Original Article

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The effect of DHEA on apoptosis and cohesin levels in oocytes in aged mice

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Summary **Female fertility declines with age as the number of ovarian follicles decreases and aneuploidy increases. Degradation of the cohesin complex might be responsible for age-related aneuploidy. Dehydroepiandrosterone (DHEA) can improve the ovarian reserve and reduce the rate of aneuploidy, but the relationship between DHEA and cohesin levels in oocytes is still unknown. The aim of the current study was to evaluate the effect of the supplement DHEA on ovarian function, including the number of follicles and cohesin levels in oocytes. C57BL/6J mice at 3 weeks, 6 weeks, 12 weeks, 6 months, and 10 months of age were used to obtain a systematic view into follicle apoptosis and cohesin levels in oocytes. Nine-month-old C57BL/6J mice were administered saline (*n* = 5), 17β-estradiol (100 μg/kg per day, *n* = 5), or DHEA (5mg/Kg per day, *n* = 5). After 4 weeks, aged mice were weighed and sacrificed, and ovarian tissue samples were prepared. Anti-VASA staining and HE staining were used to count the number of follicles. Anti-γH_2AX staining and TUNEL were used to measure follicle apoptosis and immunofluorescent staining was used to detect the levels of three oocyte cohesin subunits: REC8, SMC1β, and SMC3. Administration of the supplements 17β-estradiol and DHEA to aged mice increased the number of primordial and primary follicles and decreased the age-related apoptosis of follicles. Levels of the cohesin subunits REC8 and SMC1β declined with age, but DHEA and 17β-estradiol tended to delay that decline. The supplement DHEA increased the number of primordial and primary follicles in aged mice by inhibiting follicle apoptosis and tended to delay the decrease in cohesin levels in oocytes.**

Keywords: Dehydroepiandrosterone (DHEA), apoptosis, cohesin, oocyte, mice

1. Introduction

As society has advanced, more and more women have received an advanced education and are working than before. The age at which women bear children has been postponed as a result, and later childbearing can be affected by the issue of the decline in female fertility. Female fertility declines with age (*1,2*), and women of advanced maternal age have a greater incidence of miscarriages and embryo aneuploidy (*3-5*). The decline in fertility is due not to uterine aging but to ovarian aging, which is the process by which both the quantity and quality of oocytes decrease with age (*6-8*).

The number of oocytes decreases from when a woman was still a fetus. Without regeneration, the number of oocytes drops from 7×10^6 to < 1,000 during

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menopause (*9*). In an adult woman, only a small proportion of oocytes are recruited during ovulation and enter meiosis from their original quiescent status; instead, more oocytes undergo apoptosis and are eliminated from the ovaries, leading to a decrease in the number of oocytes and eventually ovarian aging (*10*). Apoptosis is age-related and Hansen devised a model of reproductive aging in which the loss of oocytes and follicles gradually accelerates in women at the age of 37 (*11*). Oocyte maturation is also a process of folliculogenesis. The underlying mechanism of oocyte apoptosis and follicular atresia is still unclear, but numerous studies and different therapies have been designed to promote follicle growth and prevent follicular arteria, thus alleviating the loss of ovarian function due to the aging process (*12*).

The increased rate of aneuploidy in oocytes is considered to be a distinct manifestation of a decline in oocyte quality. Aneuploidy of oocytes is also one of the major causes of infertility, spontaneous abortions, and genetic diseases (*13,14*). The production of an oocyte involves a process called meiosis, which is an error-prone process in which a tiny mistake can result in aneuploidy (*15*). Previous studies have focused on several hypotheses of age-related aneuploidy including abnormal homologous recombination (*16*) and defective spindle assembly (*17*). However, recent studies have speculated that cohesin in chromosomes might be the main cause.

The cohesin complex is a ring-structured complex containing four subunits, and SMC1β, SMC3, REC8, and STAG3 are the specific proteins in mice (*18*). Cohesin complexes are loaded on chromosomes during the replication of DNA in oocytes, with little to no turnover after loading (*19*). Cohesin complexes have multiple functions in cell life including chromosome segregation, DNA repair, and gene expression, and their most important function is to maintain the cohesion of chromosomes during mitosis and meiosis, which means that these complexes could be the chief culprits of age-related aneuploidy. Cohesin-specific subunit knockout mice have a high rate of aneuploidy, and that rates increases greatly with age (*20*). Different cohesin subunits and their functions have been summarized in Table 1. In senescence-accelerated mice (SAM), immunostaining of REC8 between the chromosome arms and centromeres in aged oocytes was either absent or disrupted (*21*). In oocytes of women in their 40s, levels of the cohesin complex subunits REC8 and SMC1β decreased substantially in comparison to levels in women age 20 (*22*). In the process of maternal aging, ovarian function changes markedly in line with the loss of cohesin complexes in oocytes. However, the specific mechanism of the decrease in cohesin is still unknown.

Dehydroepiandrosterone (DHEA) is a type of androgenic steroid that is mainly produced in the adrenal cortex (*23*). The androgenic activity of DHEA is relatively weak and is estimated to be only 10% of that of testosterone. DHEA is a precursor to sex steroids including testosterone and estradiol. The level of DHEA and dehydroepiandrosterone sulfate (DHEAS) declines with age, reaching its nadir at 80 years of age. This suggests that DHEA might be involved in the ovarian aging process. Moreover, the development of follicles requires androgen-based hormonal support and the production of DHEA could also be affected by the number of follicles, which makes the relationship of DHEA and ovary functions more complicated (*24*).

DHEA supplementation has also been used in clinical therapy to improve the fertility outcomes in *in-vitro* fertilization (IVF) treatment. DHEA was first used in an experiment by Casson *et al*. involving IVF (*25*). The supplement DHEA improved embryo quality and increased the chance of pregnancy in women with a poor ovarian reserve (*26*). When follicular growth was detected with transvaginal ultrasound after administration of DHEA, an increased antral follicle count (*27*) and more retrieved follicles and a greater number of follicles >17 mm were noted (*28*). A recent study suggested that DHEA reduces oocyte aneuploidy (*29*), thus improving outcomes for IVF patients. DHEA has been widely used around the world, but its effect on ovarian function and the mechanism of that action are still unclear. Whether DHEA could affect the quantity and quality of oocytes and how it would do so are still topics of speculation.

The aim of this study was to ascertain the effect of DHEA on ovary function, including the quantity and quality of oocytes in naturally aging mice. The number of follicles and follicular atresia were detected after administration to show how the supplement DHEA affected folliculogenesis. Detecting the levels of oocyte cohesin complexes would reveal whether DHEA affects oocyte quality. As a preliminary investigation of how DHEA combats ovarian aging, the current study might provide new insight into the effect that the supplement DHEA has on ovarian function and the mechanism of that action.

2. Materials and Methods

2.1. *Animals*

In total, 40 female C57BL/6J mice were purchased from the Laboratory Animal Facility of Chinese Academy of Science (Shanghai, China) and raised in the Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, China. Mice were given a fresh supply of sterile drinking water and food and exposed to a 12-h day and 12-h night photoperiod during the experiment. This study as approved by the animal ethics committee of the Obstetrics and Gynecology Hospital of Fudan University. To facilitate a systemic study of the ovarian aging process, five mice were sacrificed at the ages of 3 weeks, 6 weeks, 3 months, 6 months, and 10 months. Ovaries were collected for subsequent examination.

Table 1. Properties and functions of cohesin complexes in mammals

Category	Name	Molecular Weight (kDa)	Gene location in human	Principal function in meiosis/mitosis	Related human disease	Effect in knock-out mice	Changes with age in humans	Changes with age in mice
Structural maintenance of chromosomes (SMC)	SMC1α	121.1	Xp11.22	Mitotic cohesion, G2/M cell cycle, restructuring the DNA double helix	CdLS, Cancer, Epileptic encephalopathy, Acute myeloid leukemia	–	No SD	No SD
	SMC1β*	116.1	22q13.31	Sister chromatid cohesion, assembly of axial elements during prophase, synapsis, recombination, chromosome movement	-	Males and females sterile	Decreases	Decreases
	SMC3	121.7	10q25.2	Sister chromatid cohesion, restructuring the DNA double helix	CdLS, Cancer, PCOS, AML in Down Syndrome infants, AML	Lethal to embryos (E14.5)	No SD	No SD
Stromalin	STAG1 (SA1)	122.1	–	Telomere replication, telomere cohesion	Cancer	Lethal to embryos (E11.5)	–	–
	STAG2 (SA2)	123.1	Xq25	Centromere cohesion, sister chromatid cohesin, repair of kinetochore-microtubule attachments	Cancer	–	–	–
Kleisin	STAG3 (SA3)*	122.5	7q22.1	Sister chromatid cohesion, centromeric cohesion, chromosome pairing and synapsis, DNA repair, progression of meiosis, axis formation	Male infertility, Premature failure, Williams-Beuren syndrome	Lethal to embryos (E11.5)	No SD	No SD
	RAD21	64.3	8q24.11	Axis formation, synaptonemal complex maintenance, monopolar attachment of sister kinetochores	CdLS, Cancer, Acute myeloid leukemia	–	No SD	No SD
	RAD21L*	55.6	17q21.31	Normal clustering of pericentromeric heterochromatin, axis formation, SC maintenance, DNA repair	-	Males infertile. Females are fertile but experience age-dependent sterility	–	–
	REC8*	54.7	14q12	Sister chromatid cohesion, centromeric cohesion, axis formation	Cancer, Male infertility	Males and females sterile.	Decreases	Decreases

* stands for meiosis-specific cohesin subunits.

2.2. *Agent administration*

Nine-month-old female C57BL/6 mice were used in this study and corresponded to women around 40 years of age (*30*). Fifteen mice were randomly divided into three groups: the control group, the E2 group, and the DHEA group. All of the mice were intragastrically administered the corresponding agent. The control group mice were administered saline (n = 5). Mice in the E2 group were administered 17β-estradiol (100 μg/kg per day, n = 5) as a positive control (*31*). The DHEA group was administered DHEA (5mg/Kg per day, n = 5) (*32*). The dosages of the two hormones were calculated according to the dosages used in postmenopausal hormone therapy (*33*). After 4 weeks, all mice were sacrificed in order to collect blood and ovarian tissue samples.

2.3. *Serum hormone measurements*

Blood samples were collected from each mouse *via* cardiac puncture prior to sacrifice. After centrifugation, the serum samples were prepared to measure the concentration of AMH. EIA kits for AMH were used according to the manufacturer's protocol. The experiment was repeated three times.

2.4. *Histological sample preparation and staining*

Ovarian tissues were fixed overnight in 4% paraformaldehyde and then washed and stored in 70% ethanol at 4ºC for embedding in paraffin. Eight-micrometer sections were processed for further use. Slides were stained with hematoxylin and eosin (HE) and then scanned using the Olympus Scanner (Tokyo, Japan). After staining, the follicles were classified into four stages: primordial, primary, secondary, and antral. The classifications of follicles was according to the features of granulosa cells (GCs) surrounding oocytes as described by Gougeon (*34*). Primordial follicles consisted of primordial oocytes surrounded by a single layer of GCs. When the single surrounding layer of GCs became cuboidal, these follicles were classified as primary follicles. Follicles were classified as secondary based on the presence of a visible follicular antrum. When several layers of cuboidal GCs without a visible atrium surrounded oocytes, the follicles were categorized as secondary follicles. When an antral cavity with follicular fluid appeared around oocytes, the follicles were categorized as antral follicles. Despite the size differences in each group, the total number of follicles in each ovary was manually counted by two independent researchers. At least three slides per mouse were used to analyze the number of follicles in each group. Data are expressed as the average number of follicles per ovary.

2.5. *TUNEL staining*

The atresia of follicles was detected using TdT-mediated dUTP nick-end labeling (TUNEL). The In Situ Death Detection Kit was purchased from POD, Roche Molecular Biochemicals, Mannheim, Germany. TUNEL was performed according to the manufacturer's instructions. Briefly, the prepared ovarian tissues were incubated with reaction mixtures including biotinylated nucleotides and terminal deoxynucleotidyl transferase (TdT) at 37°C for 1 h. After incubation with a converter-POD solution for 30 min at 37°C, DAB was added. At least 30 slides from 5 mice in each group were stained with TUNEL to obtain data on follicular atresia. Follicles were considered TUNEL-positive if either the oocyte alone, the oocyte and GCs, or oocytes were negative but > 50% of GCs were positive (*35*). The percentage of TUNEL-positive follicles was manually calculated by two independent blinded examiners using the Olympus Scanner.

2.6. *Immunohistochemistry*

Vasa primary antibodies were purchased from Merck Millipore, Massachusetts, USA. γH_2AX primary antibodies were purchased from Cell Signaling Technology, Boston, USA. The immunohistochemical staining steps were strictly performed with standard protocols. Briefly, the prepared slides were washed three times and then stained with two primary antibodies: anti-VASA, and anti-γH_2AX antibody. After incubation with the primary antibodies overnight, the slides were washed with PBS three times and then incubated with biotinylated-conjugated secondary antibody (Zymed Laboratories-Invitrogen, San Francisco, CA, USA) for 15 minutes at room temperature. Follicles were considered γH_2AX-positive if the oocyte or > 50% of GCs were positive (*36*). Images were obtained using the Olympus Scanner. At least six slides per mouse were used to analyze the number of follicles that were positive for different types of staining from 5 mice in each group. γH_2AX-positive follicles and VASA-positive cells were counted manually and confirmed by at least two blinded researchers. Data are expressed as the average number of positive cells or follicles per ovary.

2.7. *Immunofluorescent staining*

Since the density of the cohesin subunit STAG3 changes little in the ovarian aging process (*22*), the current study only detected the levels of three cohesin subunits: REC8, SMC1β, and SMC3. Ovarian slides were blocked and then incubated with primary and secondary antibodies as described (*37*). Briefly, slides were first incubated with blocking buffer (5% normal donkey serum and 5% normal goat serum in PBS), and then with the primary antibodies diluted in PBS at 4°C overnight at the following dilutions: rabbit anti-REC8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1,000;

mouse anti-SMC1β (Rockland Antibodies & Assays, Limerick, PA, United States) and SMC3 (Cell Signaling Technology, Boston, USA). The slides were then washed in PBS three times and incubated with the secondary antibodies Alexa 594-conjugated donkey anti-rabbit IgG and Alexa 488-conjugated goat anti-guinea-pig IgG (Invitrogen) or aminomethylcoumarin acetate-conjugated donkey anti guinea-pig IgG (Jackson Immuno Research, West Grove, PA, USA) at room temperature for 30 minutes. Finally, the slides were washed three times in PBS.

The slides were observed with a confocal laser microscope (LSM510, Zeiss, Oberkonchen, Germany) and images were captured with the LSM510 microscope. Densitometric analysis of the oocyte immunofluorescence signals was performed using Image-Pro Plus 6.0. The green signal intensity (REC8, SMC1β, and SMC3) was defined as described in a previous study, except that DAPI was used as a control (*22*).

2.8. *Statistical analysis*

All data are expressed as the mean ± SD. Variance was analyzed with SPSS database using one-way analysis of variance (ANOVA) or the Student's *t* test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. *The number of follicles decreased with age, but the supplement DHEA increased the number of follicles in aged mice*

In the aging process, female fertility in rodents and humans declines, and the quantity and quality of follicles serve as indices of this process. The current study detected the number of follicles in female C57BL/6J mice of different ages and then used VASA and HE staining to measure changes in the number of follicles in aged mice that were administered different agents for 4 weeks.

VASA protein is an oocyte-specific marker. VASA staining helped with follicle counting while VASA staining together with HE staining provides more objective results than HE staining alone. In ovarian sections from 3-week-old mice, the number of vasa-positive cells was as high as 90, but the number decreased rapidly with age from 3 weeks to 10 months (Figure 1a and Table 2). The supplements DHEA and 17β-estradiol increased the number of vasa-positive cells in aged mice (Figure 1b and Table 3).

HE staining is used to distinguish different stages of follicles. The number of primordial follicles from mice declined rapidly with age from 3 weeks to 10 months (Figure 2a and Table 2). The number of primary follicles increased from 3 weeks to 3 months. The number of primary follicles peaked at 3 months and then sharply decreased from 3 months to 10 months. The number of secondary follicles also decreased markedly from 3 weeks to 3 months and plateaued at 10 months (Figure 2a and Table 2). The number of antral follicles increased with age from 3 weeks to 6 weeks and plateaued at 10 months (Figure 2a and Table 2). The supplements DHEA and 17β-estradiol increased the number of primordial and primary follicles in aged mice with little effect on follicles in other stages (secondary and antral) (Figure 2b and Table 3).

In order to compare the levels of follicular atresia in mice and the effect of DHEA on follicular atresia, ovarian sections were stained using TUNEL. The percentage of apoptotic follicles increased greatly with age from 3 weeks to 10 months (Figure 3a and Table 2). After the supplements DHEA and 17β-estradiol were administered to aged mice for 4 weeks, the level of oocyte apoptosis was measured again. The level of oocyte apoptosis decreased significantly compared to the level in the control group (Figure 3b and Table 3).

γH_2AX staining was used to detect damage to DNA where the Ser of H_2AX was phosphorylated, resulting in γH_2AX (*38*). The percentage of γH_2AX-positive follicles increased greatly with age from 3 weeks to 10 months (Figure 4a and Table 2). The supplements DHEA and 17β-estradiol decreased the percentage of γH_2AX-positive follicles in aged mice (Figure 4b and Table 3).

3.2. *DHEA had little effect on the age-related decrease in cohesins in aged mice*

In order to compare the cohesin levels in oocytes from mice of different ages, meiosis cohesin subunits REC8, SMC1β, and SMC3 were detected from the dictate oocytes of mice of different ages, and the relative density of each protein was measured using immunofluorescence. After the supplement DHEA or 17β-estradiol was administered for 4 weeks, the cohesin levels in oocytes from aged mice were measured again.

The density of the cohesin subunit REC8 decreased with age in mice. The density declined with age from 3 weeks to 10 months (Figure 5a and Table 3). Administration of E2 tended to delay the decrease in levels of REC8 in oocytes, but the levels did not differ significantly compared to those in the control group. Administration of DHEA had a similar effect on levels of REC8 in oocytes, and again the levels did not differ significantly (Figure 5b and Table 3). The cohesin subunit SMC1β decreased with age in mice from 3 weeks to 10 months (Figure 6a and Table 3) and administration of DHEA and 17β-estradiol to aged mice tended to delay that decrease (Figure 6b and Table 3). However, levels of SMC1β did not differ significantly compared to those in the control group. In contrast, levels of SMC3 appeared to remain unchanged in the current study. No age-related changes in those were noted during the aging process (Figure 7a and Table 4), and DHEA and 17β-estradiol

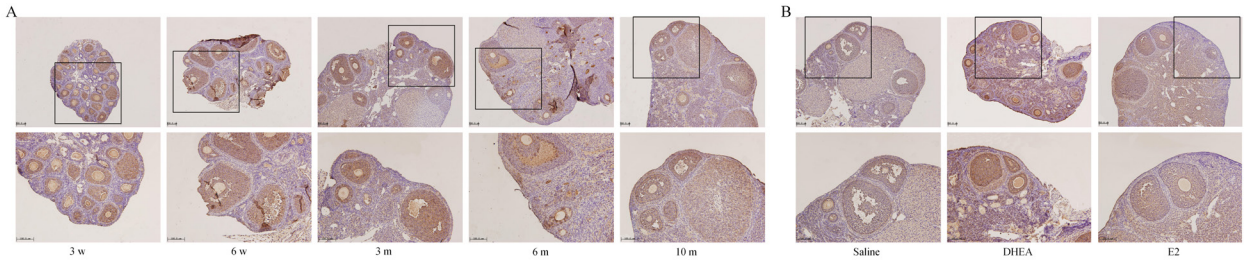

Figure 1. The elimination of oocytes in mice can be reversed by the supplement DHEA. Representative images of anti-VASA-stained ovary sections from female C57BL/6J mice of different ages **(A)** and aged mice administered saline, E2 (100 μg/kg per day), or DHEA (5 mg/Kg per day, $n = 5$) for four weeks **(B)**. Bar = 100 μm.

Table 2. Number of follicles and follicular atresia in mice of different ages

Type of follicle	3 w	6 w	3 m	6 m	10 m
Vasa-positive cells	98.75 ± 1.053	79.67 ± 1.269*	63.08 ± 0.86578*	51.00 ± 0.7177*	46.33 ± 1.082*
Primordial Follicles	59.54 ± 1.233	48.85 ± 1.709*	30.15 ± 1.686*	19.69 ± 0.8799*	11.58 ± 1.264*
Primary Follicles	25.00 ± 1.638	35.00 ± 1.255*	48.08 ± 0.9806	30.50 ± 1.091*	16.25 ± 0.5383*
Secondary Follicles	17.17 ± 0.6945	11.75 ± 0.6867*	8.833 ± 0.4234	8.417 ± 0.3128	8.333 ± 0.6195
Antral Follicles	2.000 ± 0.4264	5.583 ± 0.4345*	5.917 ± 0.4516	6.750 ± 0.5919	7.250 ± 0.9545
%γH_2AX-positive Follicles	21.49 ± 1.243	24.66 ± 0.5394*	42.07 ± 1.383*	49.82 ± 3.036*	61.16 ± 2.288*
%TUNEL-positive Follicles	11.25 ± 0.6593	16.79 ± 1.038*	31.83 ± 1.348*	39.03 ± 1.921*	45.59 ± 1.653*

Mice of different ages were sacrificed and ovarian slides were used to count the number of follicles and detect follicular atresia. Data are expressed as the mean ± standard deviation (SD). The *P*-value was determined with ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the previous one, respectively.

Table 3. Number of follicles and follicular atresia in aged mice administered different agents

Type of follicle	Saline	DHEA	E2
Vasa-positive cells	46.33 ± 1.082	49.58 ± 0.8021*	51.17 ± 0.8947*
Primordial Follicles	11.58 ± 1.264	14.77 ± 0.6994*	15.92 ± 0.7291*
Primary Follicles	16.25 ± 0.5383	18.42 ± 0.7732*	19.50 ± 1.270*
Secondary Follicles	8.333 ± 0.6195	8.583 ± 0.8744	10.17 ± 0.7470
Antral Follicles	7.250 ± 0.9545	6.333 ± 0.5412*	6.417 ± 0.3128*
%γ H_2AX-positive Follicles	61.16 ± 2.288	53.77 ± 1.424*	53.49 ± 2.275*
%TUNEL-positive Follicles	45.59 ± 1.653	40.77 ± 1.382*	40.18 ± 1.803*

Aged mice that were treated with saline, E2 (100 μg/kg per day), or DHEA (5 mg/Kg per day, $n = 5$) for four weeks were sacrificed and ovarian slides were used to count the number of follicles and detect follicular atresia. Data are expressed as the mean± standard deviation (SD). The P-value was determined with ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the group administered saline.

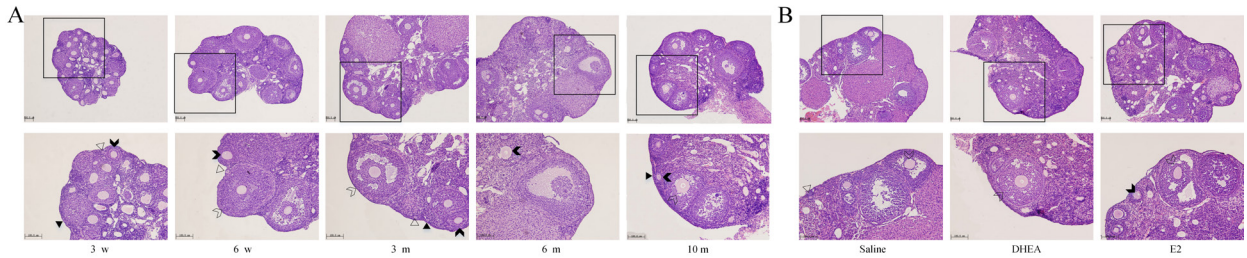

Figure 2. The number of follicles in different stages that were eliminated with age in mice. Representative images of HE-stained ovary sections from female C57BL/6J mice of different ages **(A)** and aged mice administered different agents for four weeks **(B)**. Bar = 100 μm. "▲" represents primordial follicles, "Δ" represents primary follicles, "⩓" represents secondary follicles, and "⩔" represents antral follicles.

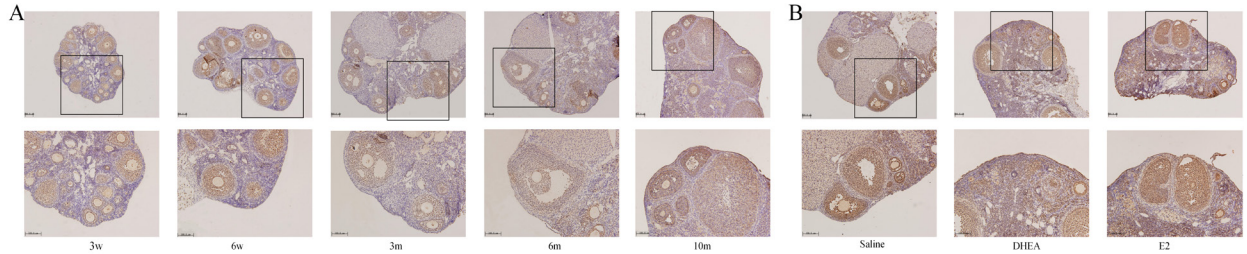

Figure 3. The increased apoptosis of oocytes in mice can be partly reversed by the supplement DHEA. Representative images of TUNEL-stained ovary sections from female C57BL/6J mice of different ages **(A)** and aged mice administered different agents for four weeks **(B)**. Bar = 100 μm.

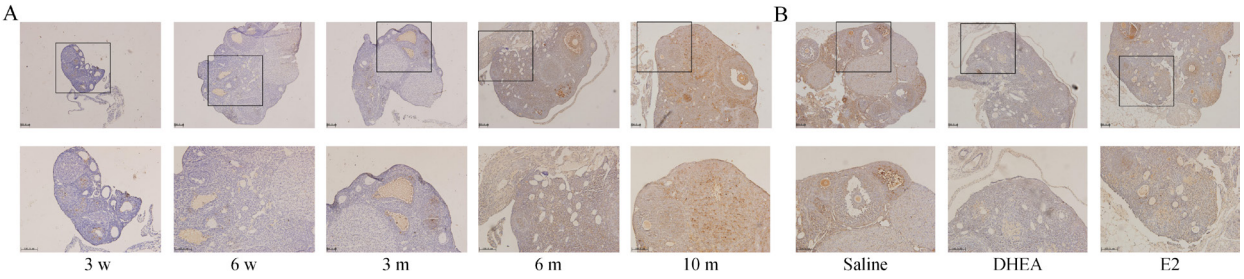

Figure 4. The increase in DNA double-strand breaks in oocytes in mice can be partly reversed by the supplements 17β-estradiol and DHEA. Representative images of anti-γH_2AX-stained ovary sections from female C57BL/6J mice of different ages **(A)** and aged mice administered different agents for four weeks **(B)**. Bar = 100 μm.

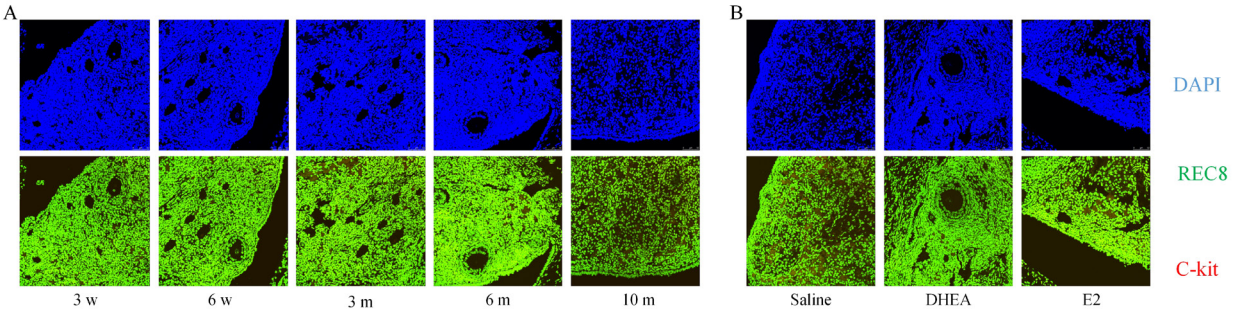

Figure 5. The decrease in levels of the cohesin complex subunit REC8 in mice can be partly delayed by the supplement DHEA. Representative images of immunofluorescence-stained ovary sections from female C57BL/6J mice of different ages **(A)** and aged mice administered different agents for four weeks **(B)**. Bar = 50 μm.

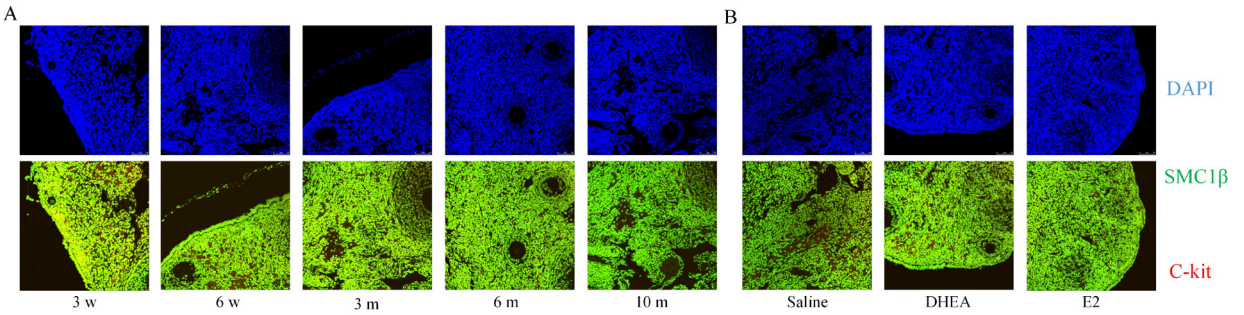

Figure 6. The decrease in cohesin complex subunits-SMC1β in mice showed a trend to be delayed by the supplement DHEA. Representative images of immunofluorescence-stained ovary sections from female C57BL/6J mice of different ages **(A)** and aged mice administered different agents for four weeks **(B)**. Bar = 50 μm.

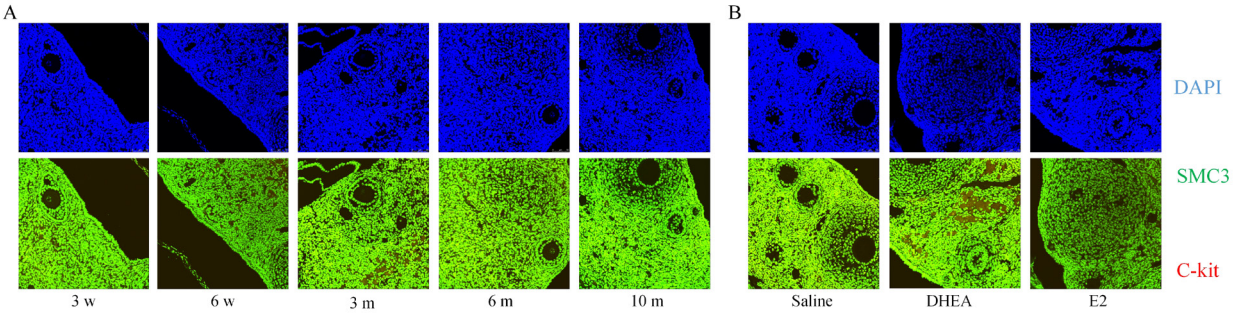

Figure 7. Levels of the cohesin complex subunit SMC3 in mice of different ages and different agents. Representative images of immunofluorescence-stained ovary sections from female C57BL/6J mice of different ages **(A)** and aged mice administered different agents for four weeks **(B)**. Bar = 50 μm.

Table 4. Relative density of cohesin subunits in mice of different ages

Cohesin subunit	3 w	6 w	3 m	6 m	10 m
REC8	0.7239 ± 0.05156	0.5501 ± 0.04139*	0.4045 ± 0.01736*	0.3226 ± 0.01668*	0.2660 ± 0.01933*
SMC1β	1.193 ± 0.06215	1.038 ± 0.03060*	0.9221 ± 0.04221*	0.7619 ± 0.03218*	0.6664 ± 0.02739*
SMC3	1.015 ± 0.03451	0.9433 ± 0.05124*	0.9305 ± 0.03390*	0.9246 ± 0.04650*	0.9145 ± 0.04117*

Mice of different ages were sacrificed and ovarian slides were used to detect the relative density of cohesin subunits. Data are expressed as the mean± standard deviation (SD). The P-value was determined with ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the previous one, respectively.

Table 5. Relative density of cohesin subunits in aged mice administered different agents

Cohesin subunit	Saline	DHEA	E2
REC8	0.2660 ± 0.01933	0.3003 ± 0.02564	0.3187 ± 0.01981
SMC1β	0.6664 ± 0.027398	0.7402 ± 0.02528	0.7519 ± 0.03066
SMC3	0.9145 ± 0.04117	0.9275 ± 0.03268	0.9050 ± 0.02581

Aged mice that were administered saline, E2 (100 μg/kg per day), or DHEA (5 mg/Kg per day, $n = 5$) for four weeks were sacrificed and ovarian slides were used to detect the relative density of cohesin subunits. Data are expressed as the mean ± standard deviation (SD). The *P*-value was determined with ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the group administered saline.

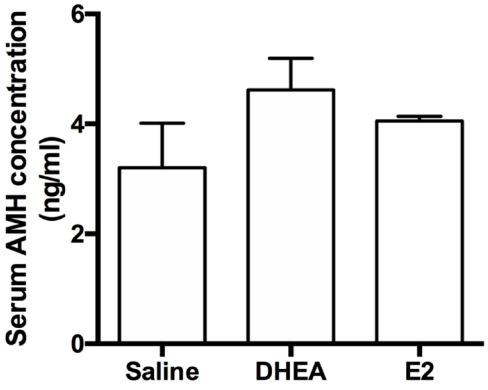

Figure 8. Administration of DHEA did not increase serum levels of AMH in aged mice. Aged mice were sacrificed after 4 weeks of administration, and sera were collected to determine concentrations of AMH. Steroid concentrations in sera were measured using an AMH enzyme immunoassay kit. Bars denote the mean±standard deviation (SD). Agents are listed below the X-axis. The *P*-value was determined with ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$.

had no effect on the density of SMC3 (Figure 7b and Table 5).

3.3. *Administration of DHEA increased serum E2 but not AMH*

DHEA is a precursor to sex steroids including testosterone and estradiol. Hence, the question was whether DHEA improved ovarian function directly or indirectly by increasing concentrations of estrogens. The administration of DHEA and E2 had no effect on AMH (Figure 8).

4. Discussion

Ovarian aging is a protracted process and more than 99.9% of follicles, including oocytes and GCs, are programmed to die during this process (*39*). The current study indicated that DHEA and E2 increased the number of follicles and alleviated follicular atresia in aged mice. This study also provided a view into the changes in the number of follicles and follicular atresia in the aging process.

DHEA is a C19 androgenic steroid that has been used as a therapy in several areas, and especially in IVF. The use of DHEA in patients could improve embryo quality and live birth rates and thus increase the pregnancy rate in patients. The effect also seems to be time- and dose-dependent (*40*). Some clinical studies have also suggested that this effect could be achieved by reducing aneuploidy, increasing the antral follicle count, and improving follicular steroidogenesis (*25,41,42*). Although one study failed to obtain positive results (*43*), DHEA has consistently been a focus of research. These clinical trials may be less persuasive due to the number of patients and individual differences in different studies, and more studies have instead involved animal experiments.

Narkwichcean *et al*. (*44*) used a sheep model to explore effect and mechanism of the supplement DHEA *in vivo*. As the first *in vivo* study to explore the effect of DHEA on early follicle development, it suggested that DHEA could increase antral follicle population by increasing the rates of primordial follicle initiation and preantral follicle development. Rats that underwent a unilateral oophorectomy were also used as a DOR model and were administered DHEA in one study (*45*). The ovaries of these rats had a greater number of follicles in all stages, including primordial, primary, and growing (preantral and antral) stages, and a decreased rate of atresia after administration of the supplement DHEA for 45 days.

The current study used aged female C57BL/6J mice as a model to investigate the probable effect of DHEA

and its potential mechanism. In previous studies, DHEA was also used to induce PCOS-like syndromes in rodents. However, the dose of DHEA to induce PCOS in a mouse model is at least 30 mg/Kg (*46*), while the DHEA dose in the current study was only 5 mg/Kg (*32*). The supplement DHEA increased the number of follicles in aged mice, and particularly the number of primordial and primary follicles. This effect was presumably achieved by inhibiting follicular atresia (*35*). Anti-γH_2AX staining indicated that atretic follicles were rescued by suppressing DNA double-strand breaks (*36*). DNA double-strand breaks play a major role in the ovarian aging process and the age-related decline in fertility. In some of the cells with damaged DNA, that DNA is repaired; if not, the cells undergo apoptosis (*47*). The current study suggested that DHEA decreases the extent of DSBs and it inhibits the apoptosis of oocytes, thus increasing the number of follicles.

The underlying mechanism of DHEA and folliculogenesis has been studied but is nonetheless still unknown. DHEA is a prohormone of androgen and may be beneficial for follicle maturation like androgen (*48*). Androgen receptors were seen in stroma and GCs in ovaries, and especially in primary follicles or other more advanced follicles. Excessive androgen levels may upregulate follicle formation, and Sen et al. contended that androgen signaling affected folliculogenesis *via* androgen receptors (*49*). They also found that androgen receptors in GCs and not in any other cells were the most important cells in this process. By stimulating the androgen receptors in GCs, androgen may promote preantral follicle development and could even prevent follicular atresia. DHEA treatment may increase androgen levels in GCs (*50*). As a result, DHEA may also promote preantral follicle growth and even rescue atretic follicles (*51,52*), suppressing the DNA double-strand breaks in follicles and thus inhibiting apoptosis (*53*).

The segregation of chromosomes is a process that requires high fidelity. The rate of a trisomy increases greatly from nearly 2% for women in their 20s to 35% for women in their 40s (*13*). A recent study has suggested that the deterioration of cohesins might be responsible for age-related aneuploidy (*22*). In mice, this phenomenon is thought to be related to the strain. Age-related aneuploidy and loss of cohesin subunits have been observed in C57BL-related strains (*54,55*), which led the current authors to choose C57/BL6J as a natural model for further study.

Several studies have noted a sharp decrease in cohesin levels in oocytes from aged mice in comparison to those in young mice (*56,57*). However, those studies failed to systemically reveal a specific relationship between age and cohesin levels in oocytes. The current study noted an almost linear decrease in cohesin levels in dictyate oocytes from mice of different ages, and the current study found that the supplement DHEA partly alleviated the age-related decline in cohesin levels. To the extent known, this is the first report to systematically describe the age-related decrease in cohesin levels in oocytes and the effect of DHEA on cohesin levels. These findings may help to better understand the principle of cohesin deterioration in dictyate oocytes.

Cohesin complexes are a type of protein with a ring-like structure and are located at the centromeres of chromosomes as well as at the arms. The main function of cohesin complexes is to maintain the cohesion of chromosomes, so a lack of cohesion in chromosomes may be the main cause of oocyte aneuploidy. Unlike in mitosis, meiosis involves low or even minute levels of cohesin in oocytes. Cohesin complexes in oocytes are highly susceptible to changes in conditions (*58*) and undergo no turnover over the lifespan of females. Several *in vitro* studies have examined the mechanism of cohesin deterioration but failed to draw specific conclusions (*59,60*).

DHEA is an essential substrate in the process of steroidogenesis. Previous studies indicated that DHEA improved the outcomes of IVF by reducing aneuploidy and improving ovarian reserve (*25,61*). The current study found that administration of DHEA tended to delay the loss of cohesin in oocytes and thus reduce the rate of oocyte aneuploidy. The current study might provide other researchers with a new tack for the study of the underlying decline in cohesin levels in oocytes.

The use of DHEA in aged mice increased the quantity of oocytes and tended to improve the quality of oocytes, suggesting that the quality of oocytes might be influenced by cohesin levels. DHEA tended to delay the age-related loss of cohesins, perhaps by increasing the number of follicles. A previous study has also suggested that the oocyte pool might have be related to aneuploidy by affecting cohesion in chromosomes (*62*). The study in question also suggested a possible relationship between the oocyte pool and cohesin levels in oocytes. More studies of the effects of cohesin levels and *in vitro* studies need to be conducted to confirm this hypothesis.

The main limitation of the current study is that *in vivo* experiments were performed to reveal how DHEA combats ovarian aging. The mechanisms and pathways involved in this process were not fully investigated in this study. However, this study was a preliminary investigation of how DHEA combats ovarian aging, and the current findings may provide new insight into this subject.

The current study suggested that the supplement DHEA improved ovary function by increasing the number of follicles, inhibiting follicular atresia, and tending to delay the decrease in cohesin levels in oocytes in aged mice. The underlying mechanism and pathways involved in this process will be investigated further in the future.

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