Advance Publication

Experimental Animals

Received: 2023.12.20

Accepted: 2024.3.9

J-STAGE Advance Published Date: 2024.3.16

Category: Reproduction

Type: Original paper

Dehydroepiandrosterone Modulates the PTEN/PI3K/AKT Signaling Pathway to Alleviate 4-Vinylcyclohexene Diepoxide-Induced Premature Ovarian Insufficiency in Rats

RUNNING HEAD: DHEA MODULATES OVARIAN INSUFFICIENCY

Cihan CAKIR[1], Goktan KUSPINAR[1], Kiper ASLAN[2], Cengiz BOZYIGIT[3], Isil KASAPOGLU[2], Melahat DIRICAN[4], Gurkan UNCU[2], Berrin AVCI[1]

[1] *Department of Histology and Embryology, Bursa Uludag University School of Medicine, Bursa, Turkey*
[2] *Department of Obstetrics and Gynecology, Bursa Uludag University School of Medicine, Bursa, Turkey*
[3] *Department of Medical Biochemistry, Bursa City Hospital, Bursa, Turkey*
[4] *Department of Medical Biochemistry, Bursa Uludag University School of Medicine, Bursa, Turkey*

Cihan CAKIR- https://orcid.org/0000-0002-8332-7353
Goktan KUSPINAR- https://orcid.org/0000-0002-0338-8368
Kiper ASLAN- https://orcid.org/0000-0002-9277-7735
Cengiz BOZYIGIT- https://orcid.org/0000-0003-2215-6973
Isil KASAPOGLU- https://orcid.org/0000-0002-1953-2475
Melahat DIRICAN- https://orcid.org/0000-0002-4956-5278
Gurkan UNCU- https://orcid.org/0000-0001-7660-8344
Berrin AVCI- https://orcid.org/0000-0001-8135-5468

Corresponding author: Cihan Cakir
Address: Bursa Uludag Universitesi Tıp Fakültesi Histoloji ve Embriyoloji AbD., Görükle, Bursa 16059, Türkiye
Phone: +902242952525
e-mail: cihancakir@uludag.edu.tr

This study was supported by grants from The Scientific and Technological Research Council of Turkey (TUBITAK). Grant Number: 218S810. Authors declare no conflicts of interest.

ABSTRACT

Dehydroepiandrosterone (DHEA) is frequently integrated as an adjuvant in over a quarter of controlled ovarian hyperstimulation (COH) protocols, despite the ongoing debate regarding its impact. This study aimed to evaluate the efficacy and mechanism of action of DHEA on ovarian follicular development and ovarian response in rats with varying ovarian reserves. The study involved 75 rats categorized into 15 distinct groups. The ovarian tissues of rats in both the normal ovarian reserve group and the premature ovarian insufficiency (POI) group, induced by 4-vinylcyclohexene diepoxide (VCD) injection, were subjected to histomorphological and biochemical analyses following the administration of DHEA, either alone or in combination with COH. Follicle counting was performed on histological sections obtained from various tissues. Serum concentrations of AMH and the quantification of specific proteins in ovarian tissue, including PTEN, PI3K, AKT, COX-2, caspase-3, as well as assessments of total antioxidant status and total oxidant status, were conducted employing the Enzyme-Linked Immunosorbent Assay (ELISA) method. The impact of DHEA exhibited variability based on ovarian reserve. In the POI model, DHEA augmented follicular development and ovarian response to the COH protocol by upregulating the PTEN/PI3K/pAKT signaling pathway, mitigating apoptosis, inflammation, and oxidative stress, contrary to its effects in the normal ovarian reserve group. In conclusion, it has been determined that DHEA may exert beneficial effects on ovarian stimulation response by enhancing the initiation of primordial follicles and supporting antral follicle populations.

Key words: Controlled ovarian hyperstimulation, dehydroepiandrosterone, premature ovarian insufficiency, PTEN/PI3K/pAKT signaling pathway, 4-vinylcyclohexene diepoxide

1. INTRODUCTION

Infertility is a prevalent social concern that impacts a considerable number of couples within the reproductive age demographic [1]. While the etiology of infertility is multifaceted, encompassing both male and female factors, a considerable proportion (32%) of infertile couples are attributed to cases of diminished ovarian reserve (DOR) [2]. Ovarian reserve denotes the quantitative and qualitative status of ovarian follicles, serving as a determinant of ovarian potential [3,4]. The decline in both the quantity and quality of oocytes, typically occurring around the mid-40s, represents a natural physiological phenomenon known as DOR. However, certain women may encounter this reduction at an earlier age, leading to premature infertility, a condition referred to as pathologic DOR, also known as premature ovarian insufficiency (POI). The investigation of POI is imperative due to the contentious nature of its diagnostic and therapeutic protocols, its limited clinical success rates subsequent to intervention, and its escalating prevalence. The decline in follicular count and oocyte quality precipitates reduced rates of fertilization, implantation, clinical pregnancy, and live births, concomitant with an augmented incidence of aneuploidy among developing embryos and an elevated frequency of miscarriages post-embryo transfer [5].

The therapeutic strategies employed to enhance clinical outcomes in cases of POI are a subject of controversy. Currently, a definitive optimal treatment approach remains undefined [6]. Prior investigations have indicated that diverse protocols for controlled ovarian hyperstimulation (COH) yielded comparable clinical results in the management of POI [7,8]. Furthermore, it has been observed that the utilization of distinct forms of gonadotropins did not exert an influence on clinical outcomes [9], and increasing the dose of gonadotropins demonstrated no impact on clinical results [10]. In order to attain the requisite quality and quantity of oocytes, pivotal for augmenting clinical success, it is imperative to elucidate the efficacy and mechanisms of action inherent in both the COH protocol and complementary adjunctive interventions [11].

The absence of an established treatment protocol for patients with POI has prompted researchers to explore alternative strategies. Among these approaches, dehydroepiandrosterone (DHEA), frequently discussed as an adjuvant agent integrated into treatment protocols, is an androgen synthesized in the adrenal glands, gonadal tissue, and brain. Notably, DHEA serves as a precursor for androstenedione, testosterone, and estradiol. Additionally, it has been recognized for its involvement in the initiation of puberty and its gradual decline with advancing age [12,13]. Despite ongoing debates surrounding the potential advantages of DHEA administration in POI cases, its utilization is progressively gaining momentum in assisted

reproductive technology (ART) centers worldwide. Anticipations are high that DHEA could yield favorable impacts on the response to ovarian stimulation and subsequent clinical outcomes. A comprehensive study encompassing 124,700 in vitro fertilization (IVF) cycles conducted across 196 centers spanning 45 countries indicated a notable clinical utilization rate of DHEA at 25.8% [14].

Research findings indicate that the utilization of 4-vinylcyclohexene diepoxide (VCD), an industrial chemical, over a specific duration through repeated administrations, results in a targeted reduction of primordial and primary follicles through the activation of apoptosis [15–17]. VCD is widely employed to curtail ovarian reserve, provoke perimenopausal hormonal shifts, and establish a model stimulating experimental POI [18–21].

In our study, our primary objective was to assess the impacts of using DHEA either alone or in conjunction with the COH protocol. DHEA functioned as an adjuvant agent within the COH protocol, although its definitive efficacy remains uncertain. Our focus was to examine its influence and molecular mechanisms on ovarian follicular development in rats presenting varying ovarian reserves. To achieve this, we examined the modulatory effects of DHEA on apoptosis, inflammation, oxidative stress, and the PTEN/PI3K/AKT signalling pathway.

2. MATERIALS AND METHODS

2.1. Experimental Animals and Study Design

A total of one hundred unmated *Sprague Dawley* female rats, aged 60 days and weighing 160-180 g, were procured from the Experimental Animal Breeding Applied Research Centre of Bursa Uludag University (DEHYUAM). Ethical protocols were strictly adhered to, aligning with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Approval for the study was granted by the Local Ethics Committee on Experimental Animal Research of Bursa Uludag University (Approval ID: 2018-09/02). A seven-day acclimatization period was provided in the laboratory environment, during which the rats were screened for signs of any health-related issues. The rats were housed in standard cages at DEHYUAM, maintaining controlled temperature (24 ± 2 °C), humidity (50–60%), and a 12/12-hour light/dark cycle, with ad libitum access to food and water. To establish homogeneity in ovarian reserves, serum anti-Müllerian hormone (AMH) levels were quantified at the study's outset via the enzyme-linked

immunosorbent assay (ELISA) method. Seventy-five rats with serum AMH values ranging from the 25th to the 75th percentiles were selected for inclusion in the study. Consequently, all subjects initially enrolled in the study were classified as having a normal ovarian reserve (NOR).

The study participants were allocated randomly into 15 distinct groups, categorized according to their status of ovarian reserve; POI or NOR. Each group consisted of five subjects, as illustrated in Figure 1.

- **Control Group (n=5):** Subjects were sacrificed after measuring their serum AMH levels on day 0 of the experiment.
- **VCD Group (n=5):** Subjects were sacrificed after receiving VCD injections for a duration of 15 days.
- **VCD+DHEA Group (n=5):** Subjects were sacrificed after receiving VCD injections for 15 days, followed by DHEA injections for 45 days.
- **VCD+DHEA+COH Group (n=5):** Subjects were sacrificed after a 15-day regimen of VCD injections, followed by DHEA injections for 45 days and a COH protocol for 9 days.
- **VCD+DHEA+Saline Group (n=5):** Subjects were sacrificed after a 15-day regimen of VCD injections, followed by DHEA injections for 45 days and saline injections for 9 days.
- **VCD+DMSO Group (n=5):** Subjects were sacrificed after a 15-day regimen of VCD injections, followed by injections of dimethyl sulfoxide (DMSO) for 45 days
- **VCD+DMSO+COH Group (n=5):** Subjects were sacrificed after a 15-day regimen of VCD injections, followed by DMSO injections for 45 days, and a COH protocol for 9 days.
- **VCD+DMSO+Saline Group (n=5):** Subjects were sacrificed after a 15-day regimen of VCD injections, followed by DMSO injections for 45 days, and saline injections for 9 days.
- **DMSO Group (n=5):** Subjects were sacrificed after receiving DMSO injections for 15 days.
- **DMSO+DHEA Group (n=5):** Subjects were sacrificed after a 15-day regimen of DMSO injections, followed by DHEA injections for 45 days.
- **DMSO+DHEA+COH Group (n=5):** Subjects were sacrificed after a 15-day regimen of DMSO injections, followed by DHEA injections for 45 days, and a COH protocol for 9 days.
- **DMSO+DHEA+Saline Group (n=5):** Subjects were sacrificed after a 15-day regimen of DMSO injections, followed by DHEA injections for 45 days, and saline injections for 9 days.

- **DMSO+DMSO Group (n=5):** Subjects were sacrificed after a 15-day regimen of DMSO injections, followed by DMSO injections for 45 days.
- **DMSO+DMSO+COH Group (n=5):** Subjects were sacrificed after a 15-day regimen of DMSO injections, followed by DMSO injections for 45 days, and a COH protocol for 9 days.
- **DMSO+DMSO+Saline Group (n=5):** Subjects were sacrificed after a 15-day regimen of DMSO injections, followed by DMSO injections for 45 days, and saline injections for 9 days.

In each experimental group, the body weights of the rats were assessed both at the onset and conclusion of the injection regimen. Changes in body weight were calculated based on these measurements. Furthermore, subsequent to sacrifice, weight measurements were conducted on the dissected ovarian tissues.

2.2. Experimental Induction of Premature Ovarian Insufficiency Model

This study employed a rat model to induce POI using VCD. Rats were selected randomly for each treatment group, their weights recorded, and subsequently administered either VCD (n = 35) for POI induction, or DMSO (n = 35) for a NOR condition, over a course of 15 days. VCD (≥96% purity) (Sigma-Aldrich, Cat.No: 94956) and DMSO (Sigma-Aldrich, Cat.No: 47230) were stored at -20°C, and precautions were taken to prevent skin contact. The rats were subjected to daily subcutaneous injections (sc) of VCD at a dose of 160 mg/kg/day (diluted in DMSO; 1 ml/kg), or vehicle (1 ml/kg/day DMSO). The chosen dose was guided by earlier research [22,23], and the subcutaneous route was preferred due to its minimal impact on rat well-being compared to intraperitoneal or intramuscular administration [24].

2.3. DHEA Administration

DHEA, was administered after VCD or vehicle (DMSO) injections. DHEA (≥99% purity) (Sigma-Aldrich, Cat.No: D4000) was dissolved in 0.2ml of DMSO and subcutaneously administered (sc) at a dose of 60 mg/kg/day over a 45-day duration [18]. Rats designated as controls for the DHEA injections received DMSO injections in the same 0.2ml/day/sc volume.

2.4. Controlled Ovarian Hyperstimulation Protocol

Ovarian stimulation was carried out on subjects with regular oestrus with the GnRH agonist long protocol. The initiation of the COH protocol was scheduled on the third day of the estrous cycle, specifically during the proestrus stage, guided by vaginal smear findings. Over the span of days 3 to 9 of the cycle, a single daily intraperitoneal (ip) injection of 1.5 µg/100g/day GnRH agonist (Lucrin, Abbott) was administered. On the ninth day of the cycle, a single injection of 5 IU/100 g/ip pregnant mare serum gonadotropin (PMSG) (ProSpec Bio, Cat.No: HOR-272) was administered. Ovulation was induced by a 10 IU/100g/ip human chorionic gonadotropin (hCG) (Ovitrelle, Merck Serono) injection, administered 28 hours after the final dose of injections. For the COH-sham groups, instead of the agents used in the COH protocol, saline in equivalent volume was administered to the rats during days 3 to 9 of the estrous cycle according to vaginal smear findings, and 28 hours after the last dose [25].

2.5. Oestrus cycle assessment

Rats that exhibited two consecutive normal estrus cycles were included in the study. Regularity of the estrous cycle was evaluated throughout the study using exfoliative vaginal cytology. Vaginal smears were taken consistently at 09:00 each morning. Exfoliative cells were collected using saline-moistened cotton-tipped swabs, transferred to glass slides, and air-dried. Dried smears were fixed with 70% ethanol and stained with methylene blue [26]. The stage of the estrous cycle was identified by microscopic examination of the prevailing cell type in the vaginal smear [27,28], categorized as follows: (1) Proestrus, nucleated epithelial cells; (2) Estrus, cornified squamous epithelial cells lacking a discernible nucleus; (3) Metestrus, a mixture of nucleated, cornified, and leukocytes; (4) Diestrus, leukocytes.

2.6. Collection of Blood Samples and Biochemical Evaluation of Serum Samples

Blood samples were collected from the subjects retro-orbitally under sevoflurane anesthesia. The rats' heads were steadied by gripping the skull and jaw, the upper eyelid was gently retracted, and a 500 µl blood sample was collected from the orbital venous plexus using capillary action and a middorsal approach [29]. The blood samples were centrifuged at 4000rpm for 10 minutes at 4 °C, and the serum was isolated. The serum samples were subsequently stored at −80 °C until AMH levels were analyzed. AMH was assessed in serum samples using commercially available ELISA kit (Cat.No: EA0083Ra; BT Lab Co.).

Spectrophotometric measurements (mQuant, Biotek, Winooski, VT) were conducted in accordance with the kit protocol.

2.7. Tissue Extraction and Histomorphological Analysis

The subjects were sacrificed during the estrus phase of their ovarian cycles following the administered injections. Following induction of sevoflurane inhalation anesthesia, the abdominal cavity and thorax were opened. Unilateral right oophorectomy was conducted for subsequent use in the ELISA technique, and the obtained samples were promptly preserved in a -80°C ultra-deep freezer. Subsequently, the perfusion fixation method was employed prior to the left oophorectomy, facilitating histomorphological analysis. This method involved inserting a catheter into the aorta connected to a perfusion pump, followed by the administration of saline at an appropriate pressure to clear blood from circulation. Subsequently, 4% paraformaldehyde solution was introduced into circulation for fixation purposes. Ovarian tissues were then excised, fixed in 4% paraformaldehyde, and subjected to routine histological processing. The tissues were eventually embedded in paraffin. Sections with a thickness of 5µm were obtained from the paraffin blocks using a rotary microtome (Leica RM2245, Leica, Wetzlar, Germany). These sections were stained using a standard hematoxylin and eosin (H&E) staining protocol (Merck, Darmstadt, Germany), and follicle count was conducted under a microscope (Olympus BX50, Tokyo, Japan) [30]. The number of corpora lutea was also recorded. For the paraffin blocks, the initial section was chosen as the section containing at least 2 secondary follicles. Follicle counting was executed across 5 sections, starting from the initial section, with a gap of 10 sections between each section designated for evaluation. Only ovarian follicles containing oocytes were included in the count to prevent recounting of the same follicle. Morphological classification of follicles was achieved by considering characteristics such as granulosa cell shape, number of cell layers, and presence of an antrum. Follicles were categorized into five developmental stages: (1) primordial follicles (oocytes surrounded by a layer of flattened pregranulosa cells); (2) unilaminar primary follicles (oocyte encircled by a single layer of cuboidal granulosa cells); (3) multilaminar primary follicles (two or more layers of granulosa cells surrounding the oocyte without a visible cavity); (4) secondary follicles (one or more cavities within the granulosa layer without a visible antrum); and (5) Graafian follicles (large antral cavity filled with secretory fluid). In order to demonstrate both follicular recruitment and growth, we categorized ovarian follicles at all developmental stages into two groups: preantral (including primordial

follicles, unilaminar and multilaminar primary follicles) and antral (including secondary and Graafian follicles) follicles (Figure 2). Atretic follicles were defined by granulosa cells detached from the follicle edge with intact oocytes, or by disruption/loss of both granulosa and theca cells with damaged or absent oocytes [31] (Figure 3).

2.8. Biochemical Evaluation of Ovarian Tissue Samples

To explore the impact of DHEA treatment on oxidative stress, inflammation, apoptosis, and the PTEN/PI3K/AKT pathway; levels of the total antioxidant status (TAS) (Cat.No: MBS1600693; Mybiosource Co.), the total oxidant status (TOS) (Cat.No: MBS1600508; Mybiosource Co.), caspase-3 (Cat.No: E-EL-R0160; Elabscience Co.), cyclooxygenase 2 (COX-2) (Cat.No: E1373Ra; BT Lab Co.), phosphatase and tensin homolog of chromosome 10 (PTEN) (Cat.No: MBS452729; Mybiosource Co.), Phosphoinositide 3-kinase (PI3K) (Cat.No: MBS702819; Mybiosource Co.), and phosphorylated protein kinase B (pAKT) (Cat.No: EA1201Ra; BT Lab Co.) were assessed in tissue homogenates using commercially available ELISA kits. Spectrophotometric measurements (mQuant, Biotek, Winooski, VT) were conducted in accordance with the kit protocols to determine these parameters.

2.9. Calculation of Oxidative Stress Index (OSI)

The ratio of TOS to TAS was accepted as the oxidative stress index (OSI). OSI value was calculated according to the following formula: OSI = TOS/(TASx100) [32].

2.10. Statistical Analysis

The distribution characteristics of variables were assessed using the Shapiro-Wilk test. Descriptive statistics were employed to summarize the study data. Continuous variables were presented as mean±standard deviation based on their distribution characteristics. The Student's t-test was employed for comparing two groups, while for scenarios encompassing more than two groups, data were subjected to one-way ANOVA, followed by the post hoc Tukey test. Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 28.0, SPSS Inc., Chicago, Illinois, USA). A significance level of $p<0.05$ was adopted to indicate statistical significance.

3. Results

3.1. Effects on body and ovarian tissue weight

Data on the body weights recorded on the initial and final days of injections, the corresponding weight changes during the injection period, and the ovarian tissue weights obtained post-sacrifice across all groups are presented in *Supplemental Table 1*. Notably, administration of VCD *(p=0.84)*, DHEA *(p=0.91)*, or the COH protocol *(p=0.56)* did not elicit a significant alteration in body weight. Ovarian tissue weights exhibited no statistically significant changes following VCD injections *(p=0.17)*. Conversely, DHEA injections and the applied COH protocol triggered a notable increase in ovarian tissue weights *(p=0.02 and p=0.03, respectively)*.

3.2. Confirmation and Biochemical Evaluation of POI Model

The ovarian follicle counts within the Control, VCD, and DMSO groups, formed for morphometric assessments, were subjected to comparative analysis. Remarkably, following VCD administration, a statistically significant reduction was observed in the number of follicles across all developmental stages *(p<0.001)*. However, additional DMSO injections demonstrated no discernible influence on follicular development (Table 1, Figure 2 and Figure 4).

Furthermore, blood samples taken on the 15th day of the experiment unveiled a statistically significant decline in serum AMH levels within the VCD-treated groups compared to the DMSO-treated groups *(p=0.031)*. (Table 2).

Ovarian tissue homogenates were assessed across groups with respect to the PTEN/PI3K/pAKT signaling pathway, recognized as a marker for follicular activation. Within the VCD group, a significant increase in PTEN levels *(p=0.001)* and concurrent increases in PI3K *(p<0.001) and* pAKT concentrations *(p=0.006)* were observed in comparison to both the control and DMSO groups (Table 3 and Figure 7). Furthermore, tissue samples from the VCD group displayed statistically significant reductions in TAS levels *(p=0.007)* and increases in caspase-3 *(p<0.001)*, TOS *(p<0.001)*, OSI *(p<0.001)*, and COX-2 *(p<0.001)* concentrations, relative to the Control and DMSO groups (Table 3 and Figure 7).

3.3. Evaluation of the Effects of DHEA in Different Ovarian Reserve Groups in Terms of Histomorphometric and Biochemical Parameters

The administration of DHEA injections to rats in the POI model resulted in a significant decrease in atretic follicle count, accompanied by a significant increase in all other follicle counts (VCD+DHEA vs. VCD+DMSO) (Table 1 and Figure 2). Additionally, in the VCD+DMSO group, which assessed for long-term VCD effects, a statistically significant decrease was observed in primordial and primary follicle numbers, coupled with a noteworthy increase in atretic follicles when contrasted with the VCD group (Table 1 and Figure 2). In contrast, in rats with normal ovarian reserve, DHEA injections induced a statistically significant reduction in primordial and atretic follicles, while all other follicle types remained unchanged (DMSO+DHEA vs. DMSO+DMSO) (Table 1, Figure 2 and Figure 5). Furthermore, irrespective of ovarian reserve (VCD+DHEA vs. VCD+DMSO and DMSO+DHEA vs. DMSO+DMSO), injections of DHEA or DMSO over a 45-day span had no impact on serum AMH levels (Table 2).

After administering DHEA injections, a reduction in PTEN protein levels was observed alongside an elevation in PI3K and pAKT levels within ovarian tissues, both in subjects exhibiting a POI model and those with a normal ovarian reserve. Moreover, while DHEA injections did not induce alterations in the levels of caspase-3, TAS, TOS, and COX-2 in ovarian tissue among subjects with a normal ovarian reserve (DMSO+DHEA group), statistically significant differences were evident in subjects with a POI model (VCD+DHEA) (Table 3 and Figure 7). In comparison to the VCD+DMSO group, diminished levels of caspase-3, TOS, OSI, and COX-2 were discerned in ovarian tissue in the VCD+DHEA group, accompanied by a notably higher TAS level (Table 3 and Figure 7).

3.4. Assessment of COH Protocol Efficacy in Different Ovarian Reserve Groups

In rats subjected to the COH protocol without prior DHEA injections, a statistically significant augmentation was observed in the number of secondary follicles, Graafian follicles, and corpora lutea across both POI and NOR groups, regardless of ovarian reserve (VCD+DMSO+COH vs. VCD+DMSO+Saline) (DMSO+DMSO+COH vs. DMSO+DMSO+Saline) (Table 1 and Figure 2).

Furthermore, among rats with POI, administering DHEA prior to the COH protocol (VCD+DHEA+COH vs. VCD+DMSO+COH) heightened the effectiveness of the COH protocol, leading to a statistically significant increase in the number of follicles across all developmental stages. Conversely,

unlike rats with POI, only secondary follicles, Graafian follicles, and corpora lutea displayed a significant increase in NOR groups when DHEA injections were administered before the COH protocol (DMSO+DHEA+COH vs. DMSO+DMSO+COH) (Table 1, Figure 2 and Figure 6).

Evaluating the ovarian tissue ELISA outcomes, discernible disparities were noted. Specifically, the VCD+DHEA+COH group exhibited statistically significantly elevated levels of PI3K, pAKT, and TAS, alongside decreased TOS, OSI, caspase-3, and COX-2 protein levels compared to the VCD+DMSO+COH group. Conversely, within cohorts characterized by normal ovarian reserve, administration of DHEA injections prior to the COH protocol yielded no discernable alterations in most biochemical parameters, except for elevated PI3K levels and diminished COX-2 levels. Moreover, irrespective of ovarian reserve and DHEA injections, the COH protocol administered to rats did not induce alterations in biochemical parameters (PTEN, PI3K, pAKT, caspas-3, TAS, TOS) with the exception of an elevation in COX-2 (Table 3 and Figure 7).

4. Discussion

This study marks the first examination of the potential impacts of administering DHEA prior to the COH protocol on treatment success within a standardized experimental animal model. Furthermore, it presents a comprehensive assessment of DHEA's effects on follicular development, encompassing both diminished and normal ovarian reserve scenarios. The inclusion of a substantial sample size and various groups contributes to the robustness of our findings. Our results indicate divergent outcomes of DHEA treatment in subjects with differing ovarian reserves. In the context of POI model rats, DHEA administration exhibited a positive influence on ovarian reserve and the targeted antral follicle count post-COH protocol. In contrast, among rats with normal ovarian reserve, while solitary utilization of DHEA did not elicit alterations in ovarian function, its preceding use prior to ovarian stimulation notably heightened the efficacy of the COH protocol.

In delineating the mechanism of action based on our current findings concerning DHEA, several key observations offer insight into its effects. To begin with, DHEA demonstrated a significant augmentation in follicular activation, as evidenced by the upregulation of the PTEN/PI3K/AKT signaling pathway. This signaling cascade plays a pivotal role in promoting the development and survival of ovarian follicles. Androgens have consistently demonstrated associations with heightened follicular recruitment

and growth across various studies [33]. These effects seem to be mediated through an enhancement of the actions of both insulin-like growth factor 1 (IGF-1) and follicle stimulating hormone (FSH) [34,35]. In a notable investigation, DHEA was reported to augment IGF-1 within follicles, thereby potentially amplifying the gonadotropin effect [36]. Moreover, androgens were found to induce an elevated expression of FSH receptor mRNA in preantral follicles [37]. In our investigation, DHEA exhibited a substantial increase in follicular activation, as indicated by the upregulation of the PTEN/PI3K/AKT signaling pathway. This signaling cascade plays a pivotal role in supporting the development and survival of ovarian follicles. While our study did not encompass control over signal pathways at all stages of follicular development, the observed augmentation in the overall number of follicles under DHEA influence suggests a potential impact on primary follicles and beyond. Notably, the escalated count of Graafian follicles, representing the final stage of follicular development, particularly when DHEA is employed in isolation or preceding the KOH protocol, serves as evidence of its influence across all developmental stages. It is imperative to acknowledge that these findings warrant further validation through advanced molecular techniques. Moreover, our study unveiled a concurrent reduction in apoptosis, as demonstrated by a decrease in caspase-3 levels in ovarian tissue. This anti-apoptotic effect suggests that DHEA contributes to the preservation and viability of ovarian cells, creating a more favorable environment for follicular development. Additionally, DHEA administration resulted in a mitigation of oxidative stress within the ovarian tissue. This was characterized by an increase in TAS and lower levels of TOS and OSI. These findings imply that DHEA may possess antioxidative properties, alleviating the adverse impact of oxidative stress on ovarian function. Finally, our investigation indicated a reduction in inflammation in the ovarian tissue of rats with POI following DHEA injections. This anti-inflammatory effect was evidenced by decreased levels of COX-2, suggesting that DHEA modulates inflammatory processes associated with ovarian dysfunction. The observed divergent response between groups with normal ovarian reserve and those with POI can be attributed to inherent disparities in the ovarian microenvironment and hormonal profiles within these distinct populations. In individuals with normal ovarian reserve, the endogenous production of hormones and finely tuned regulatory mechanisms governing ovarian function may already be optimized. Consequently, the introduction of exogenous DHEA may not exert a discernible effect on the pre-existing well-balanced system. The physiological equilibrium in individuals with normal ovarian reserve may render them less susceptible to additional interventions, including DHEA supplementation.

Conversely, in the POI group, the addition of DHEA may act as a compensatory factor, potentially ameliorating or enhancing deficient pathways associated with follicular development. The presence of compromised endogenous hormonal milieu and regulatory mechanisms in individuals with POI makes them more responsive to the beneficial effects of DHEA supplementation. In this context, DHEA may serve to repair or improve specific deficiencies, thereby contributing to the observed positive outcomes in follicular development within the POI group.

Numerous studies have demonstrated similar effects of DHEA in tissues exhibiting heightened inflammation, highlighting its role in modulating the inflammatory response. For instance, DHEA administration has been shown to mitigate inflammation in cartilage tissue among rheumatoid arthritis patients [38]. In a study by Malik et al. [39], cortical tissue inflammation decreased following DHEA injections in female Sprague-Dawley rats subjected to traumatic brain injury, which induced an augmented systemic inflammatory response. Moreover, 7-Hydroxy-DHEA, a metabolite of DHEA, has been found to modulate inflammation in experimental colitis model rats [40]. Additionally, numerous investigations across diverse tissues have evidenced DHEA's capacity to reduce pro-inflammatory cytokines [41–43]. Moreover, similar to DHEA, antioxidant and antiapoptotic agents such as Coenzyme Q10 [44] and melatonin [45,46] exhibit tissue-specific effects.

Previous studies have documented limited clinical success rates in cases of POI, regardless of the adopted ovarian stimulation protocol [47–49]. However, the presence of non-standardized diagnostic criteria in these studies and the limited statistical significance of meta-analyses due to sample size constraints are pertinent limitations [50]. The absence of a universal definition greatly complicates the comparison of treatment protocols adopted in various studies [51]. Consequently, establishing a standardized animal model to evaluate the impact of DHEA emerges as a pivotal step in addressing these challenges. In our present investigation, rats were selected as the standardized animal model. The choice of rats in scientific research is supported by multiple factors. Firstly, rats have been widely employed in animal modeling, as extensively documented in the literature. Secondly, their prompt sexual maturation, hormonal regulation resembling that of humans, and the absence of a seasonal menstrual cycle, but rather exhibiting cyclic estrous cycles from puberty to menopause, align them closely with human reproductive characteristics. Additionally, rats exhibit advantageous attributes for research purposes. Their capacity for superovulation induction and larger size in comparison to mice facilitate the process of ovarian tissue

sampling and evaluation. These characteristics collectively render rats a favored model in reproductive and physiological studies.

Findings from our study corroborate prior research indicating that both VCD [52,53] and DHEA [54,55] injections do not exert an impact on body weight. This observation suggests that injections of VCD and DHEA do not impact food consumption or subject metabolism. Given that body mass index is a pivotal determinant in adjusting gonadotropin doses and regulating the follicular development process within ovarian stimulation protocols [56], the lack of statistically significant variance among the groups in terms of body weight measurements contributes to standardization, enabling a proper assessment of the COH protocol's efficacy. Furthermore, consistent with existing literature [57], no statistically significant shift in ovarian tissue weights emerged following VCD injections. This alignment supports the notion that VCD injections possess a specific effect on primordial and primary follicles, rather than a generalized impact on ovarian tissue. Conversely, DHEA injections and the COH protocol prompted a noteworthy upswing in ovarian tissue weights. This escalation in ovarian weight was attributed to the steroidogenic effects induced by DHEA, functioning as a precursor to estradiol and testosterone [58,59]. Additionally, it is challenging to draw direct comparisons from individual histological sections; however, the provided figures offer comprehensive representations of tissue integrity for each respective group. Although some individual sections may not inherently embody the entire group characteristics, they serve as a basis for inter-group comparisons. Within the scope of our investigation, variations in the observation of ovarian medullary areas were contingent upon the section plane, precluding a thorough stromal assessment. Furthermore, it is noteworthy that an augmented presence of vascular structures, particularly evident in groups subjected to the COH protocol, was observed in the medulla layer (Figure 6B). A precedent study attested to the impact of ovarian stimulation on altering ovarian tissue physiology, concomitantly augmenting blood flow and ovarian volume [60]. Blood flow assumes a pivotal role in both the folliculogenesis and steroidogenesis stages within ovarian tissue [61]. Primordial and primary follicles sustain their viability through the support of stromal vascular structures [62]. Our findings align with this, demonstrating heightened folliculogenesis and increased vascular structures following the COH protocol, substantiating the efficacy of the employed COH protocol.

In the context of primordial and primary follicles, the KITLG/KIT pathway exerts a pivotal role in fostering oocyte viability and persistence [63,64]. Previous investigations have revealed that VCD

induces a reduction in KIT mRNA expression while elevating KITLG mRNA expression, thereby exerting toxic effects on the ovary through inhibition of the KITLG/KIT signaling pathway [65,66]. In the realm of POI models within existing literature, employing VCD at the identical dosage and timing as our study has yielded variable outcomes concerning follicle reserve and developmental progression. In a study by Mayer et al., a decrease in primordial and primary follicle counts post VCD exposure was noted, with secondary follicles, antral follicles, and corpora lutea numbers remaining unchanged [53]. Conversely, other studies have documented decreases across all follicular developmental stages [18,67]. Our findings align with studies showcasing VCD's impact on follicle counts across all developmental stages, a result reinforced by serum AMH concentrations within our investigation. Thus, the establishment of a standardized, reproducible experimental POI model via VCD injections gains validation. The incorporation of the VCD+DMSO group within our experimental lineup, aimed at neutralizing the influence of advancing age on follicle reserve, unearthed a statistically significant decrease in primordial and primary follicle numbers, coupled with a noteworthy increase in atretic follicles post injections. These insights corroborate the enduring negative impact of VCD, compounded by advancing age, on primordial and primary follicles. Furthermore, in our investigation, DMSO served as the solvent for VCD and DHEA. DMSO, commonly employed as a cryoprotectant despite concerns regarding its cytotoxicity, has been extensively utilized for both VCD [68–70] and DHEA [71,72] in the existing literature. Furthermore, a separate study demonstrated that the administration of DMSO at a dosage of 5 ml/kg per day for one month was well-tolerated [73], thereby excluding any toxic effects associated with the DMSO dosage employed in our investigation. The consistency noted in ovarian morphology, tissue biochemical analyses, and follicular development between the DMSO group and the control group in our study is in concordance with and strengthens findings from previous research.

In the ovarian tissue sections of the control group, each follicle exhibited an oocyte with euchromatic nuclei and granulosa cells in contact, displaying normal morphological features. The resemblance of the DMSO group sections, considered the sham group, to the control group implied that DMSO injections did not induce any discernible toxicity in the ovarian tissue. Conversely, the VCD group demonstrated an increase in atretic follicles, indicative of diminished ovarian reserve. This was accompanied by features such as acidophilia, fragmentation, and vacuolization in oocytes, along with an elevated presence of pyknotic-looking nuclei in granulosa cells—consistent with morphological

characteristics associated with apoptosis in VCD, as reported in previous studies [74,75]. Furthermore, ovarian tissue sections from the VCD group revealed atretic follicles exhibiting a glassy membrane appearance. Numerous studies have corroborated the assertion that VCD promotes apoptosis in follicles across various developmental stages [76–78]. In the current investigation, the histological observations and caspase-3 protein levels supported and reinforced findings from prior research. Notably, it was observed that the administration of DHEA in the POI group resulted in a reduction of apoptotic cells in primary oocytes, granulosa cells, theca cells, and stromal cells.

In the management of patients with POI, the application of assisted reproductive techniques becomes necessary to enhance the quantity, quality, and sufficiency of follicles contributing to folliculogenesis and steroidogenesis in the ovaries. Nonetheless, the challenging response of these cases to ovarian stimulation presents a dilemma [11]. In the 2019 report by the US Centers for Disease Control and Prevention (CDC), it was highlighted that the live birth rate stood at 37.2% across all IVF cycles, displaying a decline as per the underlying causes of infertility. Specifically, the lowest rate of 14.7% was recorded in patients diagnosed with DOR [79]. The same report also noted oocyte donation as a predominant treatment avenue for DOR patients. Moreover, a distinct study established a significant link between the count of oocytes retrieved during IVF cycles and the likelihood of a live birth, underscoring that an additional oocyte in cases with DOR, where 2 or 3 oocytes are attainable, results in a twofold rise in the live birth rate. These observations underline the critical role of treatments applied in POI scenarios [80]. In light of these insights, devising a COH protocol alongside complementary interventions aimed at augmenting oocyte retrieval in POI cases promises to elevate clinical success rates. To this end, our study centers on the impact of DHEA on ovarian function. The primordial follicle reservoir represents a paramount indicator in assessing ovarian potential [3,81]. Correspondingly, serum AMH levels stand as another pivotal marker for determining ovarian reserve [82,83]. In alignment with this premise, the effectiveness of DHEA was evaluated both histomorphologically through ovarian follicle counts and via biochemical analyses. Early studies in this field reported increased ovarian response [36], enhanced oocyte retrieval [84], and heightened clinical pregnancy rates [85] in IVF cycles following DHEA administration. In a subsequent meta-analysis, DHEA's presumed role in antral follicle development was associated with an increase in follicular responsiveness during ovarian stimulation [86]. Nevertheless, the limited population size and heterogeneity within these studies have spurred debates about their outcomes. Our study yielded diverse results in relation

to DHEA treatment across subjects with varying ovarian reserves. Notably, DHEA administration demonstrated a safeguarding effect on ovarian reserve in those with POI, as evidenced by a reduction in the loss of primordial and developing follicles, along with a decrease in the number of atretic follicles. Existing literature also documents increased primordial and developing follicles, and reduced atretic follicles in POI animal models subject to similar intraperitoneal or oral DHEA doses and timings, aligning with our findings [18,87,88]. Furthermore, administering DHEA before the COH protocol to subjects with POI was linked with heightened counts of primordial and all developing follicles, in addition to a decrease in atretic follicles. These outcomes signify that pre-protocol DHEA injections elevate folliculogenesis and the COH protocol's efficiency for those with POI. In contrast, for subjects with normal ovarian reserve, while DHEA injections independently led to a decrease in primordial follicle reserve and atretic follicle count, no notable alteration was observed in developing follicles. Unlike the POI model, the drop in primordial follicle numbers among normal reserve subjects was attributed to DHEA's propensity for expediting the transition from primordial to developing follicles through accelerated folliculogenesis. This result was proven by the upregulation of the ovarian tissue PTEN/PI3K/AKT signaling pathway in our study. This situation was corroborated by the isolated increase in multilaminar primary follicles, accompanied by no change in primordial follicle numbers between the DMSO+DHEA+Saline and DMSO+DMSO+Saline groups, a consistency with extant literature [89]. Moreover, the higher count of Graafian follicles in both the VCD+DHEA+COH and DMSO+DHEA+COH groups compared to the control group can be attributed to the influence of the DHEA+COH protocol. Particularly in the comparisons between the (VCD+DHEA+COH vs. VCD+DMSO+COH) and (DMSO+DHEA+COH vs. DMSO+DMSO+COH) groups, it was observed that the administration of DHEA before the COH protocol amplified this effect.

The activation of the PI3K/AKT signaling pathway by dehydroepiandrosterone (DHEA) has been observed in various extragonadal tissues, including the prostate [90], liver [91], and testis [92]. However, the precise mechanisms underlying DHEA's influence on the PTEN/PI3K/pAKT signaling pathway remain ambiguous, whether through genetic or epigenetic interactions, or via receptor or protein-level interactions. Subsequent investigations into cellular signal transduction networks and molecular biology are poised to elucidate these mechanisms further.

This study presents some inherent limitations, including the utilization of an animal model and the absence of direct clinical outcomes. One notable limitation of our study is the reliance on ELISA analysis for assessing the PTEN/PI3K/AKT signaling pathway at the tissue level. Given that these molecules serve as intracellular signal transducers, a more precise and specific evaluation of their expression levels in oocytes can be achieved through the immunostaining method applied to tissue sections. In future investigations, the intention is to employ tissue immunohistochemical staining methods to enhance the accuracy and specificity of the results. However, its notable strengths encompass a comprehensive investigation involving a substantial number of subgroups, encompassing diverse ovarian reserve classifications. Despite the constraints found in existing literature, compounded with the paucity of robust randomized controlled trials, the administration of DHEA remains widely adopted within POI patient cohort. This popularity is attributed to its benign side-effect profile, cost-effectiveness, and apparent augmentation of spontaneous pregnancies. The varying effects of DHEA in groups with normal and diminished ovarian reserve may be attributed to the baseline differences in the ovarian microenvironment and hormonal profiles. DHEA's impact may be more pronounced in situations where there is an underlying deficiency or imbalance, potentially explaining the observed positive outcomes in individuals with POI.

In summary, our study indicates that DHEA exerts a positive influence on ovarian reserve and follicular development during COH by upregulating the PTEN/PI3K/AKT signaling pathway, inhibiting apoptosis, mitigating oxidative stress, and suppressing inflammation within the ovarian microenvironment. Our results strongly propose that DHEA may yield positive effects on ovarian stimulation response, achieved through augmenting the initiation of primordial follicles and bolstering antral follicle populations.

Acknowledgment This study was supported through grants from The Scientific and Technological Research Council of Turkey (TUBITAK), under Grant Number: 218S810. The authors declare no conflicts of interest.

References

1. Thoma ME, McLain AC, Louis JF, King RB, Trumble AC, Sundaram R, et al. Prevalence of infertility in the United States as estimated by the current duration approach and a traditional constructed approach. Fertil Steril. 2013;99(5):1324-1331.e1.
2. Report NS. Assisted Reproductive Technology 2016 National Summary Report. Natl Cent Chronic Dis Prev Heal Promot Div Reprod Heal. 2018;1–74.
3. Lass A. Assessment of ovarian reserve - is there a role for ovarian biopsy? Hum Reprod. 2001 Jun 1;16(6):1055–7.
4. Seifer DB, Lambert-Messerlian G, Hogan JW, Gardiner AC, Blazar AS, Berk CA. Day 3 serum inhibin-B is predictive of assisted reproductive technologies outcome. Fertil Steril. 1997 Jan;67(1):110–4.
5. Broekmans FJ, Soules MR, Fauser BC. Ovarian aging: Mechanisms and clinical consequences. Vol. 30, Endocrine Reviews. 2009. p. 465–93.
6. Ubaldi F, Vaiarelli A, D'Anna R, Rienzi L. Management of poor responders in IVF: Is there anything new? Vol. 2014, BioMed Research International. 2014. p. 352098.
7. Madani T, Ashrafi M, Yeganeh LM. Comparison of different stimulation protocols efficacy in poor responders undergoing IVF: A retrospective study. Gynecol Endocrinol. 2012;28(2):102–5.
8. Pandian Z, McTavish AR, Aucott L, Hamilton MP, Bhattacharya S. Interventions for "poor responders" to controlled ovarian hyper stimulation (COH) in in-vitro fertilisation (IVF). Cochrane Database Syst Rev. 2010;20:(1):CD004379.
9. Van Wely M, Kwan I, Burt AL, Thomas J, Vail A, Van der Veen F, et al. Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. A cochrane review. Hum Reprod Update. 2012;18(2):111.
10. Lekamge DN, Lane M, Gilchrist RB, Tremellen KP. Increased gonadotrophin stimulation does not improve IVF outcomes in patients with predicted poor ovarian reserve. J Assist Reprod Genet. 2008;25(11–12):515–21.
11. Reed BG, Babayev SN, Bukulmez O. Shifting paradigms in diminished ovarian reserve and advanced reproductive age in assisted reproduction: Customization instead of conformity. Semin Reprod Med. 2015;33(3):169–78.
12. Harper AJ, Buster JE, Casson PR. Changes in adrenocortical function with aging and therapeutic implications. Vol. 17, Seminars in Reproductive Endocrinology. 1999. p. 327–38.
13. Labrie F. DHEA, important source of sex steroids in men and even more in women. Prog Brain Res. 2010;182.
14. Patrizio P, Vaiarelli A, Levi Setti PE, Tobler KJ, Shoham G, Leong M, et al. How to define, diagnose and treat poor responders? Responses from a worldwide survey of IVF clinics. Reprod Biomed Online. 2015;30(6):581–92.
15. Hu X, Christian P, Sipes IG, Hoyer PB. Expression and Redistribution of Cellular Bad, Bax, and Bcl-xL Protein Is Associated with VCD-Induced Ovotoxicity in Rats1. Biol Reprod.

2001;65(5):1489–95.
16. Pestana-Oliveira N, Carolino ROG, Kalil-Cutti B, Leite CM, Dalpogeto LC, De Paula BB, et al. Development of a chemical reproductive aging model in female rats. Bio-protocol. 2021;11(8).
17. Liu W, Wang LY, Xing XX, Fan GW. Conditions and possible mechanisms of VCD-induced ovarian failure. ATLA Altern to Lab Anim. 2015;43(6).
18. Hassa H, Aydin Y, Ozatik O, Erol K, Ozatik Y. Effects of dehydroepiandrosterone (DHEA) on follicular dynamics in a diminished ovarian reserve in vivo model. Syst Biol Reprod Med. 2015;61(3):117–21.
19. Li J, Fan S, Han D, Xie J, Kuang H, Ge P. Microarray gene expression profiling and bioinformatics analysis of premature ovarian failure in a rat model. Exp Mol Pathol. 2014;97(3):535–41.
20. Kuang H, Han D, Xie J, Yan Y, Li J, Ge P. Profiling of differentially expressed microRNAs in premature ovarian failure in an animal model. Gynecol Endocrinol. 2014;30(1):57–61.
21. Mayer LP, Pearsall NA, Christian PJ, Devine PJ, Payne CM, McCuskey MK, et al. Long-term effects of ovarian follicular depletion in rats by 4-vinylcyclohexene diepoxide. Reprod Toxicol. 2002;16(6):775–81.
22. Mayer LP, Devine PJ, Dyer CA, Hoyer PB. The Follicle-Deplete Mouse Ovary Produces Androgen1. Biol Reprod. 2004 Jul 1;71(1):130–8.
23. Lohff JC, Christian PJ, Marion SL, Arrandale A, Hoyer PB. Characterization of cyclicity and hormonal profile with impending ovarian failure in a novel chemical-induced mouse model of perimenopause. Comp Med. 2005;55(6).
24. Muhammad FS, Goode AK, Kock ND, Arifin EA, Cline JM, Adams MR, et al. Effects of 4-vinylcyclohexene diepoxide on peripubertal and adult sprague-dawley Rats: Ovarian, clinical, and pathologic outcomes. Comp Med. 2009;59(1):46–59.
25. Gong X, Tong Q, Chen Z, Zhang Y, Xu C, Jin Z. Microvascular density and vascular endothelial growth factor and osteopontin expression during the implantation window in a controlled ovarian hyperstimulation rat model. Exp Ther Med. 2015;9(3):773–9.
26. Mohammed S, Sundaram V. Comparative study of metachromatic staining methods in assessing the exfoliative cell types during oestrous cycle in Sprague-Dawley laboratory rats. Int J Morphol. 2018;36(3).
27. Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: Some helpful considerations. Brazilian J Biol. 2002 Nov;62(4 A):609–14.
28. McLean AC, Valenzuela N, Fai S, Bennett SAL. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp. 2012;(67).
29. Sharma A, Fish BL, Moulder JE, Medhora M, Baker JE, Mader M, et al. Safety and blood sample volume and quality of a refined retro-orbital bleeding technique in rats using a lateral approach. Lab Anim (NY). 2014;43(2).
30. Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissueand cell sections. Cold Spring Harb Protoc. 2008;3(5).

31. Taşdemir M, Ekiz-Yılmaz T, Uğur-Yılmaz C, Orhan N, Doğan MA, Arıcan N, et al. The effects of CLP-induced sepsis on proliferation and apoptosis of granulosa and theca cells in rat ovary: A histochemical and ultrastructural study. Reprod Biol. 2020;20(3).
32. Akbaş N, Suleyman B, Mammadov R, Yazıcı GN, Bulut S, Süleyman H. Effect of taxifolin on cyclophosphamide-induced oxidative and inflammatory bladder injury in rats. Exp Anim. 2022;71(4):460–7.
33. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. Androgens stimulate early stages of follicular growth in the primate ovary. J Clin Invest. 1998;101(12):2622–9.
34. Vendola K, Zhou J, Wang J, Bondy CA. Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. Hum Reprod. 1999;14(9):2328–32.
35. Weil S, Vendola K, Zhou J, Bondy CA. Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. J Clin Endocrinol Metab. 1999;84(8):2951–6.
36. Casson PR. Dehydroepiandrosterone supplementation augments ovarian stimulation in poor responders: a case series. Hum Reprod. 2000;15(10):2129–32.
37. Xue K, Liu JY, Murphy BD, Tsang BK. Orphan nuclear receptor NR4A1 is a negative regulator of DHT-induced rat preantral follicular growth. Mol Endocrinol. 2012;26(12):2004–15.
38. Sun JS, Wu CX, Tsuang YH, Chen LT, Sheu SY. The in vitro effects of dehydroepiandrosterone on chondrocyte metabolism. Osteoarthr Cartil. 2006;14(3):238–49.
39. Malik AS, Narayan RK, Wendling WW, Cole RW, Pashko LL, Schwartz AG, et al. A novel dehydroepiandrosterone analog improves functional recovery in a rat traumatic brain injury model. J Neurotrauma. 2003;20(5):463–76.
40. Hennebert O, Pelissier MA, Le Mee S, Wülfert E, Morfin R. Anti-inflammatory effects and changes in prostaglandin patterns induced by 7β-hydroxy-epiandrosterone in rats with colitis. J Steroid Biochem Mol Biol. 2008;110(3–5):255–62.
41. Straub RH, Konecna L, Hrach S, Rothe G, Kreutz M, Schölmerich J, et al. Serum dehydroepiandrosterone (DHEA) and DHEA sulfate are negatively correlated with serum interleukin-6 (IL-6), and DHEA inhibits IL-6 secretion from mononuclear cells in man in vitro: Possible link between endocrinosenescence and immunosenescence. J Clin Endocrinol Metab. 1998;83(6):2012–7.
42. Ben-Nathan D, Padgett DA, Loria RM. Androstenediol and dehydroepiandrosterone protect mice against lethal bacterial infections and lipopolysaccharide toxicity. J Med Microbiol. 1999;48(5):425–31.
43. Weiss EP, Villareal DT, Fontana L, Han Dong-Ho DH, Holloszy JO. Dehydroepiandrosterone (DHEA) replacement decreases insulin resistance and lowers inflammatory cytokines in aging humans. Aging (Albany NY). 2011;3(5):533–42.
44. Darabi Z, Basir Z, Tabandeh MR, Ghotbeddin Z. Coenzyme Q10 improves ovarian histology and attenuates the expression of angiogenesis-associated proteins in the ovary of rats with experimental hyperstimulation syndrome. Iran J Basic Med Sci. 2022;26(8):989–96.

45. Feng J, Ma WW, Li HX, Pei XY, Deng SL, Jia H, et al. Melatonin prevents cyclophosphamide-induced primordial follicle loss by inhibiting ovarian granulosa cell apoptosis and maintaining AMH expression. Front Endocrinol (Lausanne). 2022;13:895095.
46. Xing F, Wang M, Ding Z, Zhang J, Ding S, Shi L, et al. Protective Effect and Mechanism of Melatonin on Cisplatin-Induced Ovarian Damage in Mice. J Clin Med. 2022;11(24):7383.
47. Yu R, Jin H, Huang X, Lin J, Wang P. Comparison of modified agonist, mild-stimulation and antagonist protocols for in vitro fertilization in patients with diminished ovarian reserve. J Int Med Res. 2018;46(6).
48. Boza A, Cakar E, Boza B, Api M, Kayatas S, Sofuoglu K. Microdose flare-up gonadotropin-releasing hormone (GnRH) agonist versus GnRH antagonist protocols in poor ovarian responders undergoing intracytoplasmic sperm injection. J Reprod Infertil. 2016;17(3).
49. Papamentzelopoulou M, Stavros S, Mavrogianni D, Kalantzis C, Loutradis D, Drakakis P. Meta-analysis of GnRH-antagonists versus GnRH-agonists in poor responder protocols. Arch Gynecol Obstet. 2021;304(2):547–57.
50. Esteves SC, Roque M, Bedoschi GM, Conforti A, Humaidan P, Alviggi C. Defining low prognosis patients undergoing assisted reproductive technology: POSEIDON criteria-the why. Vol. 9, Frontiers in Endocrinology. 2018. p. 17:9:461.
51. Cohen J, Chabbert-Buffet N, Darai E. Diminished ovarian reserve, premature ovarian failure, poor ovarian responder—a plea for universal definitions. J Assist Reprod Genet. 2015;32(12):1709–12.
52. Berger T, Horner CM. In vivo exposure of female rats to toxicants may affect oocyte quality. Reprod Toxicol. 2003;17(3):273–81.
53. Mayer LP, Devine PJ, Dyer CA, Hoyer PB. The follicle-deplete mouse ovary produces androgen. Biol Reprod. 2004;71(1).
54. Medina MC, Souza LC, Caperuto LC, Anhê GF, Amanso AM, Teixeira VPA, et al. Dehydroepiandrosterone increases β-cell mass and improves the glucose-induced insulin secretion by pancreatic islets from aged rats. FEBS Lett. 2006;580(1):285–90.
55. Mauriège P, Martel C, Langin D, Lacaille M, Després JP, Bélanger A, et al. Chronic effects of dehydroepiandrosterone on rat adipose tissue metabolism. Metabolism. 2003;52(3):264–72.
56. Maged AM, Fahmy RM, Rashwan H, Mahmood M, Hassan SM, Nabil H, et al. Effect of body mass index on the outcome of IVF cycles among patients with poor ovarian response. Int J Gynecol Obstet. 2019;144(2).
57. Flaws JA, Doerr JK, Sipes IG, Hoyer PB. Destruction of preantral follicles in adult rats by 4-vinyl-1-cyclohexene diepoxide. Reprod Toxicol. 1994;8(6):509–14.
58. Neunzig J, Bernhardt R. Dehydroepiandrosterone sulfate (DHEAS) stimulates the first step in the biosynthesis of steroid hormones. PLoS One. 2014;9(2).
59. Chimote BN, Chimote NM. Dehydroepiandrosterone (DHEA) and Its Sulfate (DHEA-S) in Mammalian Reproduction: Known Roles and Novel Paradigms. In: Vitamins and Hormones. 2018. p. 223–50.
60. Pan HA, Wu MH, Cheng YC, Wu LH, Chang FM. Quantification of ovarian Doppler signal in

hyperresponders during in vitro fertilization treatment using three-dimensional power Doppler ultrasonography. Ultrasound Med Biol. 2003;29(7):921–7.

61. Abulafia O, Sherer DM. Angiogenesis of the ovary. Am J Obstet Gynecol. 2000;182(1):240–6.

62. Findlay JK. Angiogenesis in reproductive tissues. Vol. 111, Journal of Endocrinology. 1986. p. 357–66.

63. Jin X, Han CS, Yu FQ, Wei P, Hu ZY, Liu YX. Anti-apoptotic action of stem cell factor on oocytes in primordial follicles and its signal transduction. Mol Reprod Dev. 2005;70(1):82–90.

64. Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology. 1999 Sep 1;140(9):4262–71.

65. Keating AF, J. Mark C, Sen N, Sipes IG, Hoyer PB. Effect of phosphatidylinositol-3 kinase inhibition on ovotoxicity caused by 4-vinylcyclohexene diepoxide and 7, 12-dimethylbenz[a]anthracene in neonatal rat ovaries. Toxicol Appl Pharmacol. 2009;241(2):127–34.

66. Keating AF, Fernandez SM, Mark-Kappeler CJ, Sen N, Sipes IG, Hoyer PB. Inhibition of PIK3 Signaling Pathway Members by the Ovotoxicant 4-Vinylcyclohexene Diepoxide in Rats. Biol Reprod. 2011;84(4):743–51.

67. Ahmadian S, Sheshpari S, Pazhang M, Bedate AM, Beheshti R, Abbasi MM, et al. Intra-ovarian injection of platelet-rich plasma into ovarian tissue promoted rejuvenation in the rat model of premature ovarian insufficiency and restored ovulation rate via angiogenesis modulation. Reprod Biol Endocrinol. 2020;18(1).

68. Lukefahr AL, Frye JB, Wright LE, Marion SL, Hoyer PB, Funk JL. Decreased bone mineral density in rats rendered follicle-deplete by an ovotoxic chemical correlates with changes in follicle-stimulating hormone and inhibin A. Calcif Tissue Int. 2012;90(3):239–49.

69. Mark-Kappeler CJ, Sen N, Keating AF, Sipes IG, Hoyer PB. Distribution and responsiveness of rat anti-Müllerian hormone during ovarian development and VCD-induced ovotoxicity. Toxicol Appl Pharmacol. 2010;249(1):1–7.

70. Frye JB, Lukefahr AL, Wright LE, Marion SL, Hoyer PB, Funk JL. Modeling perimenopause in sprague-dawley rats by chemical manipulation of the transition to ovarian failure. Comp Med. 2012;62(3):193–202.

71. Ozatik FY, Ozatik O, Yigitaslan S, Kaygısız B, Erol K. Do resveratrol and dehydroepiandrosterone increase diminished ovarian reserve? Eurasian J Med. 2020;52(1):6–11.

72. Hu Y, Cardounel A, Gursoy E, Anderson P, Kalimi M. Anti-stress effects of dehydroepiandrosterone: Protection of rats against repeated immobilization stress-induced weight loss, glucocorticoid receptor production, and lipid peroxidation. Biochem Pharmacol. 2000;59(7):753–62.

73. Gad SC, Cassidy CD, Aubert N, Spainhour B, Robbe H. Nonclinical vehicle use in studies by multiple routes in multiple species. Int J Toxicol. 2006;25(6):499–521.

74. Springer LN, McAsey ME, Flaws JA, Tilly JL, Sipes IG, Hoyer PB. Involvement of apoptosis in 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats. Toxicol Appl Pharmacol. 1996;139(2):394–401.

75. Kao SW, Sipes IG, Hoyer PB. Early effects of ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats and mice. Reprod Toxicol. 1999;13(1):67–75.
76. Devine PJ, Sipes IG, Skinner MK, Hoyer PB. Characterization of a rat in vitro ovarian culture system to study the ovarian toxicant 4-vinylcyclohexene diepoxide. Toxicol Appl Pharmacol. 2002;184(2):107–15.
77. Hu X, Flaws JA, Sipes IG, Hoyer PB. Activation of Mitogen-Activated Protein Kinases and AP-1 Transcription Factor in Ovotoxicity Induced by 4-Vinylcyclohexene Diepoxide in Rats1. Biol Reprod. 2002;67(3):718–24.
78. Springer LN, Tilly JL, Sipes IG, Hoyer PB. Enhanced expression of bar in small preantral follicles during 4-vinylcyclohexene diepoxide-induced ovotoxicity in the rat. Toxicol Appl Pharmacol. 1996;139(2):402–10.
79. Centers_for_National_Disease_Control_and_Prevention. Assisted reproductive technology 2009: Success rates [Internet]. National Summary and Fertility Clinic Report. 2009.
80. Sunkara SK, Rittenberg V, Raine-Fenning N, Bhattacharya S, Zamora J, Coomarasamy A. Association between the number of eggs and live birth in IVF treatment: An analysis of 400 135 treatment cycles. Hum Reprod. 2011;26(7):1768–74.
81. Findlay JK, Hutt KJ, Hickey M, Anderson RA. How is the number of primordial follicles in the ovarian reserve established? Vol. 93, Biology of Reproduction. 2015. p. 93(5):111.
82. Deeks ED. Elecsys® AMH Assay: A Review in Anti-Müllerian Hormone Quantification and Assessment of Ovarian Reserve. Mol Diagnosis Ther. 2015;19(4).
83. Peluso C, Fonseca FLA, Rodart IF, Cavalcanti V, Gastaldo G, Christofolini DM, et al. AMH: An ovarian reserve biomarker in assisted reproduction. Vol. 437, Clinica Chimica Acta. 2014. p. 175–82.
84. Barad D, Gleicher N. Effect of dehydroepiandrosterone on oocyte and embryo yields, embryo grade and cell number in IVF. Hum Reprod. 2006;21(11):2845–9.
85. Barad D, Brill H, Gleicher N. Update on the use of dehydroepiandrosterone supplementation among women with diminished ovarian function. In: Journal of Assisted Reproduction and Genetics. 2007. p. 629–34.
86. Sunkara SK, Pundir J, Khalaf Y. Effect of androgen supplementation or modulation on ovarian stimulation outcome in poor responders: A meta-analysis [Internet]. Vol. 22, Reproductive BioMedicine Online. Reproductive Healthcare Ltd.; 2011. p. 545–55.
87. Mahmoud YI, Mahmoud AA, Abo-Zeid FS, Fares NH. Effects of dehydroepiandrosterone on the ovarian reserve and pregnancy outcomes in perimenopausal rats (DHEA and fertility in perimenopausal rats). Life Sci. 2018 Apr;199:131–8.
88. Sozen B, Ozekinci M, Erman M, Gunduz T, Demir N, Akouri R. Dehydroepiandrosterone supplementation attenuates ovarian ageing in a galactose-induced primary ovarian insufficiency rat model. J Assist Reprod Genet. 2019;36(10):2181–9.
89. Narkwichean A, Jayaprakasan K, Maalouf WE, Hernandez-Medrano JH, Pincott-Allen C, Campbell BK. Effects of dehydroepiandrosterone on in vivo ovine follicular development. Hum

Reprod. 2014;29(1):146–54.

90. Sun HZ, Yang TW, Zang WJ, Wu SF. Dehydroepiandrosterone-induced proliferation of prostatic epithelial cell is mediated by NFKB via PI3K/AKT signaling pathway. J Endocrinol. 2010;204(3):311–8.

91. Li L, Zhao J, Ge C, Yu L, Ma H. Dehydroepiandrosterone rehabilitate BRL-3A cells oxidative stress damage induced by hydrogen peroxide. J Cell Physiol. 2018;233(8):6262–72.

92. Ding X, Wang D, Li L, Ma H. Dehydroepiandrosterone ameliorates H2O2-induced Leydig cells oxidation damage and apoptosis through inhibition of ROS production and activation of PI3K/Akt pathways. Int J Biochem Cell Biol. 2016;70:126–39.

Figures and Tables

Figure 1: Experimental groups of the study

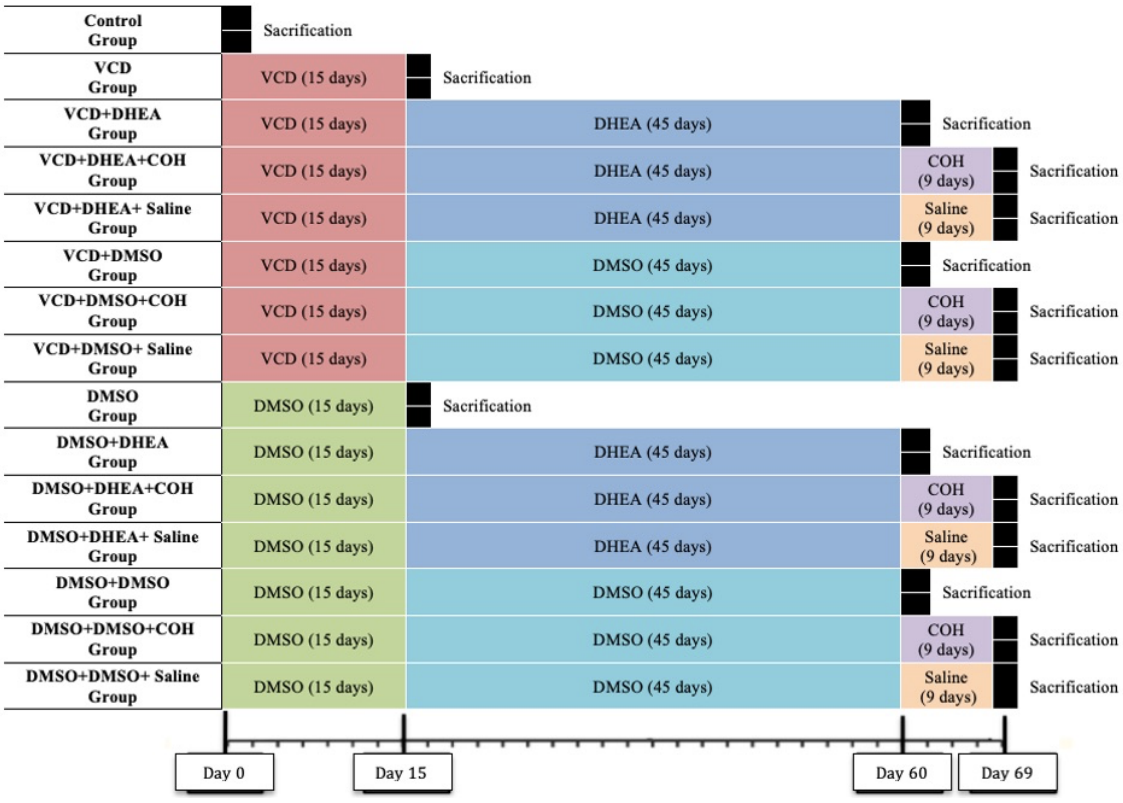

Figure 2: Comparison of preantral and antral follicles counts of the groups.

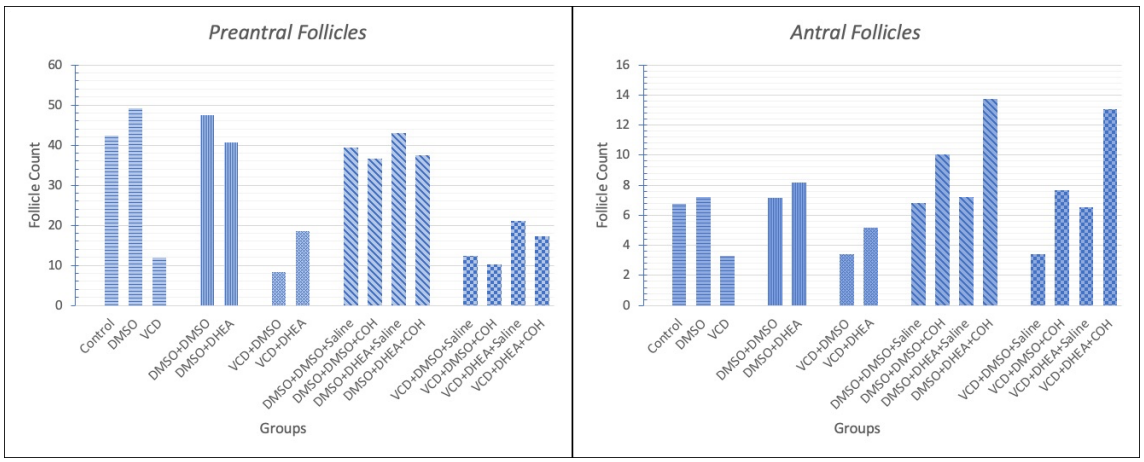

Figure 3: Histomorphologic observation of normal and atretic ovarian follicles across various stages

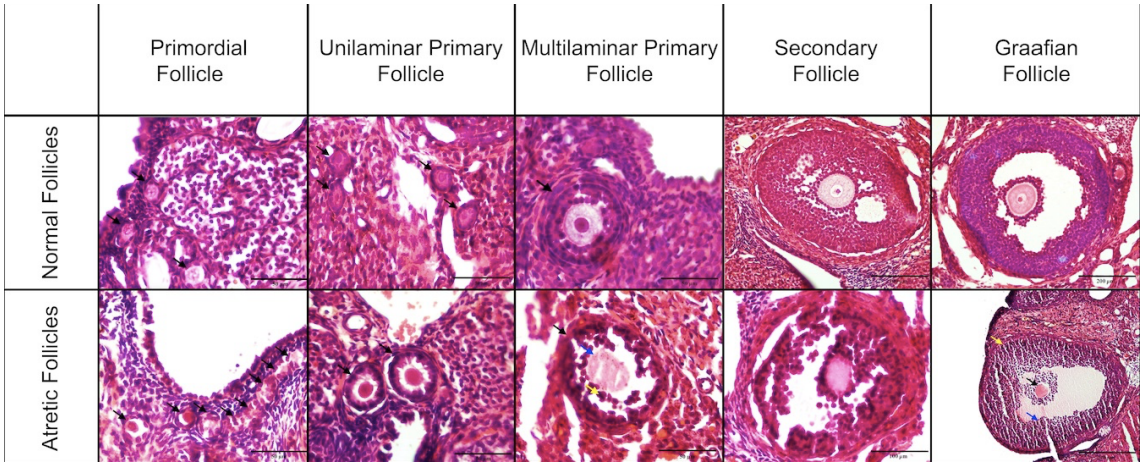

Figure 4: (A) General view of the ovarian tissue section in the DMSO group. (B) General view of the ovarian tissue section in the VCD group

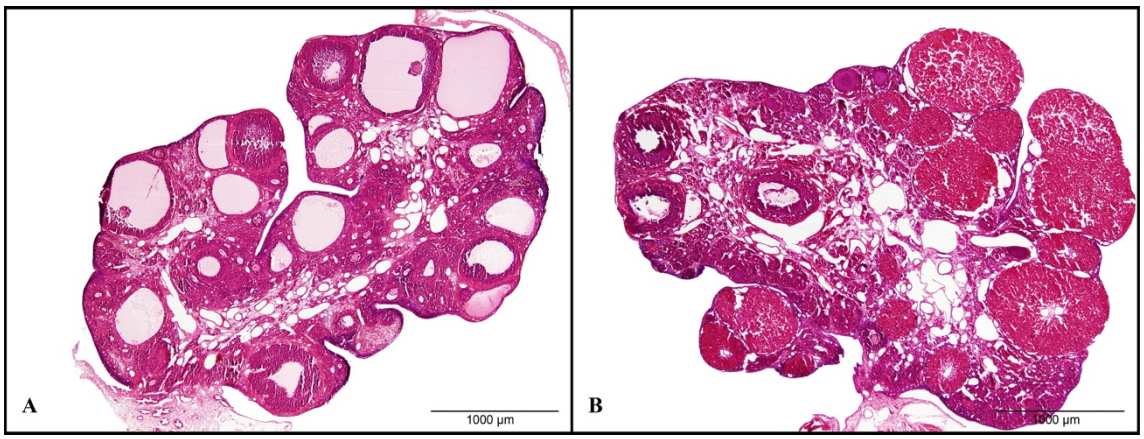

Figure 5: (A) General view of the ovarian tissue section in the DMSO+DHEA group. (B) General view of the ovarian tissue section in the VCD+DHEA group

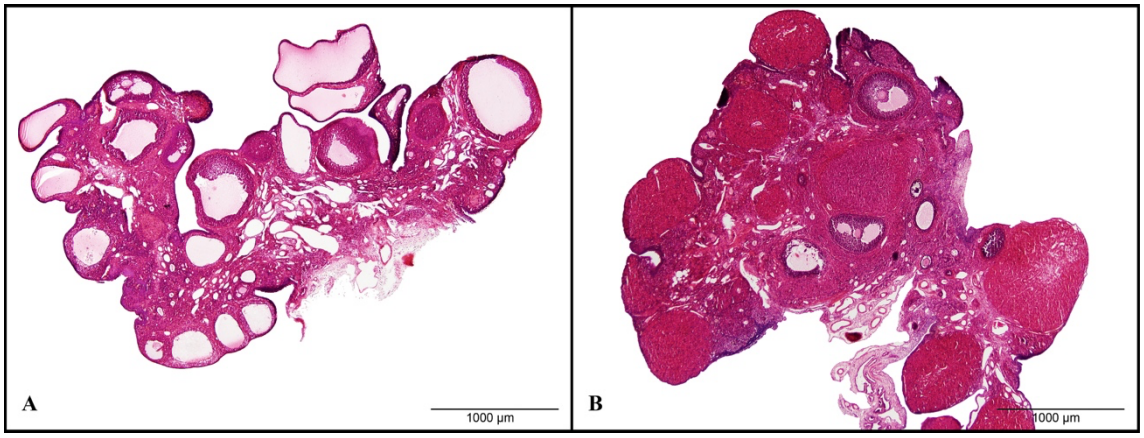

Figure 6: (A) General view of the ovarian tissue section in the DMSO+DHEA+COH group. (B) General view of the ovarian tissue section in the VCD+DHEA+COH group

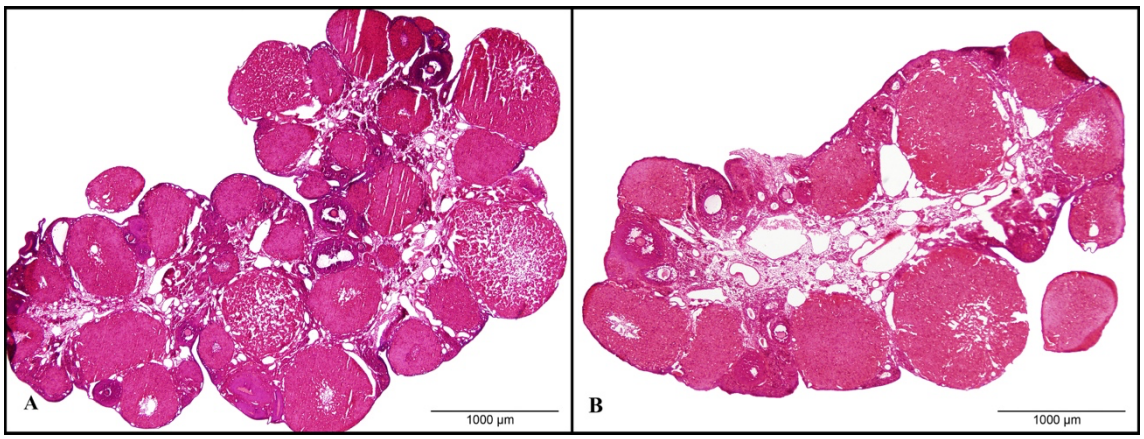

Figure 7: Biochemical evaluation of the subjects in each group: Each distinct dot on the graph represents the protein concentration value of an individual subject. Additionally, each solid line on the graph signifies the median protein concentration value for the respective group.

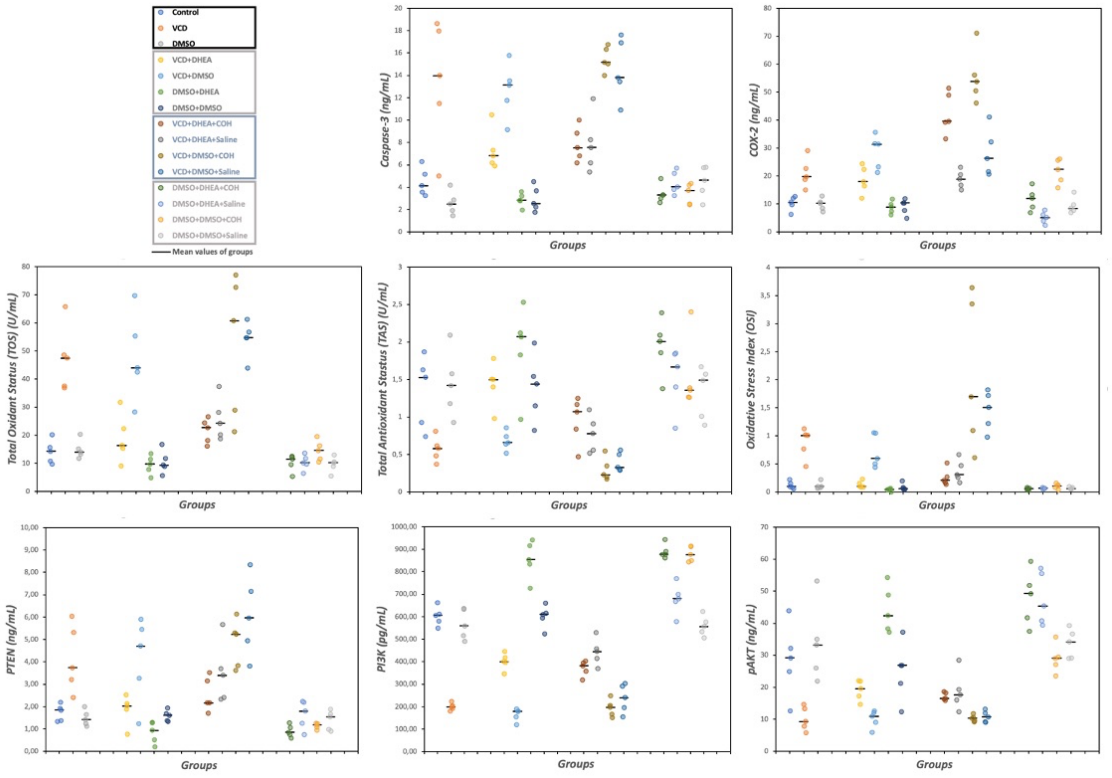

Table 1: Comparison of the ovarian follicles counts of the groups.

	Primordial Follicles	Unilaminar Primary Follicles	Multilaminar Primary Follicles	Secondary Follicles	Graafian Follicles	Atretic Follicles	Corpora Lutea
Control	21.59±1.37[a]	10.80±0.81[a]	9.96±0.74[a]	3.64±0.36[a]	3.12±0.21[a]	1.28±0.15[a]	9.16±0.67[a]
VCD	3.60±0.83[b]	3.53±20.27[b]	4.88±0.80[b]	2.16±0.09[b]	1.12±0.08[b]	3.72±0.41[b]	2.96±0.35[b]
DMSO	25.8±3.29[a]	9.52±0.98[a]	13.76±1.17[a]	4.60±0.42[a]	2.60±0.31[a]	1.16±0.18[a]	7.56±0.92[a]
***p* ***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***0.001***	***<0.001***	***<0.001***
VCD	3.60±0.83[a]	3.53±20.27[a]	4.88±0.80[a]	2.16±0.09[a]	1.12±0.08[a]	3.72±0.41[a]	2.96±0.35[a]
VCD+DHEA	5.24±0.37[b]	6.12±0.41[b]	7.16±0.65[b]	3.16±0.25[b]	2.04±0.18[b]	1.76±0.25[b]	5.92±0.54[b]
VCD+DMSO	2.24±0.30[c]	2.12±0.33[c]	4.04±0.44[a]	2.12±0.25[a]	1.28±0.16[a]	4.94±0.18[c]	2.80±0.14[a]
***p* ***	***<0.001***	***0.006***	***0.004***	***0.007***	***0.001***	***<0.001***	***<0.001***
DMSO	25.81±3.29[a]	9.52±0.98	13.76±1.17	4.60±0.41	2.60±0.31	2.46±0.18[a]	7.56±0.67
DMSO+DHEA	16.92±1.04[b]	8.64±0.90	15.03±0.61	4.56±0.37	3.62±0.37	1.68±0.21[b]	7.16±0.26
DMSO+DMSO	25.76±2.48[a]	9.01±0.91	12.60±1.20	3.44±0.51	3.72±0.50	3.76±0.21[c]	6.56±0.79
***p* ***	***0.01***	*0.87*	*0.2*	*0.6*	*0.3*	***0.012***	*0.23*
VCD+DHEA+COH	6.01±0.48[a]	6.04±0.53[a]	5.20±0.62[a]	6.16±0.63[a]	6.88±0.26[a]	2.08±0.21[a]	6.12±0.57[a]
VCD+DHEA+Saline	5.96±0.31[a]	6.52±0.38[a]	8.56±0.83[b]	3.88±0.27[b]	2.68±0.11[b]	3.62±0.23[b]	4.76±0.48[b]
VCD+DMSO+COH	2.84±0.32[b]	4.32±0.25[b]	3.16±0.35[c]	4.4±0.23[b]	3.24±0.18[b]	3.24±0.15[b]	5.28±0.18[a]
VCD+DMSO+Saline	3.08±0.46[b]	5.08±0.22[b]	4.16±0.38[c]	2.24±0.26[c]	1.16±0.25[c]	3.52±0.40[b]	3.40±0.32[c]
p* *	***<0.001***	***0.001***	***<0.001***	***<0.001***	***<0.001***	***0.004***	***<0.001***
DMSO+DHEA+COH	17.12±0.17	8.36±1.13	11.96±0.51[a]	6.60±0.48[a]	7.12±0.43[a]	1.82±0.20	8.60±0.25[a]
DMSO+DHEA+Saline	17.31±0.86	9.21±1.05	16.44±0.62[b]	3.75±0.45[b]	3.44±0.44[b]	1.52±0.16	2.56±0.27[b]
DMSO+DMSO+COH	18.44±2.58	9.24±1.12	9.01±0.92[a]	4.96±0.21[c]	5.08±0.19[c]	1.39±0.21	6.04±0.73[c]
DMSO+DMSO+Saline	20.28±2.07	8.40±1.31	10.61±1.23[a]	3.04±0.51[b]	3.76±0.53[b]	1.48±0.23	2.92±0.62[b]
p* *	*0.48*	*0.8*	***<0.001***	***<0.001***	***<0.001***	*0.75*	***<0.001***

All values are given as mean±standard deviation.

* One-way ANOVA test was used for comparisons between three groups and the p value represents the comparison between three groups.

** One-way ANOVA test was used for comparisons between four groups and the p value represents the comparison between four groups.

[a,b,c,d] Significant differences were indicated by Post Hoc Tukey test using distinct superscript letters for pairwise comparisons, with no corresponding presentation of p values.

Table 2: Comparison of serum AMH levels. All values are given as mean±standard deviation.

	Day 1	Day 15	Day 60
	AMH Value (ng/mL)	AMH Value (ng/mL)	AMH Value (ng/mL)
Control	3.16±1.02[a]		
VCD	2.94±1.06[a]	0.16±0.01[b]	
DMSO	3.15±1.11[a]	4.02±1.47[a]	
p	*0.986 **	***0.031*** [#]	
VCD+DHEA	3.02±1.21[a]	0.33±0.04[b]	0.45±0.05[b]
VCD+DMSO	3.17±1.22[a]	0.56±0.16[b]	0.12±0.01[b]
DMSO+DHEA	3.37±1.29[a]	4.03±1.43[a]	3.42±1.28[a]
DMSO+DMSO	3.49±1.32[a]	4.22±1.36[a]	3.86±1.18[a]
p	*0.993 ***	***0.017 *****	***0.035 *****
VCD+DHEA+COH	3.72±1.40[a]	0.15±0.03[b]	0.47±0.12[b]
VCD+DHEA+Saline	3.81±1.42[a]	0.44±0.17[b]	0.69±0.16[b]
VCD+DMSO+COH	4.18±1.62[a]	0.53±0.13[b]	0.32±0.09[b]
VCD+DMSO+Saline	4.31±1.64[a]	0.65±0.22[b]	0.49±0.16[b]
DMSO+DHEA+COH	4.06±1.40[a]	4.07±1.33[a]	4.11±1.43[a]
DMSO+DHEA+Saline	4.13±1.38[a]	3.21±1.13[a]	3.17±1.04[a]
DMSO+DMSO+COH	4.22±1.41[a]	2.57±1.14[a]	2.78±1.10[a]
DMSO+DMSO+Saline	4.17±1.33[a]	4.28±1.59[a]	3.91±1.52[a]
p	*0.998 ****	***0.008 ******	***0.010 ******

* One-way ANOVA test was used for comparisons between three groups and the p value represents the comparison between three groups.

** One-way ANOVA test was used for comparisons between four groups and the p value represents the comparison between four groups.

*** One-way ANOVA test was used for comparisons between eight groups and the p value represents the comparison between eight groups.

Student's t-test was used for comparisons of two groups.

[a,b,c,d] Significant differences were indicated by Post Hoc Tukey test using distinct superscript letters for pairwise comparisons, with no corresponding presentation of p values.

Table 3: Biochemical evaluation of ovarian tissue samples. All values are given as mean±standard deviation.

	PTEN (ng/mL)	PI3K (pg/mL)	pAKT (ng/mL)	Caspas3 (ng/mL)	TAS (U/mL)	TOS (U/mL)	OSI TOS/(TASx100)	COX2 (ng/mL)
Control	1.73±0.37 [a]	601.76±41.99 [a]	28.55±11.36 [a]	4.49±1.25 [a]	1.34±0.48 [a]	14.16±4.18 [a]	0.12±0.06 [a]	10.35±2.53 [a]
VCD	4.14±1.50 [b]	200.06±15.69 [b]	10.20±3.73 [b]	15.98±3.09 [b]	0.57±0.16 [b]	47.28±11.68 [b]	0.87±0.27 [b]	23.12±4.16 [b]
DMSO	1.48±0.35 [a]	567.28±67.29 [a]	33.89±12.05 [a]	2.59±1.04 [a]	1.44±0.44 [a]	14.95±3.29 [a]	0.11±0.06 [a]	9.93±2.15 [a]
p*	***0.001***	***<0.001***	***0.006***	***<0.001***	***0.007***	***<0.001***	***<0.001***	***<0.001***
VCD+DHEA	1.87±0.65 [a]	401.96±36.28 [a]	19.13±3.16 [a]	7.34±1.85 [a]	1.43±0.29 [a]	18.99±8.54 [a]	0.13±0.06 [a]	18.67±4.90 [a]
VCD+DMSO	4.11±1.89 [b]	164.67±28.04 [b]	10.1±2.67 [b]	12.6±2.43 [b]	0.68±0.13 [b]	47.97±15.41 [b]	0.73±0.31 [b]	28.65±6.04 [b]
DMSO+DHEA	0.84±0.48 [a]	854.77±84.02 [c]	44.19±7.24 [c]	2.89±0.61 [c]	1.90±0.58 [a]	9.47±3.30 [a]	0.05±0.01 [a]	8.83±2.23 [c]
DMSO+DMSO	1.59±0.25 [a]	601.07±50.18 [d]	24.93±9.06 [d]	2.95±1.12 [c]	1.39±0.44 [ab]	10.51±4.11 [a]	0.09±0.07 [a]	9.11±2.77 [c]
p	***0.002***	***<0.001***	***<0.001***	***<0.001***	***0.002***	***<0.001***	***<0.001***	***<0.001***
VCD+DHEA+COH	2.54±0.76 [a]	411.11±68.25 [a]	17.13±1.21 [a]	7.88±1.55 [a]	0.96±0.31 [a]	21.59±4.33 [a]	0.26±0.15 [a]	42.53±7.45 [a]
VCD+DHEA+Saline	3.50±1.35 [a]	443.20±58.65 [a]	18.75±6.02 [a]	7.86±2.54 [a]	0.77±0.24 [a]	25.79±7.43 [a]	0.37±0.19 [a]	18.86±3.18 [b]
VCD+DMSO+COH	4.82±1.07 [ab]	194.50±38.07 [b]	10.28±1.05 [b]	15.46±1.11 [b]	0.30±0.16 [b]	52.16±25.53 [b]	2.08±1.35 [b]	55.50±9.45 [a]
VCD+DMSO+Saline	6.04±1.78 [b]	236.85±62.85 [b]	10.83±1.81 [b]	14.54±2.74 [b]	0.40±0.13 [b]	54.26±6.37 [b]	1.45±0.35 [b]	28.36±8.46 [c]
p	***0.003***	***<0.001***	***0.001***	***<0.001***	***<0.001***	***0.002***	***0.002***	***<0.001***
DMSO+DHEA+COH	0.91±0.26 [a]	889.65±31.36 [a]	47.96±8.59 [a]	3.42±0.81 [a]	1.95±0.37 [a]	10.28±2.96 [a]	0.06±0.02 [a]	11.74±3.96 [a]
DMSO+DHEA+Saline	1.65±0.64 [a]	879.39±33.23 [a]	47.64±8.28 [a]	4.42±1.03 [a]	1.52±0.42 [a]	10.41±2.65 [a]	0.07±0.01 [a]	5.14±2.09 [b]
DMSO+DMSO+COH	1.14±0.13 [a]	626.75±81.93 [b]	28.96±4.40 [a]	3.45±0.91 [a]	1.54±0.49 [a]	14.53±3.68 [a]	0.10±0.04 [a]	21.73±4.47 [c]
DMSO+DMSO+Saline	1.40±0.43 [a]	559.31±44.43 [b]	33.64±4.53 [a]	4.47±1.42 [a]	1.32±0.35 [a]	9.65±2.71 [a]	0.07±0.02 [a]	9.43±2.88 [ab]
p	***0.060***	***<0.001***	***<0.001***	***0.254***	***0.151***	***0.079***	***0.064***	***<0.001***

* One-way ANOVA test was used for comparisons between three groups and the p value represents the comparison between three groups.

** One-way ANOVA test was used for comparisons between four groups and the p value represents the comparison between four groups.

[a,b,c,d] Significant differences were indicated by Post Hoc Tukey test using distinct superscript letters for pairwise comparisons, with no corresponding presentation of p values.

Supplemental Table 1: Comparison of the body weights and ovarian tissue weights of the groups. All values are given as mean±standard deviation.

	Body Weights on the First Day of the Injections (g)	Body Weights on the Last Day of the Injections (g)	Body Weight Change (g)	Ovarian Tissue Weight (g)
VCD/DMSO Injections				
Control	174.3±7.7			0.139±0.019
VCD	182.3±8.5	201.4±7.3	19.1±7.6	0.131±0.011
DMSO	177.8±8.1	199.4±6.7	21.6±7.1	0.136±0.014
p	*0.62 **	*0.54*[#]	*0.84* [#]	*0.17 **
DHEA/DMSO Injections				
VCD+DHEA	202.1±5.9	245.1±8.6	43.0±5.3	0.206±0.014[a]
VCD+DMSO	198.3±7.2	239.3±6.9	41.0±5.9	0.142±0.019[a]
DMSO+DHEA	204.2±6.3	241.6±7.1	37.4±6.6	0.214±0.021[b]
DMSO+DMSO	201.8±4.3	237.9±7.6	36.1±6.1	0.144±0.012[b]
p	*0.32 ***	*0.46 ***	*0.91 ***	***0.02 *****
COH Protocol/Saline Injections				
VCD+DHEA+COH	244.1±7.9	249.3±5.1	5.2±5.8	0.241±0.023[a]
VCD+DHEA+Saline	246.6±4.1	251.3±5.3	4.7±7.3	0.209±0.019[a]
VCD+DMSO+COH	240.7±7.8	243.4±5.2	2.7±7.3	0.182±0.027[b]
VCD+DMSO+Saline	239.1±5.1	242.1±6.2	3.0±7.9	0.142±0.019[b]
DMSO+DHEA+COH	243.3±6.9	246.6±4.3	3.3±7.1	0.254±0.018[c]
DMSO+DHEA+Saline	238.7±5.1	242.3±6.8	3.6±6.5	0.214±0.021[c]
DMSO+DMSO+COH	240.3±8.1	244.1±6.4	3.8±7.9	0.179±0.025[d]
DMSO+DMSO+Saline	238.6±6.5	245.6±4.9	7.0±5.8	0.144±0.012[d]
p	*0.24 ****	*0.52 ****	*0.56 ****	***0.03 ******

* One-way ANOVA test was used for comparisons between three groups and the p value represents the comparison between three groups.

** One-way ANOVA test was used for comparisons between four groups and the p value represents the comparison between four groups.

*** One-way ANOVA test was used for comparisons between eight groups and the p value represents the comparison between eight groups.

[#] Student's t-test was used for comparisons of two groups.

[a,b,c,d] Significant differences were indicated by Post Hoc Tukey test using identical superscript letters for pairwise comparisons, with no corresponding presentation of p values.