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Research Article

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Evaluation of the Effectiveness of vitamin D compared to metformin on letrozole Induced Polycystic Ovary Syndrome (PCOS) in Female Rats

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Abstract

Vitamin D is an old supplementation with a new mode of action as an encouraging alternative to current therapies for PCOS, and this research is designed to analyze the ability of vitamin D to rebalance hormonal abnormalities, antioxidant capacity, and ovarian morphology in letrozole-induced rats. Thirty-five female Sprague Dawley rats were used, and then they separated into five groups with seven animals each: the first group received only water, the second group received DMSO, the third group signed as PCOS group, Group IV received metformin, Group V received Topferol (vitamin D). PCOS was induced by administration of a daily dose of 1 mg per weight of letrozole for 21 days; after induction, the animals were treated with metformin and vitamin D for 19 days, and at the end of the experiment blood samples and ovarian tissues were taken for analysis. The results show that vitamin D normalized the estrus cycle in rats, restored the normal follicular development process, decreased the high levels of LH and testosterone, and enhanced the total antioxidant capacity. In conclusion, vitamin D is as effective as metformin in the reduction of several hormonal and biochemical features that are usually associated with PCOS mainly by restoring normal estrus cycle, renovating morphological features and folliculogenesis, and organizing abnormalities in hormone levels; however, it showed superior action regarding the antioxidative effect.

Keywords: Histopathology; Infertility; letrozole; Metformin; Polycystic Ovary Syndrome; Vitamin D.

1. Introduction

Polycystic ovary syndrome (PCOS) is a widespread endocrinological inherited disease and is correlated with 20% of patients with infertility situations [1]. As been set by the Rotterdam Consensus [2], the diagnosis can be made based on the presence of hyperandrogenism, metabolic disturbances, and polycystic ovaries, which are considered the salient characteristics of PCOS [3] and the major contributors to its most disturbing complications such as cardiovascular diseases (CVD), type2 diabetes mellitus (TDM) and reproductive system dysfunction [4]. Although several investigations have been made, the exact etiology could not be brought to light; however, hormonal disturbance (i.e., Luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone, estrogen, and testosterone) and abnormal

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insulin levels considered to be the possible causative factors of the pathophysiology of PCOS [5]. Normally, gonatropine hormones (LH and FSH) are secreted in females due to the stimulation of Gonadotropin-releasing hormone (GnRH) in the pituitary. These hormones play a critical role in the reproductive cycle and ovulation by exerting their effect on the ovaries to regulate steroidogenesis and folliculogenesis [6]. However, in women with PCOS, there is a lack of coordination between these hormones, as there is a high level of LH and excess of androgen production, which is responsible for devastating cosmetic features associated with PCOS, such as hirsutism, acne, and seborrheic dermatitis [7]. Abnormal androgen levels create disturbance in the LH to FSH ratio in addition to a decrease in progesterone secretion [8]. The whole event will harm the normal follicle cycle, ovulation, and fertility [9].

Traditionally, insulin sensitizers such as metformin have been extensively utilized for the treatment of PCOS to ameliorate metabolic features, which represent the fundamental element in the etiology of PCOS; however, long-term use is linked with obnoxious side effects that motivate researchers to find a better alternative, yet with fewer side effects [10, 11].

Over the last few years, vitamin D has gained interest as a possible replacement therapy for PCOS. This is in part not only due to its rule to replenish the deficiency in vitamin D concentration usually found in serum tests of women who are suffering from PCOS and responsible for their defective folliculogenesis [12]. However, due to its rebalancing action on insulin abnormalities, vitamin D deficiency can cause hypocalcemia, negatively impacting folliculogenesis, menstruation, and fertility [13]. Since intracellular calcium represents an integral part of the signaling pathway in oocyte maturation [14], vitamin D's antioxidant effect can also contribute to reducing the oxidative stress status found in PCOS [15]. Thus, we aimed to investigate the possible effectiveness of vitamin D as an alternative therapy to metformin in letrozole-induced PCOS female rats.

2- Materials and Methods

2.1. Material

Letrozole 2.5mg tablet (Accord Healthcare, UK), dissolved in Dimethyl sulfoxide(DMSO) and given at a once-daily dose of 1 mg per kg by gavage tube for 21 days [16].

Topferol® (cholecalciferol) 5000IU tablet (Pharma International Co. Amman-Jordan) dissolved in DMSO, the giving dose of 1000 IU /kg/day daily by oral gavages [17].

Glucophage® (metformin) 500mg tablet (Merck Santé, France) dissolved in distilled water, given at a dose of 300mg per kg daily by oral gavages [18].

2.2. Animals

After approval of the study by the Ethical Committee of the University of Kufa with the Registration number 6681 on 10/3/2024, Thirty-five female Sprague Dawley rats at the age of 15 weeks and about 200 grams' weight kept and acclimatized under controlled circumstances (at room temperature 25 ± 5°C and humidity of 50± 5%) with the administration of the confirmed food and water were ad libitum in the animal house of the faculty of Pharmacy-University of Kufa for one week, estrus cycle was detected by vaginal smear [19], which have been performed at the beginning of the experiment to confirm reproductively for all animals, those with three consecutive cycles were included in the study, and also performed after letrozole administration to confirm disturbance in the cycle resemble that found in PCOS, and eventually performed at the end of the experiment to verify the effectiveness of the treatment by returning of the cycle to its normal pattern [19, 20].

2.3. The Experimental Design

After seven days of acclimation in the standard condition, the experimental animals were randomly divided into two sets. The first set was divided into groups I and II (n=7 each), considered as a control group with no treatment received from the vehicles (distilled water and DMSO, respectively). At the same time, the second set (n=21) was induced to develop PCOS by receiving 1 mg per weight of letrozole daily for 21 days [21]. After induction, these animals were subdivided into three groups: Group III (n=7), signed as PCOS group, received the only vehicle, Group IV received metformin, and Group V received Topferol (vitamin D). The next day after the last treatment, all animals were euthanized by using 4 mg/kg of body weight Lidocaine injection, then blood samples were obtained and saved at (-20°C) for analysis. In addition, all the obtained ovarian tissues were collected and fixed in 10% formalin for histopathological analysis, as shown in **Table 1**.

Table 1: The Experimental Design**.**

Groups	Treatment
Group I (control)	distilled water
Group II (DMSO only)	DMSO
Group III (PCOS non-treatment group)	letrozole 1 mg/kg dissolved in DMSO
Group IV (metformin)	Metformin300mg per kg dissolved in distilled water to PCOS-induced rats
Group V (vitamin D)	Vitamin D 1000 IU /kg/day daily dissolved in DMSO to PCOS-induced rats

2.4. The Biochemical Analysis

2.4.1. Serum Hormone Assessment

The assessment of serum Luteinizing Hormone (LH), follicle-stimulating hormone (FSH), and testosterone was performed using ELISA kits obtained from Nanjing Pars Biochem CO., Ltd-China.

2.4.2. Serum Antioxidant Assessment

Serum total antioxidant capacity(TAOC) was assessed using an ELISA kit obtained from Nanjing Pars Biochem CO., Ltd-China.

2.4.3. Histopathological Assessment of the Ovaries

Following a 24-hour formalin fixation, the ovarian tissues were embedded in paraffin, then sliced the tissues longitudinally at a thickness of 4-5µm using a microtome, and finally by using hematoxylin and eosin stain to be recognized for analyzation under a light microscope. Then, the number of primary, secondary, and mature follicles and corpus luteum was counted for each treatment group.

2.5. Statistical Analysis

Graph Pad Prism 8.0.1. were used for statistical analyses in this study, the results for each parameter were shown as mean ± Standard Error Mean (SEM)and compared by using the One-Way Analysis of Variance (ANOVA) and the Bonferroni multiple comparison test. $P < 0.001$ was used as the significance threshold to achieve statistical significance.

3. **Results and Discussion**

3.1. Evaluation of the Estrous Cycle

Before using any treatment, the estrus cycle was normal in all rats; after a period of letrozole induction, group III (PCOS group) showed a delay in the cycle while it returned to normal in animals treated with metformin and vitamin D.

3.2. Effect on Serum Hormonal Parameters

The results in **Figure 1a** and 1.c. showed that the PCOS group had a significant increase(P=*0.*0001) in LH level and testosterone concentration (P=0.01) compared to the Control group and DMSO group; conversely, as presented in **Figure 1a** a significant decrease was noticed in the level of LH in metformin group (P=*0.*0001) and vitamin D group(P=*0.*0001) in comparison with PCOS group, also, as it presented in **Figure 1c**. Testosterone had a significant decrease in the metformin group (P=0.1) and in the vitamin D group (P=*0.*0001) compared to the PCOS group; however, Treatment with metformin had non-significant differences compared to treatment with vitamin D regarding the levels of LH and testosterone, while as it has been shown in **Figure 1b**, the non-significant difference in the FSH level was observed in all treated groups.

3.3. Effect on Antioxidant Activity

The results in **Figure 1d** showed that letrozole significantly reduced TAOC level in the PCOS group (P=0.002) when compared with the control group and DMSO group; results also showed that there is an elevation in TAOC in group Vitamin D treated groups as compared with the PCOS group (P=0.001), while no difference was noticed in Metformin group compared with PCOS group**.** Additionally, no significant difference was noticed between vitamin D and metformin groups.

The isolated ovaries of the control group showed a regular texture and multitudinous follicles at their variant stages: primary, secondary, mature, and corpus luteum; the same was presented in the DMSO non-PCOS group. While the PCOS group had enlarged-sized ovaries with multiple cystic follicles and a reduction in the number of

corpus luteum, this abnormality seemed to be diminished after administration of the treatment in the metformin group and vitamin D group, which showed normal ovarian morphology and reduction in the cystic follicles. Regarding comparing the number of primary, secondary, and mature follicles, our results showed no significant difference in value among all groups. The result showed that the PCOS group significantly reduced the corpus luteum (P=0.001) compared to the control group. Metformin was given as standard treatment in group IV and showed a significant elevation of corpus luteum compared to group PCOS group). Although the mean value of corpus luteum seemed higher after administering vitamin D in group V, there was no significant difference compared to the PCOS group, as shown in **Table 2** and **Figure 2**.

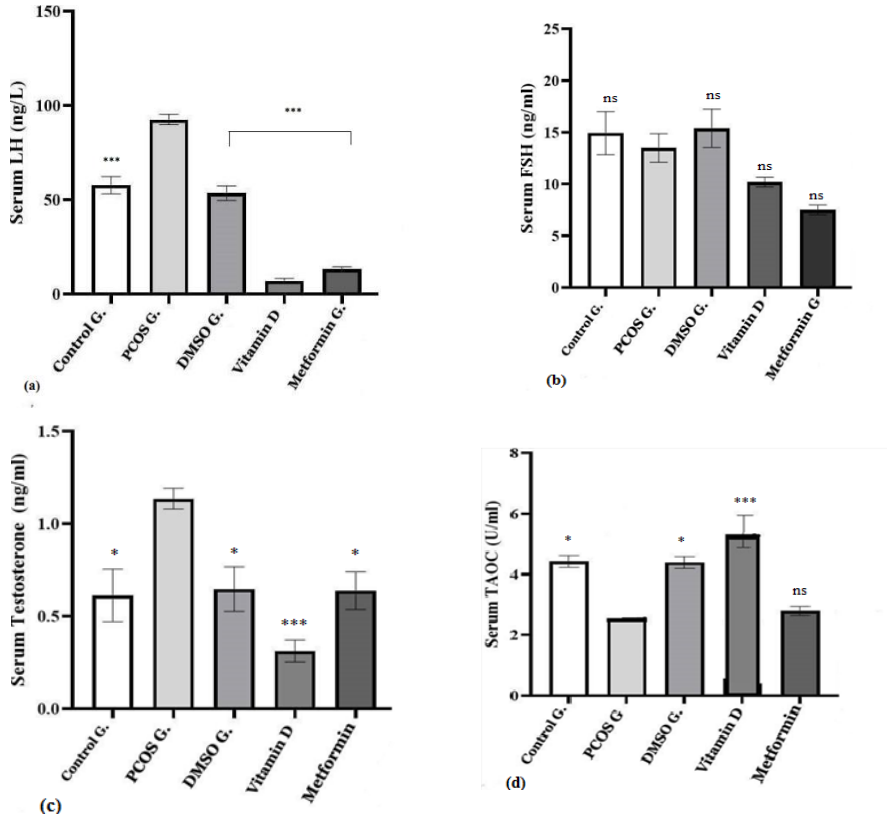

Figure 1: Effect of different groups on serum hormonal parameters and antioxidant activity. a. Serum LH level of the experimental groups. b. Serum FSH level of the experimental groups. c. Serum testosterone level of the experimental groups. d. Serum TAOC level of the experimental groups.
* =comparison between the PCOS group and the rest groups
Ns=no significant

Table 2: Data presented as Mean ±Std. Error of Mean SEM (n=7), ANOVA evaluation was followed by multiple comparisons in mean values (ng/ml) among different study groups using Bonferroni's test of primary follicles, secondary follicles, mature follicles and corpus luteum.

Parameters / Groups	primary follicles (ng/ml)	Secondary follicles(ng/ml)	Mature follicles (ng/ml)	corpus luteum (ng/ml)
Control G.	4.75±0.25	4.62±0.32	6.625±0.3239	13.13±0.4407
PCOS G.	5.37±0.84	4.125±0.2950	8.500±0.4226	8.375±0.6529
DMSO non-PCOS G.	4.75±0.45	4.625±0.3750	6.375±0.1830	12.88±0.7662
Metformin G	4.00±0.27	4.500±0.4226	0.64±0.10	12.50±0.8238
Vitamin D G.	6.25±0.92	5.375±0.4605	7.250±0.9402	10.63±0.5957

Bonferroni analysis. $p < 0.001$

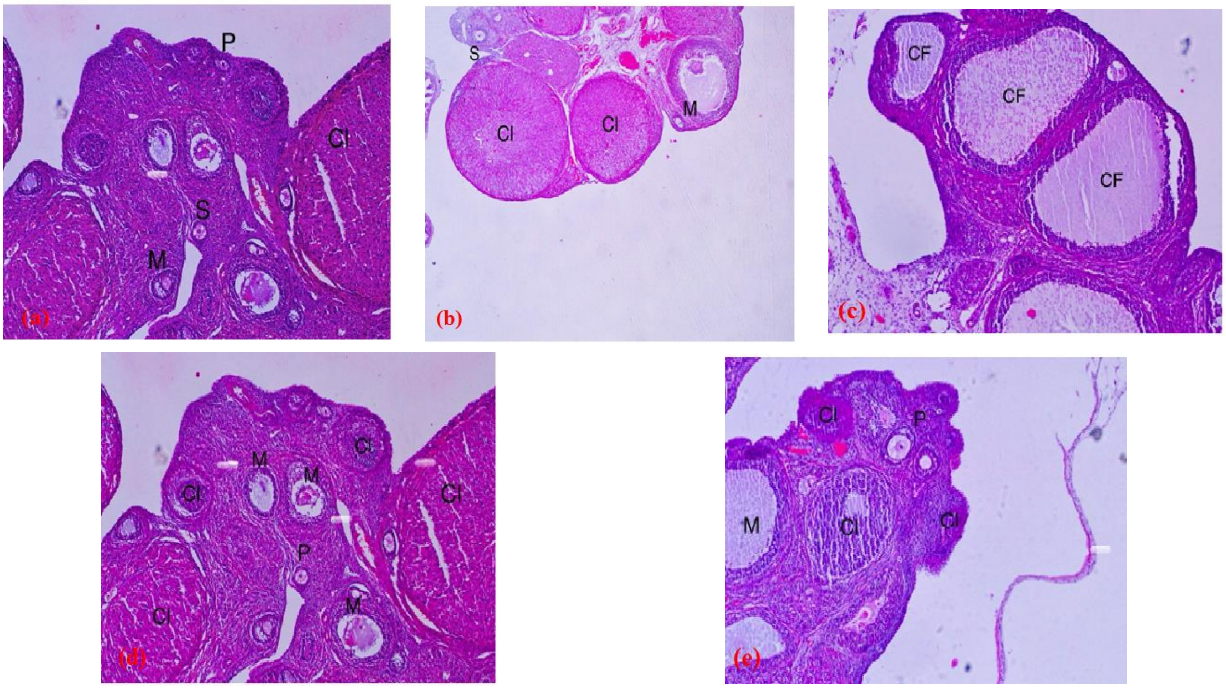

Figure 2: The cross-section of the ovarian tissue showed different stages of ovarian follicles (primary(p), secondary (s), mature follicles(M), and corpus luteum (CL). (a): The cross-section of the ovarian tissue of control G. (b): The cross-section of the ovarian tissue of DMSO non-PCOS G. (c): The cross-section of the ovarian tissue of PCOS G (d): ovarian cross-section of the group treated with metformin. (e): The ovarian cross-section of the group was treated with vitamin D. CF: cystic follicles.

3.4. Discussion

In the current study, the usage of letrozole for a 21 days induced polycystic ovarian syndrome in female rats [16], letrozole acts by inhibiting the aromatase enzyme that's responsible for the conversion of androgen into estrogen, leading to the accumulation of testosterone and decrease of estrogen, this is one of the attributed features of PCOS in human [22].

In the current study, the estrus cycle showed dysregulation after letrozole administration in the PCOS group.

The elevation of LH level in PCOS is due to the action of androgen on the hypothalamic-pituitary axis; a high level of androgen along with diminished estrogen will prevent the negative feedback effect on the hypothalamus leading to LH over secretion [23, 24]. This is consistent with Younas, A. et al. [25], who proposed that the overactivity of specific genes and enzymes results from activating the PI3K/Akt pathway upon disrupting the hypothalamic-pituitary axis. These enzymes participate in the steroidogeneses, especially the 17-α hydroxylase enzyme responsible for the conversion of progesterone into androgen, leading to an increase in androgen levels. While metformin treatment showed a decrease in LH level, which is mainly due to the regulatory rule of metformin on the hypothalamus axis in addition to its effect on the hyperinsulinemia status associated with PCOS those together will cause a decrease in LH level [26], our results is in agreement with previous studies by Malini, N. A., [27] and Abulfadle K.A [28] who reported that insulin status has a direct correlation with the level of LH and testosterone mainly by its stimulatory effect on GnRH to induce LH secretion and subsequently the production of testosterone ,moreover insulin through its action on the liver also decrease the production of sex hormone binding globulin (SHBG) produce a further augmentation in the concentration of LH and testosterone ,thus metformin through its effect on insulin lower the elevated levels of LH and testosterone , On the other hand, vitamin D administration led to reduction in LH with mean value lower than that with metformin, this is consistent with results obtained by Abdelsalam, H. M.,[29] the mechanism behind this due to the regulatory

action on the GnRH by the vitamin D which in turn will cause a decrease in LH secretion [30] another possible cause could be due to the restoring of the normal level of vitamin D in the body, since the deficiency in vitamin D considered to be a possible participant in PCOS pathogenesis [31].

The elevation of serum testosterone levels is found in PCOS due to abnormalities in LH and insulin, which in turn cause overproduction of testosterone [32]. This resembles the results from a previously published article by Manuel Maliqueo [33], who stated that aromatase inhibition by letrozole administration produced a disruption in the negative feedback on the hypothalamus, causing a further release of LH and subsequently overproduction of testosterone .in the current study the administration of metformin cause a reduction in testosterone level which is similar to results obtained by Ayyadurai, [34] who have explained the reason due to metformin regulatory effect at a molecular level on the enzyme involved in the testosterone formation by interfering with pathways associated with cAMP signaling.

Vitamin D in our study led to a decrease in testosterone levels similar to results obtained by Helal, B. A. F., Ismail [17], who stated that vitamin D has a beneficial effect in the regulation of the abnormalities associated with PCOS not only due to its regulatory effect on hyperinsulinemia status but also due to regulation of hypothalamus axis.

Several studies [35, 36, 37] demonstrated that hormonal and metabolic abnormalities associated with PCOS could lead to oxidative stress and reduction in the antioxidant formation that can cause exacerbation of the disease; this had been emphasized in the current study that showed a decrease in the total antioxidant capacity in PCOS group which in agreement with previous study [38]. Treatment with metformin showed no significant difference in TAOC level compared with the PCOS group, which disagrees with Naseri L.[39], who found that metformin caused an elevation in TAOC level while there was a decrease in TAOC level in a study by Janati S [40], who explained it due to diminishing of the enhancing action of metformin on antioxidant effect due to Hyperhomocysteinemia following prolonged use of metformin .vitamin D however exhibit superior action regarding TAOC level by increase its level which in agreement with the results obtained from other researches [41, 42] This is in part due to the scavenging effect of vitamin D on the free radical associated with stimulation of oxidative stress [43, 44].

Histopathological analysis of the PCOS group showed abnormality in the folliculogenesis process as there is an elevation in cystic follicles and a decline in corpus luteum number; previous studies indicated the rule of androgen in the promoting the maturation of early follicles to the following stages of follicular development while the hyperandrogenism state found in PCOS will inhibit oocyte maturation, and hence ovulation, thus corpus luteum formation will be decreased [45]. These results support the findings obtained by Khosrowpour Z [46]. Treatment with metformin showed an increase in corpus luteum formation compared to the PCOS group due to the normalizing action of metformin on androgen level [47]. Also, it could be due to the effect of metformin in the upregulation of specific enzymes found in theca cells responsible for ovulation [48]; similar findings were reported in previous articles [47, 49]. However, in the current study, vitamin D did not show any superior significance regarding the improvement in the number of corpus luteum, which is dissimilar to findings obtained by Kyei, G. et al. [50], who reported that there is an enhancing effect of vitamin D on folliculogenesis and corpus luteum formation.

4. Conclusion

The study showed that vitamin D was as effective as metformin in the reduction of several hormonal and biochemical features that are usually associated with PCOS mainly by restoring normal estrus cycle, renovating morphological features and folliculogenesis, and organizing abnormalities in hormone levels; however, it showed superior action regarding the anti-oxidative effect.

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Using artificial intelligence chatbots

There was no use of artificial intelligence in the making of this article.

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