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The Effect of Retinoic Acid and Bevacizumab on Ovarian Hyperstimulation Syndrome

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ABSTRACT

Objective: Ovarian hyperstimulation syndrome (OHSS) is an undesired outcome of controlled ovarian stimulation, commonly used in infertility treatment during *in vitro* fertilization and intrauterine insemination cycles. This study aimed to investigate whether retinoic acid and bevacizumab have an effect on the prevention of OHSS.

Materials and Methods: A hyperstimulation model was created using 40 Wistar albino rats, each 22 days old. The rats were divided into four groups. Group 1 was the negative control group, Group 2 was the positive control group, Group 3 was the bevacizumab-administered group after ovarian hyperstimulation, and Group 4 was the retinoic acid-administered group after ovarian hyperstimulation. Serum estradiol and vascular endothelial growth factor (VEGF) levels were evaluated. Peritoneal fluid was collected using a syringe, and the presence of peritoneal fluid was recorded observationally. Hematoxylin and eosin staining and Masson's trichrome staining were performed on the ovaries. Immunohistochemical staining for VEGF and estrogen receptors was conducted.

Results: Retinoic acid treatment significantly reduced peritoneal fluid and inhibited fluid leakage, whereas bevacizumab treatment resulted in a slight reduction of peritoneal fluid. The number of tertiary and secondary follicles remained unaffected by both bevacizumab and retinoic acid treatment. However, the number of primary follicles significantly decreased in the bevacizumab and retinoic acid treatment groups. Retinoic acid treatment significantly reduced estrogen receptor (ER) in the stromal compartment.

Conclusion: Both retinoic acid and bevacizumab appear to be effective in treating OHSS. Additionally, retinoic acid and bevacizumab influence the initial stages of follicular development. Future studies are essential to determine the optimal dosage and timing for these therapeutic agents.

Keywords: Bevacizumab, ovarian hyperstimulation syndrome, retinoic acid.

INTRODUCTION

A couple is considered infertile if they are unable to conceive after one year of consistent, unprotected sexual activity. Infertility is a condition that significantly impacts individuals' lives, placing them under physical, social, and financial burdens.[1]

Controlled ovarian stimulation (COS) is one of the most commonly used methods in infertility treatment. Gonadotropins used in COS stimulate the ovaries, leading to the development of multiple follicles. Ovulation is then triggered by the administration of human chorionic gonadotropins. Once multiple eggs are produced, they can be retrieved and fertilized using *in vitro* fertilization (IVF).[2]

Ovarian hyperstimulation syndrome (OHSS) is an undesired consequence of COS, which is used in infertility treatment during IVF and intrauterine insemination (IUI) cycles.[3] OHSS is characterized by multiple follicle development in both ovaries, ovarian enlargement, increased vascular permeability, and a fluid shift into the third space. Moderate to severe OHSS can lead to serious clinical complications requiring hospitalization, including ascites, pleural effusion, renal function impairment, electrolyte imbalances, hypercoagulability, and thrombosis. In severe or critical cases, OHSS can be life-threatening.[4]

A sudden increase in human chorionic gonadotropin (hCG) levels triggers granulosa-lutein cells to release vascular endothelial growth factor (VEGF), which increases vascular permeability.[5]

Furthermore, the prevalence of OHSS is associated with elevated blood estradiol (E2) levels during COS. Strong evidence suggests that a blood E2 concentration exceeding 3,500 pg/mL significantly increases the risk of OHSS. The underlying mechanism is that estrogens enhance cyclic adenosine monophosphate (cAMP), which subsequently activates cAMP-dependent protein kinase A, ultimately stimulating VEGF transcription. The expression of aquaporin 1 (AQP1) and cystic fibrosis transmembrane conductance regulator (CFTR) in peritoneal epithelial cells may also be upregulated by elevated E2 levels. The process of peritoneal fluid buildup and effusion is significantly influenced by the synergistic interaction between CFTR and AQP1. Therefore, inhibiting excessive E2 synthesis during COS or the early luteal phase may serve as an effective strategy for preventing OHSS.[4,6]

Retinoic acid (RA) and estrogen signaling are closely interrelated, with RA influencing estrogen metabolism and the expression of estrogen receptors. Vitamin A has protective effects against the adverse reproductive tract changes caused by estrogenic endocrine disruptors. RA treatment can prevent permanent epithelial changes induced by exposure to estrogenic endocrine-disrupting chemicals, suggesting that RA plays a protective role in counteracting developmental and hormonal disruptions caused by such chemicals.[7] Moreover, RA, an active metabolite of vitamin A, plays a central role in cell development, differentiation, regulation, and specific gene expression.[8,9] There is evidence from breast cancer studies that retinoic acid derivatives influence estrogen biosynthesis and metabolism by acting on the 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) enzyme, suggesting that RA may serve as an antiestrogenic treatment alternative.[10]

All these molecular properties make RA a plausible candidate for the treatment of OHSS.

Bevacizumab is a monoclonal antibody that inhibits all isoforms of VEGF, making it an important therapeutic agent in the treatment of various tumors, including breast, colon, ovarian, non-small cell lung, and renal cell carcinoma.[11]

This study aimed to investigate the effect of bevacizumab and retinoic acid in the prevention of OHSS using a rat model.

KEY MESSAGES

- Ovarian hyperstimulation syndrome is a serious and potentially fatal complication of infertility treatment, and its development can be prevented or alleviated by targeting its formation mechanisms.
- Retinoic acid may be effective in preventing OHSS through its effects on estrogen metabolism.
- If bevacizumab is administered at the correct timing and dose, it may provide a limited contribution to the prevention of OHSS by targeting VEGF.

MATERIALS AND METHODS

Immature rats were selected to create an OHSS model because the formation of corpora lutea has hormonal effects on subsequent cycles. The OHSS model was established based on the methods previously described by Ishikawa K et al.[12]

Before initiating the study, approval was obtained from the Local Ethics Committee of Animal Experiments of Erciyes University (no: 18/056). A total of 40 Wistar albino rats, each 22 days old, were used to establish the OHSS model. These rats were obtained from the Experimental Animal Investigations Center of Erciyes University, Kayseri, Türkiye, where the study was conducted. This research was carried

out in accordance with the Erciyes University Affirmed Guidelines for the care and use of animals, which align with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Washington, DC. Professional laboratory technicians provided the animals with a standard diet, and the rats had free access to water under 12-hour light and dark cycles. The weight of the rats was recorded at two time points: on day 22 of life and just before the operation on day 27 of life. This study consisted of four groups, with 10 rats in each group.

Group 1: Ten rats received 0.9% saline solution between days 22 and 26 of life for five days. No additional treatment was applied to this group, which was designated as the negative control group. Ketamine (75 mg/kg) and Xylazine (10 mg/kg) were administered before the operation, and the rats were operated on day 27 of life.

Group 2: Ten rats received pregnant mare serum gonadotropin (0.1 mL of 0.9% saline) between days 22 and 26 of life for five days. On the fifth day of medication, 30 IU of hCG subcutaneously (SC) was administered. This group was designated as the positive control group. Ketamine (75 mg/kg) and Xylazine (10 mg/kg) were administered before the operation, and the rats were operated on day 27 of life.

Group 3: Ten rats received pregnant mare serum gonadotropin (0.1 mL of 0.9% saline) between days 22 and 26 of life for five days. On the fifth day of medication, 30 IU of hCG SC was administered. On the same day as hCG SC administration, Bevacizumab (Altuzan, 100 mg/4 mL, Roche, İstanbul, Türkiye) was administered intraperitoneally at 5 mg/kg body weight in saline. Ketamine (75 mg/kg) and Xylazine (10 mg/kg) were administered before the operation, and the rats were operated on day 27 of life.

Group 4: Ten rats received pregnant mare serum gonadotropin (0.1 mL of 0.9% saline) between days 22 and 26 of life for five days. On the fifth day of medication, 30 IU of hCG SC was administered. On the same day as hCG SC administration, these rats received 10 mg/kg of RA (Roaccutane, Roche) in 0.5 mL olive oil orally. Ketamine (75 mg/kg) and Xylazine (10 mg/kg) were administered before the operation, and the rats were operated on day 27 of life.

After anesthesia was administered during the operation, the abdomen was opened with a midline incision. Peritoneal fluid was collected using a syringe from rats exhibiting peritoneal fluid accumulation, and its presence was recorded observationally. Subsequently, bilateral ovaries were excised and stored for histological and immunohistochemical analyses.

Biochemical Evaluation

Biochemical parameters, including serum estradiol and VEGF, were measured using an enzyme-linked immunosorbent assay (ELISA) kit for estradiol and VEGF (Wuhan USCN Business Co., Ltd., China). Tail vessel blood samples were used to detect biochemical parameters.

Histopathological Examination and Ovarian Follicle Counting

Surgically excised ovaries were dissected, and adipose tissue was removed. The tissue was then embedded in paraffin wax after being fixed in a 10% formalin solution for 24 hours. Routine hematoxylin and eosin (H&E) staining and Masson's trichrome (MT) staining were performed on 5-micrometer tissue sections. An Olympus microscope (Japan) was used to examine the slides under light microscopy. Follicular activity was assessed by analyzing five randomly selected samples from each ovary. The examiner was blinded to the treatment groups. Follicle counts were conducted on serially sectioned slices following Taskin's method.[13]

Using recognized morphological criteria, healthy follicles were classified and counted.[14] Oocytes surrounded by a single layer of squamous granulosa cells were classified as primordial follicles; those surrounded by a single layer of cuboidal granulosa cells were classified as primary follicles; those surrounded by two or more layers of granulosa cells were classified as secondary follicles; and those surrounded by multiple layers of granulosa cells with a fluid-filled cavity were classified as antral follicles.

Immunohistochemical Staining

After being sectioned into 5-µm slices, the tissue sections were placed on positively charged slides. After dewaxing in xylene and rehydrating in 99.9%, 95.9%, and 70% ethanol, the sections were washed in phosphate-buffered saline (PBS) and treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity. For antigen retrieval, the slides were microwaved for 20 minutes in a citrate buffer (pH 6.0). Primary anti-VEGF antibodies (Bioss Inc., Woburn, MA, USA; dilution 1/100) or anti-estrogen receptor (ER) antibodies (Bioss Inc., Woburn, MA, USA; dilution 1/100) were incubated overnight, followed by PBS washing. A biotinylated secondary antibody was applied for 30 minutes. After rinsing in PBS, the slides were incubated for 30 minutes with the streptavidin-biotin complex reagent. Following another rinse, the slides were treated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and then rinsed in distilled water. Finally, the slides were counterstained with hematoxylin, dried, cleaned, mounted, and covered for microscopic analysis.

Table 1. Comparison of OHSS variables among experimental groups

OHSS variables	Experimental groups				p
	Negative control (n=10)	Positive control (n=10)	Bevacizumab treatment (n=10)	Retinoic acid treatment (n=10)	
Weight					
Before	59.50±1.65	58.20±1.99	59.30±3.34	60.90±1.97	0.097
After	62.00±2.40[a]	71.90±3.84[b]	72.40±4.74[b]	71.10±5.17[b]	<**0.001**
Difference	2.50±1.43[a]	13.70±3.89[b]	13.10±3.45[b]	10.20±5.35[b]	<**0.001**
p-value	<**0.001**	<**0.001**	<**0.001**	<**0.001**	
Peritoneal fluid (present)	0 (0.0)[a]	10 (100.0)[b]	8 (80.0)[bc]	5 (50.0)[c]	<**0.001**

OHSS: Ovarian hyperstimulation syndrome. Values are expressed as n (%), mean±standard deviation, or median (1st–3rd quartiles). All significant p values are in bold. Different superscripts in the same row indicate a statistically significant difference between groups.

Light Microscopic Examinations

The sections were examined under a light microscope at 400x magnification. Each slide was evaluated independently in a blinded manner. Tissue sections were tested for VEGF and ER, and cells exhibiting staining were classified as positively stained. The intensity of immunoreactivity was measured in ten high-power, randomly selected, non-overlapping frames for each specimen in every group using Image J software (ImageJ, Bethesda, USA) at 400x magnification. The examiner was unaware of the different treatment groups.

Power Analysis

The sample size was determined based on the hypothesis that VEGF blood levels would show significant differences among the experimental groups. Initially, a pilot study was conducted, and the effect size was estimated using samples from three mice per group. Based on this, it was calculated that at least eight mice per group were needed, considering an effect size=0.7193, alpha=5%, and power=90%. To account for potential losses during the experiment, the study was conducted with ten mice per group, for a total of 40 mice. Power analyses were performed using PASS 11.0 (Power Analysis Statistical System, NCSS Inc., USA).

Statistical Analysis

The biostatistician of the study (G. Z.) examined histograms and q-q plots. To assess data normality, the Shapiro-Wilk test was applied, while Levene's test was used to assess variance homogeneity. For continuous variables, one-way analysis of variance (ANOVA), Welch ANOVA, or Kruskal-Wallis H tests were used to compare data among Groups 1, 2, 3, and 4. For categorical variables, the Pearson chi-square test was applied. For multiple comparisons, Tukey, Tamhane T2, and Bonferroni-adjusted z-tests were considered. Additionally, a paired T-test was used to compare changes in animal weights among Groups 1, 2, 3, and 4. We used our institution's statistical software, TURCOSA (Turcosa Analytics Ltd. Co., Türkiye, www.turcosa.com.tr), for all analyses. A p value <0.05 was considered statistically significant.

Patient and Public Involvement

None.

RESULTS

The weight of rats, the presence of peritoneal fluid, and weight differences after COS were the primary outcomes in the OHSS rat model. The weights of the rats before and after COS, as well as the presence of peritoneal fluid, are presented in Table 1.

Retinoic acid treatment significantly reduced peritoneal fluid and inhibited fluid leakage, whereas bevacizumab treatment resulted in a slight reduction of peritoneal fluid.

The effects of bevacizumab and retinoic acid treatment on follicle count are shown in Table 2.

The numbers of tertiary and secondary follicles were not affected by bevacizumab and retinoic acid treatment. However, the number of primary follicles significantly decreased in the bevacizumab and retinoic acid treatment groups.

A comparison of ER variables, VEGF blood and tissue levels, and E2 blood levels among the experimental groups is presented in Table 3.

Retinoic acid treatment significantly reduced ER in the stromal compartment. However, ER in the corpus luteum did not show significant changes following retinoic acid and bevacizumab treatment. Additionally, VEGF blood levels significantly increased in the positive control, retinoic

Table 2. Comparison of follicle count variables among experimental groups

Follicle count	Experimental groups				p
	Negative control (n=10)	Positive control (n=10)	Bevacizumab treatment (n=10)	Retinoic acid treatment (n=10)	
Primordial	29.50±15.32	22.10±9.78	16.50±9.85	17.17±6.79	0.128
Primary	50.00±13.09[a]	40.70±14.69[a]	12.50±3.02[b]	14.83±4.96[b]	<**0.001**
Secondary	19.25±8.28	19.70±8.03	14.83±3.87	24.50±6.41	0.053
Tertiary	5.88±3.60[a]	15.30±9.33[b]	12.50±3.83[b]	16.50±5.61[b]	**0.004**
Atretic	15.00±8.55[a]	32.80±13.64[b]	22.67±7.17[ab]	24.00±10.68[ab]	**0.041**

Values are expressed as mean±standard deviation. All significant p values are in bold. Different superscripts in the same row indicate a statistically significant difference between groups.

Table 3. Comparison of ER variables, VEGF blood and tissue levels, and E2 blood levels among experimental groups

	Experimental groups				p
	Negative control (n=10)	Positive control (n=10)	Bevacizumab treatment (n=10)	Retinoic acid treatment (n=10)	
Stromal ER					
N (%)	51	49	17	21	<**0.001**
Mean±SD	169.24±3.38[a]	174.32±4.83[b]	172.70±5.19[b]	170.07±5.44[a]	
Corpus luteum ER					
N (%)	48	47	21	30	0.101
Mean±SD	172.36±5.53[ab]	172.79±5.03[ab]	171.21±6.70	171.74±6.09	
VEGF blood	17.60 (15.86–26.69)[a]	54.57 (35.81–58.56)[b]	64.58 (32.33–78.96)[b]	70.79 (53.87–99.24)[b]	<**0.001**
E2 blood	110.91 (81.67–131.62)	130.56 (92.60–204.96)	148.24 (100.42–196.08)	102.70 (96.56–151.48)	0.219
VEGF tissue	164.69±9.57	160.94±13.24	165.49±11.53	165.69±13.99	0.190

ER: Estrogen receptor; VEGF: Vascular endothelial growth factor; E2: Estradiol; SD: Standard deviation. All significant p values are in bold. Different superscripts in the same row indicate a statistically significant difference between groups. Values are expressed as mean±standard deviation.

acid, and bevacizumab treatment groups. Micrographs showing VEGF and ER immunoreactivity in ovarian tissue are presented in Figure 1.

Following COS, ovarian damage is an expected event. Newly formed collagen serves as a marker of fibrotic areas in the ovary. Masson's trichrome stain was used to investigate fibrotic areas in the ovary. The positive control group exhibited significantly increased fibrotic areas, whereas both treatment groups demonstrated a reduction in fibrotic areas. An illustration of newly formed collagen can be seen in Figure 2.

DISCUSSION

Despite the development of preventive strategies, OHSS remains a clinical risk during COS. Approximately 20% of high-risk women may develop OHSS while undergoing gonadotropin treatment.[6,15]

This study aimed to investigate the effect of bevacizumab and retinoic acid in the prevention of OHSS using a rat model. The positive control group, bevacizumab treatment group, and retinoic acid treatment group showed significantly greater weight and weight gain compared to the negative control group. This finding suggests that vascular leakage occurred, confirming that the OHSS rat model was successfully established. Moreover, all rats in the positive control group exhibited peritoneal fluid accumulation, whereas the retinoic acid treatment group showed a significant reduction in peritoneal fluid (5 out of 10 rats). In the bevacizumab treatment group, 8 out of 10 rats had peritoneal fluid, suggesting that bevacizumab treatment slightly reduced peritoneal fluid accumulation.

We found that the number of primary follicles decreased following retinoic acid and bevacizumab treatment. This finding

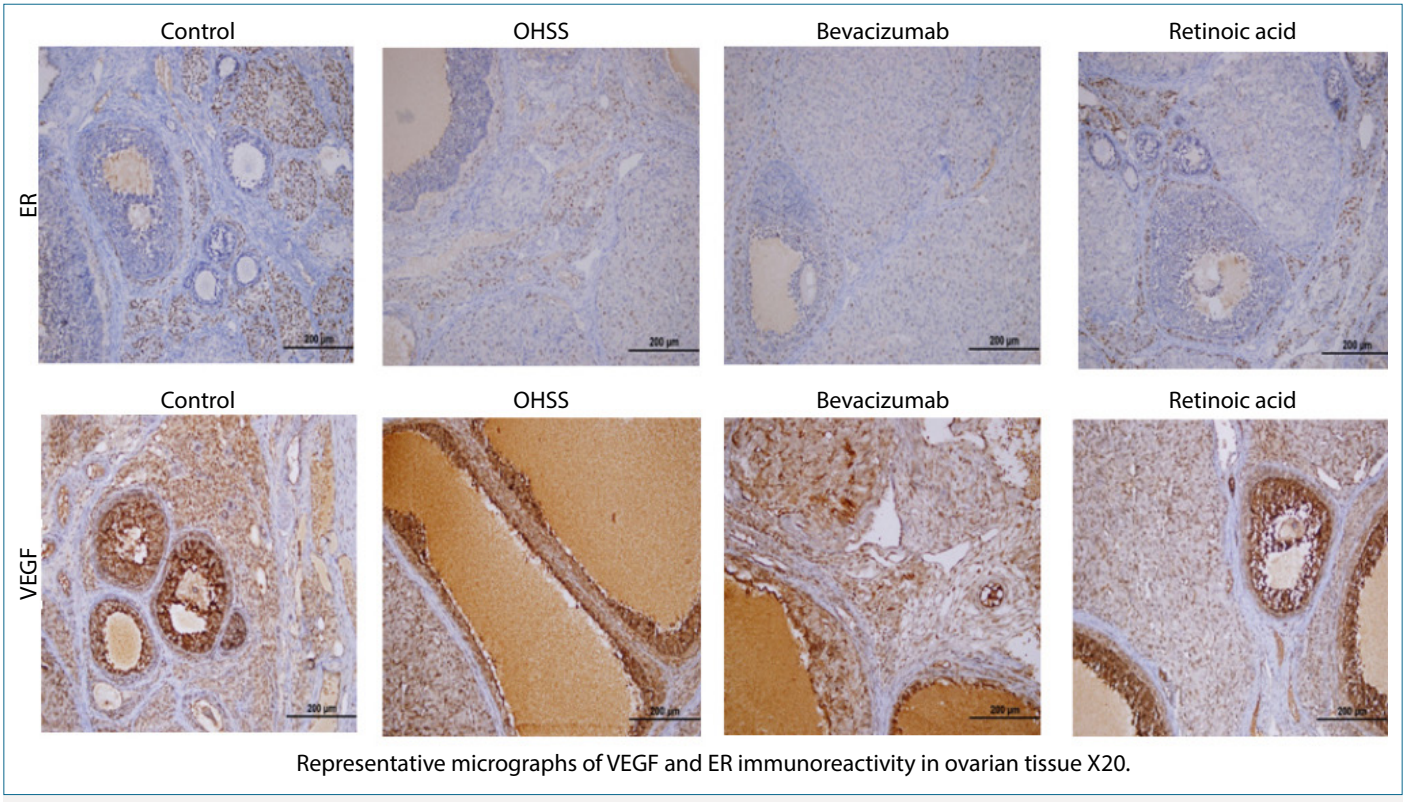

Figure 1. Vascular endothelial growth factor (VEGF) and estrogen receptor (ER) immunoreactivity in ovarian tissue.

suggests that both retinoic acid and bevacizumab influence the initial stages of follicular development, likely through local growth factors. However, neither retinoic acid nor bevacizumab treatment affected the number of tertiary follicles.

Follicular development occurs in distinct stages. The initial stage, known as the resting stage, includes primordial follicles and small primary follicles. These follicles lack functional follicle-stimulating hormone (FSH) receptors and constitute 90-98% of all follicles. At this stage, local factors play an essential role in facilitating the passage of nutrients, ions, and other essential molecules between cells through connexins and intercellular membrane channels. Therefore, follicular growth in this stage primarily depends on local factors.[16,17]

As follicular development progresses, granulosa cell proliferation increases, and oocyte size expands. In the secondary follicle stage, granulosa cells form two or more layers around the follicle, and the follicle develops one or two arterioles. This stage is important because the follicle becomes responsive to circulating agents in the bloodstream.[18]

Bevacizumab is an important component of combined oncologic therapies and was the first approved angiogenesis inhibitor. This molecule has been approved for the treatment of various solid tumors, including breast, cervical, ovarian, non-small cell lung, and renal cell cancers. Because bevacizumab targets VEGF, it is now recognized for its immune-modulatory role.[19] Another therapeutic agent, retinoic acid, has been investigated in a dose- and time-dependent manner for its hormonal effects. Studies have shown that retinoic acid increases gonadotropin-releasing hormone (GnRH) secretion.[20] An increased frequency of GnRH stimulation leads to a decrease in gonadotropin receptors, which is considered an anti-estrogenic effect.[21]

We found that retinoic acid treatment significantly decreased ER in the stromal compartment of ovarian tissue. However, no effect of retinoic acid treatment was observed on ER in the corpus luteum. Similarly, bevacizumab treatment resulted in a slight decrease in ER in the stromal compartment of ovarian tissue, but no effect was observed in the corpus luteum. Additionally, retinoic acid treatment led to a decrease in estradiol levels.

RA and estrogen signaling are closely related, as RA influences estrogen metabolism and the expression of estrogen receptors. RA derivatives may play a protective role against endocrine disorders caused by hyperestrogenism, exhibiting anti-estrogenic activity.[4] Moreover Ombra MN et al.[22] suggested

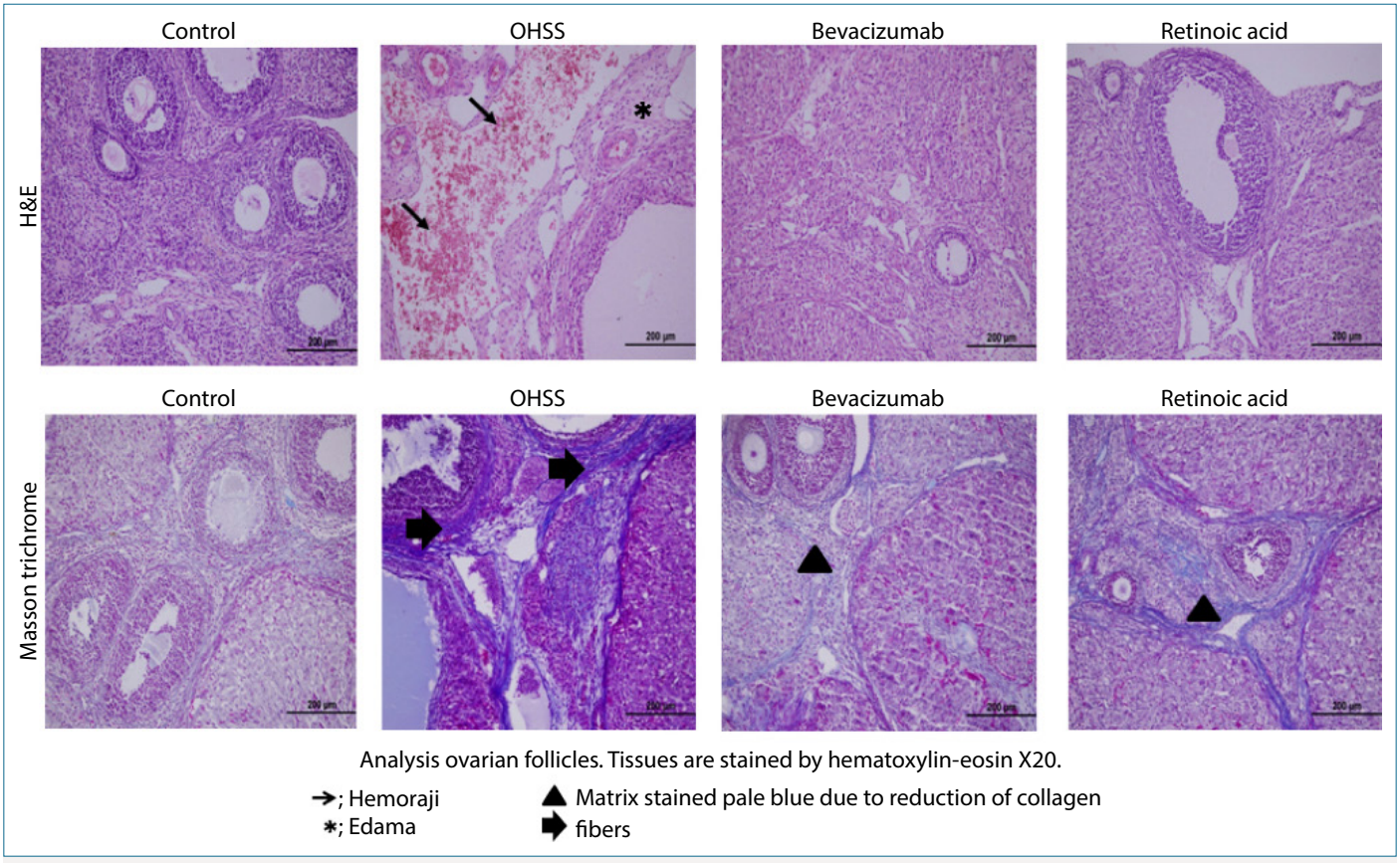

Figure 2. Masson's trichrome staining and hematoxylin and eosin (H&E) staining of ovarian follicles.

that estradiol exerts its effect via binding to ER alpha in breast cancer. The proliferative effect of estradiol is inhibited by retinoic acid through its binding to ER alpha promoters.

Contrary to these anti-estrogenic effects, Kirschenbaum A et al.[23] investigated prostate tissue obtained from eight patients with prostate cancer. They found that in four patients who had been treated with GnRH analogs for four months, ER mRNA expression was increased.

This study was conducted on a male population, with each group consisting of four members. We believe that the differences in study results were likely due to variations in study protocols and the use of a male population.

CONCLUSION

Both retinoic acid and bevacizumab treatment appear to be effective in treating OHSS by targeting the initial stage of follicular development. The administration of retinoic acid and bevacizumab at an appropriate dosage and correct timing before ovarian induction may help prevent OHSS in high-risk patients. Future studies are essential to determine the optimal dosage and timing for these drugs.

Limitations of the study include the lack of evaluation of the long-term effects of these drugs on the endometrium. Additionally, pregnancy rates in the rats were not taken into consideration.

Ethics Committee Approval: The Erciyes University Clinical Research Ethics Committee granted approval for this study (date: 09.05.2018, number: 18/056).

Author Contributions: Concept – FO, GA, AY, HA, OMC, GZ, BA, IM, SM, EK, KS; Design – FO, GA, AY, HA, OMC, GZ, BA, IM, SM, EK, KS; Supervision – FO, GA, AY, HA, OMC, GZ, BA, IM, SM, EK, KS; Resource – AY, HA, OMC, KS; Materials – FO, GA, AY, OMC, SM; Data Collection and/or Processing – FO, BA, EK, OMC, SM; Analysis and/or Interpretation – GZ, IM, BA, SM; Literature Search – FO, GA, BA, EK; Writing – FO, GA, BA; Critical Reviews – IM, SM, GZ, AY.

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