Do Resveratrol and Dehydroepiandrosterone Increase Diminished Ovarian Reserve?

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ABSTRACT

Objective: In this study, the aim is to observe changes induced by dehydroepiandrosterone (DHEA) and resveratrol (RES) in diminished ovarian follicles that was induced by 4-vinylcyclohexenediepoxide (VCD).

Materials and Methods: Twenty four Wistar albino female rats were divided into 3 groups: control, DHEA and RES. Unilateral oophorectomy was performed in control group to remove the right ovary of 4 rats and the left ovary of 4 rats. After administration of 160 mg/kg VCD, remaining ovaries were removed. Following the same VCD treatment, in DHEA and RES groups, 60 mg/kg DHEA and 20 mg/kg RES were given for 45 days respectively and residual ovaries were removed. Hematoxylin-eosin and TUNEL staining were performed. Follicle stimulating hormone (FSH), estradiol (E2) and anti-mullerian hormone (AMH) values were measured.

Results: In control group, VCD-induced apoptosis in follicles increased the TUNEL-positive cell counts ($p<0.001$) with decreased number of follicles. On the other hand, DHEA significantly increased all three follicle types in the ovaries and decreased apoptosis ($p<0.001$). The decreased follicle number in all three follicle types after VCD treatment were found to be significantly increased after RES treatment ($p<0.001$). Apoptosis in the follicles was significantly decreased by RES administration ($p<0.001$). FSH values were found to be increased with VCD and to reach control values with DHEA and RES. E2 values significantly decreased with VCD, but significantly increased with RES and DHEA.

Conclusion: Both DHEA and RES may improve VCD-induced diminished ovarian reserve. DHEA and RES increased the number of primary, primordial and growing follicles, with no significant difference between them.

Keywords: Dehydroepiandrosterone, resveratrol, ovarian follicle, Vinylcyclohexene diepokside

Introduction

After the fetal period, it is thought the ovaries do not produce new oocytes. Negative conditions during the intrauterine period and childhood affect follicular production in the first trimester and may change the follicular capacity. The initially formed follicular pool is limited, and in this situation, women may be faced with earlier follicular consumption [1]. In principle, if follicular atresia is reduced and oocyte quality is preserved, the fertile period may lengthen and ovarian aging may be delayed [2]. Recent in vitro and in vivo studies with production of oocyte-like cells from germinal epithelium in experimental animals and humans have led to the hope that this situation may be resolved. Oocytes sourced in germinal epithelium may sustain fertility in women who have entered early menopause or the postmenopause period and will be effective in terms of ameliorating postmenopausal syndromes [3].

Androgens influence follicle growth through various mechanisms, and they play a very effective role in female infertility and folliculogenesis. There are important studies about the efficacy of dehydroepiandrosterone (DHEA), which is an important androgen for female infertility treatment, and about increasing pregnancy rates in women with diminished ovarian reserve (DOR) entering the early menopause [4, 5].

Resveratrol (RES) is a polyphenolic compound found in red fruit, especially grapes and red wine. It is known to have anticarcinogenic, anti-inflammatory, analgesic, cardioprotective, neuroprotec-

tive, and antitumoral effects. Due to the structural similarity of RES to the synthetic estrogen of diethylstilbestrol, it is proposed to have estrogenic activity [6]. Some studies in recent times have identified that RES protects, and even increases, primordial, antral, and atretic follicle numbers against oxidative stress [7, 8].

Vinylcyclohexene diepoxide (VCD) is a chemical used in production of tires, plastic, rubber, and insecticides [9]. VCD is the only chemical to cause damage to primary and primordial follicles in oocytes from rats and mice. VCD is known to cause autotoxicity by inhalation, dermal, and oral intake in animals. With this aim, it is used as a model in animal studies [9]. VCD shows this effect in both animals and rats [10, 11].

Studies have clearly made the age-linked reduction in follicle numbers a target for new treatment trials. However, it is more important to sustain fertility in women who have entered early menopause due to DOR. Changing the intraovarian environment in these cases will provide hopeful results. There are studies of DHEA and RES in this area. Our study aimed to observe changes in the three follicular groups (primary, primordial, and developing) with DHEA and the phytoestrogen RES in patients with DOR due to 4-vinylcyclohexene diepoxide and to compare the efficacy of DHEA and RES on folliculogenesis.

Materials and Methods

The study included 24 female Wistar Albino rats aged 75 days weighing 125-175 g. The rats were obtained from Eskisehir Osmangazi University, Faculty of Medicine Experimental Animals Laboratory. Animals were housed at room temperature (24±2°C) with 55±15% relative humidity with 12-h light/12-h dark cycles with fixed limits. Water and standard rat feed were administered ad libitum. The study received ethics committee permission from Eskisehir Osmangazi University Animal Experiments Ethics Committee.

Rats were divided into three groups with eight animals in each group.

Group 1: Rats in this group had unilateral oophorectomy performed without any other procedure with the right ovary taken from four rats and the left ovary taken from four rats. The first eight ovaries removed were assessed as control. Blood was sampled from the tail vein of rats. Later, after wound healing, rats were administered 160 mg/kg dose of i.p. VCD (Fluca, Buchs, Switzerland) for 15 days. Relaparotomy was performed, and the other ovaries were removed. Later, animals were euthanized by taking high amounts of intracardiac blood under anesthesia.

Group 2: Rats in this group were administered 160 mg/kg dose of i.p. VCD for 15 days. Then unilateral oophorectomy was performed with the right ovary taken from four rats and the left ovary taken from four rats. Blood was sampled from the tail vein of rats. After wound healing, 60 mg/kg dose of DHEA (Sigma Aldrich Co, Turkey) was administered s.c. for 45 days. As solvent for DHEA, 0.1 mL dimethyl sulfoxide (DMSO) (Sigma Aldrich Co, Turkey) was used [12]. Relaparotomy was performed, and the other ovaries were removed. Later, animals were euthanized by taking high amounts of intracardiac blood under anesthesia.

Group 3: Rats in this group were administered 160 mg/kg dose [6] of i.p. VCD for 15 days. Then unilateral oophorectomy was performed with the right ovary taken from four rats and the left ovary taken from four rats. Blood was sampled from the tail vein of rats. After wound healing, 20 mg/kg dose of RES (Sigma Aldrich Co, Turkey) [6] was administered by oral gavage for 45 days. Relaparotomy was performed, and the other ovaries were removed [5]. Later, animals were euthanized by taking high amounts of intracardiac blood under anesthesia.

When removing ovaries, 40-80 mg/kg dose of ketamine (Pfizer, Turkey) and 5-10 mg/kg dose of xylazine (Ata-fen, Turkey) was used. The ovaries were removed with laparotomy under sterile conditions and fixated in formalin for preparation for histologic studies. All blood samples were centrifuged at 3000 rpm for 3 min with serum samples stored at -40°C for biochemical studies.

Histologic study

For general assessment and identification of follicle numbers on sections, hemtoxylin and eosin (H&E) staining was used. To investigate apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling immunohistochemical staining (TUNEL) was performed.

Sections with 5-µm thickness were prepared from paraffin blocks containing tissue. Five sequential sections were taken for investigation and stained with H&E staining [13, 14]. A total of 2218 sections were taken from 48 ovaries. Selected sections were investigated at 40X magnification. Apart from granulosa, cells with pycnosis and oocyte invasion with deformation and follicles with deformation showing eosinophilia and other follicles with health appearance were assessed as normal follicles [15]. For follicles to be assessed as healthy, the criteria were granulosa cells without granular appearance having a non-dense nucleus [16] (Figure 1). Follicles were classified in accordance with Geugeon's classification [17]. According to this classification,

1- Primordial (Pmdf) (35.0 mm): formed of primary oocyte surrounded by needle-like granulosa cells.
2- Primary follicle (Prmf) (46 mm): formed of primary oocyte surrounded by granulosa cell layer with first signs of zona pellucida around the oocyte.
3- Secondary follicle (77 mm): formed of primary oocyte surrounded by more than one granulosa cell layer.
4- Antral follicle: follicle characterized by formation of an antral cavity due to collection of fluid and glycoprotein structure.

In this classification, secondary and antral follicles are named "growing follicles (Gf)." Apart from this, the total follicle number including atretic

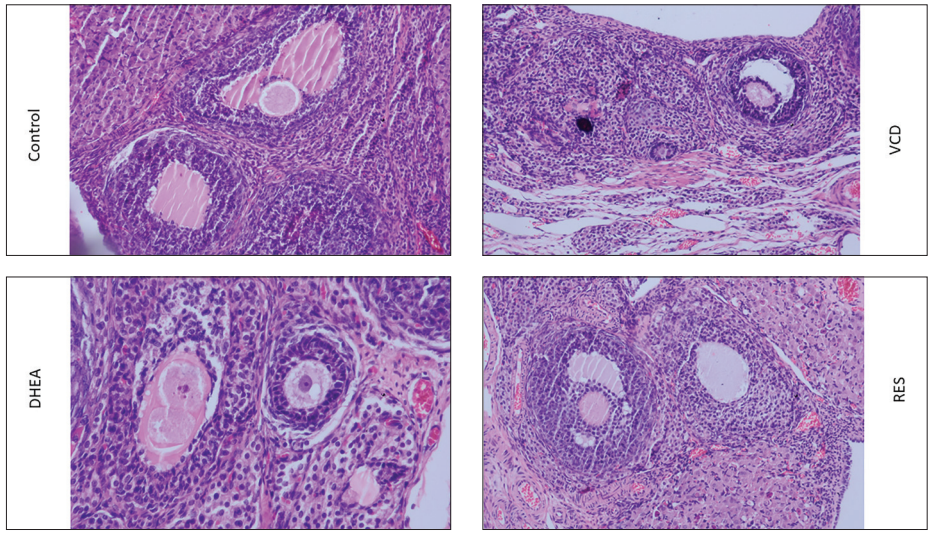

Figure 1. Hematoxylin-eosin (H&E) stained sections showing ovarian follicle of control, VCD, DHEA, and RES. Primordial, primary, and growing follicles are seen in over tissues stained with H&E.

and normal follicles was calculated for each follicular group. The total follicle number was statistically analyzed. Later, this value was multiplied by five with the aim of finding a value for the whole ovary [13]. In each of the three groups, the ovaries were compared with the ovaries of their own control group. For ovaries in all study and control groups, Pmdf, Pmrf, and Gf mean values were calculated.

For assessment of immunohistochemical staining (TUNEL), 25 randomly chosen sections were counted in 50 areas at 40X magnification. Cells stained brown in all follicles at 40X magnification on randomly chosen sections with TUNEL staining were counted, and the mean was calculated (Figure 2).

Biochemical Study

Follicle-stimulating hormone (FSH), estradiol (E2), and anti-Müllerian hormone (AMH) measurement: Using a commercial kit (Elabscience Rat FSH, E2 and AMH ELISA Kit) (BMG Labtech Spectrostar Nano, GmbH, Ortenderg, Germany), first serum samples stored at -40°C had measurements for FSH, E2, and AMH performed. Results are given as ng/mL.

Statistical Analysis

Continuous quantitative variables are given as n, mean, and standard deviation; while qualitative or score variables are given as n, median, and 25% and 75% percentage values. Independent groups with continuous variables and normal distribution were analyzed with the independent samples t-test and one-way analysis of variance, while dependent groups with variables and normal distribution had the paired samples t-test applied. Probability values $p<0.05$ were accepted as significant. All data analyses were performed with the The Statistical Package for the Social Sciences (SPSS) 21 program (IBM Corp., Armonk, NY, USA).

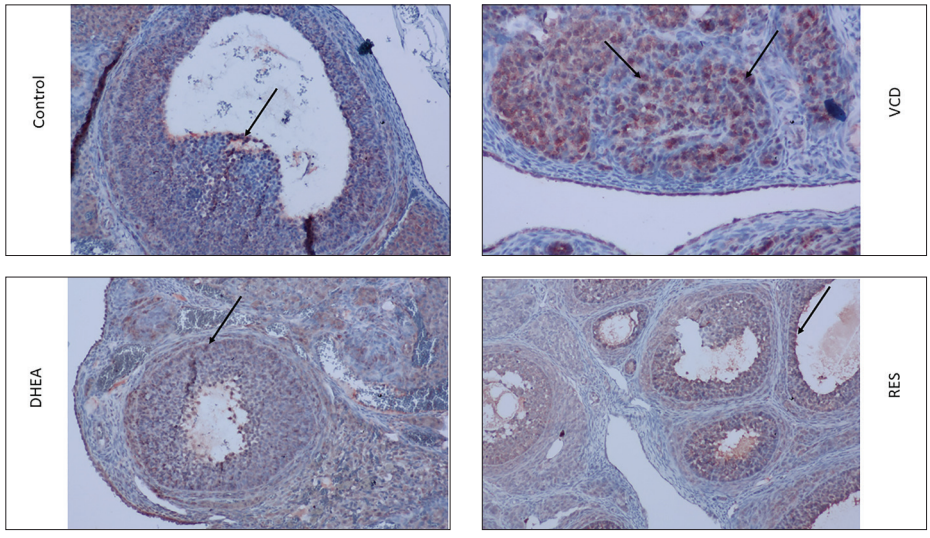

Figure 2. Histologic sections showing ovarian follicle and apoptotic cells of Control, VCD, DHEA, and RES. In the study groups, some of the tunnel-positive stained cells were shown with arrows to indicate apoptosis.

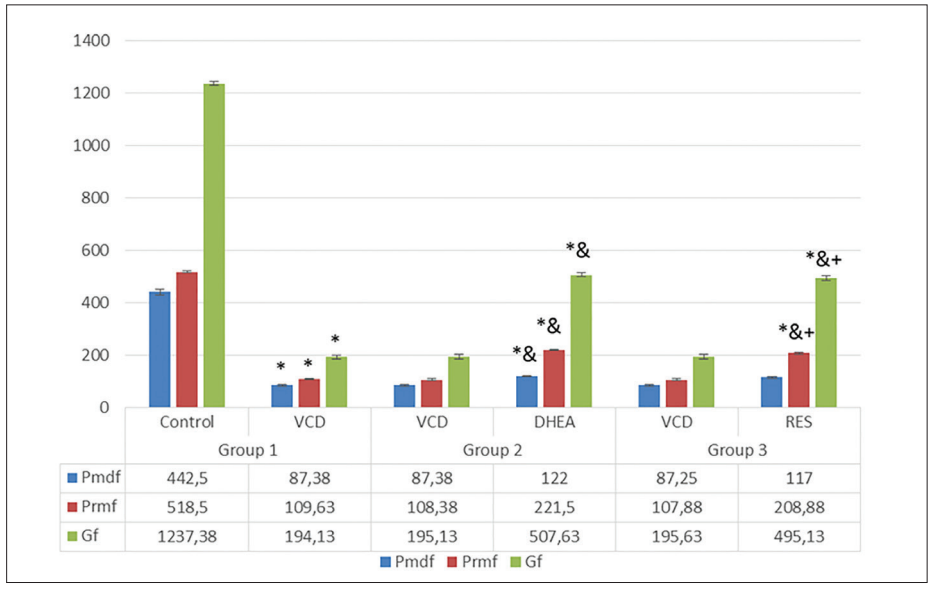

Figure 3. Comparison of primary, primordial, and growing follicle counts.
(*): Different from control, (&): Different from VCD, (+): Different from DHEA ($p<0.05$)

Results

The efficacy of the administered VCD dose on follicles used to form DOR in Group 1 was shown. The follicle numbers (primordial, primary, and growing) found in the first ovaries taken from experimental animals in this group were compared with the follicle numbers obtained from ovaries taken after VCD administration. A statistically significant reduction was identified in follicle numbers in the VCD administration group ($p<0.001$) (Figure 3). Examination of TUNEL positive cells with immunohistochemical staining to investigate apoptotic changes occurring in ovarian follicles due to VCD showed increased apoptosis in all three follicle types with VCD ($p<0.001$) (Figure 4).

After inducing DOR with VCD in Group 2, histologic results from the ovaries were compared with histologic results from ovaries obtained after DHEA administration. Significant differences were observed in terms of follicle numbers for all three follicle types ($p<0.001$). With DHEA, the follicle numbers increased compared to the VCD group (Figure 3). The apoptosis of follicles was significantly reduced with administration of DHEA ($p<0.001$) (Figure 4). When results from ovaries obtained from Group 1 and assessed as control (first eight ovaries) were investigated with results for ovaries in Group 2 after DHEA administration, there was a statistically significant difference in numbers from all three follicle types ($p<0.001$) (Figure 3). Comparison of follicles obtained from ovaries after DHEA administration with the control ovary group (first eight ovaries from Group 1) in terms of apoptosis found no significant difference in terms of primordial and primary follicles ($p=0.175$, $p=0.617$). However, a statistically significant difference was found when investigated in terms of developing follicles ($p<0.001$) (Figure 4).

In Group 3, the histologic results of ovaries after DOR was induced with VCD were compared with those from ovaries after RES was administered. Significant differences were observed for follicle numbers in each of the three follicle types ($p<0.001$). RES was observed to increase the number of follicles compared to the VCD group (Figure 3). It was observed that apoptosis in follicles significantly reduced with RES administra-

tion (p<0.001) (Figure 4). Results were compared between ovaries obtained from Group 1 and assessed as controls (first eight ovaries) and ovaries after administration of RES in Group 3. Statistically significant differences were observed in numbers of each type of follicle (p<0.001) (Figure 3). Follicles obtained from ovaries after RES administration were compared with the control ovary group (first eight ovaries in Group 1) in terms of apoptosis; and no significant difference was present in terms of primordial and primary follicles (p=0.175, p=0.671). However, when compared in terms of developing follicles, a statistically significant difference was observed (p<0.001) (Figure 4).

The FSH values rose with VCD and were identified to reach control values with administration of DHEA and RES. The E2 values decreased with VCD and significantly increased with administration of RES and DHEA (Table 1). No statistically significant difference was observed between the groups for AMH values.

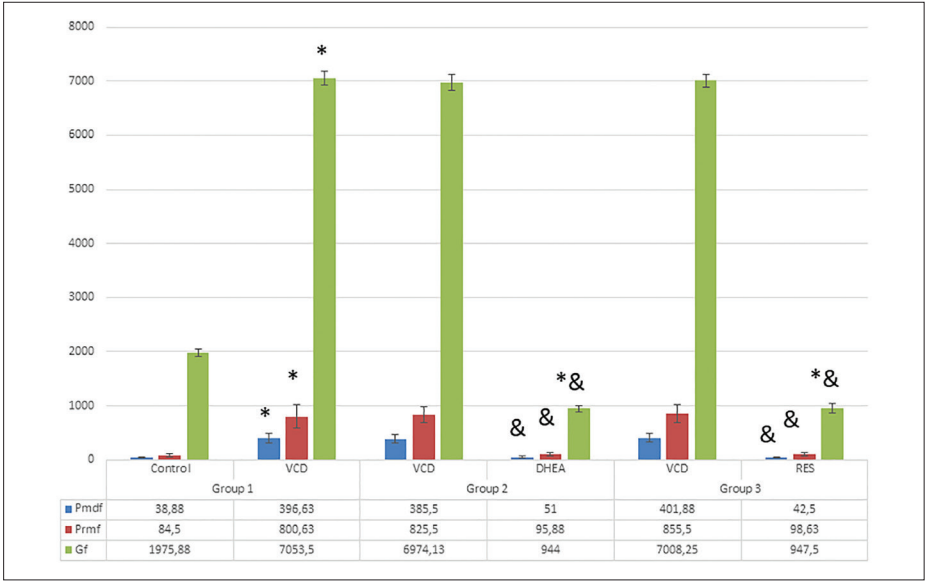

Figure 3. Comparison of primary, primordial, and growing follicle apoptotic cell numbers.
(*): Different from control, (&): Different from VCD (p<0.05)

Table 1. Serum FSH, E2, AMH values

	N	Mean±Std. Deviation	Median (25%-75%)	p
Grup1-FSH-Control	8	0.29±0.09	0.29 (0.22–0.36)	0.014
Grup1-FSH-VCD	8	0.46±0.12	0.41 (0.36–0.59)	0.044
Grup2-FSH-VCD	8	0.47±0.09	0.47 (0.40–0.56)	
Grup2-FSH-DHEA	8	0.34±0.11	0.37 (0.23–0.44)	
Grup3-FSH-VCD	8	0.47±0.06	0.47 (0.42–0.52)	<0.001
Grup3-FSH-RES	8	0.28±0.03	0.28 (0.26–0.32)	
Grup1-E2-Control	8	738.13±88.31	759.50 (651.25–810.00)	<0.001
Grup1-E2-VCD	8	169.88±54.89	156.50 (128.50–196.25)	
Grup2-E2-VCD	8	150.25±31.80	140.50 (124.25–184.75)	<0.001
Grup2-E2-DHEA	8	501.88±77.60	480.50 (440.00–578.25)	
Grup3-E2-VCD	8	149.13±27.19	140.00 (127.75–176.50)	<0.001
Grup3-E2-RES	8	637.88±44.47	631.00 (600.00–664.75)	
Grup1-AMH-Control	8	1.05±0.13	1.03 (0.97–1.17)	0.174
Grup1-AMH-VCD	8	1.02±0.11	1.04 (0.93–1.11)	
Grup2-AMH-VCD	8	1.02±0.09	1.00 (0.94–1.13)	0.846
Grup2-AMH-DHEA	8	1.01±0.08	1.02 (0.93–1.08)	
Grup3-AMH-VCD	8	1.02±0.13	1.00 (0.90–1.16)	0.777
Grup3-AMH-RES	8	1.04±0.12	1.05 (0.93–1.15)	

p<0.05: significant

Discussion

Induction of DOR with VCD in rats was definitely shown in our control group called Group 1. Later, we researched the effects of DHEA and RES on DOR in study groups Group 2 and Group 3, respectively. In Group 2 (administered DHEA after VCD), the follicle numbers in all three follicle types were identified to increase. We observed similar results in Group 3. However, none reached the levels of control group. Identification of apoptotic cells with TUNEL staining showed that apoptotic cell numbers increased after VCD administration, and reduced significantly, though not to control levels, after DHEA and RES administration. Interestingly, there was a reduction in the FSH levels and an increase in the E2 levels after DHEA and RES administration. The E2 levels increased more in the group given RES compared to the group given DHEA.

DOR is a very significant problem in young women and women undergoing in vitro fertilization (IVF) treatment. It has severely negative effects on becoming a parent. Especially for IVF treatment, clinicians are continuously searching for methods to improve DOR [18].

Androgens are effective on folliculogenesis; and there is evidence they may increase the effects of gonadotropins. In recent years, DHEA originating in the zona reticularis of the adrenal cortex and from theca cells in ovaries has attracted attention in studies about increased ovarian response in women dealing with DOR. DHEA is reported to regulate androgen receptor transcription, increase FSH receptor expression, and increase follicle numbers affecting early follicle maturation [19-21]. A study by Barad and Gleicher [4] found that DHEA improved the response to ovarian stimulation in women with DOR, and this was clearer in young women. In our study, histologic assessment found that the group administered DHEA was different compared to the group administered VCD in terms of the three types of follicles. DHEA significantly increased the follicle numbers (Figure 3). A significant difference was identified with rats with DOR induced with VCD. Similarly, it was observed that DHEA ameliorated the apoptosis caused by VCD (Figure 4). Again, DHEA was identified to significantly improve FSH and E2 values. Because of these findings, it may be concluded that DHEA may improve DOR, increase

follicle numbers, and positively affect pregnancy.

RES is a phytoalexin abundantly found in the skin of red grapes and red wine. It has anti-inflammatory, antioxidant, and immune modulatory effects. It is known that RES has estrogenic activity [2]. A study aiming to assess the effects of RES on age-linked infertility by Liu et al. [22] added RES at 30 mg/L dose to the drinking water of rats for 6- and 12-month periods and assessed the ovarian function and fertility of RES and the control groups. In this study, RES was shown to play a protective role against age-linked fertility changes, caused positive variations in amount and quality of oocytes and preserved healthy follicle numbers. In our study, we observed that RES significantly increased the number of follicles compared to the VCD group. Again, RES administration was observed to significantly reduce apoptosis in follicles. Comparison of follicles from ovaries taken after RES and DHEA administration with the ovarian group assessed as control (first eight ovaries from Group 1) found no significant difference in terms of primordial and primary follicles. However, when investigated in terms of developing follicles, a statistically significant difference was found (Figure 4). When the effects of DHEA and RES on primary, primordial, and developing follicles are compared, the increase in primary and developing follicle numbers with DHEA was observed to be more effective compared to RES. However, when examined in terms of apoptotic cell numbers, no statistical significance was identified. In fact, Narkwichea et al. [23] stated that DHEA may be effective by increasing the speed of primordial follicle formation and development of preantral follicles in ovarian folliculogenesis. In addition, it has been found in recent studies that androgens play an effective role in early follicle development [24]. In a prepubertal rat study of peroxisome proliferator-activated receptor gamma (PPARG) and DHEA, Velez et al. [24] found that DHEA is effective in early follicle development but not in atretic follicle formation. All the results of these studies are similar to that of our study. Again in recent years, in their study, Mahmoud YI et al. [25] have studied the effects of DHEA at pharmacological doses on ovarian reserve changes, follicular development, reproductive function, and pregnancy outcomes of perimenopausal rats and concluded that DHEA represents a promising therapeutic option improving the pregnancy outcomes. Histomorphometric studies revealed a dose dependent significant increase in all follicles, which also support our findings. RES caused a significant change in follicle numbers compared to the VCD group, though not as much as DHEA. A study by Özcan et al. [7] identified that RES reduced the toxic effects induced in ovaries by cisplatin, with increased follicle numbers compared to the group with cisplatin. Additionally, they identified a significant reduction in atretic follicle numbers. Chen et al. [26] in a study of tea polyphenols, quercetin, genistein, and RES identified that RES and genistein may increase ovarian reserve and lengthen the ovarian life in rats, and that these effects were not just in the transition from primordial to primary follicle but also reduced atretic follicles. A study by Morita et al. [27] found sirtuin 1 (SIRT 1) localized in granulosa cells of human ovaries. SIRT 1 is in the NAD+ dependent, class III histone deacetylase family included in a range of cellular processes like DNA repair, recombination, and aging. Morita et al. [27] assumed RES affected SIRT 1 to play a role in ovarian physiology. In our study, similar to results from these studies, we identified significant reductions in apoptotic cell numbers in the group administered DHEA and the group administered RES. We observed a significant reduction in atretic follicle numbers after administration of DHEA and RES. However, we did not identify any statistical significance when we compared DHEA and RES. This may be assessed as DHEA and RES having the same degree of effect on apoptotic cells. There are studies about the effects of DHEA and RES on folliculogensis. However, no study compares the effects of DHEA and RES on folliculogenesis.

In our study, the variation in the FSH and E2 levels caused by DHEA and RES is interesting. The FSH levels nearly reached control levels in the groups administered DHEA and RES (Table 1). When DHEA and RES are compared, we observe that RES brought the FSH levels to values close to control levels. In our previous study inducing testicular toxicity in male rats with VCD, we observed that 20 mg/kg dose of RES brought the FSH levels to control values [28]. A study by Lin et al. [29] of rats with DOR identified that the FSH levels and follicle numbers reached control levels in the group administered DHEA. In addition, Furat Rençber S et al. [30] have investigated the effects of the combined treatment of RES and metformin on the ovaries of rats with DHEA-induced polycystic ovary syndrome (PCOS) in rats and found that the high levels of FSH in rats were significantly decreased with RES. These are similar to our results. Both DHEA and RES affect the hypothalamus pituitary axis lowering the FSH levels elevated by VCD.

The main source of estrogens after menopause is the extragonadal region. They form by biotransformation of androgens, testosterone, and androstenedione by aromatase enzymes in adipose tissue, bones, skin, and vaginal epithelium [31]. Teixerira et al. [31] examined DHEA effects in obese rats in the late postmenopausal period and showed that DHEA increased the E2 levels in ovariectomized rats; however, it did not ameliorate atrophy in the uterus. They explained this as DHEA transformation being unique to cells and tissue contrary to estrogen replacement. In our study, the E2 levels significantly increased after administration of RES and DHEA. However, we did not investigate the effects of menopause, DHEA and RES on the uterus. The elevation in the E2 levels in the study is in accordance with the fall in FSH levels.

Small antral follicles in granulosa cells in adult ovaries primarily produce AMH. AMH helps protect the dormant primordial follicles. AMH was first defined due to causing regression of the Mullerian channel during fetal development of males. It is expressed by ovarian granulosa cells in developing preantral and antral cells. It reduces the FSH response in developing follicles [32]. According to previous studies in rats, primordial and small primary follicles do not express AMH; however, AMH shows maximum expression from granulosa cells in mature large primary follicles and in small preantral follicles [33]. In the large antral follicle stage, follicular development begins linked to FSH and AMH expression reduces and finally becomes unidentifiable. A study by Lin et al. [29] investigating the effects of DHEA in rats with induced DOR identified a significant fall in the AMH levels in the group with induced DOR compared to the control group, while administration of DHEA did not cause any change in the AMH levels. In our study, we did not encounter any significant change in the AMH levels in groups with induced DOR or groups administered DHEA or RES. However, Lin et al. [29] used pregnant mare gonadotropin, human chorionic gonadotropin, and prostaglandin F5 alpha to induce DOR. Previous studies inducing DOR with VCD identified significant reductions in follicle numbers. However, the study by Sahambi SK et al. [34], especially, used 240 mg/kg VCD. Tran DN et al. [35] found that the AMH levels were reduced in rats with VCD-induced DOR. Again, in the study by Furat Rençber S et al. [30], the AMH levels were significantly higher in the PCOS group compared to the control group of rats. On the other hand, the AMH levels were found to be decreased in the group treated with RES. These results do not support the results we found in our study. However, in a clinical study of people with DOR, Wong QHY et al. [36] found similar results with our results. The authors had treated the patients with DOR by DHEA for 12 months, but observed no change in the AMH levels. The

authors suggested that DOR is a condition in which primordial ovarian pool is severely depleted, and that AMH is a hormone produced only by the ovarian gronulosa cells, which can explain the lack of a significant difference in serum AMH levels. The findings of this study also support our findings.

In conclusion, DHEA and RES administered after DOR is induced with VCD caused an increase in the follicular pool, with no significant difference between their effects. RES and DHEA could be used as treatment tools for DOR to increase primary, primordial, and growing follicle numbers.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Eskisehir Osmangazi University Animal Experiments Ethics Committee.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – F.Y.O., O.O., K.E.; Design – F.Y.O., O.O., S.Y.; Supervision - S.Y., B.K., K.E.; Resources – F.Y.O., O.O., S.Y.; Materials – F.Y.O., O.O.; Data Collection and/or Processing – F.Y.O., O.O., S.Y., B.K; Analysis and/or Interpretation – F.Y.O., O.O., B.K.; Literature Search – F.Y.O., S.Y., B.K., O.O.; Writing Manuscript – F.Y.O., O.O., ; Critical Review – K.E., S.Y., B.K.

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