

## Research Article

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# Effects of myo-inositol plus folic acid on ovarian morphology and oocyte quality in PCOS mouse model

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**Summary**

Although the role of myo-inositol (MYO) in promoting the oocyte quality of PCOS patients has been documented in human studies; the cellular effects of this supplement on oocytes have not been directly examined due to ethical limitations. In the first phase of this study, MYO dosimetry was carried out simultaneously with the PCOS model development. An effective dose was obtained following the assessment of fasting insulin and testosterone levels using ELISA and ovarian morphology appraisal by histopathology. In the second phase, following the continuous administration of the effective dose of MYO and dehydroepiandrosterone (DHEA), cellular evaluation was performed. The quality of oocytes from superovulation was analyzed by examining maturity and normal morphology percentage using a stereomicroscope, intracellular reactive oxygen species (ROS) and glutathione (GSH) levels using fluorometry, and ATP count evaluation using ELISA. The results revealed that, among the four different MYO concentrations, the 0.36 mg/g dose compared with the DHEA group reduced testosterone levels and large atretic antral follicles (LATAnF) diameter. This dose also increased the corpus luteum count and the granulosa:theca (G/T) layer thickness ratio in antral follicles. Furthermore, this dose increased mature oocytes and normal morphology percentage, ATP count, and GSH levels; however, it decreased ROS levels in mature oocytes. Our findings provide the grounds for further cellular and molecular studies on the PCOS mouse model, suggesting that the improvement in mitochondrial function and its antioxidant properties is probably one of the mechanisms by which MYO increases oocyte quality.

**Introduction**

Polycystic ovary syndrome (PCOS) is one of the most common endocrine diseases with uncertain aetiology and causes infertility in 10–15% of women of reproductive age (Eini *et al.*, 2017). Several factors, including hormonal, genetic, metabolic, and environmental, are involved in PCOS (Laganà *et al.*, 2016). It has been characterized by multiple disorders such as hyperandrogenism, insulin resistance, obesity, type 2 diabetes, hyperlipidaemia, increased levels of LH to FSH, chronic anovulation, increased immature oocytes, and decreased oocyte quality, fertilization, and implantation rate (Patel and Carr, 2008; Qiao and Feng, 2011; Hayes *et al.*, 2015; Amini *et al.*, 2016).

Hyperinsulinemia in PCOS patients develops androgen hormones in two different ways. On the one hand, stimulating thecal cells increases androgen secretion; on the other hand, a decrease in sex hormone-binding globulin levels raises the free androgen level in the blood. These changes eventually create a vicious cycle of hyperandrogenism and hyperinsulinemia (Kokosar *et al.*, 2016). Hyperandrogenism impairs the folliculogenesis process, increasing immature oocytes and decreasing the quality of the mature oocytes. Lack of the ovarian follicle's proper response to FSH is another consequence of hyperinsulinemia in PCOS, which provides a route to ovulation disorders and reduces corpus luteum presence (Patel and Carr, 2008). Hyperandrogenism plays a critical role in oxidative stress and apoptosis, increasing the antral follicle diameter while decreasing the G/T ratio and making the follicles go into atresia. Consequently, the increased apoptosis in granulosa cells and oocytes has a negative effect on the IVF results (Karuputhula *et al.*, 2013; Ikeda *et al.*, 2014).

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Mitochondria are vital organs in oocytes, and their function is one of the parameters affecting oocyte quality and development. Mitochondrial function can be assessed by ATP content examination (Zhang *et al.*, 2019). DHEA impairs mitochondrial function by acting on complex I of the respiratory chain and inhibiting cellular respiration, which ultimately increases the production of reactive oxygen species (ROS) (Safiulina *et al.*, 2006), followed by a decrease in the ATP content (Huang *et al.*, 2015). Conversely, hyperandrogenism decreases the intracellular glutathione (GSH) level in PCOS (Eini *et al.*, 2017), which is a critical antioxidant. Inofolic is one of the supplements used to improve the fertility of PCOS women, and contains 2 g MYO and 200 µg folic acid. MYO plays a key role in many cellular functions (Vitale *et al.*, 2016).

In ovarian follicles, a direct relationship between the MYO concentration in the follicular fluid and the oocyte quality has been observed (Lewin *et al.*, 1973, Chiu *et al.*, 2002). Moreover, increasing the ratio of MYO to D-chiro-inositol (DCI) in the follicular fluid promotes blastocyst quality, implantation, and pregnancy rate (Ravanos *et al.*, 2017). The use of MYO in PCOS patients increases estradiol (Zeng and Yang, 2018) and reduces prolactin, testosterone, the LH/FSH ratio, androstenedione, insulin concentration, the glucose:insulin ratio (Artini *et al.*, 2013), and insulin resistance (Papaleo *et al.*, 2011; Artini *et al.*, 2013). In addition, it improves metabolic status (Laganà *et al.*, 2016). MYO also increases the number of mature oocytes and decreases the number of immature oocytes received in the IVF cycles (Ciotta *et al.*, 2011).

Research on human oocytes is not possible due to ethical issues. Previous clinical studies have examined the effect of MYO on oocyte quality in granulosa cells and follicular fluid (Akbari Sene *et al.*, 2019). This study is the first to assess the direct effect of the optimal dose of MYO on the oocyte quality parameters, including oocyte maturity and morphology, oxidative stress, intracellular GSH, and oocyte ATP content.

## Materials and methods

### Experimental design

In this study, 110 female prepubertal (21-day-old) NMARI mice provided by the Pasteur Institute of Iran were used. The animals were housed in fibreglass cages in a room with a light/dark cycle of 12 h, an ambient temperature of 22–24°C, proper ventilation, and free access to water and food. To determine the effective dose of MYO plus folic acid (Inofolic: LO.LI.pharma-Italian) supplement and its use in the later stages, this study was designed in two phases. First phase: the development and confirmation of the PCOS model along with the determination of MYO plus folic acid effective dose. Second phase: oocyte retrieval and cellular evaluation. The animals used in both stages were subjected to identical conditions. In the first phase, 36 mice were randomly divided into six groups after weighing.

#### *First phase: The development and confirmation of the PCOS model along with the determination of MYO plus folic acid effective dose*

The DHEA group consisted of female prepubertal (21-day-old) mice that received a daily dose of 6 mg/100 g of body weight of dehydroepiandrosterone (DHEA; Sigma Aldrich, St Louis, MO, USA) subcutaneously (s.c.) for 20 days continuously. For injection preparation, 0.01 ml of 95% ethanol was used to dissolve the DHEA powder (Aragno *et al.*, 1997, 2002). Then the resulting

solution was well mixed with 0.09 ml sesame oil (Sigma Aldrich, St Louis, MO, USA) (Henmi *et al.*, 2001; Zhu *et al.*, 2010; Hakimpour *et al.*, 2022). The control group received a mixture of 0.09 ml of sesame oil and 0.01 ml of 95% ethanol subcutaneously (s.c.) for 20 continuous days. All treatment groups received daily DHEA (6 mg/100 g body weight, s.c.) and MYO plus folic acid supplement intraperitoneally (i.p.) in four different doses of: 1.2 mg/g body weight (DI4) (Croze *et al.*, 2013), 0.73 mg/g body weight (DI3) (through conversion of human to animal dose by article formula) (US Food and Drug Administration, 2005), 0.36 mg/g body weight (DI2) (half dose of the DI3), and 0.184 mg/g body weight (DI1) (half dose of the DI2), for 20 days continuously. To confirm the PCOS model, vaginal smear, hormonal examination, and histopathological examination were performed.

**Oestrous cycle assessment.** From the 10th day of the DHEA injection until the end of the study, vaginal smears were taken for cytological examination, daily at a specified time. Cell collections were performed from the beginning of the vagina by swab impregnated with saline lavage. After transferring the cells to glass slides and drying, the samples were stained with crystal violet (Sigma Aldrich, St Louis, MO, USA) and observed under a light microscope (Nikon inverted light microscope ECLIPSE Ti-U, Japan). The dominant cells of each smear represented the stage of the cycle. Proestrus stage with nucleated cells, oestrus stage with cornified epithelium cells, metestrus stage with cornified epithelial cells and leukocytes, and diestrus stage mainly with leukocytes were detected in a vaginal smear (Ubaldi and Rienzi, 2008). To validate the results and eliminate the effect of different stages of the oestrus cycle, in the control group, only the mice that were in the oestrous stage were killed and assessed.

**Hormone assay.** After 8 h of fasting, the mice were anaesthetised with ketamine (100 mg kg<sup>-1</sup>) then 750–1000 µl of blood was taken from the heart and centrifuged at 3000 g for 10 min. After separation, the serum was immediately stored at –80°C for hormonal analysis. Fasting insulin and testosterone levels were assessed by ELISA (catalogue nos. 80552 and 90080, respectively; Crystal Chem, USA). Reagents were added according to the manufacturer's instructions and examined using a spectrophotometer (Bio Teck, Highland Park, USA) at a wavelength of 450 nm.

**Ovarian morphology.** Ovaries were carefully removed from various tissues and placed in 10% formalin for fixation then embedded in paraffin. After routine histological stages, tissue sections with a thickness of 5 µm were cut and placed on a clean glass slide. Followed by dehydration and paraffinization with different concentrations of alcohol, tissue sections were stained with Harris' haematoxylin and eosin (HandE; DAKO, CA, USA) and then were observed and photographed under an inverted light microscope (Nikon ECLIPSE Ti-U inverted microscope, Japan) that was equipped with a camera (Nikon Digital camera D5100, Japan) with ×4 and ×10 magnifications.

During microscopic observation, primordial follicles (oocytes surrounded by a row of squamous granulosa cells), preantral follicles (PAF; oocytes surrounded by multiple layers of cubic granulosa cells), early antral follicles (EAF; the presence of multiple cavities among granulosa cells), antral follicles (AF; the presence of a large and fluid-filled cavity between granulosa cells), corpus luteum (CL), large atretic antral follicles (LAtAnF; degenerated oocytes and sometimes degenerated granulosa cells that collapse in the antral space), and cystic follicles (CF: a thin wall covered with

flat granulosa cells in the absence of oocytes) were examined for their specific histological features. ImageJ software (v.1.50; National Institutes of Health, Bethesda, MD, USA) was used to measure the diameter of follicles and layers. In this study, for the accurate evaluation of follicles, tissue sections from all over the ovaries (beginning to end) were prepared, stained, and examined. To evaluate the diameter of the follicle, the average diameters of small and large follicles were measured from the outer theca layer. To measure the thickness of the theca and granulosa layers of the AF, each layer with different thicknesses in each follicle was measured and their average was presented. The diameter measurement of the theca layer was carried out from areas of the follicle that were not adjacent to another follicle to differentiate theca cells from two adjacent follicles.

#### *Second phase: The oocyte retrieval and cellular evaluation*

**Confirmation of PCOS mouse model.** An effective dose of MYO plus folic acid (0.36 mg/g body weight), which was obtained in the first phase, was used to continue the study. So, 70 female pre-pubertal (21-day-old) NMRI mice were randomly divided into four groups: Control group received (s.c.) a mixture of 0.09 ml of sesame oil and 0.01 ml of 95% ethanol per day ( $n = 15$ ), Sham group injected (i.p.) with 0.36 mg/g body weight MYO plus folic acid per day ( $n = 15$ ), DHEA group received (s.c.) 6 mg/100 g body weight DHEA per day ( $n = 25$ ), and treatment group injected daily with (s.c.) 6 mg/100 g body weight DHEA along with 0.36 mg/g body weight MYO plus folic acid (i.p.), ( $n = 15$ ). All injections were performed for 20 continuous days. Vaginal smears were taken every day from the 10th day onwards. At the end of the study, four mice from the DHEA group were anaesthetised with ketamine (100 mg kg<sup>-1</sup>) to confirm the PCOS model, and the ovaries were removed for histopathological examination. Ovulation stimulation was performed in the remaining mice of the four groups to release mature oocytes.

#### *Oocyte collection and investigation of maturity and morphology.*

To obtain mature oocytes, 10 IU of pregnant mare serum gonadotropin (PMSG) hormone (Gestyl; Organon, Oss, The Netherlands) was injected i.p. and 48 h later 5 IU human chorionic gonadotrophin (hCG) (Pregnyl, Organon, Oss, The Netherlands) was injected in the same way. Mice were killed 14–16 h later by cervical dislocation. Then under a stereomicroscope (Luxeo 6Z Stereozoom Microscope, Labo America, Inc, Roseville, CA, USA) oviducts were opened in a dish containing HTCM culture medium (HEPES-buffered TCM199; Sigma Aldrich, St Louis, MO, USA) supplemented with 0.5% fetal bovine serum (FBS; GIBCO BRL Invitrogen). To isolate oocytes from cumulus cells, the cumulus–oocyte complexes (COCs) were pipetted in HTCM medium containing 100 IU ml<sup>-1</sup> hyaluronidase. Finally, all oocytes of the four groups were counted in three categories of abnormal oocytes, meiosis I (MI) stage oocytes, and MII stage oocytes. Subsequently, MII oocytes were selected for the cellular study taking into consideration the normal morphological parameters such as having spherical oocyte, round and smooth zona pellucida, small perivitelline space (PVS), and cytoplasm with low and uniform granulation (Ubaldi and Rienzi, 2008). For imaging oocytes, a stereomicroscope equipped with a camera was used.

**Detection of intracellular ROS levels.** Intracellular ROS levels were detected as green fluorescence in a dark environment using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen, Barcelona, Spain). From each group, 35–40 MII oocytes were washed

in phosphate-buffered saline (PBS) containing 1 mg ml<sup>-1</sup> polyvinyl alcohol (PBS-PVA) droplets to remove the previous culture medium. Then, the oocytes were washed in several PBS-PVA drops containing 10 μM H2DCFDA and incubated for 30 min at 37°C. Afterwards, the washed oocytes were imaged by fluorescence microscopy (Olympus fluorescence microscope, Tokyo, Japan) with a 460 nm filter and saved as graphic files in TIFF format (Wang *et al.*, 2014). Using ImageJ software, the fluorescence pixel intensity was analyzed in different groups.

#### *Measurement of the intracellular glutathione (GSH) level.*

Intracellular GSH levels were detected as blue fluorescence in a dark environment using 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker Blue CMF2HC Molecular Probes; Invitrogen, Barcelona, Spain). After washing, 35–40 MII oocytes from each group in PBS drops containing 1 mg ml<sup>-1</sup> PVA, they were washed several times in PBS-PVA drops containing 10 μM Cell Tracker Blue and incubated for 30 min at 37°C. Finally, the washed oocytes were rapidly photographed under a fluorescence microscope with a 370 nm filter (Wang *et al.*, 2014). The fluorescence intensity of the images, as graphic files in TIFF format, was analyzed by ImageJ software.

#### *Measurement of ATP content.*

To evaluate the ATP content in this study, a colorimetric-based commercial mouse adenosine triphosphate ELISA kit (AB Clonal Technology, USA) was used. In total, 15 mature oocytes from each group were loaded into the microtubes with 20 μl of ultrapure water and stored at –80°C until the ATP was evaluated. For cell lysis, 100 μl of schizolysis (centrifugal separation technique based on component density) solution was added to each microtube containing oocytes and vortexed on ice for 1 min. To obtain a supernatant, the resulting mixtures were centrifuged at 10,000 g for 10 min at 4°C. After this step, the supernatant was loaded into microplates and reagents were added according to the manufacturer's instructions. Finally, light absorption was read using an ELISA reader (Bio Teck, Highland Park, USA) with a wavelength of 450 nm. In each assay, a six-point standard curve obtained from different concentrations of standard solution (0 to 5 ng ml<sup>-1</sup>) and their light absorption readings were used. The ATP content of oocytes in four groups was calculated using the linear formula obtained from the standard curve.

#### *Statistical analysis*

The entire experiments were repeated three times and the results were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 25.0 statistical software (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, USA), whereas a *P*-value < 0.05 was considered as statistically significant. The obtained data were analyzed via one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc multiple comparisons test. An independent-sample *T*-test was used for analysis between the two groups.

## **Results**

### *The first phase: The development and confirmation of the PCOS mouse model along with determination of MYO plus folic acid effective dose*

#### *The effect of DHEA and MYO on oestrous cycle and ovarian tissue morphology*

The investigations showed that the mice in the DHEA group became acyclic and remained at the oestrus stage (predominant



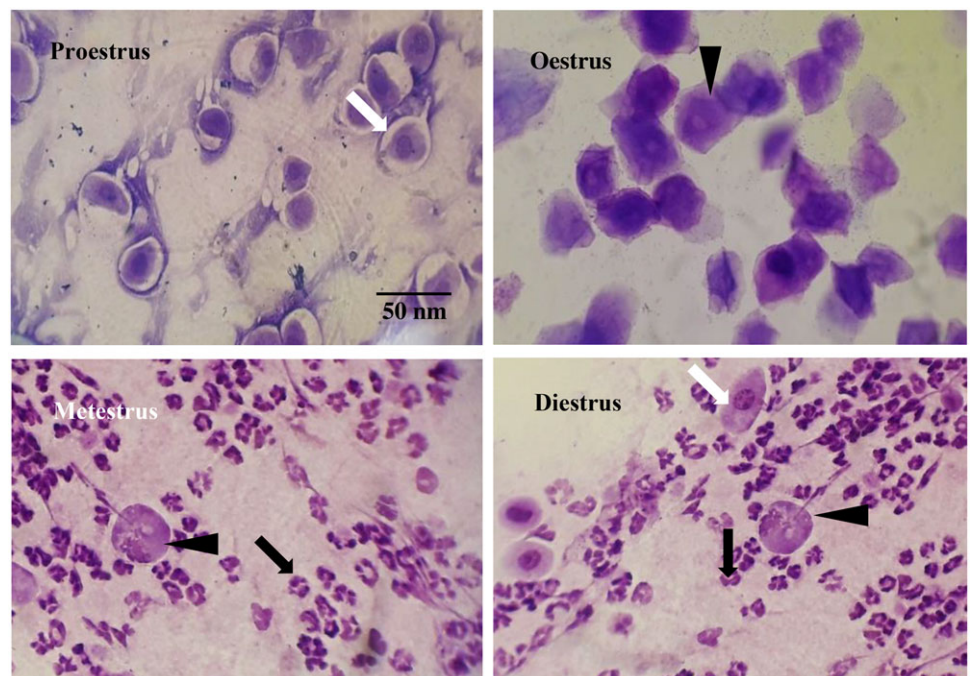
**Table 1.** Comparison of follicles types number in the study groups

Number of variables	Group 1 (control)	Group 2 (DHEA group)	Group 3 (DI1)	Group 4 (DI2)	Group 5 (DI3)	Group 6 (DI4)	P-value
Preantral follicles	5 ± 0.41	11.2 ± 1.2	10.75 ± 0.75	10 ± 1.30	9 ± 1.53	13.8 ± 1.93	0.003
Early antral follicles	4 ± 0.41	5.6 ± 0.51	4.5 ± 0.64	4.2 ± 0.48	5 ± 0.58	4.6 ± 0.51	0.325
Antral follicles	1.5 ± 0.29	4 ± 0.32	2.5 ± 0.64	3 ± 0.41	3 ± 0.58	2.4 ± 0.24	0.008
Cystic follicles	0	1.2 ± 0.37	0.75 ± 0.25	0.5 ± 0.29	0.33 ± 0.33	1 ± 0.45	0.174
Large atretic antral follicles	0	1.8 ± 0.58	2 ± 0.41	1 ± 0.41	1.6667 ± 0.33	2.4 ± 0.24	0.006
Corpus luteum	8.75 ± 0.85	0	0.25 ± 0.25	1.5 ± 0.65	0.75 ± 0.48	0.25 ± 0.25	000

Data are presented as mean ± SEM and *P*-value < 0.05 was significant (variance analysis and LSD) *n* = 6.

Table 1 presents the obtained values for the six variables in the six studied groups. Group 1 was the control, group 2 as the DHEA group, and the remaining four groups (groups 3–6) are groups treated with DHEA and different concentrations of myo-inositol plus folic acid (Inofolic) including: 0.184 mg/g (DI1: group 3), 0.36 mg/g (DI2: group 4), 0.73mg/g (DI3: group 5), and 1.2 mg/g (DI4: group 6).

As can be seen, for the preantral follicles significant differences between group 1 with groups 2, 3, 4, and 6 (*P* < 0.01) were observed. Also, group 5 showed a significant difference with group 6 (*P* < 0.05). Regarding the early antral follicles, a significant difference was found between groups 1 and 2 (*P* < 0.05). For antral follicles significant differences between groups 1 with group 2 (*P* < 0.0001), group 1 with groups 4 and 5 (*P* < 0.05), and group 2 with groups 3 and 6 (*P* < 0.05) were observed. Regarding cystic follicles significant differences between group 1 with groups 2 and 6 (*P* < 0.05) were found. For large atretic antral follicles, significant differences between group 1 with groups 2, 3, and 6 (*P* < 0.005), group 1 with group 5 (*P* < 0.05), and group 4 with group 6 (*P* < 0.05) were obvious. Last, the corpora lutea were significantly different between group 1 with groups 2–6 (*P* < 0.000), as well as group 2 with group 4 (*P* = 0.033).

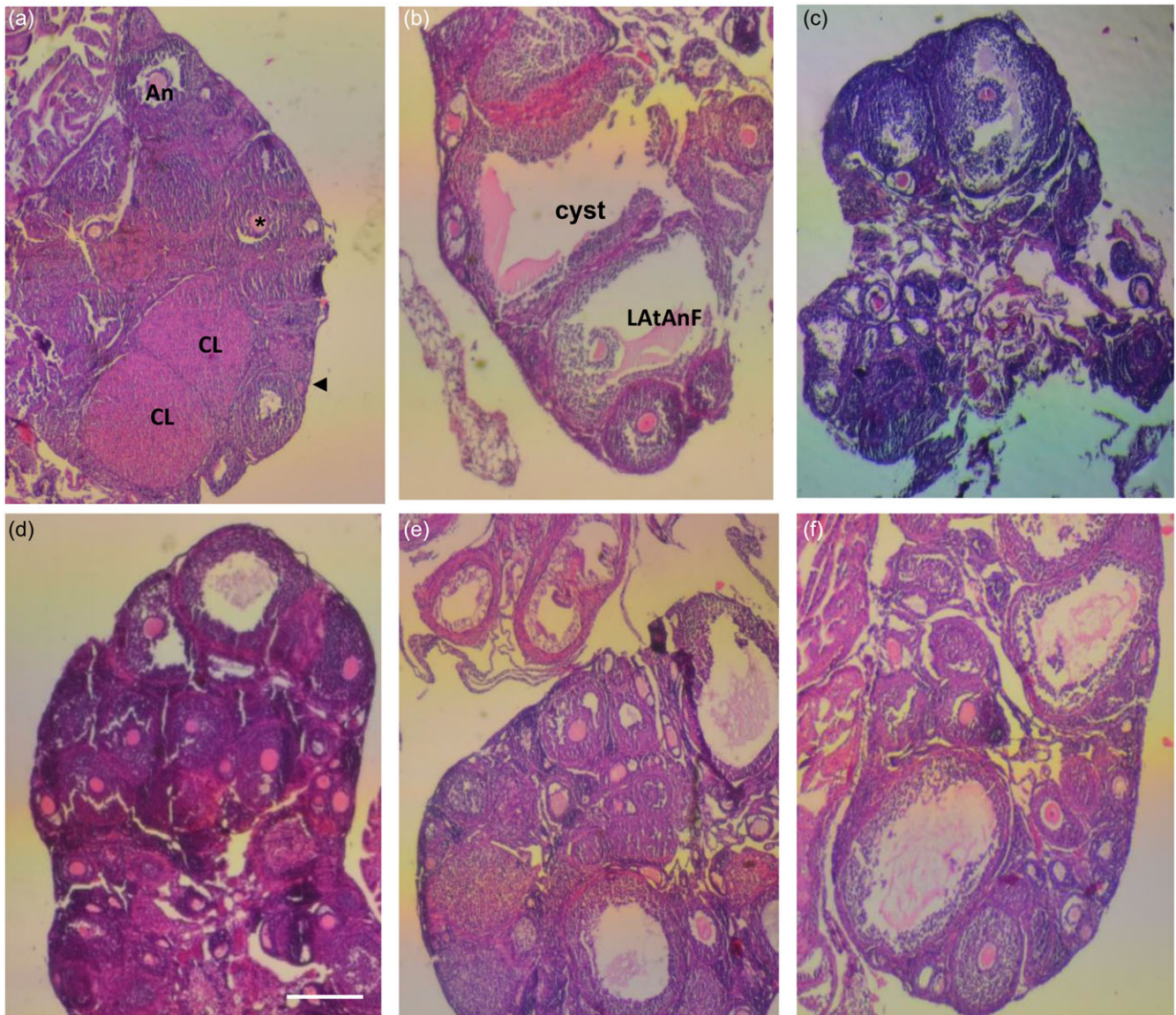


**Figure 1.** Evaluation of vaginal smear cytology to identify different stages of mice oestrous cycle: Three main cells are visible in vaginal smears. Nuclear epithelial cells (white arrow), cornified epithelial cells (black arrowheads), and leukocytes (black arrow). The predominant presence of each of these cells determines the stage of the oestrous cycle. Dominant cells in oestrus are nucleated epithelial cells, in oestrus cornified epithelial cells, in metestrus leukocyte and cornified epithelial cells, and in diestrus leukocyte.

cells were cornified epithelium) (Figure 1). Moreover, in this group, the absence of CL was significantly different compared with the control group (*P* < 0.000), which indicated anovulation in PCOS mice. In all the MYO plus folic acid recipient groups, a relative improvement in the oestrous cycle and the presence of CL in the ovaries were observed. However, compared with the DHEA group, only in the group treated with DHEA and 0.36 mg/g MYO plus folic acid (DI2), significant increases in the numbers of corpora lutea were observed (*P* < 0.033).

In the ovaries of the DHEA group mice, as in the control group mice, follicles were noticed at different stages of development. The results showed that in the DHEA group the numbers of preantral, early antral, and AF increased significantly compared with the control group (*P* < 0.01, *P* < 0.05, and *P* < 0.0001, respectively).

However, the numbers of preantral follicles in the DI3 group (0.73 mg/g MYO plus folic acid) and the numbers of AF in the DI1 group (0.184 mg/g MYO plus folic acid) were not significantly different compared with the control group. DHEA injection for 20 days led to a significant increase in the numbers of LATAnF and CF in the DHEA group compared with the control group (*P* < 0.004, *P* < 0.02, respectively). It was also observed that the numbers of CF in the groups treated with DHEA and MYO plus folic acid in the three groups DI1 (0.184 mg/g), DI2 (0.36 mg/g), and DI3 (0.73 mg/g) were not significantly different compared with the control and DHEA groups. In this study, LATAnF numbers were significantly increased in all the studied groups except for the group treated with DHEA and 0.36 mg/g MYO plus folic acid (DI2) compared with the control group (*P* < 0.01) (Table 1, Figure 2).



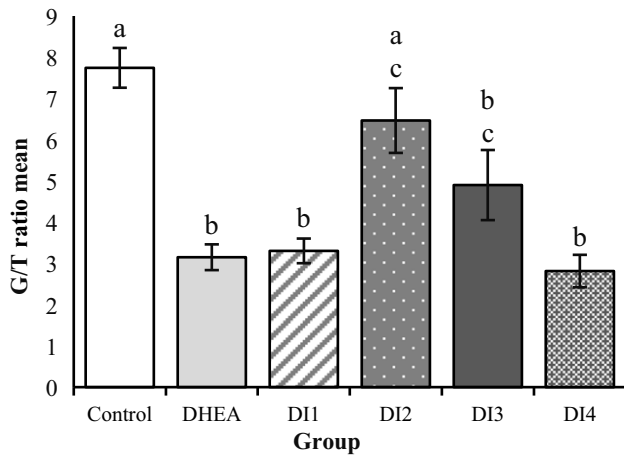
**Figure 2.** Comparison of mice ovary morphology in groups of control, DHEA group, and groups treated with DHEA and different concentrations of myo-inositol plus folic acid (Inofolic). (a) The part of the ovarian tissue in the control group that contains different categories of follicles, including the primordial follicle shown by the arrowhead, the early antral follicle (EAn) marked with an asterisk (\*), the antral follicle shown as (An), and the corpus luteum pointed as (CL). (b) Part of the ovarian tissue of the DHEA group is characterized by large cystic follicles (cyst, CF) and large atretic antral follicles (LAtAnF). (c) Part of the ovarian tissue in the DHEA-treated group with 0.184 mg/g MYO plus folic acid (DI1). (d) Part of the ovarian tissue in the DHEA-treated group with 0.36 mg/g MYO plus folic acid (DI2). (e) Part of the ovarian tissue in the DHEA-treated group with 0.73 mg/g MYO plus folic acid (DI3). (f) Part of the ovarian tissue in the DHEA-treated group with 1.2 mg/g MYO plus folic acid (DI4). All images are magnification  $\times 40$  (scale bar: 200  $\mu\text{m}$ ) ( $n = 6$ ).

The G/T ratio in the AF of the DHEA group was significantly lower than in the control group ( $P < 0.0001$ ). The G/T ratio showed a significant increase only in the AF of the group treated with DHEA and 0.36 mg/g MYO plus folic acid (DI2) compared with the DHEA group ( $P < 0.0001$ ). To evaluate the G/T ratio, 20 follicles were selected from the ovaries of six mice in each group (Figure 3). Significant reduction in LAtAnF diameter was only observed in the group treated with DHEA and 0.36 mg/g MYO plus folic acid (DI2) compared with the DHEA group ( $P < 0.0001$ ), and in the other MYO plus folic acid doses, no significant reduction was observed in diameter of LAtAnF. To examine the large antral atrial follicles, complete ovarian sections of six mice in each group were analyzed (Figure 4).

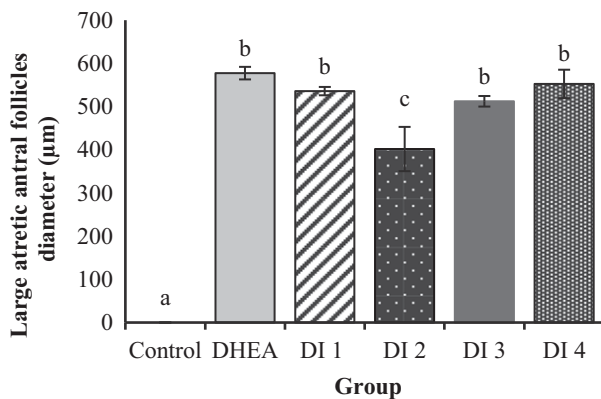
#### *Effect of DHEA and MYO on testosterone and fasting insulin levels*

The results showed that fasting insulin and testosterone concentrations were significantly increased in all groups compared with the control group ( $P < 0.0001$  and  $P < 0.01$ , respectively). As shown in Figure 5(a), it was observed that testosterone concentrations decreased in all groups receiving MYO plus folic acid and DHEA compared with the DHEA group, but only in the DI2 group with a dose of 0.36 mg/g per day, was the decrement significant ( $P < 0.05$ ). The experiment showed that fasting insulin levels in none of the groups were significantly lower than in the DHEA group. In this study, MYO plus folic acid did not cause a significant decrease in serum insulin levels (Figure 5b).





**Figure 3.** The effect of dehydroepiandrosterone (DHEA) and different concentrations of myo-inositol plus folic acid (Inofolic) on the morphometry of granulosa theca layer thickness (G/T) ratio in antral follicles of mice ovaries. The six studied groups are including the control group, the DHEA group, and the groups treated with DHEA and MYO plus folic acid at different concentrations such as 0.184 mg/g (DI1), 0.36 mg/g (DI2), 0.73 mg/g (DI3), and 1.2 mg/g (DI4). Data are the mean  $\pm$  SEM ( $n = 20$ ). As the graph presents, there was a significant reduction in DHEA recipient groups (DHEA group, DI1, DI4, and DI3) compared with the control group ( $P < 0.0001$  and  $P = 0.003$ , respectively) and significant increase in the group treated with DHEA and 0.36 mg/g MYO plus folic acid (DI2) compared with the DHEA group ( $P < 0.0001$ ). However, there was no significant difference between the control group and the DI2 group. Different lowercase letters above bars indicate significant difference.



**Figure 4.** The effect of dehydroepiandrosterone (DHEA) and different concentrations of myo-inositol plus folic acid (Inofolic) on the diameter morphometry of large atretic antral follicles (LATAnF) in the mice ovary. The six groups included the control group, the DHEA group, and four groups of treated with DHEA and MYO plus folic acid at different concentrations such as 0.184 mg/g (DI1), 0.36 mg/g (DI2), 0.73 mg/g (DI3), 1.2 mg/g (DI4). Data are presented as the mean  $\pm$  SEM ( $n = 6$ ). There was a significant increase in all groups receiving DHEA (DHEA group, DI1, DI2, DI3, and DI4) compared with the control group ( $P < 0.0001$ ) and a significant decrease in the DI2 group with the concentration of 0.36 mg/g MYO plus folic acid compared with the DHEA group ( $P < 0.0001$ ). Different lowercase letters above bars indicate significant difference.

### The second phase: The oocyte retrieval and cellular evaluation

#### Confirmation of PCOS mouse model

In the second phase of the study, the cessation of the oestrous cycle in the vaginal smear and the presence of CF in the DHEA group ovaries were observed ( $n = 3$ ). The numbers of CF increased significantly compared with the control group ( $P < 0.01$ ), which confirmed the PCOS model (Figure 6).

#### The effect of DHEA and MYO on oocyte maturity and morphology

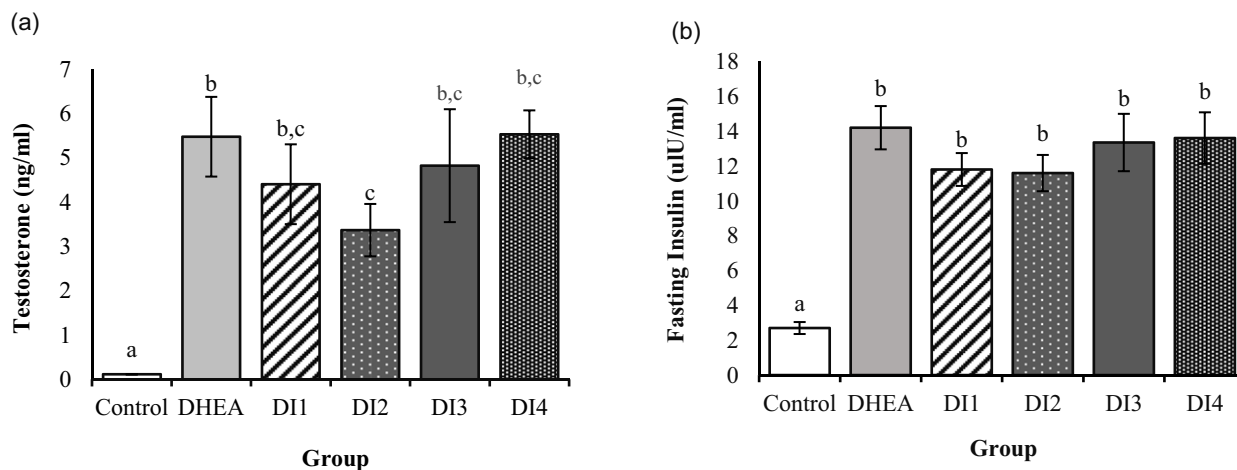
The results showed that DHEA led to a significant increase in the percentage of abnormal morphology (fragmented or giant polar body, large PVS, and fragmented cytoplasm) and immature oocytes (MI) in the DHEA group compared with the control, sham, and DHEA-treated with MYO plus folic acid (DI2) groups (MI:  $17.71 \pm 1.89$  vs  $4.60 \pm 1.07$  and  $5.79 \pm 0.99$ ;  $P < 0.0001$  and  $10.33 \pm 1.35$ ;  $P = 0.001$ , respectively); abnormal morphology:  $22.28 \pm 2.74$  vs  $5.00 \pm 1.36$  and  $5.10 \pm 1.27$ ;  $P < 0.0001$  and  $12.00 \pm 1.77$ ;  $P = 0.001$ , respectively). Conversely, there was a significant decrease in the percentage of mature oocytes (MII) in the DHEA group compared with the control, sham, and DHEA-treated with MYO plus folic acid (DI2) groups (MII:  $54.14 \pm 5.01$  vs  $83.83 \pm 4.00$  and  $84.83 \pm 2.721$ ;  $P < 0.0001$ , and  $69.66 \pm 5.51$ ;  $P = 0.02$ , respectively). The results showed that the supplement of MYO plus folic acid in the DI2 group significantly increased the percentages of normal morphology and mature oocytes compared with the DHEA group. Conversely, despite the use of this supplement, the maturity and normal morphology percentages of the oocytes decreased significantly compared with the control and sham groups (MII:  $69.66 \pm 5.51$  vs  $83.83 \pm 4.00$  and  $84.83 \pm 2.72$ ; abnormal morphology:  $12.00 \pm 1.77$  vs  $5.00 \pm 1.36$  and  $5.10 \pm 1.27$ , respectively;  $P < 0.05$ ) (Figure 7).

#### The effect of DHEA and MYO on intracellular levels of ROS and GSH

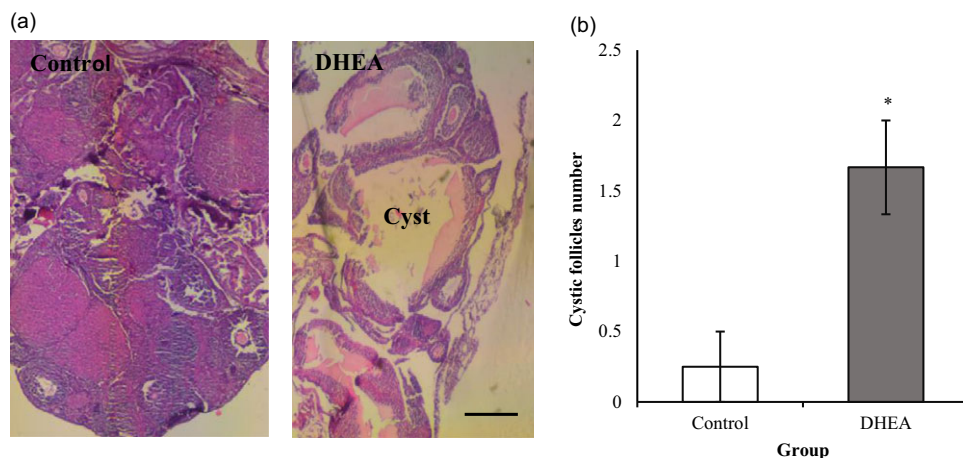
The addition of DHEA led to a significant increase in intracellular ROS ( $H_2O_2$ ) levels in the DHEA group compared with the control and sham groups ( $80.89 \pm 7.63$  vs  $17.49 \pm 2.23$  and  $15.8 \pm 2.23$  pixels per MII oocyte, respectively;  $P < 0.0001$ ; Figure 6a–c). MYO plus folic acid at the 0.36 mg/g concentration in the DI2 group led to a significant decrease in intracellular ROS levels compared with the DHEA group ( $59.96 \pm 6.07$  vs  $80.89 \pm 7.63$  pixels per MII oocyte,  $P < 0.05$ ; Figure 6c,d). Nevertheless, intracellular ROS levels in this group had a significant increase compared with the control and sham groups ( $59.96 \pm 6.07$  vs  $17.49 \pm 2.23$  and  $15.8 \pm 2.23$  pixels per MII oocyte groups, respectively;  $P < 0.0001$ ; Figure 6a,b,d). Outcomes showed that the level of intracellular GSH was significantly reduced in MII oocytes in the DHEA group compared with the control and sham groups ( $82.69 \pm 5.99$  vs  $150.15 \pm 8.22$  and  $153.1 \pm 7.146$  pixels per MII oocyte groups, respectively;  $P < 0.0001$ ; Figure 6e–g). However, MYO plus folic acid at the 0.36 mg/g concentration in the DI2 group led to a significant increase in intracellular GSH levels compared with the DHEA group ( $110.72 \pm 12$  vs  $82.69 \pm 5.99$  pixels per MII oocyte groups;  $P < 0.05$ ; Figure 6g,h) (Figure 8).

#### The effect of DHEA and MYO on ATP content

DHEA with mitochondrial dysfunction caused a significant reduction in ATP content in the DHEA group compared with the control and sham groups ( $33.08 \pm 9.43$  vs  $216.06 \pm 37.28$  and  $175.69 \pm 2.85$  ng/ml;  $P < 0.0001$ ). In the group receiving DHEA and MYO plus folic acid (DI2), ATP content was markedly higher than in the DHEA group ( $93.31 \pm 18.09$  vs  $33.08 \pm 9.43$  ng/ml;  $P < 0.05$ ) but, in this group, ATP content still had a significant decrement compared with the control and sham groups ( $93.31 \pm 18.09$  vs  $216.06 \pm 37.28$  and  $175.69 \pm 2.85$  ng/ml;  $P < 0.001$ ) (Figure 9).



**Figure 5.** Effect of dehydroepiandrosterone (DHEA) and different concentrations of myo-inositol plus folic acid (Inofolic) on testosterone and fasting insulin serum levels. Serum levels of testosterone (a) and fasting insulin (b) in the control group, DHEA group, and four groups treated with DHEA and MYO plus folic acid at different concentrations including 0.184 mg/g (DI1), 0.36 mg/g (DI2), 0.73 mg/g (DI3), 1.2 mg/g (DI4). Data are presented as the mean  $\pm$  SEM ( $n = 6$ ). As panels (a) and (b) show, significant increases in testosterone and fasting insulin concentrations were observed in all groups compared with the control group ( $P < 0.01$  and  $P < 0.0001$ , respectively). Testosterone levels decreased significantly only in the DI2 group compared with the DHEA group ( $P < 0.05$ ). No significant decrease in fasting insulin concentration was observed in the groups DHEA-treated with MYO plus folic acid compared with the DHEA group. Different lowercase letters above bars indicate significant difference.



**Figure 6.** Part of ovarian tissue in control and DHEA group. (a) Presence of cyst follicles in the DHEA group. (b) Effect of dehydroepiandrosterone on the cystic follicles' formation in control and DHEA group groups (scale bar: 200  $\mu$ m). Data are presented as the mean  $\pm$  SEM ( $n = 3$ ). \* $P < 0.01$  compared with the control group with the DHEA group.

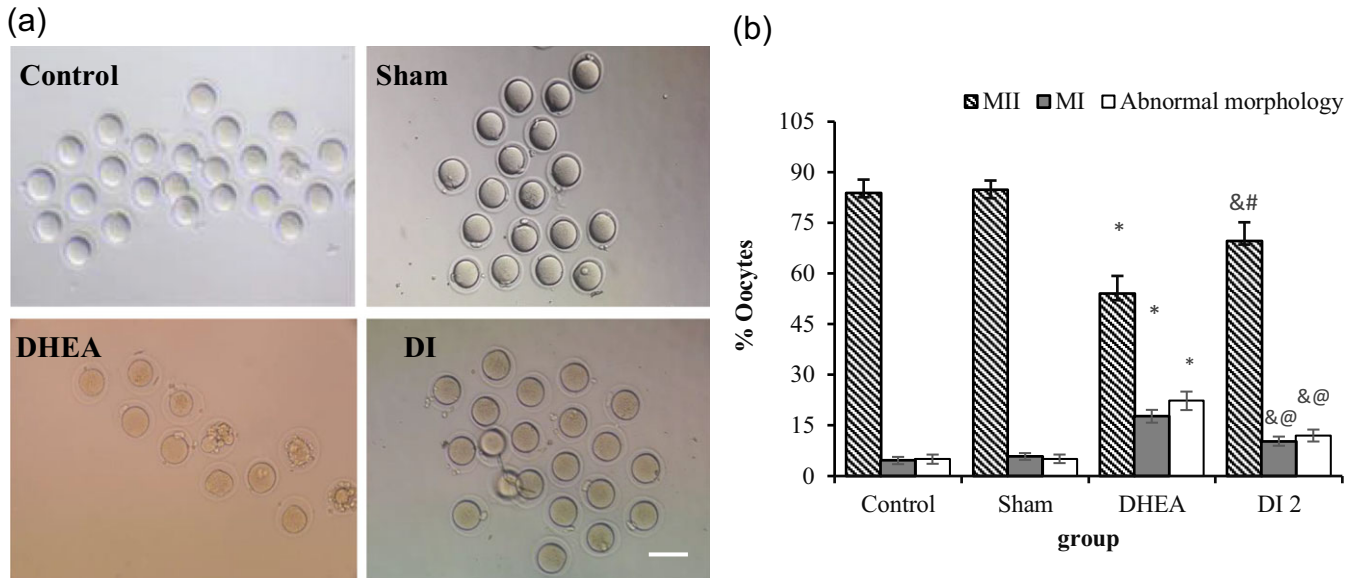
## Discussion

In the present study, which followed MYO dosimetry in a PCOS mice model, the morphology of the ovary and the number of immature oocytes, fasting insulin and testosterone levels, oxidative stress, and ATP production were examined. The findings showed that MYO plus folic acid supplementation could improve almost all parameters, except for fasting insulin.

Hyperandrogenism affects the growth of preantral follicles until early antral by increasing the expression of androgen receptors on these follicles (Gleicher *et al.*, 2011). Previous studies have indicated that the continuous administration of DHEA in mice with increasing plasma androgen concentration led to the abnormal development of growing follicles, failure to select the dominant follicle for ovulation, and the formation of CF (Huang *et al.*, 2015; Nikmard *et al.*, 2017) and LAtAnF (Kim *et al.*, 2003). Similarly, our findings indicated that the injection of DHEA into mice significantly increased the numbers of preantral, early antral, antral, cystic, and LAtAn follicles compared with the control group. Moreover, it led to oestrous cycle disorders, anovulation, and the absence of the CL in the ovaries. In this study, for the first time,

the effects of MYO plus folic acid on different types of ovarian follicles, G/T ratio in AF, and the diameter of large atretic AF in the PCOS mice model were evaluated.

In PCOS patients, MYO is converted to DCI by the enzyme insulin-dependent epimerase, and the increased DCI enhances androgen production in theca cells (Unfer *et al.*, 2014). In the first phase of the present study (dosimetry), the administration of MYO plus folic acid reduced the numbers of preantral (DI3) and antral (DI1) follicles in some groups, which were not significantly different from the control group. Moreover, there was no significant difference between the control group and the three PCOS groups receiving MYO plus folic acid (DI1, DI2, DI3) regarding the number of CF. Also, in the DI2 group (0.36 mg/g), no significant difference was observed in the numbers of LAtAnF compared with the control group, which illustrated the positive effect of this supplement on ovarian morphology, as shown by a relative decrease in the number of growing follicles, CF and LAtAnF. MYO administration reduced DCI-induced androgen synthesis in theca cells by increasing the MYO/DCI ratio in the ovary (Unfer *et al.*, 2017, 2020). It seems that a reduction in the intracellular androgen levels



**Figure 7.** The effect of dehydroepiandrosterone (DHEA) and myo-inositol plus folic acid (Inofolic) on maturation and oocyte morphology in a PCOS mice model. (a) Images of oocyte maturation and morphology in the control, sham, DHEA, and DHEA-treated with MYO plus folic acid (DI2) groups. (b) Quantified data. Data are presented as the mean  $\pm$  SEM [ $n = 217, 203, 83,$  and  $134$  in the control, sham, DHEA, and DHEA-treated with MYO plus folic acid (DI2) groups, respectively]. In the DHEA group, a significant decrease in the MII oocytes percentage and increase in the percentage of MI oocytes and abnormal morphology were observed (\*). In the DI2 group significant increases in the MII oocytes percentage ( $^{\#}P = 0.02$ ) and decrease in MI oocytes and abnormal morphology percentage ( $^{\textcircled{a}}P = 0.001$ ) were found compared with the DHEA group;  $^{\textcircled{\&}}P < 0.05$ , in the DI2 group compared with the control and sham groups (scale bars:  $100 \mu\text{m}$ ).

led to a relative improvement in the development of growing follicles and a relative decrease in ovarian follicular atresia.

MYO also improves ovulation and the menstrual cycle by reducing testosterone levels (Zacchè et al., 2009; Unfer et al., 2017) and enhancing FSH signalling (Bizzarri et al., 2016; Zeng and Yang, 2018; Kamenov and Gateva, 2020). In this research, CL was observed in the ovaries of four groups receiving MYO plus folic acid. However, only in the DI2 group, the numbers of corpora lutea were significantly larger than in the DHEA group, indicating the positive effect of this supplement with the  $0.36 \text{ mg/g}$  dose on the oestrous cycle and ovulation relative recovery compared with the other MYO doses. An increased plasma androgen concentration elevates the oxidative stress and apoptosis in the oocyte and inner layer of granulosa cells of follicles, which diminishes the G/T ratio and the oocyte quality (Maurya et al., 2014; Mikaeili et al., 2016; Azhary et al., 2019). Furthermore, Kim et al. (2003) showed that, following DHEA administration, apoptosis in the ovarian increased the diameter of the AF. Eventually, the follicles shifted to atresia, in which ovarian morphology is characterized by the appearance of cystic and large atretic AF.

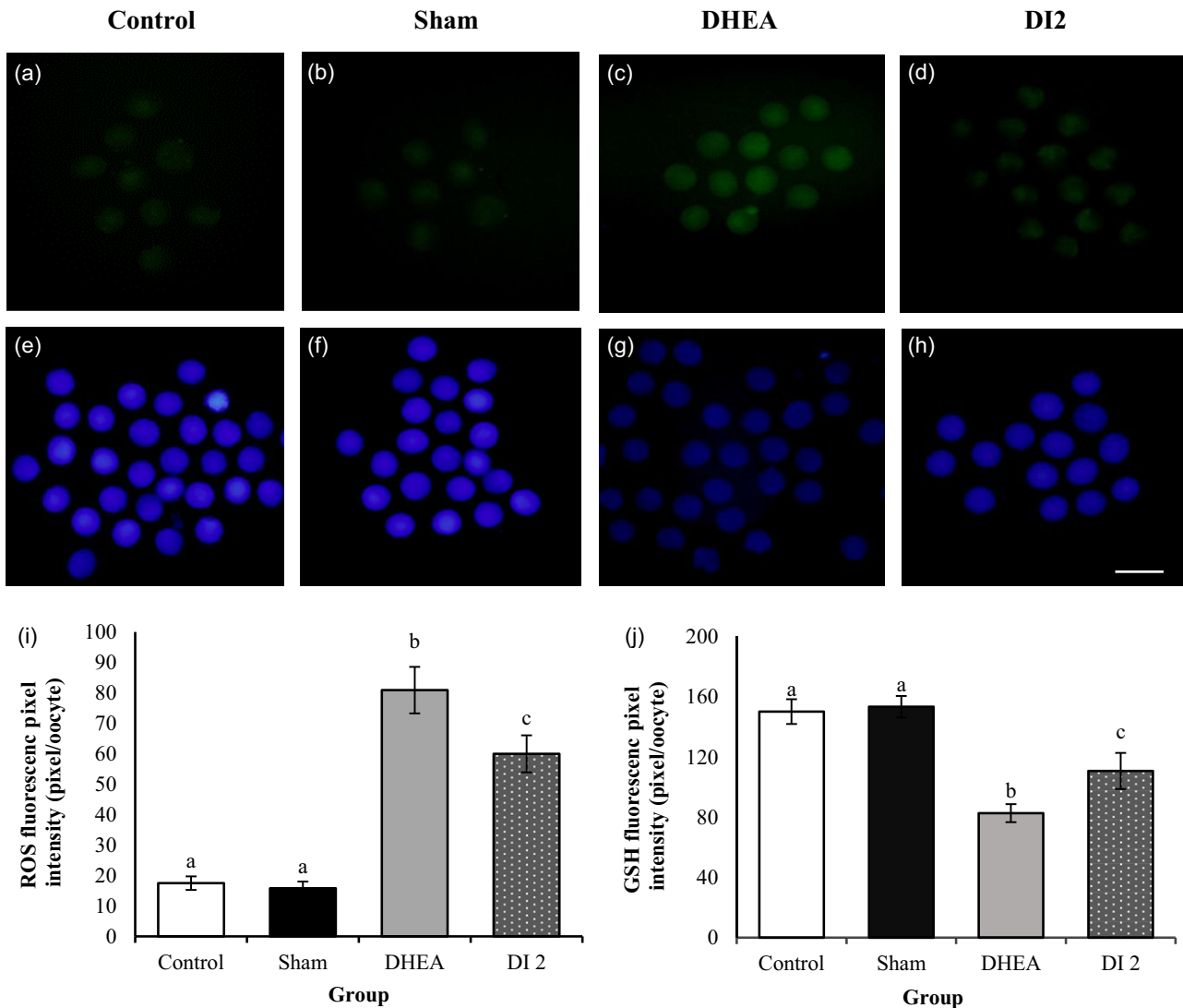
Similarly, the G/T ratio in the DHEA group decreased significantly, whereas the diameter of the AF increased. In PCOS patients, MYO increased *CDC42* gene expression, thereby reducing granulosa cell apoptosis (Akbari Sene et al., 2019). Similarly, the G/T ratio of AF in the DI2 group markedly increased compared with the DHEA group and was not significantly different from the control group. Moreover, the diameter of LAtAn follicles was significantly smaller only in the DI2 group compared with other groups. According to these findings, MYO plays an effective role in enhancing the G/T ratio and reducing the diameter of the LAtAn follicle by diminishing apoptosis in the ovary.

Nikmard et al. (2017) reported that the daily injection of DHEA in mice increased plasma testosterone levels. Song et al. (2018) reported a significant increase in fasting serum insulin levels in

mice receiving DHEA. Similarly, our findings revealed increased levels of testosterone and fasting insulin in the DHEA-treated groups. In PCOS patients, MYO reduces testosterone levels (Zacchè et al., 2009; Unfer et al., 2017); however, several studies have revealed conflicting findings regarding its effect on fasting insulin levels (Kamenov and Gateva, 2020). Some studies have documented that MYO is ineffective in PCOS patients who have a high BMI (Gerli et al., 2007), and some others have indicated its benefits in the same group of patients with high fasting insulin levels (Genazzani et al., 2012). In the present study, MYO supplementation did not significantly reduce fasting serum insulin in any dose; however, it significantly reduced testosterone levels in the DI2 group compared with the DHEA group. We speculate that the DHEA hormonal signal is too strong to be fully counteracted by MYO supplementation, which was not sufficient to recover the insulin levels of the control group, as well as testosterone, despite the significant decrease observed in testosterone levels.

Evaluating the effects of the four doses of MYO plus folic acid on the PCOS mice demonstrated that the most effective dose of MYO plus folic acid was  $0.36 \text{ mg/g}$  because of the relative improvement of ovarian morphology and serological parameters. Accordingly, this dose was considered in the second phase of the study (cellular evaluation). Patel and Carr (2008) reported that hyperandrogenism with impaired folliculogenesis led to the cessation of follicle growth before maturity, resulting in low-quality oocytes and a high ratio of immature oocytes (GV and MI) to mature oocytes (MII) (Tamura et al., 2008). In the DHEA group, as in previous studies (Eini et al., 2017), there was a significant decrease in MII oocytes and a significant increase in abnormal morphology and immature oocytes (MI) compared with the control group. MYO reduces hyperandrogenism (Zacchè et al., 2009; Unfer et al., 2017) and plays a key role in calcium homeostasis. MYO is converted to inositol triphosphate ( $\text{IP}_3$ ) by phospholipase C, and  $\text{IP}_3$  can bind to  $\text{IP}_3$  receptors ( $\text{IP}_3\text{R}$ ) on the endoplasmic



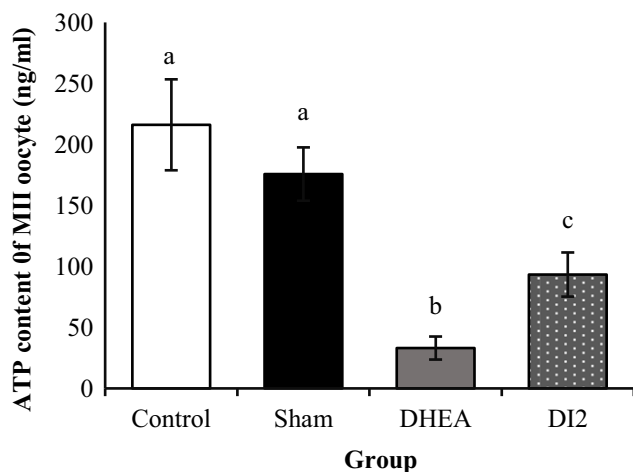


**Figure 8.** Effect of dehydroepiandrosterone (DHEA) and myo-inositol plus folic acid (Inofolic) on intracellular ROS and GSH levels of mouse MII oocytes. (a–d) Images of intracellular ROS and (e–h) intracellular GSH levels of MII oocytes in the control, sham, DHEA, and DHEA-treated with MYO plus folic acid (DI2) groups that were stained by H2DCFDA and Cell Tracker Blue, respectively. Scale bar, 100  $\mu$ m. (i, j) Data were quantified by ImageJ software and showed the fluorescence intensity of MII oocytes stained by (i) H2DCFDA and (j) Cell Tracker Blue. Data are presented as the mean  $\pm$  SEM and  $P < 0.05$  ( $n = 40$ ). In the DHEA group, fluorescence intensity significantly increased and decreased in ROS and GSH, respectively, compared with the control and sham groups ( $P < 0.0001$ ). In the DHEA-treated with MYO plus folic acid (DI2) group, fluorescence intensity showed a significant decrease and increase in ROS and GSH, respectively ( $P < 0.05$ ) compared with the DHEA group. Different lowercase letters above bars indicate significant difference.

reticulum and induce calcium release. The increased concentration of intracellular and mitochondrial calcium plays a special role in meliorating calcium homeostasis. Numerous studies have documented that IP<sub>3</sub>-induced calcium release plays a vital role in oocyte maturation during oocyte maturation (Mehlmann and Kline, 1994; Goud *et al.*, 1999). As in previous studies (Ciotta *et al.*, 2011; Zeng and Yang, 2018), the present study showed that the injection of an effective dose of MYO plus folic acid (DI2) improved folliculogenesis and significantly increased the percentages of normal morphology and MII oocytes, compared with the DHEA group.

Mitochondria is the main source of calcium homeostasis, ATP (Cardozo *et al.*, 2011), and intracellular ROS production, and it is the target organ of ROS (Huang *et al.*, 2015). Hyperandrogenism plays a critical role in oxidative stress and apoptosis (Karuputhula *et al.*, 2013; Ikeda *et al.*, 2014). Accordingly, hyperandrogenism-induced ROS production directly disrupts mitochondrial function

by impairing calcium homeostasis (Wesson and Elliott, 1995) and initiates a defective cycle of ROS production in mitochondria (Huang *et al.*, 2015). Consistent with Ying Huang and co-worker's findings (Huang *et al.*, 2015), this study showed that the DHEA-induced PCOS group had increased intracellular ROS levels and decreased GSH levels compared with the control group. In general, GSH, as a ROS scavenger, plays a significant role in maintaining redox homeostasis (Wang *et al.*, 2014). In this study, the effects of MYO plus folic acid on the ROS, GSH, and ATP levels of mature oocytes were investigated for the first time in the PCOS mouse model. Our findings indicated that, in the group receiving MYO plus folic acid with an effective dose of 0.36 mg/g, the intracellular levels of ROS and GSH significantly decreased and increased, respectively, compared with the DHEA group. Due to its antioxidant role (Lewin *et al.*, 1973), MYO could partly reduce intracellular oxidative stress, maintain the balance of oxidants and antioxidants, and improve mitochondrial function in oocytes.



**Figure 9.** Effect of dehydroepiandrosterone (DHEA) and myo-inositol plus folic acid (Inofolic) on ATP content in mouse MII oocytes. ATP content of MII oocytes in the control, sham, DHEA, DHEA-treated with MYO plus folic acid (DI2) groups. Data are presented as the mean  $\pm$  SEM ( $n=15$ ). As the diagrams show, ATP content in the DHEA group had a significant decrease compared with sham and control groups ( $P < 0.0001$ ), and in the DHEA-treated with MYO plus folic acid (DI2) group this showed a significant increase compared with the DHEA group ( $P < 0.05$ ). Different lowercase letters above bars indicate significant difference.

Any disorder in the mitochondria function affects the ATP production cycle. Safiolina *et al.* (2006) reported that the administration of DHEA in Wister rats disrupted mitochondrial function and reduced ATP production by inhibiting cellular respiration and transferring the metabolic pathway from oxidative phosphorylation (OXPHOS) to glycolysis. Similar to the findings of Huang *et al.* (2015), our findings indicated that, in DHEA-treated mice, the ATP levels markedly decreased compared with the control group (Huang *et al.*, 2015). MYO might reduce mitochondrial stress by decreasing androgen levels (Zacchè *et al.*, 2009; Unfer *et al.*, 2017) and also increase cytosolic and mitochondrial calcium levels by increasing IP<sub>3</sub> concentrations, thereby stimulating oxidative metabolism and ATP production (Kockskämper *et al.*, 2008; Vitale *et al.*, 2016). Our findings also suggested that MYO plus folic acid in the DI2 (0.36 mg/g) group had a positive effect on mitochondrial function, causing a significant increase in the ATP count levels compared with the DHEA model group. Finally, MYO may act via multiple mechanisms to improve mitochondrial function and oocyte quality.

Due to the different physiological conditions between humans and animals, the PCOS model on mice does not fully reflect the status of patients with PCOS. However, our research provided clues to the direct effect of MYO on oocyte cell changes. Finally, the effective dose of MYO determined in the PCOS mouse model in this study provides the grounds for further cellular and molecular studies on this model as a hotbed of research.

## Conclusion

In this study, the cellular effects of MYO in the PCOS mouse model were investigated due to the ethical limitations of working on human oocytes. Our findings show that DHEA affects the ovarian morphology of mice by disrupting folliculogenesis and apoptosis. It also increases fasting insulin and testosterone levels, decreases the normal morphology of oocytes, and increases the numbers of immature oocytes compared with mature ones. Moreover, it increases oxidative stress levels while decreasing the GSH levels

and the ATP counts. After evaluating several doses of MYO supplementation, the dose of 0.36 mg/g was found to be the most effective due to the positive effects on the return of the oestrous cycle, ovulation, increased G/T ratio, and reduced LAtAnF diameter. This dose increases the quality, normal morphology, and maturation percentage of oocytes. Moreover, the decreased ROS level improves GSH and relieves the decreased ATP levels. However, it does not reduce the level of fasting serum insulin.

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**Conflict of interest.** The authors declare none.

**Ethical standards.** The authors assert that all procedures contributing to this work comply with the ethical standards of the National Research Council guidelines (National Research Council, 2011) (Zimmermann, 1983) on the care and use of laboratory animals. Advised by the Independent Ethics Committee of IUMS, all protocols used in this study received the necessary approvals with the number IR.Iums. Fmd. Rec.1396.9323113001 from the Internal Affairs Department of Laboratory Animals and Research.

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