MARMARA
MEDICAL JOURNAL

Original Article

https://dergipark.org.tr/tr/pub/marumj

The ameliorating effects of apigenin and chrysin alone and in combination on polycystic ovary syndrome induced by dehydroepiandrosterone in rats

Buket BERK[1], Nevin ILHAN[1], Solmaz SUSAM[2], Fatma TEDIK[1], Nalan KAYA TEKTEMUR[3]

[1] Department of Medical Biochemistry, Faculty of Medicine, Firat University, Elazig, Turkey
[2] Department of Medical Biochemistry, Faculty of Medicine, Adiyaman University, Adiyaman, Turkey
[3] Department of Histology and Embryology, Faculty of Medicine, Firat University, Elazig, Turkey

Corresponding Author: Buket BERK
E-mail: buketberk23@hotmail.com

Submitted: 31.05.2023 **Accepted:** 23.01.2024

ABSTRACT

Objective: Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women of reproductive age and is one of the main causes of ovulation infertility, affecting 5-10% of women. Inflammation, hormonal imbalances, and disruption of the oxidant-antioxidant balance are the main factors in the pathophysiology of PCOS. This study was designed to answer the question of whether apigenin and chrysin have therapeutic effects on the dehydroepiandrosterone (DHEA)-induced rat model of PCOS.

Materials and Methods: The experimental PCOS model was created by administering 6 mg/100g DHEA subcutaneously to 21-day-old female Wistar rats for 28 days, followed by treatment with natural agents 50 mg/kg apigenin and 50 mg/kg chrysin by oral gavage twice a week for one month. The predominant cell type was determined by microscopic analysis in vaginal smears daily from day 10 to day 28 of the experiment. In tissue supernatants, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities, and malondialdehyde (MDA) levels were obtained by spectrophotometric method with appropriate manual methods; follicle-stimulating hormone (FSH), luteinizing hormone (LH), progesterone, interleukin (IL)-18, IL-1β, and IL-13 levels were determined by enzyme-linked immunosorbent assay (ELISA) method. In addition, histological sections obtained from ovarian tissue samples were stained with hematoxylin-eosin and examined under a light microscope.

Results: The results showed that treatment with apigenin and chrysin alone and in combination reduced MDA, LH, FSH, progesterone, IL-1β, IL-13, and IL-18 levels compared with PCOS rats. Furthermore, enzymatic activities of antioxidants including CAT, SOD, and GPx in the ovaries increased in therapeutic groups compared to the PCOS group.

Conclusion: In conclusion, this study demonstrates the potential therapeutic efficacy of apigenin and chrysin, either alone or in combination, in alleviating the hormonal imbalances, inflammation, and oxidative stress in DHEA-induced PCOS rats. Apigenin, in particular, emerges as a promising agent for PCOS treatment, showing superiority over chrysin and combination treatments in ameliorating cystic follicles and improving various parameters associated with PCOS pathophysiology. These findings suggest that apigenin holds promise as a novel therapeutic agent for PCOS and warrants further investigation in clinical settings.

Keywords: Polycystic ovary syndrome, Dehydroepiandrosterone, Apigenin, Chrysin

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is a gynecological and endocrine disease that negatively affects fertility and paves the way for the formation of many different diseases [1]. Individuals suffering from PCOS may experience menstrual irregularities, infertility, skin problems such as acne, hirsutism, and an increase in the number of small cysts in the ovaries [2]. If PCOS is left untreated for a long time, it can lead to many important metabolic abnormalities such as dyslipidemia, insulin resistance, type 2 diabetes mellitus, fatty liver, hypertension, infertility, depression, etc [3]. Although, the exact cause of PCOS is not known, it is suggested that genetic and environmental factors are effective in its emergence [4]. In addition, obesity is among the factors that paves the way for the formation of this disease. Excess weight and as a result insulin resistance cause hyperinsulinemia, which increases the synthesis of androgen hormones in the body. As a result, the balance of sex hormones in the body is disturbed, and ovulation disorders and the problems of inability to ovulate, called anovulation, occurs. Cyst formation in the ovaries is

How to cite this article: Berk B, Ilhan N, Susam S, Tedik F, Tekdemur Kaya N. The ameliorating effects of apigenin and chrysin alone and in combination on polycystic ovary syndrome induced by dehydroepiandrosterone in rats. Marmara Med J 2024: 37(2):198-207. doi: 10.5472/marumj.1479311

ISSN: 1309-9469

http://doi.org/10.5472/marumj.1479311
Marmara Med J 2024;37(2): 198-207

observed with the disruption of the ovulation pattern, and this situation progresses and leads to the emergence of PCOS [5].

An experimentally generated rodent model of PCOS helps us understand mechanisms and focus on clinical therapies for PCOS. In order to develop an experimental PCOS model in animals, the dehydroepiandrosterone (DHEA) application was first tried in the 1960s. In the study conducted by Roy et al., cystic degeneration and anovulation were observed in the ovaries after DHEA administration in guinea pigs with normal menstrual cycles [6]. In general, after DHEA treatment, female rats/mice form follicular cysts and become anovulatory like the human PCOS feature [7]. Hence, DHEA has been commonly preferred to cause PCOS-like phenotypes [8].

Although, there are options such as birth control pills, lifestyle changes, weight control, and surgery in the treatment of PCOS [9], there is a need for natural products that do not have side effects, are at a low cost, and that we can easily access and consume in daily life [10]. Traditional herbal compounds have been widely used to cure gynecological disorders [11]. Apigenin is a flavonoid and is found in many vegetables and fruits like parsley, celery and chamomile tea being the most common sources. It is known that apigenin exhibits antioxidant, anti-inflammatory, anti-tumor, anti-allergic, neuro-protective, anti-microbial properties and has therapeutic properties against many diseases [8]. Chrysin, another natural agent, is found in the foods we consume, such as parsley, thyme, bell pepper, celery, honey, and propolis. This natural agent is known to exhibit antioxidant, anti-inflammatory, anti-diabetic, anti-bacterial, and anti-tumor properties. The mechanisms most affected by chrysin are oxidative stress, inflammatory responses, autophagy, and apoptosis [12,13].

In this study, it was aimed to investigate the potential therapeutic effects of apigenin and chrysin alone and in combination in rats with PCOS induced by DHEA, and to reveal the level changes in selected hormones and inflammatory and oxidative stress pathway parameters in the pathophysiology and treatment of PCOS.

2. MATERIALS and METHODS

Animals and Ethics Statement

Female Wistar Albino rats of 21 days were kept in groups with free access to food and water and a controlled temperature of 22±2°C and in a reversed 12h light-dark cycle. Animals were allowed to acclimatize for one week before treatment. This study was carried out in accordance with the local ethics committee of Firat University, which approved the experimental procedure and protocols (approval number: 16/04/2020-389393).

Experimental Protocol and in Vivo Treatment

A total of 48 rats were randomly assigned to five groups that included the SHAM group (n=8) and PCOS group (n=10), PCOS+AGN group (n=10), PCOS+CH group (n=10) and PCOS+AGN+CH group (n=10). The doses and frequency of administration of the agents administered were summarized below:

SHAM Group (n=8): 0.02ml 95% ethanol + 0.18ml corn oil (0.2mL total) sc administered for 28 days, followed by 0.5ml dimethyl sulfoxide (DMSO) two times a week administered by oral gavage for one month.

PCOS Group (n=10): DHEA (6mg/100g body weight dose dissolved in 0.02ml 95% ethanol and mixed with 0.18ml corn oil) was administered sc for 28 days, followed by 0.5ml DMSO given twice a week by oral gavage for one month [14].

PCOS+AGN Group (n=10): DHEA (6mg/100g body weight) was administered sc for 28 days by dissolving in 0.02ml of 95% ethanol and mixing with 0.18ml of corn oil, then for 1-month AGN at a dosage of 50mg/kg twice a week was dissolved in 0.5ml DMSO and administered by oral gavage [15].

PCOS+CH Group (n=10): DHEA (6mg/100g body weight) was administered sc for 28 days by dissolving in 0.02ml 95% ethanol and mixing with 0.18ml corn oil, then for 1-month CH was dissolved in 0.5ml DMSO at a dosage of 50mg/kg twice a week and administered by oral gavage [16].

PCOS+AGN+CH Group (n=10): After DHEA is administered sc for 28 days, apigenin and chrysin were administered at the dose and application frequency mentioned above, 2 times a week for 1 month.

Biochemical Methods

Tissue and Supernatant Collection

At the end of the experiment, blood and tissue collection was performed during the diestrus stage of the estrous cycle and between 8 and 9 AM in the morning. Following the sacrification of animals, both ovaries of rats were quickly removed and cleaned. Then ovarian samples were homogenized, centrifuged at 4000 rpm for 10 minutes at +4^{0}C, and their supernatants were separated. Depending on the parameters to be studied, supernatant samples were divided into portions, put in eppendorf tubes and stored at – 80°C until the day of analysis.

Determination of Tissue Protein Levels

Protein levels in the supernatants were determined according to the Lowry method The basic principle of the Lowry method is based on the formation of a blue color by the Folin-Phenol reagent of proteins in alkaline medium [17].

Evaluation of Selected Hormones and Inflammation Markers by Using ELISA

Supernatant FSH (Catalog no: 201-11-0183), LH (Catalog no: 201-11-0180), progesterone (Catalog no: 201-11-0742), IL-18 (Catalog no: 201-11-0118), IL-1β (Catalog no: SG-20260), and IL-13 (Catalog no: 201-11-0113) levels were measured by enzyme-linked immunosorbent assay (ELISA) method in accordance with the manufacturer's instructions. FSH, LH, progesterone, IL-18, and IL-13 ELISA kits were obtained from Sunred Biological Technology Company, Shanghai, China. The IL-1β ELISA kit was obtained from SinoGeneClon Biotech, Hangzhou, China.

http://doi.org/10.5472/marumj.1479311
Marmara Med J 2024;37(2): 198-207

Determination of Tissue MDA Level

Tissue Malondialdehyde (MDA) levels and the end product of lipid peroxidation were measured spectrophotometrically as described by Ohkawa et al. [18]. Determination of lipid peroxidation is based on the spectrophotometric measurement at 532 nm of the pink complex formed as a result of incubation of tissue homogenate with 0.8% thiobarbituric acid (TBA) in a boiling water bath for 1 hour under aerobic conditions, where the pH is 3.5. 1,1,3,3 tetraethoxypropane was used as standard in the measurements.

Determination of Tissue SOD Activity

Tissue superoxide dismutase (SOD) activities were determined by the method of Sun et al., based on the inhibition of nitroblue tetrazolium [19]. In the assay, the xanthine-xanthine oxidase system was used as a superoxide generator. The absorbance of the reduction product (formazane) was measured at 560 nm. SOD activity was measured by the degree of inhibition of this reaction.

Determination of Tissue CAT Activity

Tissue Catalase (CAT) activity was determined using the UV spectrophotometric method which was developed by Aebi [20]. The principle of the test is based on the determination of the rate constant (s-1, k), or H_2O_2 decomposition rate, at 240 nm.

Determination of Tissue GPx Activity

Tissue glutathione peroxidase (GPx) activity levels were measured using the method of Paglia and Valentina in which GPx activity was coupled with the oxidation of nicotinamide adenine NADPH by glutathione reductase [21]. The oxidation of NADPH was followed spectrophotometrically at 340 nm.

Estrous Cyclicity and Vaginal Smear Test

Normal regular cyclic rats showed proestrus, estrus, metestrus, and diestrus stages for 4-5 days, whereas, acyclic rats showed the estrous cycle arrested in any one of the stages for 4 consecutive days. Hence, irregular estrous cycles and persistent vaginal cornification were used to indicate the successful formation of the PCOS model in all study groups [22].

To confirm whether the PCOS pattern was established, a cervical screening test (smear) was performed from day 10 to day 28 of the experiment, and the predominant cell type was microscopically analyzed in vaginal smears obtained daily. For this, approximately 0.2 ml of saline was drawn into the pasteur pipette, the animal to be smeared was held tightly with the abdomen facing upwards, the pipette tip was inserted into the vagina and the saline was sprayed inside. Afterward, the liquid sprayed into the vagina without removing the tip of the pipette was drawn back into the pipette and placed on the previously prepared clean slide. The liquid taken was examined under a light microscope without the need for staining, and finally, the cycle periods were determined depending on the presence and proportion of epithelial, cornified, and leukocyte cells.

Histopathological Analysis

After decapitation, the removed ovarian tissues were quickly placed in a 10% formaldehyde solution. After approximately 48 hours of fixation, the tissues that were washed under running tap water and passed through routine histological follow-up series were embedded in paraffin (Sigma-Aldrich Paraplast Embedding Media, U.S.A). Sections of 5 µm thickness were taken from the prepared paraffin blocks using a microtome. Hematoxylin-Eosin (H&E) staining was applied to the prepared preparations. Ovarian follicle count (primordial, primary, secondary follicles, and Graff follicles) was evaluated by Souza et al.'s (1986) method [23]. The classification of follicles was done as follows:

Primordial follicle: The oocyte is partially or completely surrounded by squamous granulosa cells.

Primary follicle: The oocyte is surrounded by one or more rows of cuboidal cells between squamous granulosa cells.

Secondary follicle: The oocyte contains two layers of granulosa cells or at least one layer of granulosa cells and at least one layer of granulosa cells in the second layer. Antral spaces begin to form between the granulosa cells.

Graff's follicle: The oocyte is surrounded by more than two layers of granulosa cells and the antrum has formed.

The Leica DM500 imaging system (DFC295; Leica, Wetzlar, Germany) was used to evaluate and photograph H&E staining on ovarian sections.

Statistical Analysis

To determine the method to be used in the analysis phase, the Shapiro-Wilk test was applied to determine whether the variable of interest in the data set comes from a normal distribution. In the general comparison of continuous measurements of more than two groups, One-Way Analysis of Variance was used if the assumptions were satisfied. Data are indicated as Mean ± SD. If the p-value is below 0.05, it means that there is a significant difference as a result of the comparison.

3. RESULTS

Weight and Growth Rates of Rats in the Study Groups

The weights of the rats in the study groups were regularly weighed and recorded on a weekly basis from the beginning to the end of the experiment. According to the data obtained, the initial weights of the rats in the experimental groups were close to each other and there was no statistical difference between them ($p>0.05$). When the weight of the rats before sacrification was evaluated at the end of the experiment, a statistically significant increase was found in the PCOS-induced groups compared to the SHAM group ($p<0.001$; only for the PCOS-formed group; $p<0.05$ in all groups in which the treatment protocol was applied) (Figure 1).

The growth rates of the experimental animals were calculated based on the following formula:

http://doi.org/10.5472/marumj.1479311
Marmara Med J 2024;37(2): 198-207

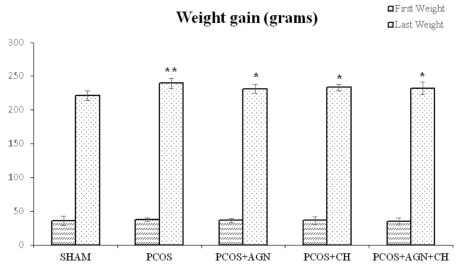

Figure 1. *Weight gains of rats in the study groups*
*A value of *p< 0.05, **p<0.001, was considered as significant versus the SHAM group.*

Growth Rate = (Final Weight-Initial Weight) / Total Experiment Time

The growth rates in the study groups are presented in Table I. According to the findings, the growth rates of the PCOS-induced groups were higher than the SHAM group. However, the growth rate increased the most only in the PCOS group (p<0.001 compared to the SHAM group). The growth rates of the PCOS+AGN and PCOS+AGN+CH treatment groups were statistically significant when compared to the SHAM group (p<0.05; for both groups).

Table I. *Growth rates in study groups*

Growth Rate	SHAM	PCOS	PCOS+AGN	PCOS+CH	PCOS+AGN+CH
	3.19±0.13	3.48±0.11**	3.36±0.96	3.39±0.71*	3.40±0.22*

*A value of *p < 0.05, **p < 0.01, was considered as significant versus the SHAM group. PCOS: Polycystic ovarian syndrome, AGN: apigenin, CH: Chrysin*

Ovarian Tissue Weights of Study Groups

When the values obtained from the measurement of the ovarian weights of the study groups were examined, it was observed that the mean ovarian weights of the rats belonging to the PCOS group were higher than those of the SHAM group, which was statistically significant only in the PCOS group (p<0.05). When the mean ovarian weights were analyzed among the groups with PCOS, it was found that the mean ovarian weight was statistically lower in the PCOS+AGN treatment group compared to the PCOS group (p<0.05) (Figure 2).

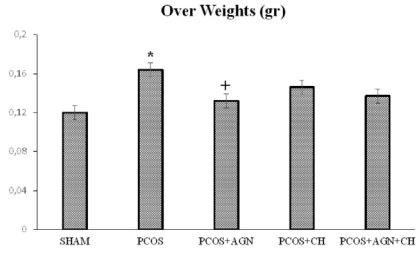

Figure 2. *A value of *p < 0.05 was considered as significant versus the SHAM group.*
A value of $^{+}p < 0.05$, was considered as significant versus the PCOS group.

Effect of Apigenin and Chrysin on Hormonal Levels

Follicle-stimulating hormone levels were higher in the supernatant samples compared to the SHAM group, which was statistically significant in the PCOS group (p<0.05; compared to the SHAM group). A decrease in supernatant FSH levels was observed in the treated groups compared to the PCOS group, but it was not statistically significant (p>0.05). Supernatant LH levels were statistically higher in the PCOS group compared to the SHAM group (p<0.05), and a statistical decrease was observed in the PCOS+AGN+CH group, which was one of the treatment agent-administered groups, compared to the PCOS group. (p<0.05). The supernatant LH/FSH ratio was increased in the PCOS-induced group compared to the SHAM group, but this increase was not statistically significant (p>0.05). A non-statistical decrease in the supernatant LH/FSH ratio was observed in the PCOS+AGN group, which is one of the treated groups, compared to the PCOS group (p>0.05). Supernatant progesterone levels were statistically lower in all PCOS-induced groups than in the SHAM group (p<0.001; for all PCOS-induced groups). On the other hand, there was a statistically insignificant decrease in progesterone levels in all groups in which the treatment agent was applied, compared to the PCOS group (p>0.05; in all treatment groups compared to PCOS group) (Table II).

Effect of Treatment Agents on Inflammation Parameters

Supernatant IL-18, IL-1β and IL-13 levels were found to be statistically higher in the PCOS group compared to the SHAM group (p<0.001; for all parameters). A statistically significant decrease was observed in the supernatant IL-18 levels in the treated groups compared to the PCOS group (p<0.05; in all treatment groups). A statistically significant decrease was observed in IL-1β levels in the PCOS+CH and PCOS+AGN+CH groups compared to the PCOS group (p<0.05). Finally, a decrease in supernatant IL-13 levels was noted in all groups treated with the treatment agent compared to the PCOS group, but this decrease was not statistically significant (Table III).

Effect of Treatment Agents on Supernatant Oxidative Stress Parameters

A statistical decrease in SOD and CAT activities and a statistical increase in MDA levels were observed in the PCOS group compared to the control group. A decrease in GPx activity was also observed compared to the control group, but this decrease was not statistically significant. When the treatment agent applied groups were compared with the PCOS group, a statistical increase in SOD activity was observed in the AGN and AGN+CH treatment groups. CAT and GPx activities were also increased in the treated groups compared to the PCOS group, but this increase was not statistically significant (p>0.05) (Table IV). Although, there was a decrease in MDA levels in all treatment groups compared to the PCOS group, this decrease was statistically significant only in the CH group.

http://doi.org/10.5472/marumj.1479311
Marmara Med J 2024;37(2): 198-207

Table II. *Effect of Apigenin and Chrysin on FSH, LH levels in DHEA-induced PCOS rats*

Factors/Groups	SHAM	PCOS	PCOS+AGN	PCOS+CH	PCOS+AGN+CH
FSH (mIU/mg protein)	31.75±2.55	39.54±5.79*	37.25±6.36	36.72±5.41	37.19±4.31
LH (mIU/mg protein)	34.30±3.03	45.91±8.30**	40.76±4.82	41.48±4.98	42.74±4.01*
LH/FSH	1.08±0.89	1.17±2.41	1.11±0.21	1.14±0.13	1.15±0.11
PROG (ng/mg protein)	112.81±15.24	79.87±16.25**	86.47±13.96**	82.48±10.27**	82.52±11.14**

*A value of *p < 0.05, **p < 0.01 was considered as significant versus the sham group. PCOS: Polycystic ovary syndrome, AGN: apigenin, CH: Chrysin, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, PROG: progesterone*

Table III. *Effect of Treatment Agents on Inflammation Parameters*

Factors/Groups	SHAM	PCOS	PCOS+AGN	PCOS+CH	PCOS+AGN+CH
IL-18 (ng/protein)	138.11±21.22	230.99±28.34**	187.83±23.47*+	193.91±34.88*+	194.54±15.28*+
IL-1β (ng/protein)	184.46±29.22	287.62±75.41**	242.27±20.76	232.45±29.77+	228.71±24.97+
IL-13 (ng/protein)	213.53±24.42	294.21±29.40**	275.02±27.35**	283.75±27.38**	278.11±31.93**

*A value of *p < 0.05, **p < 0.01 was considered as significant versus the SHAM group. PCOS: Polycystic ovary syndrome, AGN: apigenin, CH: Chrysin*

Table IV. *Effect of Treatment Agents on Supernatant Oxidative Stress Parameters*

Factors/Groups	SHAM	PCOS	PCOS+AGN	PCOS+CH	PCOS+AGN+CH
SOD activity	93.55±6.50	71.65±7.80**	84.83±9.57+	82.00±12.12	91.07±12.03++
CAT activity	6.69±1.16	4.96±1.07*	5.82±1.21	5.18±1.36	5.33±0.73
GPx activity	5179.23±364.92	4822.02±682.64	5124.46±488.66	5303.70±655.25	5070.61±559.81
MDA level	13.96±2.51	19.68±3.02**	16.94±1.86	17.60±2.40*	16.62±2.57

*A value of *p < 0.05, **p < 0.01 was considered as significant versus the SHAM group. A value of +p < 0.05, ++p < 0.001 was considered as significant versus the PCOS group. PCOS: Polycystic ovary syndrome, AGN: apigenin, CH: Chrysin. SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase, MDA: Malondialdehyde*

Histological Results

Vaginal Smear Reviews

Vaginal smear findings support the successful formation of PCOS with DHEA. When the rats in the SHAM group were examined, a regular estrous cycle was observed. In the rats in the PCOS group, the estrous cycle was found to be impaired, irregular and prolonged. It was observed that the irregular cycle started to improve in the groups that were treated with the treatment agent after PCOS occurred. Vaginal smear samples of the groups are as in Figure 3.

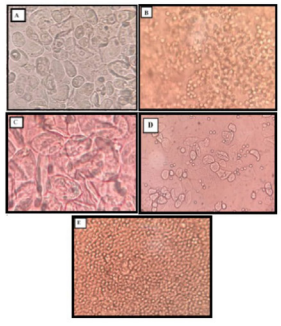

Figure 3. *Vaginal smear samples of SHAM and PCOS groups. A: Proestrous, B: Diestrous, C: Oestrous, D: Metestrous (belonging to the SHAM group), E: Diestrous (belonging to the PCOS group)*

Ovarian Follicle Count and Histological Appearance of Ovarian Tissues

When the rat ovary sections were evaluated under the light microscope, there were many corpus luteum and ovarian follicles at different stages of development (primordial, primary, secondary and Graff) in the ovarian cortex in the preparations belonging to the SHAM group. Granulosa cells and theca structures were histologically normal.Corpus luteum structures were normal and well-defined.

Similar to the SHAM group, multiple corpus luteum and follicles at different developmental stages were observed in the ovarian sections of the PCOS group. However, when the two groups were compared, it was found that the number of secondary follicles increased significantly in this group ($p<0.05$). It was also observed in this group that the number of primordial and primary follicles decreased and the number of cystic follicles increased compared to the SHAM group, but these changes were statistically insignificant ($p>0.05$). Again, in the PCOS group, excessive dilatation of the vessels in the cortical stroma was noted (Figure 4). In the PCOS+AGN, PCOS+CH and PCOS+AGN+CH groups, the secondary follicle counts were decreased compared to the PCOS group, and the Graff follicle counts were decreased compared to the SHAM group. It was determined that vascular dilatation observed in the PCOS group decreased in all treatment groups. However, vascular congestion was remarkable in the PCOS+AGN group. The follicle and corpus luteum count results of all groups are presented in Table V.

http://doi.org/10.5472/marumj.1479311
Marmara Med J 2024;37(2): 198-207

Table V. *Ovarian follicle count*

Groups	Primordial Follicle	Primary Follicle	Secondary Follicle	Graff Follicle	Corpus Luteum	Cystic Follicle
SHAM	9.50±0.64	8.25±2.05	17.75±1.47	1.50±0.95	13.75±2.86	0.25±0.25
PCOS	7.00±2.12	4.25±1.25	31.50±4.66[a]	1.25±0.25	14.00±0.91	2.00±0.70
PCOS+AGN	2.25±0.85	4.25±1.49	14.25±3.94[b]	0.00±0.00[a]	10.00±2.34	1.50±0.86
PCOS+CH	4.25±2.32	4.25±2.32	19.50±2.32	0.00±0.00[a]	13.50±1.89	1.00±0.40
PCOS+AGN+CH	8.25±2.65	3.75±0.62	10.00±1.63[b]	0.50±0.28[a]	8.50±0.93	0.50±0.28

a: Compared to the SHAM group, b: Compared to the PCOS group p<0.05 *PCOS: Polycystic ovary syndrome, AGN: Apigenin, CH: Chrysin.*

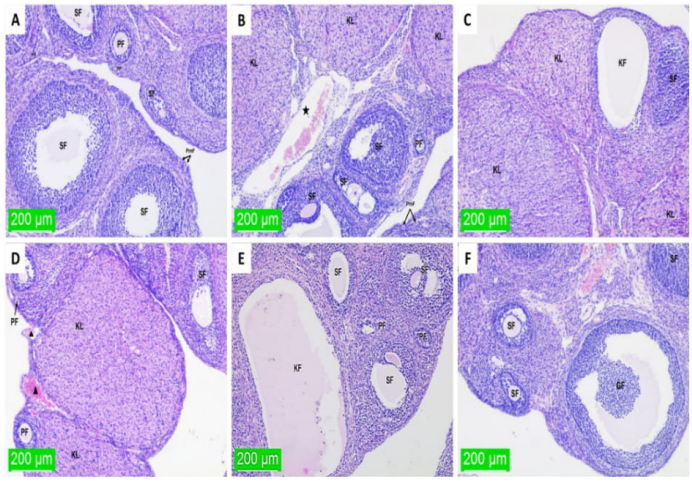

Figure 4. *Histological appearance of ovarian tissues. A: Sham group, B-C: PCOS group, D: PCOS+AGN group, E: PCOS+CH group, F: PCOS+AGN+CH group. KL: Corpus luteum; SF: Secondary follicle; PF: Primary follicle; PrmF: Primordial follicle; GF: Graff's follicle; CF: Cystic follicle. Star: Vascular dilatation; Triangle: Congestion. x100, H&E.*

4. DISCUSSION

Polycystic ovary syndrome is a very common endocrine disorder in women of reproductive age, which is characterized by enlargement of the ovaries and the formation of many small cysts, which paves the way for a number of hormonal problems in the individual, and whose exact cause is still unknown [24]. To understand the etiopathology of PCOS, the present study employed a DHEA-treated rat model, which exhibits metabolic signs similar to the human PCOS condition. To our knowledge, this is the first study using the PCOS rat model to investigate the effects of the combination of apigenin and chrysin on the estrous cycle, weight gain, and endocrine parameters such as testosterone and fasting insulin level. Combination therapies have proven to be an invaluable strategy in medical treatment ever since the inception of medicine [25]. In contemporary times, the utilization of appropriate drug combinations not only enhances therapeutic effectiveness in contrast to single-drug administration but also facilitates the attainment of positive outcomes with reduced doses of individual components. This, in turn, mitigates the occurrence of side effects, minimizes the development of drug resistance, and can also offer selective synergy against a specific target [26].

An irregular estrous cycle is used as an indicator of the successful establishment of PCOS in animal models [27] Hence, to verify PCOS in our study, the estrous cycle was noted from day 1 to 28, as well as changes in ovulation phases. The group of PCOS exhibited estrous cycle irregularity mostly at the stage of the diestrus phase, which is an indication of anovulation.

Women with PCOS have the problem of gaining weight together with hormonal imbalances. Namely, PCOS makes it difficult to use the insulin hormone and can lead to the development of insulin resistance. Increased insulin levels also increase the production of androgens, which lead to pilosity, acne, menstrual irregularity and weight gain [28]. In a study, it was reported that the body and ovarian weights of rats in the PCOS group were significantly higher than the control group [29]. In our study, it was also found that the body and ovarian weights of rats in the PCOS group increased statistically compared to the SHAM group, in line with the aforementioned study.

Follicle-stimulating hormone (FSH) is necessary for pubertal development and the function of women's ovaries and men's testes. This hormone stimulates the growth of ovarian follicles in the ovary before the release of an egg from one follicle at ovulation in women. It also increases estradiol production from the ovaries [30]. The normal level of serum FSH gradually increases from the beginning of the menstrual cycle to the middle of the cycle and decreases gradually to the basal value after ovulation [31]. LH a gonadotropin in glycoprotein structure, is responsible for inducing ovulation, preparation for fertilized oocyte uterine implantation, and the ovarian production of progesterone through stimulation of theca cells and luteinized granulosa cells [32]. Both FSH and LH play important functions in ovulation, and PCOS patients commonly show a two to three-fold increased LH/FSH ratio, which is sufficient to disrupt ovulation [33]. In a study it was reported LH, LH/FSH ratio in the PCOS model group significantly increased, in contrast, the level of FSH decreased [34]. In a letrozole-induced PCOS study, it was found that the PCOS-induced group had raised levels of LH, while the PCOS-induced group had lower levels of progesterone, estrogen, and FSH [35]. In our study, LH and FSH levels were higher in the PCOS group compared to the SHAM group, and a decrease was detected in the treatment agent-administered groups compared to the PCOS group. Progesterone is a 21-carbon steroid hormone of ovarian origin and is also secreted by the action of LH secreted from the anterior lobe of the pituitary [36]. Women with PCOS need high progesterone levels to reduce GnRH secretion because FSH synthesis is insufficient and LH synthesis is high. However, since low secretion of FSH will cause anovulation, progesterone levels may remain low [37]. It was reported in a study that in PCOS, progesterone levels were significantly lower than in the control group [38]. It was also reported that progesterone levels decreased in the PCOS model created with intramuscular estradiol valerate injection compared to the control group [39].

In our study, the supernatant progesterone levels were also statistically lower in all PCOS-induced groups compared to the control group. Apigenin treatment increased supernatant progesterone levels more than chrysin and AGN+CH treatment.

Inflammation is thought to be an important contributor to the pathogenesis of PCOS. That is, inflammation is likely to trigger events that lead to increased ovarian androgen production and reproductive dysfunction in PCOS patients [40]. Studies have reported that serum and follicular fluid IL-1β levels are elevated in women with PCOS [41,42]. It has been reported that ovarian tissue IL-1β levels in the PCOS model created with DHEA increased in the model group compared to the control group [43]. In our study, IL-1β levels were higher in the PCOS group compared to the SHAM group, and there was a decrease in the treatment agent-administered groups compared to the PCOS group. IL-18, a proinflammatory cytokine belonging to the IL-1 superfamily [44], is enhanced in PCOS patients [45]. In a study, IL-18 level was detected significantly higher in all the PCOS groups compared to the control group [46]. In our study, IL-18 levels were higher in the PCOS-induced groups than in the SHAM group. Moreover, previous studies showed that agents such as baicalin [47], and resveratrol [48] which have anti-inflammatory effects can improve the low-grade inflammation state by decreasing the levels of proinflammatory cytokines such as IL-18, including the PCOS rats. Also, in our study, a statistically significant decrease was observed in the supernatant IL-18 levels in the all-treatment groups compared to the PCOS group. Interleukin-13, a cytokine, increases the production of adhesion molecules on the endothelium and downregulates prostaglandin and E2 production [49]. In a study, it was reported that IL-12 and IL-13 levels in the follicular fluid were higher in a subgroup of patients with PCOS compared to women with normal ovulation [49]. It has been reported that serum IL-13 levels are higher in women with PCOS during pregnancy compared to the control group [50]. In our study, the selected cytokines levels were higher compared to the SHAM group. Moreover, apigenin and chrysin showed an anti-inflammation effect by decreasing of the inflammatory cytokine levels.

Oxidative stress is another factor that contributes to the pathological process of PCOS, which affects reproduction. Improving antioxidant activities, including SOD, GPx, CAT, and GSH, may be beneficial for inhibition and modulation of oxidative stress in PCOS [51]. Haslan et al., in their study of rat polycystic ovary induced by letrozole, stated that the oxidant-antioxidant balance was impaired in the PCOS group, while Ficus deltoidea improved the deteriorated balance [52]. In another PCOS study, it was reported that the activities of antioxidants such as SOD, GPx, CAT, and GSH were decreased in rats with PCOS, while they increased significantly with luteolin [51]. In our study, it was determined that MDA levels were higher and selected antioxidant enzyme activities were lower in PCOS-induced rats compared to the SHAM group, as in the studies mentioned above, which indicates an oxidant-antioxidant imbalance. Moreover, it was observed that the therapeutic agents applied decreased the MDA levels, and increased the antioxidant enzyme activities, restoring the deteriorated oxidant-antioxidant balance.

The effects of apigenin and chrysin on ovarian tissue and molecular changes in rats with PCOS induced by DHEA were examined. In a similar study, the effect of applying different amounts of apigenin on the changes in polycystic ovaries was investigated [53]. This study stated that apigenin prevented primordial follicles from turning into primary follicles. They also concluded that apigenin reduces various complications caused by PCOS and had protective effects on cystic follicles formed in the ovaries. In another study, it was observed that apigenin helped increase primary follicle, Graff follicle, and corpus luteum and decrease cystic follicles. It was stated that apigenin also played an important role in follicle development and the initiation of ovulation [54]. In our study, it was observed that apigenin, as an antioxidant and anti-angiogenic agent, reduced and improved the cystic follicles in the ovaries resulting from PCOS.

In conclusion, this study scientifically reveals the beneficial effects of apigenin and chrysin on the parameters involved in the inflammation and oxidative stress mechanisms in the pathophysiology of PCOS. Similar studies using different

http://doi.org/10.5472/marumj.1479311
Marmara Med J 2024;37(2): 198-207

treatment methods, emphasize that apigenin alone is more effective than chrysin or in combination with other treatment agents, and could be used for PCOS as an alternative therapeutic agent.

Compliance with Ethical Standards

Ethics Approval: This study was approved by the Ethics Committee of Firat University (approval number: 16/04/2020-389393).

Financial Support: This study was funded by the Firat University Scientific Research Projects Unit.

Disclosure Statement: The authors declare no conflict of interest.

Authors' Contributions: NI and SS: Data collection, data analysis, and manuscript writing, BB, FT and NKT: Data collection and data analysis, BB, FT, NI and SS: Protocol development. All authors read and approved the final version of the manuscript.

REFERENCES

[1] Yu J, Ding C, Hua Z, Jiang X, Wang C. Protective effects of berberine in a rat model of polycystic ovary syndrome mediated via the PI3K/AKT pathway. Journal of Obstetrics and Gynaecology Research 2021;47:1789-803. doi:10.1111/jog.14730.

[2] Kim JJ. Update on polycystic ovary syndrome. Clin Exp Reprod Med 2021;48:194-7. doi:10.5653/cerm.2020.04329.

[3] Azziz R. Polycystic ovary syndrome. Obstet Gynecol 2018;132:321-36. doi: 10.1097/AOG.000.000.0000002698.

[4] Fan Q, He J-F, Wang Q-R, et al. Functional polymorphism in the 5'-UTR of CR2 is associated with susceptibility to nasopharyngeal carcinoma. Oncol Rep 2013;30:11-6. doi: 10.3892/or.2013.2421.

[5] Xu Y, Qiao J. Association of insulin resistance and elevated androgen levels with polycystic ovarian syndrome (PCOS): A review of literature. J Healthc Eng 2022;2022 :2022:9240569. doi: 10.1155/2022/9240569.

[6] Roy S, Mahesh VB, Greenblatt RB. Effect of dehydroepiandrosterone and Δ4-androstenedione on the reproductive organs of female rats: production of cystic changes in the ovary. Nature 1962;196(4849):42-3. doi: 10.1038/196042a0.

[7] Ryu Y, Kim SW, Kim YY, Ku S-Y. Animal models for human polycystic ovary syndrome (PCOS) focused on the use of indirect hormonal perturbations: a review of the literature. Int J Cell Sci Mol Biol 2019;20:2720. doi: 10.3390/ijms20112720.

[8] Susam S, Çıkım G. A review on the potential pharmacological effects of apigenin. Archives Medical Review Journal 2023;32:113-9. doi:10.17827/aktd.1267942.

[9] Oguz SH, Yildiz BO. An update on contraception in polycystic ovary syndrome. Endocrinol Metab (Seoul) 2021;36:296-311. doi: 10.3803/EnM.2021.958.

[10] Iervolino M, Lepore E, Forte G, Laganà AS, Buzzaccarini G, Unfer V. Natural molecules in the management of polycystic ovary syndrome (PCOS): an analytical review. Nutrients 2021;13:1677. doi: 10.3390/nu13051677.

[11] Balamurugan S, Vijayakumar S, Prabhu S, Yabesh JM. Traditional plants used for the treatment of gynaecological disorders in Vedaranyam taluk, South India-An ethnomedicinal survey. Int J Tradit Complement Med Res 2018;8:308-23. doi: 10.1016/j.jtcme.2017.06.009.

[12] Naz S, Imran M, Rauf A, Orhan IE, Shariati MA, Shahbaz M, et al. Chrysin: Pharmacological and therapeutic properties. Life Sci 2019;235:116797. doi: 10.1016/j.lfs.2019.116797.

[13] Stompor-Gorący M, Bajek-Bil A, Machaczka M. Chrysin: Perspectives on contemporary status and future possibilities as pro-health agent. Nutrients 2021;13:2038. doi: 10.3390/nu13062038.

[14] Motta A B. Dehydroepiandrosterone to induce murine models for the study of polycystic ovary syndrome. J Steroid Biochem Mol Biol 2010;119:105-11. doi: 10.1016/j.jsbmb.2010.02.015.

[15] Adel A, Mansour A, Mohammad A, et al. Potential antioxidant activity of apigenin in the obviating stress-mediated depressive symptoms of experimental mice. Molecules 2022;27:9055. doi:10.3390/molecules27249055.

[16] Pai S A, Martis E A, Munshi R P, Gursahani M S, Mestry S N, Juvekar A R. Chrysin mitigated obesity by regulating energy intake and expenditure in rats. Int J Tradit Complement Med Res 2019:10: 577-85. doi: 10.1016/j.jtcme.2019.09.002.

[17] Classics Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75. doi: 10.1016/S0021-9258(19)52451-6.

[18] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8. doi: 10.1016/0003-2697(79)90738-3.

[19] Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988;34:497-500. doi:10.1093/clinchem/34.3.497.

[20] Aebi H. Catalase methods of enzymatic analysis. In: Hu B, editor.New York: Academic Press Inc, 1974:673-77. doi: 10.1016/S0076-6879(84)05016-3.

[21] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. JLM 1967;70:158-69. doi: 002.221.4367900765.

[22] Zhuang Z, Pan X, Zhao K, et al. The effect of Interleukin-6 (IL-6), Interleukin-11 (IL-11), signal transducer and activator of transcription 3 (STAT3), and AKT signaling on adipocyte proliferation in a rat model of polycystic ovary syndrome. Med Sci Monit 2019;25:7218. doi: 10.12659/MSM.916385.

[23] Souza AZd, Fonseca A, Izzo V, Clauzet R, Salvatore C. Ovarian histology and function after total abdominal hysterectomy. Obstet Gynecol 1986;68:847-9.

[24] Ndefo UA, Eaton A, Green MR. Polycystic ovary syndrome: a review of treatment options with a focus on pharmacological approaches. Pharmacy and Therapeutics 2013;38:336.

[25] Yuan R, Lin Y. Traditional Chinese medicine.Pharmacology and Therapeutics 2000;86:191-8. doi: 10.1016/s0163-7258(00)00039-5.

[26] Podolsky S H, Greene J A. 2011. Combination drugs—hype, harm, and hope. NEJM AI 2011;365:488-91. doi: 10.1056/NEJMp1106161.

[27] Nallathambi A, Bhargavan R. Regulation of estrous cycle by Cynodon dactylon in letrozole induced polycystic ovarian syndrome in Wistars albino rats. Anat Cell Biol 2019;52:511-7. doi: 10.5115/acb.19.114.

[28] Baptiste CG, Battista M-C, Trottier A, Baillargeon J-P. Insulin and hyperandrogenism in women with polycystic ovary syndrome. J Steroid Biochem Molec Biol 2010;122:42-52. doi: 10.1016/j.jsbmb.2009.12.010.

[29] Furat Rencber S, Kurnaz Ozbek S, Eraldemır C, et al. Effect of resveratrol and metformin on ovarian reserve and ultrastructure in PCOS: an experimental study. J Ovarian Res 2018;11:1-16. doi: 10.1186/s13048.018.0427-7.

[30] Orlowski M, Sarao MS. Physiology, follicle stimulating hormone. Follicle StatPearls Publishing, NCBI Bookshelf: 2018.

[31] Reed BG, Carr BR, Feingold KR. The normal menstrual cycle and the control of ovulation. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000

[32] Holesh JE, Bass AN, Lord M. Physiology, ovulation. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan 2023 May 1. 2017.

[33] Lee YH, Yang H, Lee SR, Kwon SW, Hong E-J, Lee HW. Welsh onion root (Allium fistulosum) restores ovarian functions from letrozole induced-polycystic ovary syndrome. Nutrients 2018;10:1430. doi: 10.3390/nu10101430.

[34] Zheng S, Chen Y, Ma M, Li M. Mechanism of quercetin on the improvement of ovulation disorder and regulation of ovarian CNP/NPR2 in PCOS model rats. J.Formosan Med.Assoc 2022;121:1081-92. doi: 10.1016/j.jfma.2021.08.015.

[35] Shrivastava VK. Turmeric extract alleviates endocrine-metabolic disturbances in letrozole-induced PCOS by increasing adiponectin circulation: A comparison with Metformin. Metabolism Open 2022;13:100160. doi: 10.1016/j.metop.2021.100160.

[36] Mihanfar A, Nouri M, Roshangar L, Khadem-Ansari MH. Therapeutic potential of quercetin in an animal model of PCOS: Possible involvement of AMPK/SIRT-1 axis. Eur J Med Chem Rep 2021;900:174062. doi: 10.1016/j.ejphar.2021.174062.

[37] Cable J, Grider M. Physiology, progesterone. In: StatPearls. StatPearls Publishing Copyright© 2022, StatPearls Publishing LLC Treasure Island: 2022.

[38] Szeliga A, Rudnicka E, Maciejewska-Jeske M, et al. Neuroendocrine determinants of polycystic ovary syndrome. Int J Environ Health Res 2022;19:3089. doi: 10.3390/ijerph19053089.

[39] Adelakun SA, Ukwenya VO, Peter AB, Siyanbade AJ, Akinwumiju CO. Therapeutic effects of aqueous extract of bioactive active component of Ageratum conyzoides on the ovarian-uterine and hypophysis-gonadal axis in rat with polycystic ovary syndrome: Histomorphometric evaluation and biochemical assessment. Metabolism Open 2022;15:100201. doi: 10.1016/j.metop.2022.100201.

[40] Khazaei F, Ghanbari E, Khazaei M. Improved hormonal and oxidative changes by Royal Jelly in the rat model of PCOS: An experimental study. Int J Reprod Biomed 2021;19:515. doi: 10.18502/ijrm.v19i6.9373.

[41] Li Y, Zheng Q, Sun D, et al. Dehydroepiandrosterone stimulates inflammation and impairs ovarian functions of polycystic ovary syndrome. J Cell Physiol 2019;234:7435-47. doi: 10.1002/jcp.27501.

[42] Amato G, Conte M, Mazziotti G, et al. Serum and follicular fluid cytokines in polycystic ovary syndrome during stimulated cycles. Obstet Gynecol 2003;101:1177-82. doi: 10.1016/s0029-7844(03)00233-3.

[43] Ebejer K, Calleja-Agius J. The role of cytokines in polycystic ovarian syndrome. Gynecology Endocrinolology 2013;29:536-40. doi: 10.3109/09513.590.2012.760195.

[44] Dinarello CA, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 binding protein. Front Immunol 2013;4:289. doi: 10.3389/fimmu.2013.00289.

[45] Yang Y, Qiao J, Li R, Li M-Z. Is interleukin-18 associated with polycystic ovary syndrome. Reprod Biol Endocrinol 2011;9:1-5. doi: 10.1186/1477-7827-9-7.

[46] Wu G, Hu X, Ding J, Yang J. The effect of glutamine on Dehydroepiandrosterone-induced polycystic ovary syndrome rats. J Ovarian Res 2020;13:1-7. doi: 10.1186/s13048.020.00650-7.

[47] Wang W, Zheng J, Cui N, et al. Baicalin ameliorates polycystic ovary syndrome through AMP-activated protein kinase. J Ovarian Res 2019;12:1-12. doi: 10.1186/s13048.019.0585-2.

[48] Brenjian S, Moini A, Yamini N, et al. Resveratrol treatment in patients with polycystic ovary syndrome decreased pro-inflammatory and endoplasmic reticulum stress markers. Am J Reprod Immunol 2020;83:e13186. doi: 10.1111/aji.13186.

[49] Gallinelli A, Ciaccio I, Giannella L, Salvatori M, Marsella T, Volpe A. Correlations between concentrations of interleukin-12 and interleukin-13 and lymphocyte subsets in the follicular fluid of women with and without polycystic ovary syndrome. Fertil Steril 2003;79:1365-72. doi: 10.1016/s0015-0282(03)00344-3.

[50] Stokkeland LMT, Giskeødegård GF, Ryssdal M, et al. Changes in serum cytokines throughout pregnancy in women with polycystic ovary syndrome. The J Clin Endocrinol Metab 2022;107:39-52. doi: 10.1210/clinem/dgab684.

[51] Huang Y, Zhang X. Luteolin alleviates polycystic ovary syndrome in rats by resolving insulin resistance and oxidative stress. Am J Physiol Endocrinol Metab 2021;320:E1085-E92. doi: 10.1152/ajpendo.00034.2021.

[52] Haslan MA, Samsulrizal N, Hashim N, Zin NSNM, Shirazi FH, Goh YM. Ficus deltoidea ameliorates biochemical, hormonal, and histomorphometric changes in letrozole-induced polycystic ovarian syndrome rats. BMC Complement Med Ther 2021;21:1-13. doi: 10.1186/s12906.021.03452-6.

[53] Koohestani Y, Abdi A, Salehiyeh S, Pourmirzaei F, Çiftci M, Emir Çoban O. Protective effect of apigenin on ovarian follicles in polycystic ovary syndrome-induced rats. Med Lab J 2022; 16:2. doi: 10.29252/mlj.16.2.7.

[54] Darabi P, Khazali H, Mehrabani Natanzi M. Therapeutic potentials of the natural plant flavonoid apigenin in polycystic ovary syndrome in rat model: via modulation of pro-inflammatory cytokines and antioxidant activity, Gynecology Endocrinology 2020; 36: 582-7. doi: 10.1080/09513.590.2019.1706084.