMH values in our study, it is quite possible that granulosa cell death may occur by more than a single pathway. As previously stated, isotretinoin induces apoptosis in different tissues. However, additional research is required to determine whether this effect extends to ovarian tissue and, if so, the specific cellular mechanisms involved.

A major problem in assessing the gonadotoxic agents or effectiveness of preventative interventions lies in the heterogeneity of the parameters used among studies in literature evaluating ovarian reserve or fertility. Serum biomarkers such as AMH, FSH, LH, and inhibin or clinical parameters, such as ultrasound-guided antral follicle number, the resumption of menses, or pregnancy rates, are widely used to determine ovarian functions. We used both hormonal and histopathological methods to detect ovarian toxicity. As a hormonal method, we preferred to use AMH as it is a reliable marker of ovarian reserve independent of the menstrual cycle and more powerfully associated with ovarian follicular status than FSH, LH, and inhibin. Histopathologically, we examined the number of healthy and atretic follicles at each stage of folliculogenesis separately.

Limitations

The findings of this study have to be seen in light of some limitations. First, the study was conducted on animals, and as such, the results may not precisely reflect the response of humans or diseases to drugs. The second limitation concerns the administered COC and GnRHa doses. The administering drugs intended for humans to animals may present difficulties with the desired dose adjustment, method, and administration schedule. Although we obtained adequate ovarian suppression OCS and GnRHa administered to animals in our study, appropriate conversion calculations between species were not made in the dose selection, and it was intended to apply a dose equal to the daily amount in humans.

Conclusions

The ovarian reserve was first evaluated after exposure to isotretinoin with a GnRH agonist or COC in an animal-randomized prospective study using the most common ovarian reserve tests, including AMH and histological examination of

ovarian follicles. Our results show that GnRH agonists and COC have no protective effects on ovarian reserve when co-administered with isotretinoin. Considering the results of our Part 1 study, we think that the long-term effect of isotretinoin on ovarian reserve should be further examined. The mechanisms by which isotretinoin causes ovarian toxicity, if any, should be revealed by molecular studies, and the development of ovarian protective methods should consider the specific causes of gonadotoxicity.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Funding

This study is supported by grants from the Research Center of Zekai Tahir Burak Women's Health Education and Research Hospital.

Ethics Approval

Ethical approval was obtained from the Ethics Committee on Animal Experimentation of the Yeditepe University Medical Faculty (Approval number: 10/2014-422).

Availability of Data and Materials

The authors give their consent for the article publication.

Informed Consent

Not applicable.

Authors' Contributions

Sevda Bas: Manuscript writing, editing.
Nilufer Cetinkaya: Project development, methodology.
Emre Ozgu: Manuscript writing, editing.
Elmas Korkmaz: Data collection.
Murat Oz: Project development, analysis of the results.
Mehmet Isikalan: Data collection.
Muzaffer Caydere: Data collection, analysis of the results.
Sema Hucumenoglu: Data collection, analysis of the results.
Umran Buyukkagnici: Data collection.
Tayfun Gungor: Review and revision of the article.

Informed Consent

Not applicable.

ORCID ID

Sevda Bas: 0000-0002-6454-6470
Nilufer Cetinkaya: 0000-0001-9183-3558
Emre Ozgu: 0000-0002-8444-9694
Elmas Korkmaz: 0009-0000-8859-4322
Murat Oz: 0000-0002-0629-5386

Mehmet Isikalan: 0000-0002-5766-7063
 Muzafer Caydere: 0000-0002-6454-6470
 Sema Hucumenoglu: 0000-0002-6898-4101
 Tayfun Gungor: 0000-0002-7869-9662
 Umran Buyukkagnici: 0009-0000-6930-3075.

References

- Eichenfield LF, Krakowski AC, Piggott C, Del Rosso J, Baldwin H, Friedlander SF, Levy M, Lucky A, Mancini AJ, Orlow SJ, Yan AC, Vaux KK, Webster G, Zaenglein AL, Thiboutot DM, American Acne and Rosacea Society. Evidence-based recommendations for the diagnosis and treatment of pediatric acne. *Pediatrics* 2013; 131 Suppl 3: S163-S186.
- Leyden JJ, Del Rosso JQ, Baum EW. The use of isotretinoin in the treatment of acne vulgaris: clinical considerations and future directions. *The Journal of Clinical and Aesthetic Dermatology* 2014; 7 Suppl 2: S3-S21.
- Choi JS, Koren G, Nulman I. Pregnancy and isotretinoin therapy. *Canadian Medical Association Journal* 2013; 185: 508.
- Abali R, Yuksel MA, Aktas C, Celik C, Guzel S, Erfan G, Sahin O. Decreased ovarian reserve in female Sprague-Dawley rats induced by isotretinoin (retinoic acid) exposure. *Reprod Biomed Online* 2013; 27: 184-191.
- Sikar Aktürk A, Abalı R, Yüksel MA, Güzel EÇ, Güzel S, Kıran R. The effects of isotretinoin on the ovarian reserve of females with acne. *Gynecol Endocrinol* 2014; 30: 30-33.
- Öztürk S, Öztürk T, Ucak H, Erden I, Demir B, Kayalı A, Cicek D. Evaluation of ovarian reserve and function in female patients treated with oral isotretinoin for severe acne: an exploratory study. *Cutan Ocul Toxicol* 2015; 34: 21-24.
- Ishizuka B. Current Understanding of the Etiology, Symptomatology, and Treatment Options in Premature Ovarian Insufficiency (POI). *Front Endocrinol (Lausanne)* 2021; 12: 626924.
- Valsamakis G, Valtetsiotis K, Charmandari E, Lambrinoudaki I, Vlahos NF. GnRH Analogues as a Co-Treatment to Therapy in Women of Reproductive Age with Cancer and Fertility Preservation. *Int J of Mol Sci* 2022; 23: 2287.
- Li ZY, Dong YL, Cao XZ, Ren SS, Zhang Z. Gonadotropin-releasing hormone agonists for ovarian protection during breast cancer chemotherapy: a systematic review and meta-analysis. *Menopause* 2022; 29: 1093-1100.
- Korkmaz E, Cetinkaya N, Oz M, Ozgu E, Bas S, Isikalan M, Buyukkagnici U, Caydere M, Hucumenoglu S, Gungor T. The Possibly Reversible Isotretinoin Effect of Decreased Ovarian Reserve in Sprague-Dawley Albino: Part I, Biochemical Analyses. *Gynecol Obstet Invest* 2017; 82: 72-77.
- Torres Mde M, Donadio N, Donadio NF, Brandão AC, Heck B. Comparison of embryo implantation in Wistar rats that underwent ovarian stimulation using exogenous gonadotropins associated with cetrorelix acetate or leuprolide acetate. *Fertil Steril* 2005; 84 Suppl 2: S1235-S1240.
- Schleif MC, Havel SL, Griswold MD. Function of Retinoic Acid in Development of Male and Female Gametes. *Nutrients* 2022; 14: 1293.
- Barber T, Esteban-Pretel G, Marín MP, Timoneda J. Vitamin a deficiency and alterations in the extracellular matrix. *Nutrients* 2014; 6: 4984-5017.
- Damdipoulou P, Chiang C, Flaws JA. Retinoic acid signaling in ovarian folliculogenesis and steroidogenesis. *Reprod Toxicol* 2019; 87: 32-41.
- Cinar SL, Kartal D, Aksoy H, Cinar E, Aydin T, Öz L, İmamoğlu H, Ferahbas A, Borlu M. Long-term effect of systemic isotretinoin on female fertility. *Cutan Ocul Toxicol* 2017; 36: 132-134.
- Turner NH, Partridge A, Sanna G, Di Leo A, Biganzoli L. Utility of gonadotropin-releasing hormone agonists for fertility preservation in young breast cancer patients: the benefit remains uncertain. *Ann Oncol* 2013; 24: 2224-2235.
- Moore HC, Unger JM, Phillips KA, Boyle F, Hitt E, Porter D, Francis PA, Goldstein LJ, Gomez HL, Vallejos CS, Partridge AH, Dakhil SR, Garcia AA, Gralow J, Lombard JM, Forbes JF, Martino S, Barlow WE, Fabian CJ, Minasian L, Meyskens FL Jr, Gelber RD, Hortobagyi GN, Albain KS; PO-EMS/S0230 Investigators. Goserelin for ovarian protection during breast-cancer adjuvant chemotherapy. *N Engl J Med* 2015; 372: 923-932.
- ISFP Practice Committee, Kim, SS, Donnez J, Barri P, Pellicer A, Patrizio P, Rosenwaks Z, Nagy P, Falcone T, Andersen C, Hovatta O, Wallace H, Meirrow D, Gook D, Kim SH, Tzeng CR, Suzuki S, Ishizuka B, Dolmans MM. Recommendations for fertility preservation in patients with lymphoma, leukemia, and breast cancer. *J Assist Reprod Genet* 2012; 29: 465-468.
- Ben-Aharon I, Gafter-Gvili A, Leibovici L, Stemmer SM. Pharmacological interventions for fertility preservation during chemotherapy: a systematic review and meta-analysis. *Breast Cancer Res Treat* 2010; 122: 803-811.
- Tan SJ, Yeh YC, Shang WJ, Wu GJ, Liu JY, Chen CH. Protective effect of a gonadotropin-releasing hormone analogue on chemotherapeutic agent-induced ovarian gonadotoxicity: a mouse model. *Eur J Obstet Gynecol Reprod Biol* 2010; 149: 182-185.
- Lehrer SB, Tekeli S, Fort FL, Cusick PK, Krasula RW, Patterson DR. Effects of a GnRH agonist on fertility following administration to prepubertal male and female rats. *Fundam Appl Toxicol* 1992; 19: 101-108.
- Guarraci FA, Davis LK, Henneman EL, Toro E, Odell SE, Le N, Navarro JM, Valdivia HS, Williams I, Credeur M, Gore AC. Daily GnRH agonist treatment delays the development of reproductive physiology and behavior in male rats. *Horm Behav* 2021; 132: 104982.
- Hasky N, Uri-Belapolsky S, Goldberg K, Miller I, Grossman H, Stemmer SM, Ben-Aharon I, Shalgi R. Gonadotrophin-releasing hormone

- agonists for fertility preservation: unraveling the enigma? *Hum Reprod* 2015; 30: 1089-1101.
- 24) Draghici CC, Miulescu RG, Petca RC, Petca A, Dumitraşcu MC, Şandru F. Teratogenic effect of isotretinoin in both fertile females and males (Review). *Exp Ther Med*. 2021; 21: 534.
- 25) Hussein MR. Apoptosis in the ovary: molecular mechanisms. *Hum Reprod Update* 2005; 11: 162-177.
- 26) Glamoclija V, Vilović K, Saraga-Babić M, Baranović A, Sapunar D. Apoptosis and active caspase-3 expression in human granulosa cells. *Fertil Steril* 2005; 83: 426-431.
- 27) Bildik G, Akin N, Senbabaoglu F, Sahin GN, Karahuseyinoglu S, Ince U, Taskiran C, Selek U, Yakin K, Guzel Y, Ayhan C, Alper E, Cetiner M, Balaban B, Mandel NM, Esen T, Iwase A, Urman B, Oktem O. GnRH agonist leuprolide acetate does not confer any protection against ovarian damage induced by chemotherapy and radiation in vitro. *Hum Reprod* 2015; 30: 2912-2925.
- 28) Clowse ME, Behera MA, Anders CK, Copland S, Coffman CJ, Leppert PC, Bastian LA. Ovarian preservation by GnRH agonists during chemotherapy: a meta-analysis. *J Womens Health* 2009;18: 311-319.
- 29) ACOG Committee Opinion No. 747: Gynecologic Issues in Children and Adolescent Cancer Patients and Survivors. *Obstet Gynecol* 2018; 132: 67-77.