

Reversal of Haemostatic Changes in Letrozole-Induced PCOS Rat Model with Vitamin D Supplementation

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine condition that appears to be associated with a prothrombotic tendency. Hypovitaminosis D is very common in PCOS patients and may exacerbate the metabolic abnormalities. Vitamin D supplementation might produce beneficial effects in these associated metabolic and haemostatic abnormalities.

Aim of Study : The present study was designed to examine the effects of vitamin D supplementation on some metabolic, oxidative stress, inflammatory and haemostatic parameters in letrozole-induced PCOS rat models.

Material and Methods: This study was conducted on 3 groups of adult female albino rats: Group-I: (Control) in which 1ml water was given orally by gavage daily for 21 days, and, group-II: (PCOS induced) in which letrozole was given orally by gavage at a dose of 1mg/kg dissolved in water daily for 21 days, and group-III: (PCOS induced treated with vitamin D) in which letrozole was given orally by gavage with cholecalciferol (200 IU/day) for 21 days. In all groups, serum levels of 25(OH) vitamin D, glucose, insulin, HOMA-IR, lipid profile parameters, Luteinizing hormone (LH), Follicle-stimulating hormone (FSH), testosterone, estradiol, progesterone, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α), plasma fibrinogen, factor VIII (FVIII), Von Willebrand factor (vWF), tissue plasminogen activator (tPA), plasminogen activator inhibitor 1 (PAI-1) activity and D-dimers levels were measured. Bleeding time (BT), whole blood clotting time (WBCT), prothrombin time (PT), activated partial thromboplastin time (aPTT) were also evaluated and ovaries were dissected and used for histopathological examination and evaluating malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels.

Results: The present study showed that letrozole-induced PCOS rat group (II) showed a statistically significant increase in the serum levels of insulin, glucose and calculated HOMA-IR, Cholesterol, Triglyceride, LDL-c, VLDL-c, testosterone, FSH, LH, TNF- α , IL-6, ovarian MDA, plasma FVIII, Vwf, D-dimers, PAI-1 activity and fibrinogen levels (with statistically significant negative correlations with serum vitamin D level) while there were a statistically significant decrease in

the serum levels of 25(OH) vitamin D and HDL-c, estradiol, progesterone, ovarian SOD and GPx, bleeding and clotting times, PT and aPTT when compared to controls (with statistically significant positive correlations with serum vitamin D level). Interestingly, all of the above measures were improved with vitamin D3 supplementation in the treated group (III). Also, vitamin D3 supplementation reduced the size and number of the cystically dilated ovarian follicles observed in the histopathological examination of letrozole-induced PCOS group.

Conclusion: PCOS are associated with hypovitaminosis D and characterized by metabolic disturbances and a prothrombotic state that can be explained by increased both insulin resistance and inflammatory mediators together with the development of oxidative stress. These changes were improved by vitamin D supplementation.

Key Words: PCOS – Haemostasis – Vitamin D.

Introduction

POLYCYSTIC ovary syndrome (PCOS), the most common endocrine abnormality in premenopausal women, is characterized by hyperandrogenism, ovulatory dysfunction, and PCO morphology [1].

Several studies pointed to a prothrombotic state in PCOS including hypofibrinolysis, hypercoagulability, and endothelial & platelet dysfunction [2,3]. Others point in the opposite direction or suggest an unaffected fibrinolysis/coagulation status [4,5].

The mechanisms of potential disturbances of the hemostatic system in women with PCOS are unknown. There is evidence that plasma levels of several hemostatic factors are modulated by hyperglycemia, insulin resistance with compensatory hyperinsulinemia, proinflammatory agents, and dyslipidemia, all of which are typical in PCOS [6].

On another note, Vitamin D is a fat-soluble vitamin that belongs to the family of steroid hormones. Its biological actions are exerted through

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the vitamin D receptor (VDR), a transcription factor located in the nuclei of target cells that mediates the genomic action of the active form of vitamin D. The presence of VDR in female reproductive tissue suggests that vitamin D is involved in female reproduction [7]. VD deficiency (VDD) is also common in women with polycystic ovary syndrome (PCOS), with the 67-85% of women with PCOS having serum concentrations of 25-hydroxy Vitamin D (25OHD) <20ng/ml [8].

Data regarding the association between VDD and PCOS are apparently conflicting; some authors showed association of low 25 hydroxy vitamins (25OHD) with metabolic derangements of PCOS [9,10] suggesting that VDD might be a causal factor in the pathogenesis of PCOS while others demonstrated no such association [11,12].

There has been a growing interest in recent years regarding non-classical effects of vitamin D, and current evidence suggests that there is increased risk of disorders like type 2 diabetes mellitus, cardiovascular disease, hypertension, IR, dyslipidaemia and obesity with VDD [13,14]. Moreover, Patients with low vitamin D levels are more prone to thrombosis but there was limited data concerning the pro-fibrinolytic, antitrombotic effect of vitamin D [15].

On the other hand, several studies had shown that the vitamin D supplementation might improve IR and reduce serum androgens in PCOS [16]. Seyyed Abootorabia et al., [17] also showed improved fasting plasma glucose, HOMA-B, adiponectin, and serum vitamin D level by Vitamin D supplementation in vitamin D deficient women with PCOS. However, another study failed to observe a positive influence of vitamin D3 supplementation on insulin-sensitivity [12].

Therefore, the aim of this study was to examine the relationship between serum level of vitamin D and PCOS metabolic and haemostatic parameters and to evaluate the effect of vitamin D3 oral supplementation on these parameters in letrozole-induced PCOS rat models.

Material and Methods

This study was conducted in Faculty of Medicine, Zagazig University in the period from June 2018 to February 2019 and involved thirty healthy adult female albino rats of local strain weighing 190-217g obtained from faculty of veterinary medicine animal house. Rats were kept under hygienic conditions in steel wire cages (5/cage) at room

temperature, maintained on a natural light/dark cycle with free access to water and adapted to the new environment for one week before the experiment going on. All experimental procedures were approved by the Institutional Research Board and Ethics Committee of Faculty of Medicine, Zagazig University.

Rats were randomly assigned to three equal groups: Group (I): (Control) in which 1ml water was given orally by gavage daily for 21 days, group (II): (PCOS induced) in which letrozole (ACDIMA international) was given orally by gavage at a dose of 1mg/kg dissolved in water daily for 21 days. This dose was chosen according to the previous study in which the cystic follicle formation was induced [18], and group (III): (Vitamin D3 treated PCOS induced) in which letrozole was given orally by gavage at a dose of 1mg/kg dissolved in water with cholecalciferol (200IU/day) [19] daily for 21 days (Vidrop oral drops 2800 IU/ml (each drop contains 100IU of vitamin D).

Determination of sexual cycle:

Vaginal smears were prepared daily by vaginal washing with saline then the fresh unstained samples were examined microscopically during the period of treatment, and cycles with duration of 4-5 days were considered regular [20]. Determination of estrus phases were done according to Goldman et al., [21].

Sample collection: Retro-orbital venous plexus blood samples (6ml) were collected and divided into three vials. The first one (2ml) was containing 3.2% sodium citrate in the ratio 1:9 with the blood and centrifuged at 2000rpm for 15min (sampling of controls were taken in the estrus phase). Plasma obtained was used for estimation of prothrombin time (PT), activated partial thromboplastin time (aPTT), plasma FVIII, vWF, (tPA), PAI-1 activity, D-dimers and fibrinogen levels. The second vial (2ml) was used to separate serum. It was obtained by allowing the blood samples to clot then centrifugation at 3000rpm for 20 minutes and kept at (-20°C) and used to measure levels of, glucose, insulin, total cholesterol (TC), triglycerides (TG), high density lipoproteins cholesterol (HDL-c), very low density lipoproteins cholesterol (VLDL-c), low density lipoproteins cholesterol (LDL-c), tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), LH, FSH, testosterone, estradiol and progesterone. The third vial was filled with fresh blood (2ml) for measuring the whole blood clotting time (WBCT). The ovaries were dissected and right ovaries were frozen immediately at -70°C until used for determination of oxidative stress markers

whereas, left ovaries were used for histopathological examination.

• *Biochemical analysis:*

Measurement of serum vitamin D: Serum vitamin D was estimated as described by Holick [22] using 25(OH) vitamin D ELISA kit (Sigma-Aldrich Co, Egypt).

Measurement of serum glucose and insulin:

Serum glucose was estimated as described by Tietz [23] using specific glucose kit (Bioscience, Egypt) and analyzed by spectrophotometer device (URIT-810, China) at 546nm wave length.

Insulin was measured as described by Temple et al., [24] using specific insulin kit (BioSource Belgium) and analyzed by spectrophotometer device at 450nm wave length.

Calculation of Homeostasis model assessment of insulin resistance (HOMA-IR): Was calculated according to the following formula:

$[HOMA-IR = \text{Insulin (}\mu\text{U/mL)} \times \text{glucose (mg/dl)} / 405]$ [25].

Measurement of serum lipids profile:

TC and TG were measured by enzymatic colorimetric method described by Tietz [23] using specific cholesterol and triglycerides kits (Spinreact Spain) and analyzed by spectrophotometer device at 500 nm wave length. HDL-c was measured by precipitating reagent method described by Tietz et al., [23] using HDL-c precipitating reagent kit (Spinreact, Spain) and analyzed by spectrophotometers device at 600nm wave length. LDL-c and VLDL-c were measured by using Friedewald et al., [26] formula.

$$LDLc = TC - HDLc - \left(\frac{TG}{5} \right) VLDc = \frac{TG}{5}$$

Measurement of serum sex hormones:

Serum FSH level was measured according to Rebar et al., [27] using Follicle-Stimulating Hormone (FSH) enzyme immunoassay test kit (Catalog Number: BC-1029, BioCheck, Inc 323 Vintage Park Dr. Foster City, CA 94404), measured by using spectrophotometer (spectronic 3000 Array, Germany) at 450nm.

Serum LH level was measured according to Tietz [23] using Luteinizing hormone (LH) enzyme immunoassay test kit (Catalog Number: BC-1031, BioCheck, Inc 323 Vintage Park Dr. Foster City, CA 94404), was measured by using spectrophotometer (spectronic 3000 Array, Germany) at 450nm. Serum testosterone level was measured

according to Tietz [23] using testosterone enzyme immunoassay test kit (Catalog Number: BC-1115, BioCheck, Inc 323 Vintage Park Dr. Foster City, CA 94404), was measured by using spectrophotometer (spectronic 3000 Array, Germany) at 450 nm. Serum estradiol Level: was measured according to the method described by Tietz [23] using rat estradiol (E2) enzyme-linked immunosorbent assay kit: (Catalog Number: 2011-11-0175, shanghai sunred biological technology, china) at 280nm. Serum progesterone Level was measured according to the method described by Tietz [23] using rat progesterone enzyme-linked immunosorbent assay Kit: (Catalog Number: Catalog Number: 2011-11-0742, shanghai sunred biological technology, china) at 254nm.

Measurement of serum TNF- a level: Serum TNF-a level was measured as described by Fernando et al., [28], using commercial ELISA kit, (Catalog Number RAB0480, provided by Sigma-Aldrich Co).

Measurement of serum IL-6 level: Serum IL-6 level was measured as described by Engvall and Perlmann [29], using IL-6 ELISA Kit (Catalog Number RAB0306 provided by Sigma-Aldrich Co).

Measurement of Ovarian malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx): These markers of oxidative stress were measured in ovarian tissue as described by Ohkawa et al., [30], Kakkar et al., [31] and Reddy et al., [32] respectively.

• *Haemostatic parameters:*

Bleeding time: It was measured according to the method described by Martin [33].

Whole blood clotting time: It was measured according to the method described by Quick, [34].

Prothrombin time: It was measured according to the method described by Arkin [35] using coagulometer (bmc coagulation analyzer 600).

Activated partial thromboplastin time: It was measured according to method of Ansell [36] using coagulometer.

Plasma fibrinogen levels: It was measured according to the method described by Cooper and Douglas [37] using coagulometer.

Plasma D-dimer level: It was measured using ELISA kit, (GenWay Biotech, Inc, ca 40-88-234402, USA) according to the method described by Declerck et al., [38].

Plasma von willebrand factor antigen (vWF) activity: It was measured using ELIZA kit, according to the method described by Declerck et al., [38].

Plasma plasminogen activator inhibitor 1 (PAI-1) level: It was measured using ELIZA kit, according to the method described by Declerck et al., [38].

Plasma coagulation factor FVIII: It was measured using ELIZA kit that was obtained from American diagnostic according to the method described by Mannucci et al., [39].

Plasma tissue-type plasminogen activator (t-PA): It was measured using t-PA ELIZA kit (catalog No: MBS2507499, provided by myobiosource) according to the method described by Ito et al., [40].

Histopathological examination:

The ovaries were dissected, fixed in 10% buffered formalin for 6 hours at room temperature and washed in a phosphate buffer saline solution. The fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene then embedded in paraffin. 5 μ m thick sections were mounted on slides previously treated with 3-aminopropyl triethoxysilane and stained with hematoxylin and eosin for light microscopy [41].

Statistical analysis:

Data were presented as mean \pm SD. The Statistical Package for the Social Sciences (SPSS), version 19.0 (SPSS Inc., Chicago, IL, United States) was used for performing the statistical analysis. Analysis of variance (ANOVA) followed by LSD post hoc test was performed to compare means of the different groups. Pearson's correlation analysis was performed to display possible relationships between serum vitamin d and the measured parameters. p -value <0.05 was considered to be statistically significant for all statistical tests done.

Results

The present study showed that while regular sexual cycles were observed in all rats at the beginning of the experiments and in control rats, letrozole-treated rats (rats with PCOS) were acyclic and showed persistent estrus.

The present study also showed that letrozole-treated rats (group II) showed a statistically significant increase in the levels of serum insulin, glucose and calculated HOMA-IR, Cholesterol, Triglyceride, LDL-c, VLDL-c, testosterone ($p<0.001$), FSH

($p<0.01$), LH, TNF- α , IL-6 and ovarian MDA ($p<0.001$) in comparison to those of control group (I), while there was a statistically significant decrease in the levels of 25(OH) vitamin D, HDL-c, estradiol, progesterone, ovarian SOD and GPx ($p<0.001$) when compared to controls.

However, PCOS rats treated with vitamin D (group III) showed a statistically significant decrease in the levels of serum insulin ($p<0.01$), glucose and calculated HOMA-IR, Cholesterol, Triglyceride, LDL-c, VLDL-c, testosterone ($p<0.001$), FSH, LH ($p<0.05$) TNF- α , IL-6 and ovarian MDA ($p<0.001$) when compared to those of untreated letrozole-induced PCOS rats, but it showed statistically significant increases in the levels of serum 25(OH) vitamin D, HDL-c, estradiol, progesterone, ovarian SOD and GPx ($p<0.001$).

In addition, serum 25(OH) vitamin D showed statistically significant negative correlations with serum insulin, glucose, calculated HOMA-IR, Cholesterol, Triglyceride, LDL-c, VLDL-c, TNF- α , IL-6, LH, FSH, testosterone and ovarian MDA in letrozole-induced PCOS studied group. However, statistically positive correlations were found between 25(OH) vitamin D serum level and HDL-c, estradiol, progesterone and ovarian SOD, GPx levels in that group.

Moreover, statistically significant decreases in bleeding and clotting times, PT and aPTT ($p<0.001$) with statistically significant positive correlations with serum 25(OH) vitamin D together with statistically significant increases in plasma FVIII, Vwf, D-dimers, PAI-1 activity and fibrinogen levels ($p<0.001$) (with statistically significant negative correlations with serum 25(OH) vitamin D) were observed in letrozole-induced PCOS rats that were improved with vitamin D supplementation in the treated group.

Additionally, histopathological examination of ovaries from the control group revealed numerous ovarian follicles at various grades of maturation surrounded by dense ovarian stroma (Photo 1). However, follicular cysts were observable on the surface of ovaries from PCOS group (II) in the form of fluid-filled sacs and histological examination revealed large cystic follicles filled by granulosa cells and surrounded by dense stroma (Photo 2). However, the number of cystic follicles was significantly decreased by vitamin D administration in vitamin D₃ treated PCOS group (letrozole + Vit. D₃) (Photo 3), but were still higher than control group.

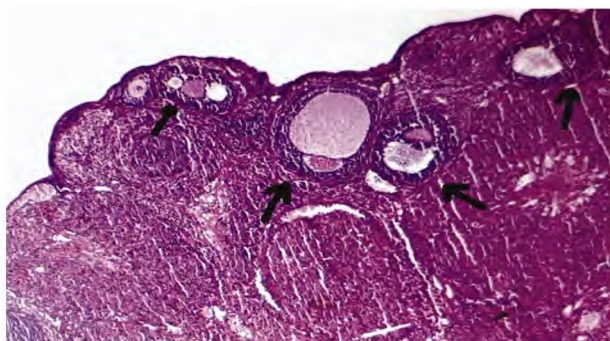


Photo (1): Photomicrograph of ovarian tissue of control group showing numerous variable sized graffian follicles (black arrows) at different stages of maturation surrounded by dense spindle shaped ovarian stroma (H&E X200).

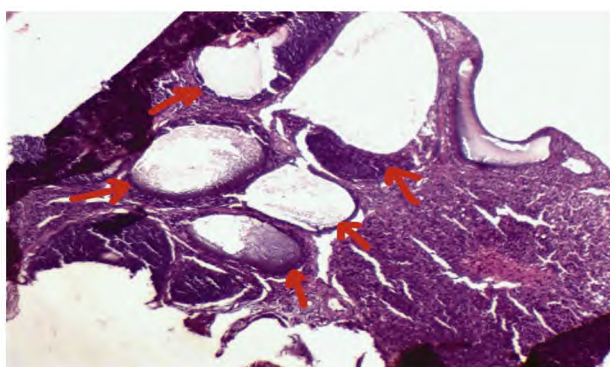


Photo (2): Photomicrograph of ovarian tissue of letrozole-induced PCOS group showing increased cystically dilated graffian follicles (red arrows) surrounded by dense ovarian stroma (H&E X200).

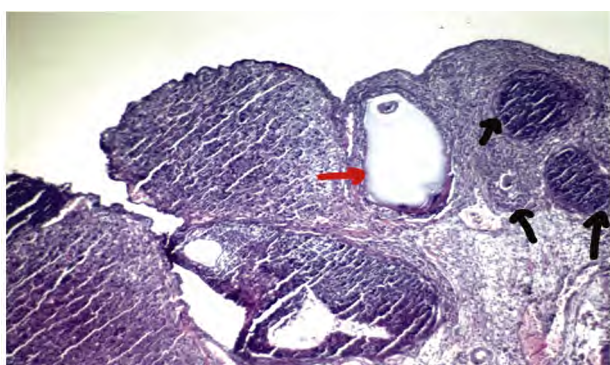


Photo (3): Photomicrograph of ovarian tissue of vitamin D 3 treated PCOS group showing reduction in the size and number of the previously cystically dilated graffian follicles (red arrows) with normal graffian follicles (black arrows) (H&E X200).

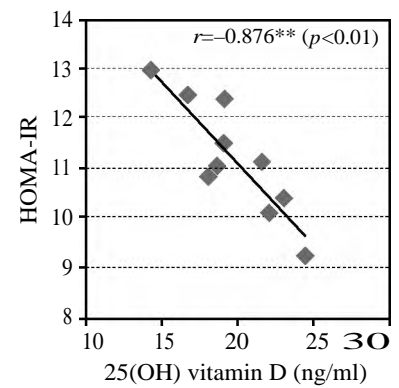
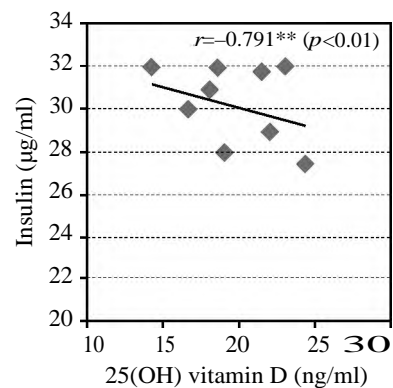
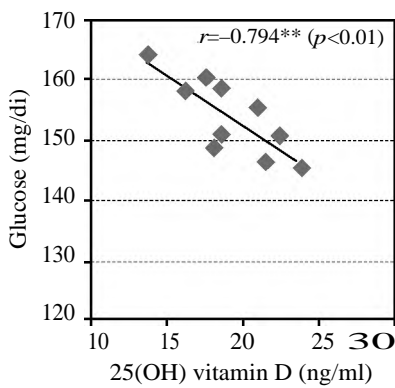
Table (1): Biochemical Parameters of all studied groups.

Parameter	Group I	Group II	Group III
25(OH) vitamin D (ng/ml)	34.65±2.36	19.6±3.16 <i>p</i> <0.001 a	32.4±2.17.4 NS a , <i>p</i> <0.001 b
Glucose (mg/dl)	81.25±6.4	153.6±6.4 <i>p</i> <0.001 a	126.65±7.3 <i>p</i> <0.001 a,b
Insulin (μIU/ml)	20.58±2.1	29.5±2.1 <i>p</i> <0.001 a	25.25±2.8 <i>p</i> <0.001 a , <i>p</i> <0.01 b
HOMA-IR	4.14±0.6	11.2±1.6 <i>p</i> <0.001 a	7.9±1.1 <i>p</i> <0.001 a,b
Cholesterol (mg/dl)	73.42±5.7	126.2±9.2 <i>p</i> <0.001 a	97.89±6.4 <i>p</i> <0.001 a,b
Triglyceride (mg/dl)	69.2±3.1	132.2±9 <i>p</i> <0.001 a	80.9±6.4 <i>p</i> <0.01 a , <i>p</i> <0.001 b
HDL-c (mg/dl)	40.47±2.5	27.61±3.2 <i>p</i> <0.001 a	34.18±2.9 <i>p</i> <0.001 a,b
LDL-c (mg/dl)	19.11±6.4	72.14±10.1 <i>p</i> <0.001 a	47.53±7.3 <i>p</i> <0.001 a,b
VLDL-c (mg/dl)	13.84±0.6	26.44±1.8 <i>p</i> <0.001 a	16.18±1.2 <i>p</i> <0.01 a , <i>p</i> <0.001 b
Testosterone (pg/ml)	1.33±0.37	2.38±0.2 <i>p</i> <0.001 a	1.72±0.2 <i>p</i> <0.01 a , <i>p</i> <0.001 b
Estradiol (pg/ml)	34.62±2.6	21.7±3.4 <i>p</i> <0.001 a	27.37±2.7 <i>p</i> <0.001 a,b
Progesterone (ng/ml)	27.82±2.6	15.78±2.3 <i>p</i> <0.001 a	21.69±2 <i>p</i> <0.001 a,b
LH (IU/ml)	0.86±0.03	0.95±0.05 <i>p</i> <0.001 a	0.90±0.02 <i>p</i> < 0.05 a ' b
FSH (IU/ml)	4.39±0.48	4.95±0.28 <i>p</i> <0.01 a	4.56±0.39 NS a , <i>p</i> <0.05 b
TNF-α (pg/ml)	24.75±4.2	47.07±4.5 <i>p</i> <0.001 a	36.63±2.9 <i>p</i> <0.001 a,b
Il-6	8.17±0.95	21.71±3.15 <i>p</i> <0.001 a	14.23±1.73 <i>p</i> <0.001 a,b
MDA (mmol/gm)	81.3±6.3	144.79±11.45 <i>p</i> <0.001 a	103.95±8.89 <i>p</i> <0.001 a,b
SOD (U/gm)	7.51±0.5	4.37±0.76 <i>p</i> <0.001 a	6.07±0.68 <i>p</i> <0.001 a,b
GPx (U/gm)	23.3±2.5	12.13±2.05 <i>p</i> <0.001 a	19.27±2.12 <i>p</i> <0.001 a,b

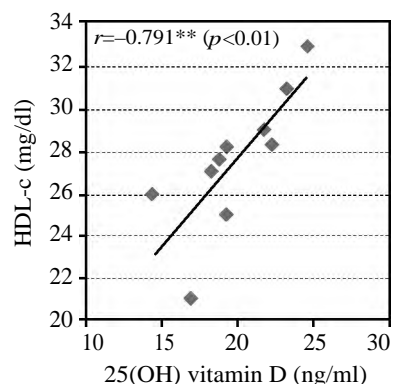
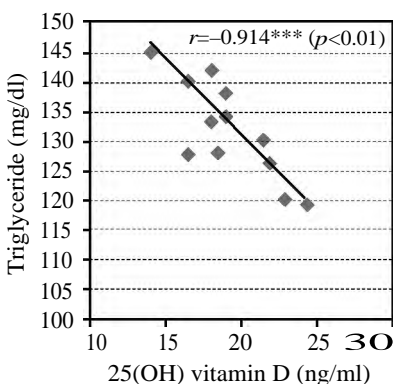
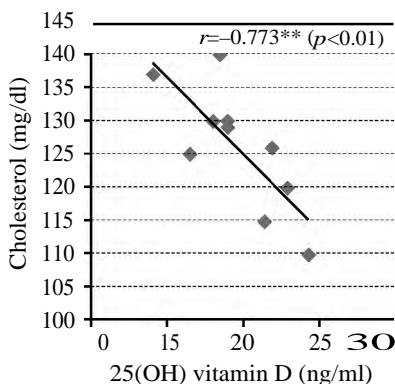
Table (2): Haemostatic parameters of all the studied groups.

Parameter	Group I	Group II	Group III
BT (sec)	93.6±3.2	74.6±3.02 <i>p</i> <0.001 a	87.2±3.25 <i>p</i> <0.001 a,b
WBCT (sec)	119.8±14.39	76.6±9.33 <i>p</i> <0.001 a	98±8.01 <i>p</i> <0.001 a,b
PT (sec)	16.4±2.01	9.5±1.9 <i>p</i> <0.001 a	12.8±1.54 <i>p</i> <0.001 a,b
aPTT (sec)	24.09±3.07	15.9±2.51 <i>p</i> <0.001 a	19.7±2.40 <i>p</i> <0.01 a,b
Plasma Fibrinogen (mg/dl)	221.1±12.55	323±27.1 <i>p</i> <0.001 a	268.7±17.35 <i>p</i> <0.001 a,b
Plasma D-dimer (mg/dl)	63.69±3.59	112.1±10.95 <i>p</i> <0.001 a	80±9.53 <i>p</i> <0.001 a,b
Plasma factor VIII (U/L)	263.09±9.56	310.3±14.75 <i>p</i> <0.001 a	285.4±11.64 <i>p</i> <0.001 a,b
Plasma Von Willebrand factor (vWF) (ng/ml)	92.08±3.69	131.38±7.69 <i>p</i> <0.001 a	103.37±6.27 <i>p</i> <0.001 a,b
Plasma tissue plasminogen activator (tPA) (ng/ml)	1.85±0.22	2.03±0.27 NS a	1.89±0.29 NS a,b
Plasma PAI-1 (ng/ml)	4.07±0.46	13.83±1.82 <i>p</i> <0.001 a	7.43±1.25 <i>p</i> <0.001 a,b

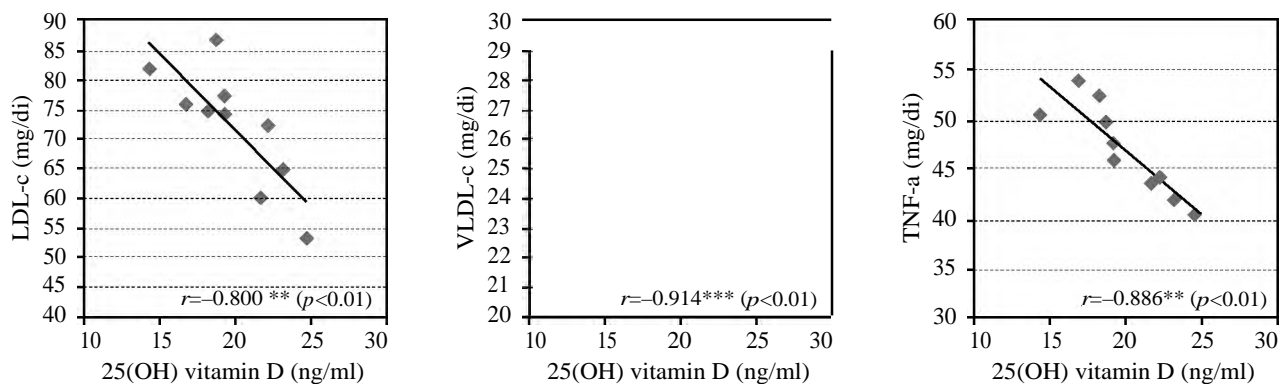
a=Versus group I. b=Versus group II. NS=Non-significant (*p*>0.05).



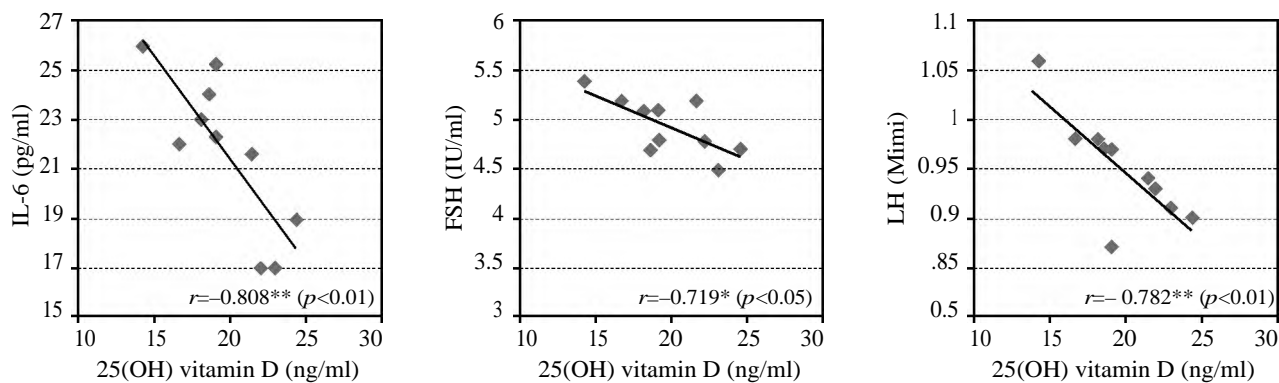
Figs. (1-3): Correlation between serum vitamin D and serum glucose, insulin and HOMA-IR respectively in group II.



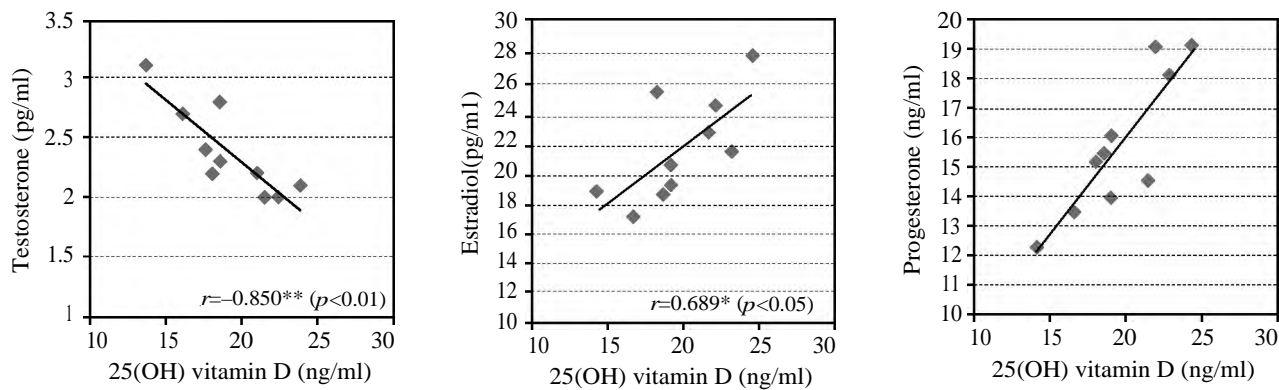
Figs. (4-6): Correlation between serum vitamin D and serum cholesterol, triglyceride and HDL-c respectively in group II.



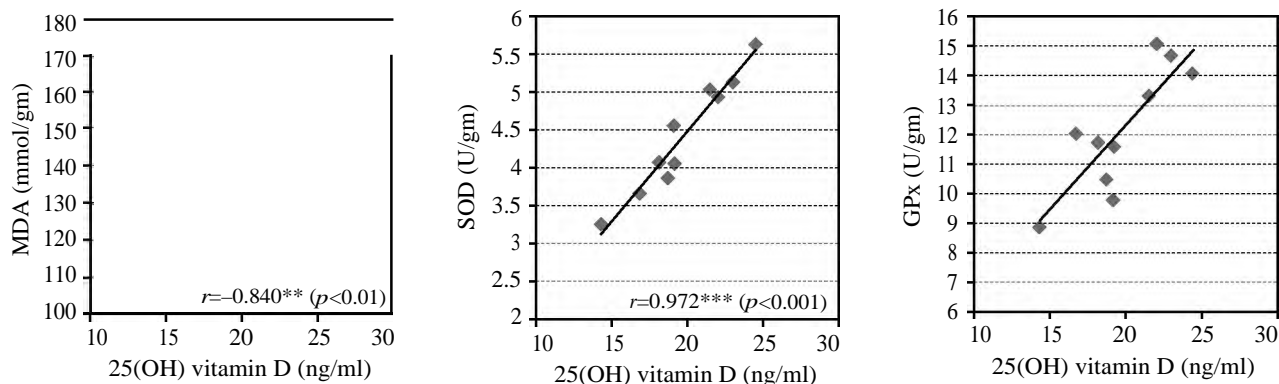
Figs. (7-9): Correlation between serum vitamin D and serum LDL-c, VLDL-c and TNF- α respectively in group II.



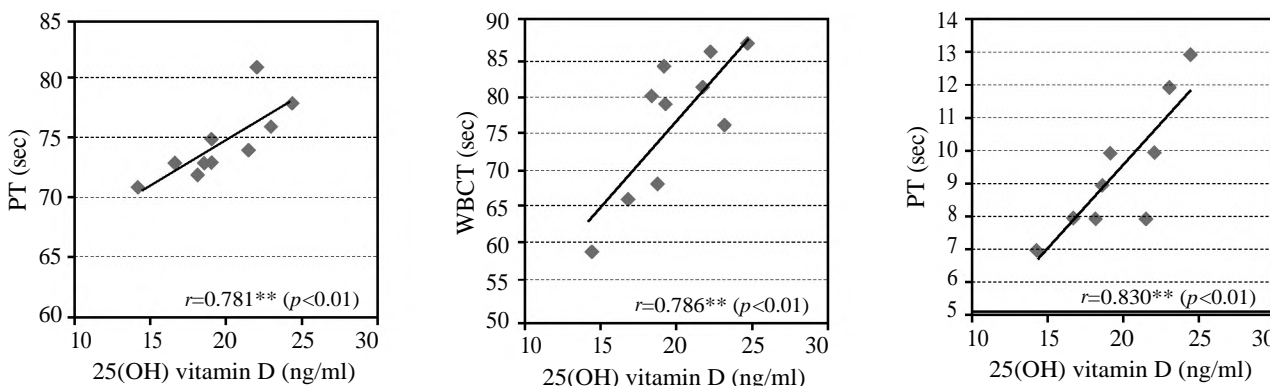
Figs. (10-12): Correlation between serum vitamin D and serum IL-6, FSH and LH respectively in group II.



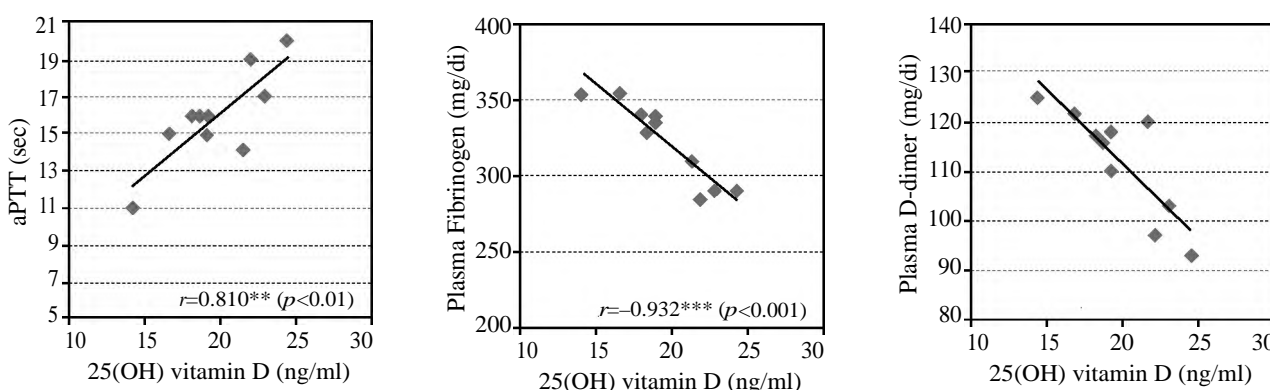
Figs. (13-15): Correlation between serum vitamin D and serum testosterone, estradiol and progesterone respectively in group II.



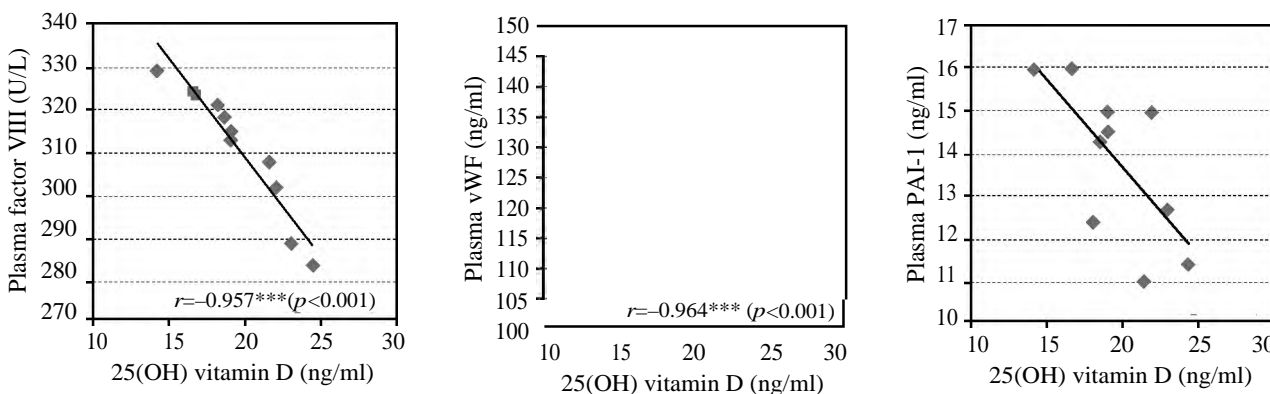
Figs. (16-18): Correlation between serum vitamin D and ovarian MDA, SOD and GPx respectively in group II.



Figs. (19-21): Correlation between serum vitamin D and BT, WBCT and PT respectively in group II.



Figs. (22-24): Correlation between serum vitamin D and aPTT, Plasma Fibrinogen and D-dimer respectively in group II.



Figs. (25-27): Correlation between serum vitamin D and Plasma factor VIII, vWF and Plasma PAI-1 respectively in group II.

Discussion

Polycystic ovary syndrome (PCOS) is a common and multifactorial disease associated with both endocrine and metabolic disorder and characterized by hyperandrogenism and ovarian abnormalities, resulting from a disruption in the hypothalamic-pituitary-ovarian axis [42].

Women with PCOS have shown dysregulation of the hemostatic system that may contribute to CV disease (CVD) [43].

The etiology and pathology of PCOS are extremely complicated and management of this disorder remains controversial. Thus, the treatment protocols are applied according to the symptoms of affected women [44]. During recent years, vitamin D supplementation is also involved in these protocols and differences in the therapeutic efficacy of vitamin D were reported in PCOS patients [45]. The number of studies with regard to effect of vitamin D treatment on ovarian morphology in PCOS is also extremely low [46].

Consequently, we examined the therapeutic effects of vitamin D supplementation and the association between its serum level and some biochemical and hemostatic parameters in letrozole-induced PCOS rat models.

Our results revealed that PCOS rats had lower serum 25(OH) vitamin D concentrations, consistent with the findings of previous studies that reported lower serum vitamin D levels in PCOS patients than in non-PCOS subjects [10,47]. However, Ganie et al., [48] did not find such difference in serum 25(OH) vitamin D levels. The effect of vitamin D deficiency on metabolic and reproductive dysfunctions in PCOS may be mediated by insulin resistance. Insulin resistance increases hyperandrogenism through insulin increasing ovarian androgen production, and reducing sex hormone-binding globulin (SHBG) production [9].

In our PCOS model, the serum FSH, LH and testosterone levels were significantly higher in the PCOS group than in the control group with significant negative correlations with vitamin D serum level. On the other hand, the serum estradiol and progesterone levels were significantly decreased with significant positive correlations with vitamin D level in the PCOS group. Letrozole, a non-steroidal aromatase inhibitor, reduces conversion of androgens to estrogens in the ovary and results in a condition termed hyperandrogenism with hyperandrogenemia, in which serum estradiol concentration significantly decreases and consequently non-aromatizable androgens such as testosterone increase. Hyperandrogenism as a key regulator in the pathogenesis of a majority of PCOS cases developed cystogenesis by impairing maturation of developing follicles in the ovaries [49]. Moreover, decreased rate of normal ovulations or anovulation and lack of developing CL have been shown to lead to decreased serum progesterone concentrations in the present model, as also seen in PCOS women [50]. Such hormonal disturbance led to constant estrus manifestation in PCO rats [51].

It was also reported that the increase of LH level probably plays an important role in the pathological mechanism of the higher androgen production in the ovaries. It is estimated that the significant increase in LH levels causes an increase in LH/FSH levels, while relative FSH deficiency causes impaired follicle maturation [52].

In this study, we also elucidated the beneficial effects of vitamin D in PCOS treated rats. We found that treatment with vitamin D alleviated the serum gonadotropins, estradiol, progesterone, and

testosterone and improved insulin resistance. These findings are in accordance with those of Çelik et al., [46] which showed that vitamin D treatment lowered FSH, LH levels, LH/FSH ratio, and testosterone levels in dehydroepiandrosterone (DHEA) treated rats. The possible mechanism may be the direct stimulatory effect of 1,25(OH)₂D₃ on the aromatase gene expression in reproductive tissues [53].

Moreover, histological examination of ovaries from the letrozole-induced PCOS rats revealed large cystic follicles filled by granulosa cells and surrounded by dense stroma. However, vitamin D₃ supplementation significantly decreased the number of these cystic follicles in the treated PCOS group (letrozole + Vit. D₃).

Similar results were reported from other studies that observed an improvement in ovarian morphology after vitamin D treatment [46]. It has been suggested that testosterone stimulates the early stages of follicle growth and vitamin D might inhibit the testosterone-induced early stages of follicular growth [54]. They also reported that the junctional complexes between granulosa cells of the follicles contain high levels of Connexin43 (Cx43) in every stage of follicle development and the expression of Cx43 was decreased during follicular atresia. In the same study it was reported that high dose testosterone administration downregulates Cx43 levels and the testosterone-induced downregulation of Cx43 can be reversed by vitamin D treatment.

VD may play an important role in reproductive functions since Vitamin D receptors (VDRs) have been found in the ovary, endometrium and placenta [55]. Several studies have shown that VD and calcium supplementation may help in ovulation and normalization of menses [56-58]. On the contrary, Ganie et al., [48] observed no relationship to menstrual cyclicity/ovulation. They also found a positive correlation between serum 25OHD and serum total testosterone levels in contrast to the negative correlation reported in our study and in the study of Yildizhan et al., [9].

Moreover, insulin resistance (IR), evidenced by the significantly increased serum glucose and insulin levels in addition to increased HOMA-IR, with significant negative correlations with vitamin D were observed in this study. Insulin excess can cause folliculogenesis disruption through stimulating androgen production by theca cells and elevating serum-free testosterone levels [59,60]. This finding is consistent with previous studies revealing

a negative association of 25OHD and HOMA-IR in PCOS women [61].

We also elucidated the beneficial effects of vitamin D3 on IR in PCOS treated rats in our study. Vitamin D might directly increase insulin sensitivity via stimulating the expression of insulin receptors in peripheral tissues [62]. Vitamin D receptors are expressed in 2776 genomic positions and modulate the expression of 229 genes in more than 30 different tissues, such as skeleton, brain, breast, pancreas, parathyroid glands, immune cells, cardiomyocytes, and ovaries [63]. Vitamin D may play a role in glucose metabolism by enhancing insulin synthesis and release, and increasing insulin receptor expression or suppression of proinflammatory cytokines that possibly contribute to the development of insulin resistance [63].

More over, in the present study, dyslipidemia manifested by significant increases in total cholesterol, triglyceride, LDL, VLDL (with a significant negative correlation versus serum 25OHD level), and a significant reduction in HDL (with a significant positive correlation versus serum 25OHD level) was observed in PCOS rats. This is probably due to excessive hypersecretion of apolipoprotein B and very low density lipoprotein (VLDL) from the liver following insulin stimulation, ultimately resulting in hypertriglyceridaemia, low levels of high-density lipoprotein (HDL) and high levels of low-density lipoproteins (LDL) [44]. A previous research has also shown that the 25OHD is inversely correlated with the total cholesterol, triglycerides, body weight, and other metabolic disturbances in PCOS patients suggesting that vitamin D deficiency might be a causal factor in the pathogenesis of PCOS [65]. However, Ganie et al., [48] and Kumar et al., [66] did not demonstrate any relationship between serum 25OHD levels and clinical, metabolic or insulin sensitivity

Vitamin D treatment also significantly improved the dyslipidemia in PCOS treated rats. Von Hurst et al., [61] and Wehr et al., [68] also showed positive effects on insulin sensitivity and IR, lipid profile, menstrual cycle and follicular development with Vitamin D supplementation. In contrast, Jia et al. [45] failed to detect any therapeutic effect of vitamin D3 supplementation in PCOS.

Moreover, the present results revealed a significant increase in serum TNF- α and IL-6 level, with a significant negative correlation versus serum 25OHD level. This is in accordance with results of Rezvanfar et al., [51], who detected higher serum TNF- α concentration in the letrozole-induced

PCOS rats when compared with controls. Vitamin D co-treatment to letrozole-induced PCO rats in this study also significantly prevented the elevation of TNF- α and IL-6 concentrations and kept it close to that of controls.

Chronic low-grade inflammation has appeared to be a key contributor to the pathogenesis of PCOS. TNF- α and IL-6 could induce insulin resistance, stimulate the production of androgen and lead to hypothalamic-pituitary-ovarian axis secretion disorder [69]. Emerging data from experimental models and from clinical studies suggest that hyperandrogenism is progenitor of chronic low-grade inflammation that in turn directly stimulates excess ovarian androgen production [70]. It can arrest follicular development via apoptosis in granulosa cells resulting to poor oocyte quality and ultimately progressive follicular atresia through higher serum TNF- α concentrations [71].

Aside from inflammation and hyperglycemia, oxidative stress also contributes to PCOS [72]. In PCOS group in our study, ovarian SOD, and GPX were decreased with significantly higher levels of ovarian MDA. These findings also co-related with the previous researches [18,73]. However, Vitamin D administration had also improved the antioxidant activities and decreased ovarian MDA level. Alqasim et al., [74] also demonstrated the beneficial antioxidant effect of vitamin D in diabetic rats. Vitamin D might improve the downregulation of nuclear factor-like 2-kelch-like ECH-associated protein 1 (Nrf2-Keap1) pathway that regulates the expression of antioxidant proteins as well as suppress nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and NADPH oxidase activity [75].

Interestingly, our results also showed a prothrombotic state in PCOS group, as reflected by decreased bleeding and clotting times, PT and aPTT together with significant increases in plasma D-dimers, PAI-1 activity and fibrinogen levels. This hypercoagulable state can be explained by insulin resistance, dyslipidemia, inflammatory mediators and oxidative stress that all have been elucidated above in our study.

Elevated levels of fibrinogen, factor VIII, and vWF could lead to hypercoagulability. Fibrinogen is mainly produced by hepatocytes in response to inflammatory cytokines [76]. Fibrinogen levels have also been reported to be elevated in PCOS in other studies [43,77]. However, Kebapcilar et al., [4] reported normal level.

PAI-1 is the principal inhibitor of fibrinolysis. Increased PAI-1 activity is associated with hypofibrinolysis and may contribute to thrombosis. Consistent with reports of Mannerås-Holm et al., [43], PAI-1 activity was found to be higher in PCOS rats than controls in this study. Low SHBG and high insulin levels were the strongest explanatory factors of high PAI-1 activity in PCOS [78].

Huang et al., [79] also noted a hypercoagulable state with higher FX levels and probable microthrombus formation in the uterine vessels in PCOS women compared with non-PCOS women, which would probably lead to poor microcirculation of endometrium in PCOS women and influence successful embryo implantation as well as subsequent maintenance of pregnancy.

The subclinical inflammation is thought to be an important underlying mechanism leading to coagulation/fibrinolysis abnormalities in PCOS. It was demonstrated that both lean and obese patients with PCOS have increased plasma C-reactive protein levels compared with BMI-matched controls, indicating that the subclinical inflammation seen in PCOS might be related with the presence of the disorder rather than with obesity [80].

It is also well established that insulin resistance/ hyperinsulinemia may alter fibrinolysis by enhancing PAI-1 secretion [81]. Treatment with insulin sensitizers is associated with a reduction in PAI-1 levels that is also paralleled by a decline in circulating androgen levels, suggesting a link between plasma androgens and PAI-1 levels [82].

Noteworthy, vitamin D₃ administration significantly improved these haemostatic parameters in the treated PCOS group. In line with these findings, Ohsawa et al., [83] found that 1,25(OH)₂D exerts anticoagulant effects by upregulating the expression of the anticoagulant glycoprotein, thrombomodulin, and downregulating the expression of a critical coagulation factor, tissue factor, and upregulates thrombomodulin expression in monocytic cells, counteracting the effects of tumor necrosis factor (TNF)-α and oxidized low-density lipoprotein cholesterol.

Moreover, vitamin D receptor (VDR) knockout mice exhibited an exacerbated multiorgan thrombus formation after lipopolysaccharide injection. These results suggest that the VDR system plays a physiological role in the maintenance of antithrombotic homeostasis [84].

Taking the present findings together, it can be concluded that letrozole-induced PCOS is characterized by a prothrombotic state, as reflected by increased PAI-1 activity, fibrinogen & D-dimers levels and decreased bleeding, clotting times, PT and aPTT that can be explained by insulin resistance, inflammatory mediators and oxidative stress

Vitamin D might be used as an interesting, economical, and safe therapeutic approach in PCOS patients especially those who had hypovitaminosis D.

Additional studies are required to detect the precise mechanism of PCOS and to investigate the potential therapeutic role of vitamin D in PCOS patients with hypercoagulable state.

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أنعكاس التغيرات فى آليات وقف النزف بالإمداد بفيتامين (د) فى نموذج الجرذان المحدث لها تكييس المبايض باستخدام الليتروزول

خلفية البحث: تعد متلازمة تكييس المبايض حالة هرمونية متباينة ويبدو أنها مرتبطة بزيادة التخثر الدموى. كما يبدو أن نقص فيتامين (د) شائعاً جداً فى حالات تكييس المبايض وقد يؤدي إلى تفاقم التغيرات الأيضية. ومن الممكن أن يكون الإمداد بفيتامين (د) له آثار مفيدة فى كل من هذه التغيرات الأيضية والتخثر الدموى المرتبط بهذا المرض.

الهدف من البحث: لقد أجريت هذه الدراسة لمعرفة تأثير إعطاء فيتامين (د) على بعض التغيرات الأيضية ومعاملات الأكسدة والإتهاب والتخثر الدموى فى نموذج تكييس المبايض المحدث تجريبياً فى الجرذان باستخدام عقار الليتروزول.

مواد وطرق البحث: أجريت الدراسة على ثلاث مجموعات من إناث الجرذان البيضاء البالغة وتم تقسيمها إلى:

مجموعة ١ (مجموعة ضابطة): أعطيت ١ ملليمتر من الماء المقطر عن طريق الفم باستخدام أنبوبة معدية مرة واحدة فى الصباح يومياً لمدة ٢١ يوماً.

مجموعة ٢: مجموعة مصابة بمرض تكييس المبايض المحدث تجريبياً بواسطة عقار الليتروزول بجرعة ١ ميلليجرام/كجم مذاباً فى ماء مقطر عن طريق الفم باستخدام أنبوبة معدية مرة واحدة فى الصباح يومياً لمدة ٢١ يوماً.

مجموعة ٣: مجموعة مصابة بمرض تكييس المبايض ومعالجة بفيتامين (د) أعطيت مكملات فيتامين (د) بجرعة ٢٠٠ وحدة دولية يومياً من عقار ليتروزول بجرعة ١ ميلليجرام/كجم مذاباً فى ماء مقطر عن طريق الفم باستخدام أنبوبة معدية مرة واحدة فى الصباح يومياً لمدة ٢١ يوماً.

فى كل هذه المجموعات تم قياس: مستوى فيتامين (د) بمصل الدم وأيضاً تم قياس مستويات الجلوكوز والأنسولين والدهون الدم وبعض هرمونات الخصوبة وأنترليوكين ٦ وعامل نخر الورم ألفا. كما تم قياس الفيبرينوجين البلازما والعامل VIII وعامل Von Willrbrand ومنشط البلازمينوجين للأنسجة وكذلك نشاط مثبط البلازمينوجين والذى دايمر (D-dimers) فى البلازما وتم تقييم زمن النزيف وزمن تخثر الدم الكامل وزمن البروثرومبين وزمن الثرومبوبلاستين الجزئى المنشط. وتم تشريح المبايض لفحص الأنسجة ميكروسكوبياً وتقييم مستويات مالون داى ألدهيد وسربر أوكسيد ديسميوتيز وجلوتاثيون بيروكسيديز.

النتائج: لقد وجد ارتفاع ذو دلالة إحصائية فى مستوى الجلوكوز والأنسولين والكوليسترول والدهون الثلاثية والبروتين الدهنى منخفض الكثافة ومنخفض الكثافة جداً وهرمون التستوسترون والهرمون المحفز للحويصلات والهرمون الملوتن وكذلك ارتفاع مستوى أنترليوكين ٦ وعامل نخر الورم ألفا فى مصل دم الجرذان المصابة بمرض تكييس المبايض، وأيضاً ارتفاع مستويات مالون داى ألدهيد فى نسيج المبيض. كما لوحظ زيادة فى الفيبرينوجين البلازما وعامل التجلط VIII وعامل Von Willrbrand وكذلك نشاط مثبط منشط البلازمينوجين، ومستويات D-dimers فى بلازما الجرذان المصابة بمرض تكييس المبايض كما وجد انخفاض فى زمن النزف وزمن تخثر الدم الكامل وزمن البروثرومبين وزمن الثرومبوبلاستين الجزئى المنشط فى الجرذان المصابة بمرض تكييس المبايض (مع وجود انخفاض ذو دلالة إحصائية مع مستوى فيتامين د بمصل الدم). كما وجد انخفاض ذو دلالة إحصائية البروتين الدهنى عالى الكثافة وهرموني الأستروجين والبروجسترون وأيضاً انخفاض مستويات سربر أوكسيد ديسميوتيز وجلوتاثيون بيروكسيديز فى نسيج المبيض فى الجرذان المصابة بمرض تكييس المبايض عند مقارنتها بالمجموعة الضابطة (مع وجود ارتباط إيجابى ذو دلالة إحصائية مع مستوى فيتامين د بمصل الدم). كما أن إعطاء فيتامين (د) للمجموعة المعالجة قد أدت إلى زيادة ذات دلالة إحصائية مع مستوى فيتامين د بمصل الدم وتحسن ملحوظ فى دلالات عملية الأيض المذكورة أعلاه وكذلك دلالات كفاءة آليات وقف النزف.

الاستنتاج: ترتبط متلازمة تكييس المبايض بنقص فيتامين (د) والتي تميزت بوجود خلل فى بعض العمليات الأيضية بالإضافة إلى زيادة قابلية الدم للتخثر وقد تعزى هذه التغيرات إلى وجود الإجهاد التأكسدى وزيادة الوسائط الأتهابية مع وجود زيادة فى مقاومة الأنسولين وقد لوحظ إعطاء فيتامين (د) له عظيم الأثر فى عودة هذه التغيرات نحو وضعها الطبيعى.