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Research paper

The influences of vitamin D and omega-3 co-supplementation on clinical, metabolic and genetic parameters in women with polycystic ovary syndrome

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of the co-administration of vitamin D and omega-3 fatty acid on clinical, metabolic and genetic parameters in women with polycystic ovary syndrome (PCOS).
Methods: This randomized, double-blinded, placebo-controlled clinical trial was conducted on 60 subjects, aged 18–40 years old with PCOS. Subjects were randomly allocated to take either 50,000 IU vitamin D every 2 weeks plus 2000 mg/day omega-3 fatty acid from fish oil ($n = 30$) or placebo ($n = 30$) for 12 weeks. Gene expression analysis of inflammatory cytokines was conducted on peripheral blood mononuclear cells (PBMCs) of PCOS women using RT-PCR method.
Results: Vitamin D and omega -3 fatty acid co-supplementation significantly decreased serum total testosterone levels (-0.2 ± 0.5 vs. $+0.1 \pm 0.4$ ng/mL, $P = 0.02$) compared with the placebo. In addition, vitamin D and omega-3 fatty acid co-supplementation resulted in a significant improvement in beck depression inventory (-1.4 ± 1.6 vs. -0.5 ± 0.6, $P = 0.01$), general health questionnaire scores (-4.5 ± 4.3 vs. -1.9 ± 2.3, $P = 0.005$) and depression anxiety and stress scale scores (-5.0 ± 5.1 vs. -2.3 ± 3.5, $P = 0.01$) compared with the placebo. Additionally, vitamin D and omega-3 fatty acid co-administration significantly decreased serum high-sensitivity C-reactive protein (hs-CRP) (-1.2 ± 1.9 vs. $+0.1 \pm 0.7$ mg/L, $P = 0.001$) and malondialdehyde (MDA) levels (-0.4 ± 0.4 vs. $+0.2 \pm 0.6$ μmol/L, $P < 0.001$), and significantly increased plasma total antioxidant capacity (TAC) levels ($+114.6 \pm 122.2$ vs. -2.4 ± 168.2 mmol/L, $P = 0.003$) compared with the placebo. Results of RT-PCR demonstrated that vitamin D and omega-3 fatty acid co-supplementation significantly downregulated gene expression of interleukin-1 (IL-1) ($P = 0.03$), and upregulated vascular endothelial growth factor (VEGF) ($P = 0.004$) in PBMCs of subjects with PCOS, when compared with placebo.
Conclusions: Overall, the co-administration of vitamin D and omega-3 fatty acid for 12 weeks had beneficial effects on mental health parameters, serum total testosterone, hs-CRP, plasma TAC and MDA levels, and gene expression of IL-1 and VEGF among women with PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS) occurs in 6% to 10% of women in reproductive age (Bozdag et al., 2016) and is characterized by hyperandrogenism, menstrual dysfunction, impaired fertility and polycystic ovaries (Setji and Brown, 2014). This common endocrine disorder is also associated with dyslipidemia (Bargiota and Diamanti-Kandarakis, 2012), inflammation (Escobar-Morreale et al., 2011; Kelly et al., 2001), oxidative stress, insulin resistance and hypertension (Goodarzi et al., 2011), all risk factors for metabolic syndrome, type 2

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diabetes mellitus (T2DM) and cardiovascular diseases (Krul-Poel et al., 2013). In addition, PCOS increases the risk of mental illnesses including, depression and anxiety (Cooney and Dokras, 2017). Due to the multitude of associated comorbidities and complications with PCOS, there is a costly multidisciplinary approach of care ranging from endocrinologists, gynecologists, psychiatrists to dermatologists.

Insulin resistance subsequent to abdominal obesity might play an important role in the pathogenesis of PCOS. This can result in ovarian and adrenal hyperandrogenism which leads to an increased accumulation of fat in the body and, ultimately, a vicious cycle of higher fat and higher androgen in the development of PCOS complications (Gambineri et al., 2002; Nasiri et al., 2015). This correlation might be mediated through the stimulation of oxidative stress pathway, triggered by fat accumulation (Vincent et al., 2007). Moreover, because of excess androgen and adipose tissue malfunction, chronic inflammation might be involved in the pathogenesis of metabolic and reproductive dysfunctions of PCOS (Spritzer et al., 2015). Impaired angiogenesis and endothelial dysfunction are also correlated with PCOS (Spritzer et al., 2015). Ovaries with PCOS upregulate vascular endothelial growth factor (VEGF) associated with increased vascularity (Pan et al., 2002; Tal et al., 2015). Intriguing recent evidence further suggests that PCOS might be associated with vitamin D. Genes involved in vitamin D level regulation are associated with glucose and lipid metabolism and blood pressure control (Wehr et al., 2011). Vitamin D may also affect inflammation and oxidative stress through its impact on preventing damaged DNA propagation in cellular level (Nair-Shalliker et al., 2012).

A combination of different nutrients and improving overall nutritional status might have beneficial effects for patients suffering from PCOS (Maktabi et al., 2018), for instance, showed that a combination of magnesium, zinc, calcium and vitamin D for 12 weeks influenced markers of inflammation, oxidative stress and impaired hormones in women with PCOS. Yet, Garg et al. (2015) showed that 4000 IU/day vitamin D for six months did not benefit insulin secretion and cardiovascular risk factors in women suffering from PCOS. We hypothesized that with co-administration of vitamin D and omega-3, and the resulting immunomodulatory and anti-inflammatory impacts, that the hormonal profile, metabolic abnormalities, mental illness, inflammation and endothelial dysfunction might be improved in women with PCOS. To our knowledge, this is the first randomized clinical trial to assess the effects of a combined vitamin D and omega-3 treatment on clinical, metabolic and genetic parameters in women with PCOS.

2. Materials and methods

2.1. Trial design and subjects

This randomized double-blinded, placebo-controlled trial registered in the Iranian website for registration of clinical trials (http://www.irct.ir: http://www.irct.ir/user/trial/6144/view) and followed the Declaration of Helsinki and Good Clinical Practice guidelines. This trial was conducted among 60 women with PCOS, diagnosed based on the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004), aged 18–40 years old whom referred to the Kosar Clinic in Arak, Iran, between February and October 2017. The study protocol was approved by the Ethics Committee of Arak University of Medical Sciences (AUMS). Written informed consent was obtained from all participants prior to the intervention. Exclusion criteria included pregnancy, adrenal hyperplasia, androgen-secreting tumors, hyperprolactinemia, thyroid dysfunction, diabetes or impaired glucose tolerance at enrollment.

2.2. Supplementation

Subjects were randomized to take either vitamin D (50,000 IU biweekly) plus 2 g/day omega-3 fatty acids from fish oil or placebo (n = 30 each group) for 12 weeks. Vitamin D, omega-3 fatty acids and the placebo were manufactured by Zahravi Pharmaceutical Company (Tabriz, Iran). The appearance of the placebo, vitamin D3 and omega 3 capsules, such as color, shape, size, and packaging, were totally similar. The compliance rate was assessed by measuring serum 25(OH) vitamin D levels, using the enzyme-linked immunosorbent assay (ELISA) method. To evaluate confounding effects of dietary intake and physical activity, all subjects completed 3-day food records (once during the weekend and on two weekdays) and three physical activity records as metabolic equivalents at weeks 0, 3, 6, 9 and 12 of the intervention. Daily macro- and micro-nutrient intakes were calculated by nutritionist IV software (First Databank, San Bruno, CA).

2.3. Anthropometric measures

A trained midwife took anthropometric measurements in the clinic at baseline and the end of the intervention. Height and weight (Seca, Hamburg, Germany) were measured light clothing with shoes removed. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

2.4. Clinical measures

Clinical assessments included hirsutism and its changes over time, using a mFG scoring system (Hatch et al., 1981).

Mental health was judged with beck depression inventory (BDI), general health questionnaire-28 (GHQ-28) and depression anxiety and stress scale (DASS) at baseline and week 12 of the intervention. BDI is a self-compiled questionnaire of 21 items in multiple choice format (Beck et al., 1961). The GHQ-28 comprises 28-item consisting of 4 subscales (Goldberg and Hillier, 1979). DASS questionnaire consists of three 14-item self-report scales that measure depression, anxiety and stress (Crawford and Henry, 2004).

2.5. Biochemical measures

Fasting blood samples (15 mL) were collected at baseline and the end of the intervention at Arak reference laboratory. Serum 25-hydroxyvitamin D levels were measured using an ELISA kit (IDS, Boldon, UK) with inter- and intra-assay coefficient of variations (CVs) of lower than 7%. Serum total testosterone with inter- and intra-assay CVs of 4.4 to 6.2%, sex hormone-binding globulin (SHBG) with inter- and intra-assay CVs of 3.7–5.6% and DHEAS concentrations with inter- and intra-assay CVs of 4.0–6.3% were measured using commercial validated kits (DiaMetra, Milano, Italy). Free androgen index (FAI) was calculated as the percentage of total testosterone/SHBG. Serum high sensitivity C-reactive protein (hs-CRP) concentrations were quantified using commercial ELISA kit (LDN, Nordhorn, Germany) with inter- and intra-assay CVs of lower than 7%. Plasma NO levels were measured using Griess method (Tatsch et al., 2011). Plasma total antioxidant capacity (TAC) concentrations were determined using the method of ferric reduction antioxidant power developed by Benzie and Strain (Benzie and Strain, 1996). Total glutathione (GSH) and malondialdehyde (MDA) concentrations also were measured using Beutler method (Beutler and Gelbart, 1985) and thiobarbituric acid reactive substances spectrophotometric test, respectively (Janero, 1990). CVs for plasma TAC, GSH and MDA were less than 5%.

2.6. Isolation of lymphocyte cells

Lymphocyte cells were extracted from blood samples using a 50% percoll (Sigma-Aldrich, Dorset, UK). Samples were taken for cell count and viability testing by trypan blue, RNA and DNA extraction (Dunkley et al., 2008).

Table 1
Specific primers used for real-time quantitative PCR.

Gene	Primer	Product size (bp)	Annealing temperature (C)
GAPDH	F: AAGCTCATTTCCTGGTATGACAACG	126	61.3
	R: TCTTCCTCTTGTGCTCTTGCTGG		
IL-1	F: GCTTCTCTCTGGTCCTTGG	174	56
	R: AGGGCAGGGTAGAGAAGAG		
IL-8	F: GCAGAGGGTTGTGGAGAAGT	150	56
	R: ACCCTACAACAGACCCACAC		
TNF-α	F: GTCAACCTCCTCTCTGCCAT	188	52
	R: CCAAAGTAGACCTGCCCAGA		
TGF-β	F: TTGAGACTTTTCCGTTGCCG	227	56
	R: CGAGGTCTGGGGAAAAGTCT		
VEGF	F: CTTCTGAGTTGCCCAGGAGA	216	54
	R: CTCACACACACACAACCAGG		

GAPDH, glyceraldehyde-3-Phosphate dehydrogenase; IL-1, interleukin-1; IL-8, interleukin-8; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor.

2.7. RNA extraction and real-time PCR (RT-PCR)

RNX-plus kit (Cinnacolon, Tehran, Iran) was used to extract RNA. RNA suspension was frozen in − 20 °C until cDNA preparation. Following the extraction of total RNAs from each sample, RNA quantification was performed using UV spectrophotometer. The OD 260/280 ratio of each sample was intended between 1.7 and 2.1 (Dunkley et al., 2008). The isolated RNA was reverse transcribed to cDNA library using moloney murine leukemia virus reverse transcriptase. Gene expression of interleukin-1 (IL-1), IL-8 and TNF-α, transforming growth factor beta (TGF-β) and vascular endothelial growth factor were determined by quantitative RT-PCR on peripheral blood mononuclear cells (PBMCs), using the LightCycler technology (Roche Diagnostics, Rotkreuz, Switzerland) with SYBR green detection and Amplicon Kit (**Table 1**). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as housekeeping gene. To design primers, we used Primer Express Software (Applied Biosystems, Foster City) and Beacon designer software (Takaposizt, Tehran, Iran). Relative transcription levels were calculated by the method of Pffafi or $2^{-\Delta\Delta CT}$.

2.8. Sample size calculation

To determine the sample size for this clinical trial, we considered type one error (α) of 0.05 and type two error (β) of 0.20 and the power of 80%. Based on a previous study (Garg et al., 2015), we used 0.11 ng/mL as SD and 0.09 ng/mL as the mean difference (d) of total testosterone concentration as the primary variable. Accordingly, 25 subjects were required in each group. Considering a dropout of 5 subjects per group, we calculated sample size as 30 subjects per treatment group.

2.9. Randomization

Randomization assignment was performed using computer-generated random numbers. Randomization and allocation concealment were conducted by the researchers and subjects and were carried out by a trained staff member at the gynecology clinic. Another person, who was not involved in the trial and not aware of random sequences, assigned the subjects to the numbered bottles of capsules.

2.10. Statistical methods

The Kolmogorov–Smirnov test was used to determine whether study variables were normally. Analyses were performed based on an intention-to-treat (ITT) principle. Independent-samples t tests were used to detect the differences in anthropometric measures, macro- and micronutrient intakes between two intervention groups. To determine the effects of vitamin D and omega-3 fatty acid co-supplementation on clinical and metabolic parameters, we used one-way repeated measures analysis of variance. To compare the effects of vitamin D and omega-3 fatty acid co-supplementation on gene expression related to inflammation, independent-samples t tests were used. Using general linear models and analysis of covariance (ANCOVA), we adjusted for the baseline values of age and BMI. The P-values of < 0.05 were considered statistically significant. All statistical analyses were conducted using the Statistical Package for Social Science version 18 (SPSS Inc., Chicago, Illinois, USA).

Table 2
General characteristics of study participants.

	Placebo group ($n = 30$)	Vitamin D plus omega-3 group ($n = 30$)	P^1
Age (y)	25.1 ± 3.7	26.8 ± 4.4	0.12
Height (cm)	163.9 ± 5.7	163.5 ± 4.4	0.80
Weight at study baseline (kg)	72.4 ± 17.1	73.1 ± 10.0	0.85
Weight at end-of-trial (kg)	72.2 ± 17.2	72.5 ± 10.0	0.93
Weight change (kg)	−0.2 ± 1.1	−0.6 ± 1.1	0.21
BMI at study baseline (kg/m^2)	27.1 ± 7.0	27.4 ± 3.9	0.85
BMI at end-of-trial (kg/m^2)	27.0 ± 7.1	27.1 ± 3.8	0.92
BMI change (kg/m^2)	−0.1 ± 0.4	−0.2 ± 0.4	0.22

Data are means ± SDs.
[1] Obtained from independent *t*-test.

3. Results

Initially, 69 subjects were screened; of whom 9 subjects were excluded due to lack of our inclusion criteria. In the current study, 4 subjects in the supplemented group and 4 in the placebo group were dropped out for personal reasons (**Fig. 1**). Finally, 52 participants [supplements ($n = 26$) and placebo ($n = 26$)] completed the trial. However, all 60 participants were included in the final analysis based on the ITT principle.

Anthropometric measures including mean age, height, weight and BMI were not statistically different between the two intervention groups at both baseline and end-of-trial (**Table 2**).

Based on the 3-day dietary records collected at baseline, end-of-trial and throughout the study, there was no significant difference in mean dietary macro- and micro-nutrient intakes between the two intervention groups (Data not shown).

Vitamin D and omega − 3 fatty acid co-supplementation significantly decreased serum total testosterone levels (− 0.2 ± 0.5 vs. + 0.1 ± 0.4 ng/mL, $P = 0.02$) compared with the placebo (**Table 3**). In addition, vitamin D and omega − 3 fatty acid co-supplementation resulted in a significant improvement in BDI (− 1.4 ± 1.6 vs. − 0.5 ± 0.6, $P = 0.01$), GHQ (− 4.5 ± 4.3 vs. − 1.9 ± 2.3, $P = 0.005$) and DASS scores (− 5.0 ± 5.1 vs. − 2.3 ± 3.5, $P = 0.01$) compared with the placebo. Additionally, vitamin D and omega-3 fatty acid co-administration significantly decreased serum hs-CRP levels (− 1.2 ± 1.9 vs. + 0.1 ± 0.7 mg/L, $P = 0.001$) and MDA levels (− 0.4 ± 0.4 vs. + 0.2 ± 0.6 μmol/L, $P < 0.001$), and significantly increased plasma TAC (+ 114.6 ± 122.2 vs. − 2.4 ± 168.2 mmol/L, $P = 0.003$) compared with the placebo. We did not observe any significant effect of combined vitamin D and omega-3 fatty acid on other hormonal profiles, plasma NO and GSH.

Analyses were controlled for baseline characteristics including age, BMI and baseline values of each biochemical parameters were significantly different between treatment groups and findings remained unchanged (**Table 4**).

RT-PCR results demonstrated that vitamin D and omega-3 fatty acid co-supplementation significantly downregulated gene expression of IL-1 ($P = 0.03$), and upregulated VEGF ($P = 0.004$) in PBMCs of women with PCOS, when compared to placebo (**Figs. 2 and 3**). There was no

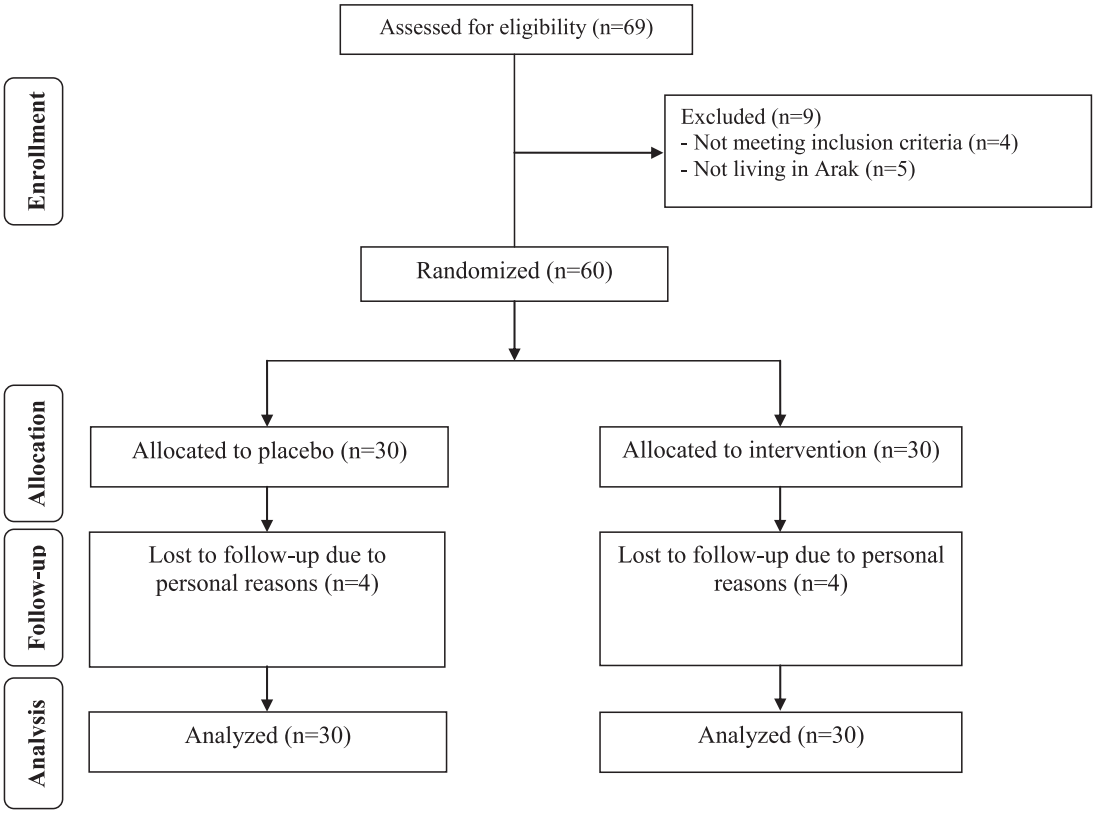

Fig. 1. Summary of patient flow diagram.

significant effect of vitamin D and omega-3 fatty acid co-supplementation on gene expression of IL-8, TNF-α and TGF-β.

4. Discussion

Current study demonstrates that 12 weeks of combined vitamin D and omega-3 fatty acid significantly alleviates depression and anxiety symptoms women with PCOS suffer from, and improves mental health status. It also decreases androgen levels helping to attenuate related symptoms. Moreover, co-administration of vitamin D and omega-3 fatty acid improves total antioxidant capacity and reduces inflammation in the body, the mainstream of PCOS comorbidities and complication. Interestingly, this combination upregulates gene expression of VEGF moderately which helps better tissue oxygenation and boosting immune system. So, the addition of omega-3 fatty acid to vitamin D might promote its anti-inflammatory and immunomodulatory effects, which are beneficial for a complex of metabolic abnormalities in PCOS.

4.1. Effect on hormonal measures

The major pathophysiology of PCOS is the excessive levels of androgens in the body. More than 75% of women with PCOS have high

Table 3
Hormonal status and inflammatory and oxidative stress markers at baseline and after the 12-week intervention in subjects with polycystic ovary syndrome.

	Placebo group (n = 30)			Vitamin D plus omega-3 group (n = 30)			P^1
	Baseline	End-of-trial	Change	Baseline	End-of-trial	Change	
25-OH-vitamin D (ng/mL)	12.4 ± 2.7	12.7 ± 3.2	0.3 ± 1.1	12.1 ± 3.1	21.9 ± 6.3	9.8 ± 5.1	<0.001
Total testosterone (ng/mL)	1.2 ± 0.7	1.3 ± 0.7	0.1 ± 0.4	1.4 ± 0.7	1.2 ± 0.6	−0.2 ± 0.5	0.02
SHBG (nmol/L)	43.5 ± 16.2	47.8 ± 26.8	4.4 ± 21.4	46.7 ± 22.9	52.7 ± 39.3	6.0 ± 24.5	0.78
FAI%	0.12 ± 0.12	0.11 ± 0.07	−0.01 ± 0.07	0.13 ± 0.13	0.11 ± 0.07	−0.02 ± 0.10	0.76
mF-G scores	11.3 ± 4.3	11.1 ± 4.1	−0.2 ± 0.6	11.8 ± 3.4	11.4 ± 3.2	−0.4 ± 0.5	0.15
BDI total scores	11.4 ± 4.3	10.8 ± 4.2	−0.5 ± 0.6	10.8 ± 5.8	9.4 ± 5.6	−1.4 ± 1.6	0.01
GHQ scores	51.1 ± 8.4	49.5 ± 8.2	−1.9 ± 2.3	47.2 ± 11.4	42.7 ± 10.9	−4.5 ± 4.3	0.005
DASS scores	103.4 ± 19.2	101.2 ± 18.8	−2.3 ± 3.5	101.3 ± 20.1	96.2 ± 19.2	−5.0 ± 5.1	0.01
hs-CRP (mg/L)	3.9 ± 1.5	4.0 ± 1.5	0.1 ± 0.7	4.2 ± 2.2	3.0 ± 1.7	−1.2 ± 1.9	0.001
NO (μmol/L)	44.6 ± 6.1	43.1 ± 8.4	−1.5 ± 6.3	39.2 ± 2.1	39.5 ± 3.1	0.3 ± 3.9	0.17
TAC (mmol/L)	946.7 ± 104.9	944.3 ± 174.7	−2.4 ± 168.2	996.0 ± 111.3	1110.6 ± 144.2	114.6 ± 122.2	0.003
GSH (μmol/L)	526.4 ± 74.7	550.6 ± 124.1	24.3 ± 116.4	559.1 ± 89.9	543.6 ± 88.2	−15.5 ± 85.8	0.13
MDA (μmol/L)	2.8 ± 0.5	3.0 ± 0.7	0.2 ± 0.6	2.9 ± 0.5	2.5 ± 0.3	−0.4 ± 0.4	<0.001

All values are means ± SDs.
BDI, beck depression inventory; DASS, depression anxiety and stress scale; GHQ, general health questionnaire; GSH, total glutathione; FAI, free androgen index; hs-CRP, high-sensitivity C-reactive protein; mF-G, modified Ferriman Gallwey; MDA, malondialdehyde; NO, nitric oxide; SHBG, sex hormone-binding globulin; TAC, total antioxidant capacity.
[1] P values represent the time × group interaction (computed by analysis of the one-way repeated measures ANOVA).

Table 4
Adjusted changes in metabolic profile of patients with polycystic ovary syndrome.

	Placebo group (n = 30)	Vitamin D plus omega-3 group (n = 30)	P^1
25-OH-vitamin D (ng/mL)	0.2 ± 0.7	9.9 ± 0.7	<0.001
Total testosterone (ng/mL)	0.1 ± 0.1	−0.1 ± 0.1	0.02
SHBG (nmol/L)	4.5 ± 4.2	5.8 ± 4.2	0.83
FAI%	−0.02 ± 0.01	−0.03 ± 0.01	0.67
mF-G scores	−0.2 ± 0.1	−0.4 ± 0.1	0.14
BDI total scores	−0.5 ± 0.2	−1.4 ± 0.2	0.006
GHQ scores	−1.7 ± 0.6	−4.8 ± 0.6	0.001
DASS scores	−2.1 ± 0.8	−5.2 ± 0.8	0.01
hs-CRP (mg/L)	0.2 ± 0.1	−0.5 ± 0.1	<0.001
NO (μmol/L)	−1.1 ± 1.1	−0.1 ± 1.1	0.50
TAC (mmol/L)	−11.4 ± 27.0	123.5 ± 27.0	0.001
GSH (μmol/L)	18.2 ± 17.9	−9.5 ± 17.9	0.28
MDA (μmol/L)	0.2 ± 0.1	−0.4 ± 0.1	<0.001

All values are means ± SEs. Values are adjusted for baseline values, age and BMI at baseline.
BDI, beck depression inventory; DASS, depression anxiety and stress scale; GHQ, general health questionnaire; GSH, total glutathione; FAI, free androgen index; hs-CRP, high-sensitivity C-reactive protein; mF-G, modified Ferriman Gallwey; MDA, malondialdehyde; NO, nitric oxide; SHBG, sex hormone-binding globulin; TAC, total antioxidant capacity.
[1] Obtained from ANCOVA.

androgens level free testosterone (Hahn et al., 2006). Current evidence is suggesting that low serum vitamin D levels are associated with high androgen levels. An inverse relationship has been reported between serum 25(OH)D levels and testosterone, DHEAS and SHBG among women with PCOS (Hahn et al., 2006; Velija-Asimi, 2014). We found that vitamin D and omega-3 fatty acid co-supplementation for 12 weeks significantly decreased total testosterone levels among women with PCOS, but did not affect other hormonal measures. Consistent with our findings, the meta-analysis conducted by Azadi-Yazdi et al. (2017) showed significant reduction in serum total testosterone, and not free testosterone level, following vitamin D supplementation in PCOS patients. Vitamin D might play an important role in glucose metabolism by inducing insulin synthesis and release, and increasing insulin receptor expression, so vitamin D deficiency possibly contributes to the development of insulin resistance (Teegarden and Donkin, 2009). The effect of vitamin D deficiency on hormonal measures in PCOS might be related to insulin resistance, which increases hyperandrogenism through insulin increasing ovarian production of androgens and reducing SHBG production. Moreover, a few vitamin D receptors gene polymorphisms might be associated with lower insulin resistance and lower testosterone levels (Plymate et al., 1988; Wehr et al., 2011). Adding omega-3 to vitamin D supplementation might promote its effect on insulin sensitivity and help improving metabolic profile of PCOS patients (Karakas et al., 2016; Newgard, 2012).

4.2. Effect on clinical symptoms

Women with PCOS experience mood dysfunction and psychiatric problems (Farrell and Antoni, 2010) which are commonly depression and anxiety and mostly related to the discomfort associated with disease presentation including hirsutism, acne, obesity and subfertility (Barry et al., 2011). We found that the co-administration of vitamin D and omega-3 fatty acid to women with PCOS for 12 weeks improved mental and general health presented by significant reduction in BDI, GHQ and DASS scores compared with the placebo. Our results were consistent with others showing that high dose vitamin D supplementation ameliorates depressive symptoms in participants suffering from depression (Jorde et al., 2008; Vieth et al., 2007). There are

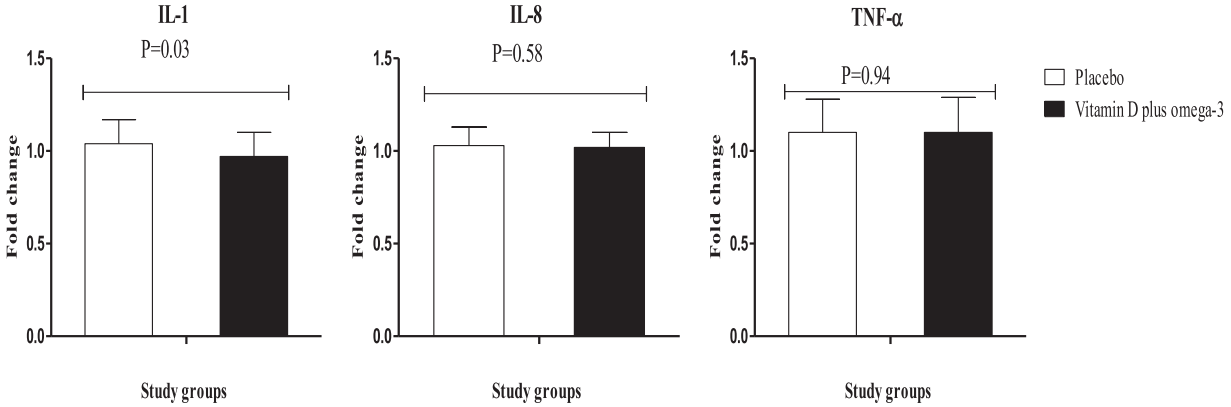

Fig. 2. Effect of the 12-week supplementation with Vitamin D plus omega-3 or placebo on expression ratio of IL-1, IL-8 and TNF-α gene in blood mononuclear cells of PCOS women.

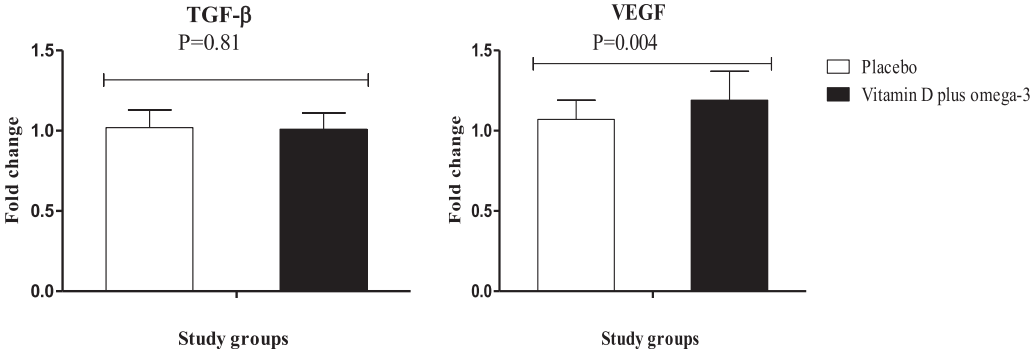

Fig. 3. Effect of the 12-week supplementation with Vitamin D plus omega-3 or placebo on expression ratio of TGF-β and VEGF gene in blood mononuclear cells of PCOS women. IL-1, interleukin-1; IL-8, interleukin-8; PCOS, polycystic ovary syndrome; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor.

several mechanisms explaining the impact of vitamin D on brain function including its direct effect on brain and serotonin uptake through vitamin D receptors distributed all over the brain (Stumpf et al., 1982), vitamin D indirect impact through its function of improving muscle strength which increases physical activity and general health (Bischoff-Ferrari et al., 2006) and its effect on decreasing PTH level which is inversely associated with brain function (Smogorzewski, 2001). In addition, unsaturated fatty acids such as omega-3 are highly distributed in neural phospholipids, the major component of the neuronal cell membrane. Omega-3 fatty acid might modulate dopaminergic and serotonergic pathways which are essential for proper brain function (Bozzatello et al., 2016). The combination of vitamin D and omega-3 fatty acid might enhance serotonin synthesis, release, and function in the brain.

4.3. Effect on inflammation and oxidative stress

Current evidence suggest that chronic inflammation resulted from abdominal obesity and adipose tissue dysfunction in PCOS patients is involved in the development of complex features of PCOS including dyslipidemia, insulin resistance, T2DM, cardiovascular risk factors, hyperandrogenism and anovulation (Nasiri et al., 2015; Spritzer et al., 2015). Inflammation in these patients might contribute to atherogenesis in PCOS (Gonzalez et al., 2012). Oxidative stress, presented as increased level of MDA and suppressed TAC, is considered as a possible pathophysiology for PCOS. Our findings documented that vitamin D and omega-3 fatty acid co-supplementation for 12 weeks significantly decreased serum hs-CRP and plasma MDA levels and gene expression of IL-1 as well as increased plasma TAC level among patients with PCOS. We also found out that the gene expression of VEGF was upregulated in women with PCOS, following the co-administration of vitamin D and omega-3 fatty acid for 12 weeks, which was not consistent with Irani et al. (2015) and Gruber et al. (2008), demonstrating decreased levels of VEGF following vitamin D supplementation. Our study did not show any effect of combined vitamin D and omega-3 on plasma NO, GSH, and gene expression related to IL-8, TNF-α and TGF-β. VEGF is a regulator of angiogenesis though its alteration might be involved in different pathologies such as preeclampsia, renal disease and PCOS (Tal et al., 2015). There is controversy regarding the effect of vitamin D supplementation on VEGF gene expression. Women with PCOS usually produce more VEGF associated with increased vascularity which might increase the risk of ovarian hyperstimulation syndrome following follicular stimulation, though improves tissue oxygenation boosts immune system in its moderate levels (Zaidi et al., 1995). The discrepancy between what we found in the current study and other clinical trials showing the negative association between vitamin D status and VEGF levels might be related to vitamin D supplementation dose and/or duration of intervention and other medical conditions might influence their relationship. Chronic diseases are usually better managed through multi-nutrients administration and improving overall nutritional status. Combining vitamin D and omega-3 fatty acid in women with PCOS produced better results because vitamin D intake improves oxidative stress through its antioxidant activities (Cetinkalp et al., 2009) and reducing the production of reactive oxygen species and pro-inflammatory cytokines (Jain and Micinski, 2013), and omega-3 fatty acid with its anti-inflammatory properties ameliorates oxidative stress (Hassan Eftekhari et al., 2013).

4.4. Limitations

The main limitation of this trial was that the circulating levels of free fatty acids were not been measured. We did not evaluate the effects of vitamin D and omega-3 fatty acids co-supplementation on gene expression related to oxidative stress and metabolic profiles. In addition, a small sample size, short duration of intervention, small doses of supplement and serum vitamin status at the beginning of the intervention may affect our findings. Therefore, these should be taken into account in the interpretation of our findings.

4.5. Conclusions

Overall, the co-administration of vitamin D and omega-3 fatty acid for 12 weeks had beneficial effects on mental health status, total testosterone level, serum hs-CRP, plasma TAC and MDA levels, and gene expression of IL-1 and VEGF among women suffering from PCOS. Our findings support the anti-inflammatory and immunomodulatory roles of combined vitamin D and omega-3 supplementation.

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Authors' contributions

ZA contributed in conception, design, statistical analysis and drafting of the manuscript. MJ, MS, NM, FA-E, EA, RT, SJ and SH-D contributed in data collection and manuscript drafting. All authors approved the final version for submission. ZA supervised the study.

Conflicts of interest

None.

Clinical trial registration number

http://www.irct.ir/user/trial/6144/view.

References

Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil. Steril. 81, 19–25.

Azadi-Yazdi, M., Nadjarzadeh, A., Khosravi-Boroujeni, H., Salehi-Abargouei, A., 2017. The effect of vitamin D supplementation on the androgenic profile in patients with polycystic ovary syndrome: a systematic review and meta-analysis of clinical trials. Horm. Metab. Res. 49, 174–179.

Bargiota, A., Diamanti-Kandarakis, E., 2012. The effects of old, new and emerging medicines on metabolic aberrations in PCOS. Ther. Adv. Endocrinol. Metab. 3, 27–47.

Barry, J.A., Kuczmierczyk, A.R., Hardiman, P.J., 2011. Anxiety and depression in polycystic ovary syndrome: a systematic review and meta-analysis. Hum. Reprod. 26, 2442–2451.

Beck, A.T., Ward, C.H., Mendelson, M., Mock, J., Erbaugh, J., 1961. An inventory for measuring depression. Arch. Gen. Psychiatry 4, 561–571.

Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70–76.

Beutler, E., Gelbart, T., 1985. Plasma glutathione in health and in patients with malignant disease. J. Lab. Clin. Med. 105, 581–584.

Bischoff-Ferrari, H.A., Orav, E.J., Dawson-Hughes, B., 2006. Effect of cholecalciferol plus calcium on falling in ambulatory older men and women: a 3-year randomized controlled trial. Arch. Intern. Med. 166, 424–430.

Bozdag, G., Mumusoglu, S., Zengin, D., Karabulut, E., Yildiz, B.O., 2016. The prevalence and phenotypic features of polycystic ovary syndrome: a systematic review and meta-analysis. Hum. Reprod. 31, 2841–2855.

Bozzatello, P., Brignolo, E., De Grandi, E., Bellino, S., 2016. Supplementation with omega-3 fatty acids in psychiatric disorders: a review of literature data. J. Clin. Med. 5. http://dx.doi.org/10.3390/jcm5080067.

Cetinkalp, S., Delen, Y., Karadeniz, M., Yuce, G., Yilmaz, C., 2009. The effect of 1alpha,25(OH)2D3 vitamin over oxidative stress and biochemical parameters in rats where Type 1 diabetes is formed by streptozotocin. J. Diab. Compl. 23, 401–408.

Cooney, L.G., Dokras, A., 2017. Depression and anxiety in polycystic ovary syndrome: etiology and treatment. Curr. Psychiatry Rep. 19, 83.

Crawford, J.R., Henry, J.D., 2004. The positive and negative affect schedule (PANAS):

construct validity, measurement properties and normative data in a large non-clinical sample. Br. J. Clin. Psychol. 43, 245–265.
Dunkley, P.R., Jarvie, P.E., Robinson, P.J., 2008. A rapid Percoll gradient procedure for preparation of synaptosomes. Nat. Protoc. 3, 1718–1728.
Escobar-Morreale, H.F., Luque-Ramirez, M., Gonzalez, F., 2011. Circulating inflammatory markers in polycystic ovary syndrome: a systematic review and metaanalysis. Fertil. Steril. 95 (1048–1058), e1041–e1042.
Farrell, K., Antoni, M.H., 2010. Insulin resistance, obesity, inflammation, and depression in polycystic ovary syndrome: biobehavioral mechanisms and interventions. Fertil. Steril. 94, 1565–1574.
Gambineri, A., Pelusi, C., Vicennati, V., Pagotto, U., Pasquali, R., 2002. Obesity and the polycystic ovary syndrome. Int. J. Obes. Relat. Metab. Disord. 26, 883–896.
Garg, G., Kachhawa, G., Ramot, R., Khadgawat, R., Tandon, N., Sreenivas, V., Kriplani, A., Gupta, N., 2015. Effect of vitamin D supplementation on insulin kinetics and cardiovascular risk factors in polycystic ovarian syndrome: a pilot study. Endocr. Connect. 4, 108–116.
Goldberg, D.P., Hillier, V.F., 1979. A scaled version of the General Health Questionnaire. Psychol. Med. 9, 139–145.
Gonzalez, F., Nair, K.S., Daniels, J.K., Basal, E., Schimke, J.M., Blair, H.E., 2012. Hyperandrogenism sensitizes leukocytes to hyperglycemia to promote oxidative stress in lean reproductive-age women. J. Clin. Endocrinol. Metab. 97, 2836–2843.
Goodarzi, M.O., Dumesic, D.A., Chazenbalk, G., Azziz, R., 2011. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. Nat. Rev. Endocrinol. 7, 219–231.
Gruber, H.E., Hoelscher, G., Ingram, J.A., Chow, Y., Loeffler, B., Hanley Jr., E.N., 2008. 1,25(OH)2-vitamin D3 inhibits proliferation and decreases production of monocyte chemoattractant protein-1, thrombopoietin, VEGF, and angiogenin by human annulus cells in vitro. Spine (Phila Pa 1976) 33, 755–765.
Hahn, S., Haselhorst, U., Tan, S., Quadbeck, B., Schmidt, M., Roesler, S., Kimmig, R., Mann, K., Janssen, O.E., 2006. Low serum 25-hydroxyvitamin D concentrations are associated with insulin resistance and obesity in women with polycystic ovary syndrome. Exp. Clin. Endocrinol. Diab. 114, 577–583.
Hassan Eftekhari, M., Aliasghari, F., Babaei-Beigi, M.A., Hasanzadeh, J., 2013. Effect of conjugated linoleic acid and omega-3 fatty acid supplementation on inflammatory and oxidative stress markers in atherosclerotic patients. ARYA Atheroscler. 9, 311–318.
Hatch, R., Rosenfield, R.L., Kim, M.H., Tredway, D., 1981. Hirsutism: implications, etiology, and management. Am. J. Obstet. Gynecol. 140, 815–830.
Irani, M., Seifer, D.B., Grazi, R.V., Julka, N., Bhatt, D., Kalgi, B., Irani, S., Tal, O., Lambert-Messerlian, G., Tal, R., 2015. Vitamin D supplementation decreases tgf-beta1 bioavailability in PCOS: a randomized placebo-controlled trial. J. Clin. Endocrinol. Metab. 100, 4307–4314.
Jain, S.K., Micinski, D., 2013. Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes. Biochem. Biophys. Res. Commun. 437, 7–11.
Janero, D.R., 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic. Biol. Med. 9, 515–540.
Jorde, R., Sneve, M., Figenschau, Y., Svartberg, J., Waterloo, K., 2008. Effects of vitamin D supplementation on symptoms of depression in overweight and obese subjects: randomized double blind trial. J. Intern. Med. 264, 599–609.
Karakas, S.E., Perroud, B., Kind, T., Palazoglu, M., Fiehn, O., 2016. Changes in plasma metabolites and glucose homeostasis during omega-3 polyunsaturated fatty acid supplementation in women with polycystic ovary syndrome. BBA Clin. 5, 179–185.
Kelly, C.C., Lyall, H., Petrie, J.R., Gould, G.W., Connell, J.M., Sattar, N., 2001. Low grade chronic inflammation in women with polycystic ovarian syndrome. J. Clin. Endocrinol. Metab. 86, 2453–2455.
Krul-Poel, Y.H., Snackey, C., Louwers, Y., Lips, P., Lambalk, C.B., Laven, J.S., Simsek, S., 2013. The role of vitamin D in metabolic disturbances in polycystic ovary syndrome: a systematic review. Eur. J. Endocrinol. 169, 853–865.
Maktabi, M., Jamilian, M., Asemi, Z., 2018. Magnesium-zinc-calcium-vitamin D co-supplementation improves hormonal profiles, biomarkers of inflammation and oxidative stress in women with polycystic ovary syndrome: a randomized, double-blind, placebo-controlled trial. Biol. Trace Elem. Res. 182, 21–28.
Nair-Shalliker, V., Armstrong, B.K., Fenech, M., 2012. Does vitamin D protect against DNA damage? Mutat. Res. 733, 50–57.
Nasiri, N., Moini, A., Eftekhari-Yazdi, P., Karimian, L., Salman-Yazdi, R., Zolfaghari, Z., Arabipoor, A., 2015. Abdominal obesity can induce both systemic and follicular fluid oxidative stress independent from polycystic ovary syndrome. Eur J Obstet. Gynecol. Reprod. Biol. 184, 112–116.
Newgard, C.B., 2012. Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell Metab. 15, 606–614.
Pan, H.A., Wu, M.H., Cheng, Y.C., Li, C.H., Chang, F.M., 2002. Quantification of Doppler signal in polycystic ovary syndrome using three-dimensional power Doppler ultrasonography: a possible new marker for diagnosis. Hum. Reprod. 17, 201–206.
Plymate, S.R., Matej, L.A., Jones, R.E., Friedl, K.E., 1988. Inhibition of sex hormone-binding globulin production in the human hepatoma (Hep G2) cell line by insulin and prolactin. J. Clin. Endocrinol. Metab. 67, 460–464.
Setji, T.L., Brown, A.J., 2014. Polycystic ovary syndrome: update on diagnosis and treatment. Am. J. Med. 127, 912–919.
Smogorzewski, M.J., 2001. Central nervous dysfunction in uremia. Am. J. Kidney Dis. 38, S122–S128.
Spritzer, P.M., Lecke, S.B., Satler, F., Morsch, D.M., 2015. Adipose tissue dysfunction, adipokines, and low-grade chronic inflammation in polycystic ovary syndrome. Reproduction 149, R219–R227.
Stumpf, W.E., Sar, M., Clark, S.A., DeLuca, H.F., 1982. Brain target sites for 1,25-dihydroxyvitamin D3. Science 215, 1403–1405.
Tal, R., Seifer, D.B., Arici, A., 2015. The emerging role of angiogenic factor dysregulation in the pathogenesis of polycystic ovarian syndrome. Semin. Reprod. Med. 33, 195–207.
Tatsch, E., Bochi, G.V., Pereira Rda, S., Kober, H., Agertt, V.A., de Campos, M.M., Gomes, P., Duarte, M.M., Moresco, R.N., 2011. A simple and inexpensive automated technique for measurement of serum nitrite/nitrate. Clin. Biochem. 44, 348–350.
Teegarden, D., Donkin, S.S., 2009. Vitamin D: emerging new roles in insulin sensitivity. Nutr. Res. Rev. 22, 82–92.
Velija-Asimi, Z., 2014. Evaluation of the association of vitamin D deficiency with gonadotropins and sex hormone in obese and non-obese women with polycystic ovary syndrome. Med. Glas. (Zenica) 11, 170–176.
Vieth, R., Bischoff-Ferrari, H., Boucher, B.J., Dawson-Hughes, B., Garland, C.F., Heaney, R.P., Holick, M.F., Hollis, B.W., Lamberg-Allardt, C., McGrath, J.J., Norman, A.W., Scragg, R., Whiting, S.J., Willett, W.C., Zittermann, A., 2007. The urgent need to recommend an intake of vitamin D that is effective. Am. J. Clin. Nutr. 85, 649–650.
Vincent, H.K., Innes, K.E., Vincent, K.R., 2007. Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity. Diab. Obes. Metab. 9, 813–839.
Wehr, E., Trummer, O., Giuliani, A., Gruber, H.J., Pieber, T.R., Obermayer-Pietsch, B., 2011. Vitamin D-associated polymorphisms are related to insulin resistance and vitamin D deficiency in polycystic ovary syndrome. Eur. J. Endocrinol. 164, 741–749.
Zaidi, J., Campbell, S., Pittrof, R., Kyei-Mensah, A., Shaker, A., Jacobs, H.S., Tan, S.L., 1995. Ovarian stromal blood flow in women with polycystic ovaries – a possible new marker for diagnosis? Hum. Reprod. 10, 1992–1996.