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Original Article

The effect of Melatonin on histological changes of ovary in induced polycystic ovary syndrome model in mice

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ABSTRACT

Background: Antioxidants can be used as adjuvant treatment of polycystic ovary syndrome (PCOS). Melatonin (MT) is one of the antioxidant that is used nowadays.
Objective: In this study, the effect of MT on the histological changes of ovary in the experimental model of polycystic ovary syndrome is investigated.
Methods: In this study 30 immature female NMRI mice were divided into 5 groups including: (1) control group received distilled water, (2) received a dose of 10 mg/kg MT for 5 days, (3) received Dehydroepiandrostenedione (DHEA) at a dose of 6 mg/kg for 20 days to induce PCOS, (4) after induction of PCOS received MT at a dose of 10 mg/kg for 5 days, and (5) received 6 mg/kg DHEA and 10 mg/kg MT for 20 days simultaneously.
Results: The evaluation of ovarian tissue characteristics such as the granulosa layer, theca, number and diameter of cysts and follicles was performed. PCOS caused a significant reduction in the number of antral follicles and corpus luteum and an increase in the number of primordial, primary, pre-antral and cystic follicles in comparison with the control group ($P < 0.05$). Moreover, MT resulted in a significant increase in the granulosa layer thickness in group 4 ($P < 0.001$), and group 5 ($P = 0.001$) and a significant reduction in the thickness of theca layer between groups 4 and 5 compared with group 3 ($P < 0.001$).
Conclusion: These findings indicate that MT have a protective effects on polycystic ovary damages induced by DHEA, although the mechanism is unclear. It is likely that this is happening by reducing oxidative damage.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common cause of infertility (20% of infertile couples) and 6–8% of women suffer from this endocrine disorder in their reproductive age; this disorder was discovered by Stein and Leventhal for the first time in 1935 [1,2]. Several studies have shown that this syndrome can cause histological abnormalities such as bilateral ovarian enlargement of more than 10 mm, presence of more than 12 follicles of less than 10 mm size in the central dense stroma, increasing the thickness of the follicular sheath and ovarian stroma due to increase in angiogenesis, vasculogenesis, ovarian blood flow and, consequently, reduction or chronic anovulation and infertility [3,4]. Women with polycystic ovary syndrome have a wide range of clinical symptoms, but usually they seek medical advice due to three disorders that include irregular menstruation, infertility and symptoms associated with an increase in androgens such as hirsutism and acne [5]. Various treatment methods have been proposed for polycystic ovary syndrome, such as changing lifestyle, surgery and taking medication. Now the most recognized way of treatment is using medications such as clomiphene citrate, metformin, Letrozole and Tamoxifen [6]. Considering the side effects of these medications, identification and preparation of alternative medicine is important. However, extensive research to find new treatments have shown that oxidative stress, inflammation and activation of endothelial cells in the ovary plays an important role in the pathogenesis of PCOS and it leads to the development of atherosclerotic lesions in the ovaries. Therefore, a positive correlation between the reduction of oxidative stress and increased oocyte maturation in women with PCOS and infertility has been found. It seems that antioxidants reduce oxidative stress in different ways and as a

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result they can improve the prognosis of PCOS [7]. Melatonin (MT) is one of the antioxidants that are in focal attention nowadays. MT and its metabolites are potent antioxidants that remove free radicals and increase the expression of several antioxidant enzymes [8]. Consuming MT in laboratory mice protects cells against oxidative damage. In fact, it reduces oxidative stress in various ways [9]. MT, N-acetyl-5-methoxy-tryptamine which is a lipophilic Indolamine is synthesized mainly by the pineal gland. MT, in a circadian rhythm, is secreted during the night with high levels in all species. In mammals, including humans, MT rhythm is produced by an endogenous circadian clock in the suprachiasmatic nucleus and it impacts the hypothalamus-pituitary-gonad axis [10]. MT receptors have been identified in the nerve cells of the hypothalamus, which governs the release of gonadotropins pituitary gland, in the pituitary gland, in both male and female gonads and other reproductive organs [11–13]. In mammals, MT can affect reproductive function through the activation of the receptors in hypothalamic hypophyseal gonadal axis [14]. MT is also found in the fluid of the ovarian follicles [15]. The Increase of MT in follicular fluid leads to a lower incidence of oxidative damage inside the follicle. Reducing oxidative stress increases the fertilization and pregnancy in women who could not be pregnant due to poor quality of oocytes [11]. MT receptor in rat, mouse and pig ovary cells has been found which suggests that it's has a direct effect on oocyte maturation [11,13]. Recently, In vitro studies have shown that MT plays an important role in optimal egg development and ovulation, and has a positive impact on early embryonic development [16]. Interestingly, MT concentration in large follicles is more than small follicles. High levels of MT in large follicles are likely to protect the granulosa cells of radicals that are induced during ovulation [17]. Considering the aforementioned issues, the inhibitory effect of MT on pituitary gonadotropin secretion and inhibiting intra follicular oxidative stress, and considering the fact that no report was found up to conducting this study, the effect of MT on the histological changes in induced polycystic ovary syndrome was analyzed.

2. Materials and methods

In this study, thirty NMRI rats which were 14 days old were used. To adapt animals, they were held in their cages for one week in laboratory conditions including 2 ± 21 C, 12 h periods of light, free consumption of food and water and good ventilation. Then, the mice were weighed and randomly divided into 5 groups (each group consisted of 6 mice). Control group: the mice in this group received distilled water intraperitoneally as MT solvent. The treatment control groups: to study the effects of MT on changes in the ovaries and the ovulation power of oocytes under normal circumstances, this group received 10 mg/kg MT intraperitoneally for 5 days. Disease group: In order to induce PCOS, this group received Dehydroepiandrostenedione (DHEA) (6 mg/kg) for 20 days intraperitoneally. Treatment group: after inducing polycystic ovarian syndrome on mice in this group, they were treated by MT intraperitoneally for 5 days. The prevention group: in order to study the effects of MT in the prevention of inducing polycystic ovary syndrome, mice in this group received at the same time 6 mg/kg DHEA and 10 mg/kg MT for 20 days. At the end of the experiment mice were weighed and killed by cervical dislocation and their abdomen was opened under sterile conditions and their ovaries were removed for morphology/morphometry study.

2.1. Polycystic ovary syndrome induction method

To induce phenotype of Polycystic ovary syndrome in immature mice (approximately 22–21 days old), they were treated for 20 days with daily injections of DHEA at a dose of 6 mg/kg of body weight intraperitoneally [18]. In order to confirm the induction of PCOS, three mice were sacrificed and their ovaries were separated from the spiral oviduct tube. Then, excessive fat was carefully dissected without damaging ovarian tissue under loop and ovaries were put inside fixative in order to do histotechnique procedure [19].

2.2. Administration of MT

MT was injected intraperitoneally for 5 days, everyday 10 mg/kg body weight. Duration and dose of MT were decided based on the previous studies conducted on rats and mice [20,21].

2.3. Histological analysis

Both mice ovaries were removed for histological examination. After fixation of tissue samples in Buin solution for 16–14 h, the samples were dehydrated with alcohol solutions with rising levels 20–100% in each for 45 min to one hour, and then were clarified in xylene alcohol (50:50), xylene (three times) and embedded in paraffin. The samples were cut with a microtome 5 μm diameter and sections were placed on slides. After Deparaffmization of sections and their yielding with alcohol solutions with lowering levels, they were painted by hematoxilina and they were differentiated with acid-alcohol solution and then they were stained [22]. In the final stage, the tissue sections taken from the ovaries were observed under an optical microscope with a magnification of 40 and different follicular groups were counted. Moreover, the thickness of antral follicles theca layer and granulosa were measured. In order to evaluate changes in ovarian tissue, ovarian structures were categorized into 6 groups based on morphology: Primordial follicles (PRIF), Primary follicles (PF), Preantral follicles (PAF), Antral follicles (AF), Cystic follicles (CF) and Corpus luteum (CL) and their changes in the ovaries were examined [23].

2.4. Statistical analysis

Kolmogorov-Smirnov test, Kruskal Wallis test, ANOVA test, Levene test, Tukey test and SPSS version 20 were used for statistical analysis of the data. P value less than 0.05 was considered significant.

3. Results

By analyzing the histological morphology of ovaries in the control group and the control treatment group different follicles were seen including graafian follicles with a thick layer of granulosa cells and corpus luteum. A number of cystic follicles with a very thin layer of granulosa which is the property of cystic follicles were seen in the ovaries of the disease group. In this group, only a few corpus luteum that indicates ovulation were observed. Therefore, based on the observations of ovarian tissue, injecting DHEA to mice for 20 days resulted in creating cystic follicles and a decrease in the number of antral follicles and corpus luteum and anovulation or very low ovulation (due to the reduction in the number of corpus luteum) in this group. Morphological studies in disease treatment group compared with disease group showed that MT during 5 days had resulted in an increase in the number of follicles, reducing the number of cystic follicles (3 ± 1.41 vs. 33.83 ± 1.60) and increase in the number of CL (7.16 ± 2.31 vs. 0.16 ± 0.40). Also, injecting MT with DHEA at the same time in the prevention group compared with disease group showed a reduction in the number of cystic follicles (5.66 ± 1.63 vs. 33.83 ± 1.60) and an increase in the number of corpus luteum (5 ± 1.41 In contrast, 0.16 ± 0.40) (Fig. 1). 10 mg/kg MT per day did not create a significant change on the

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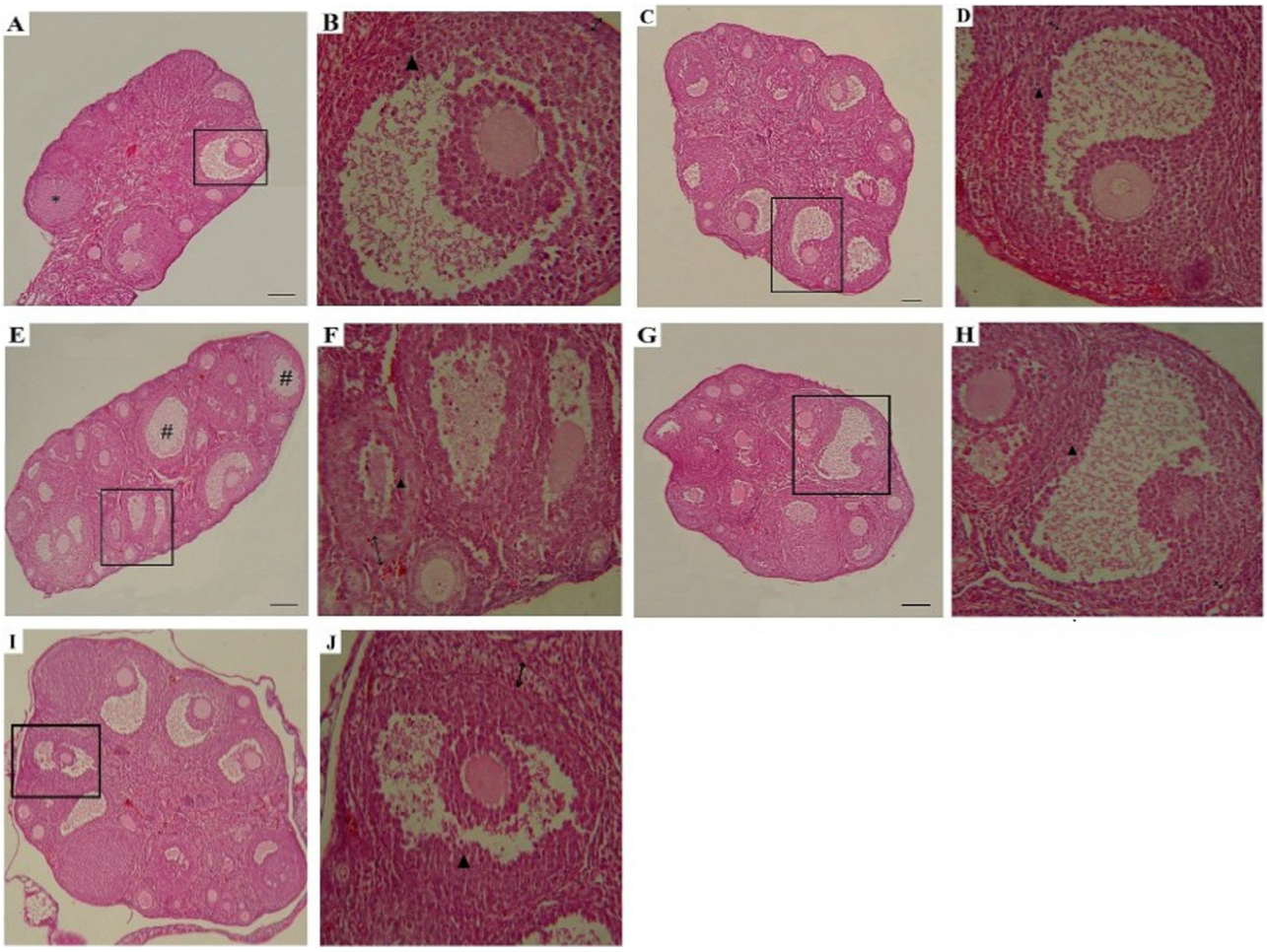

Fig. 1. Photomicrograph of ovaries in different studied groups (Hematoxilina and Eosin staining). (a) ovarian section of control group with healthy growing follicles and a corpus luteum (*) (magnification: ×40). (b) *boxed area* in a, shows the graafian follicle in the control group (magnification: × 100). (c) ovarian section of treatment group (magnification: ×40). (d) *boxed area* in c, shows the graafian follicle in the treatment group (magnification: ×100). (e) ovarian section of disease group with several cyst-like follicles (#) (magnification: × 40). (f) *boxed area* in e, represents different follicles in the disease group (magnification: ×100). (g) ovarian section of disease treatment group (magnification: ×40). (h) *boxed area* in g, observing various follicles along with CL in disease treatment group (magnification: ×100). (i) ovarian section of prevention group (magnification: ×40). (j) *boxed area* in i, observing various follicles along with CL in prevention group (magnification: ×100). Arrow showing granulosa cell layer, double-sided arrow showing a compact theca cell layer. Scale bar 100 μm.

changes of the ovary tissues in the treatment control group compared with the control group, (Table 1). Using DHEA in order to create models of polycystic ovary syndrome in laboratory mice reduced the number of AF and CL follicles and increased the number of PRIF, PF, PAF and cystic follicles, compared with a control group. These changes were statistically significant ($P < 0.05$). The average number of PF, PAF, AF and cystic follicles in disease treatment group showed a significant decrease compared to the prevention group, while the average number of CL in the disease treatment and prevention group had increase compared with the disease group ($P < 0.05$). Moreover, the number of PRIF, PAF and CL follicles in the prevention group had significantly decreased

Table 1
Comparison of index numbers of the studied variables based on the studied groups.

Number of variables	Group 1	Group 2	Group 3	Group 4	Group 5	P value
Primordial follicles	24.16 ± 1.47	29.66 ± 1.03	27.66 ± 1.36	27.16 ± 3.06	8.33 ± 2.06	0.028
Primary follicles	9 ± 1.89	10.16 ± 2.04	18.33 ± 1.63	5.83 ± 1.47	7.16 ± 1.16	0.782
Pre-antral follicles	20 ± 3.03	28.66 ± 2.25	35 ± 2	25.33 ± 1.75	21.16 ± 1.32	0.341
Antral follicles*	7.66 ± 2.16	8.16 ± 2.31	6.33 ± 1.86	2.66 ± 1.47	4 ± 1.26	0.673
Cystic follicles†	0	0.16 ± 0.04	33/83 ± 1.60	3 ± 1.41	5.66 ± 1.63	0/005
Corpus luteum	11.83 ± 1.47	13 ± 1.41	0.16 ± 0.40	7.16 ± 2.31	5 ± 1.41	0.060

Regarding primordial follicles the difference between group (3, 5) $P < 0.001$.
Regarding primary follicles the difference between group (3, 4) and (3, 5) $P < 0.001$.
Regarding antral follicles the difference between group (3, 4) $P = 0.003$, between group (3, 5) $P = 0.046$.
Regarding cystic follicles the difference between group (3, 4) and (3, 5) $P < 0.001$.
Regarding corpus luteum the difference between group (3, 4) and (3, 5) $P < 0.001$.
* The presented amounts with SD ± Mean (Variance analysis and Tukey).
† The presented amounts with Kruskal Wallis.

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compared to the disease treatment group, while the average number of cystic follicles in the prevention group showed an increase compared to the disease treatment group ($P < 0.05$). In measuring the diameter of follicles in the ovaries of different groups, significant differences were observed between the groups. A significant increase in the size of the follicles were observed between the control group ($P < 0.001$), and the disease group which is due to cysts. A significant reduction in the size of diameter of follicles in the treatment group ($P < 0.001$) and prevention ($P = 0.016$) t was observed compared with the disease group (Fig. 2). Theca layer measurements in the 5 groups showed a significant increase in the thickness of the theca between the control and disease group ($P < 0.001$). However, a significant decrease ($P < 0.001$) in the thickness of theca layer between disease group and the treatment and prevention of disease groups was observed (Fig. 3). In measuring the granulosa thickness layer in the 5 groups a significant decrease ($P < 0.001$) between disease and control group was seen and a significant increase between disease group and the treatment group ($P < 0.001$) and the prevention group ($P = 0.001$) was observed (Fig. 4).

4. Discussion

In this study, the phenotype polycystic ovary syndrome was created and the effects of MT on the development of the follicular growth and improvement of this disease on the basis of morphological and morphometric observations were investigated. Also, the effect of administering MT alone and in combination with DHEA on the ovarian tissue was examined. In order to induce PCOS phenotype, a variety of hormonal and non-hormonal methods can be used such as: Letrozole [24], Estradiol Valerate [25], Testosterone Propionate [13], and Testosterone Enanthate [26]. Moreover, it is possible to induce PCOS through exposing the laboratory animal to uniform light [27]. In some studies transgenic mice is used to induce PCOS [28]. One of the advantages of animal models that are created by androgens is that the outer androgens lead to lasting damage to ovarian tissue and reparation of Hallmark symptoms of PCOS in these models [18]. In this study, considering the positive results of Sander et al. [29] in using DHEA to induce phenotype of PCOS, this androgen was used. Therefore, the current study showed that a 6 mg/kg dose of DHEA intraperitoneally in mice, leads to changes in the follicular and morphological changes of ovary. MT as the most important epiphyseal gland secretion is a highly effective antioxidant and neutralizing free radicals [30]. Due to its small size and high lipophilic properties of MT it crosses the cell membrane very easily and disperses around the cell [31]. Its concentration is very high in the cell nucleus and keeps the structure of DNA against damaging agents [30]. The study showed that taking 10 mg/kg MT concurrently with DHEA for 5 days would decrease the adverse effects of DHEA on the ovaries. Therefore, it seems that the improvement in the treatment process of ovulation in the fifth group (prevention group) by MT and DHEA is due to the antioxidant properties of MT. Similarly, Mohammad Ghasemi et al. showed in a study that taking MT in mice treated with nicotine reduces damage to the ovary and it protects ovarian folliculogenesis [32]. Thus, it seems that MT has reduced the side effects of DHEA in this study. MT dosage in this study was based on similar studies of Ateşşahin, Guneli and Mohammadghasemi on mice [21,32,33]. The result of qualitative study in the treatment and prevention groups corresponds to the symptoms of PCOS in this group. It is likely that these changes and the process of ovulation preservation with better maturity in the fourth and fifth group in comparison with the third group can be as a result of the following mechanisms: (i) strong antioxidant properties of MT; as it can activate the activity or gene expression of antioxidant enzymes such as superoxide dismutase, glutathione reductase and glutathione peroxidase [30]. (ii) Anti-apoptotic properties of MT; its effect has been shown on different tissues in several experiments [21,32]. (iii) Anti-proliferative properties of MT [32], previous studies in mice have shown that there is an inverse relationship between MT and GnRH receptor and MT decreases the secretion of LH and FSH [34]. Moreover, previous reports indicated that MT had

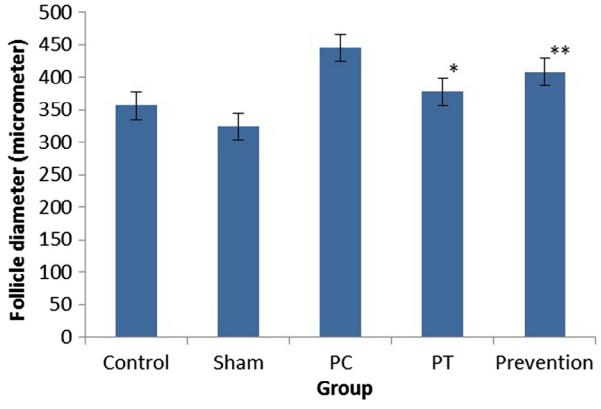

Fig. 2. The morphometry of follicular diameter in the ovary different groups' that had a significant reduction in treatment and prevention of disease group compared with disease group (n = 6) (mean ± SD). $P < 0.001$ *, $P = 0.016$ **.

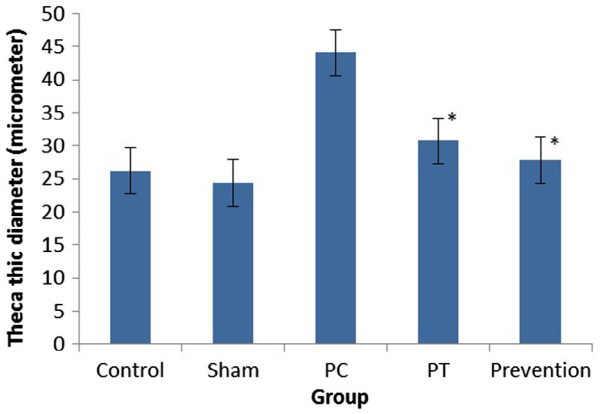

Fig. 3. The morphometry of theca layer thickness in various groups' ovary that had a significant decrease in ovary of treatment and prevention of disease group compared with disease group (6 = n) (mean ± SD). $P < 0.001$ *.

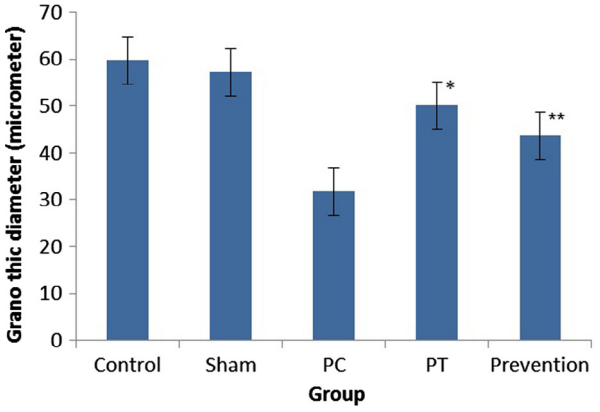

Fig. 4. The morphometry of thickness of granulosa layer in different groups' ovary that a significant increase in ovary of treatment and prevention of disease group is seen compared with disease group (6 = n) (mean ± SD). $P < 0.001$ *, $P = 0.001$ **.

anti-proliferative effects both on the productive cells [35] and other cells [36,37]. However, the above mechanisms are not proved and to prove them, further studies regarding cellular, molecular and immunohistochemistry and endocrinology is required.

5. Conclusion

In summary, the present study showed that injecting 6 mg/kg DHEA intraperitoneally, leads to changes in morphology and number of ovarian follicles to induce the polycystic ovary syndrome. Injecting 10 mg/kg MT intraperitoneally for 5 days during and after the induction of PCOS phenotype had significantly decreased adverse changes of DHEA in the morphology of ovary and considering their antioxidant properties it had an effective protective effect on the natural structure of ovary of mice. This study suggests that MT may be effective for clinical use in ovarian failure to prevent the development of PCOS and also after suffering from this syndrome.

Conflict of interest

The authors declare no conflict of interest.

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