The Effects of Calcium, Vitamins D and K co-Supplementation on Markers of Insulin Metabolism and Lipid Profiles in Vitamin D-Deficient Women with Polycystic Ovary Syndrome

Introduction
Polycystic ovary syndrome (PCOS) is the commonest hyperandrogenic and dysmetabolic disorder which affects 6–10% of women of child-bearing age [1, 2]. Subjects with PCOS have significantly higher rates of insulin resistance, impaired glucose tolerance, dyslipidemia, and metabolic syndrome than those subjects without the disease [3]. Insulin resistance and elevated lipid profiles occur in 50–75% and 70% of women with PCOS, respectively [4, 5]. Insulin resistance and dyslipidemia are central components of metabolic syndrome, and a significant risk factor for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) [6].

Recently, the role of vitamins such as vitamin D and minerals including calcium, selenium and zinc in the developing of many diseases including PCOS has been evaluated [7, 8]. We have previously shown that supplementation with 1 000 mg/day calcium plus 50 000 IU/week vitamin D for 8 weeks among vitamin D-deficient subjects with PCOS had beneficial effects on markers of insulin metabolism, serum triglycerides and VLDL-cholesterol levels, but did
not influence fasting plasma glucose and other lipid profiles [9]. In addition, vitamins D, K and calcium co-supplementation for 12 weeks among diabetic patients with coronary heart disease (CHD) improved markers of insulin metabolism and HDL-cholesterol levels, but unchanged other lipid profiles [10]. However, vitamin D and calcium co-supplementation for 6 months did not affect insulin sensitivity, insulin secretion and β-cell function among multi-ethnic adults with low vitamin D status at risk of T2DM [11]. Moreover, vitamin K administration for 36 months at a dosage of 500 µg/day decreased insulin resistance among older men [12].

Vitamins D, K and calcium supplementation may improve metabolic status through their effects on up-regulation of the insulin receptor genes [13], the regulation of insulin secretion from the pancreatic beta-cell [14], and the enhancement of β-cell proliferation and adiponectin expression [15]. However, beneficial effects of combined vitamin D and calcium on markers of insulin metabolism and lipid profiles in PCOS subjects [9] and patients with T2DM [16] have previously reported, data on the effect of calcium, vitamins D and K co-supplementation on markers of insulin metabolism and lipid profiles among vitamin D-deficient subjects with PCOS are scarce. This study aimed to determine the effects of calcium, vitamins D and K co-supplementation on markers of insulin metabolism and lipid profiles in vitamin D-deficient subjects with PCOS.

Subjects and Methods

Trial design
This was an 8-week randomized, double-blind, placebo-controlled clinical trial.

Participants
Participants of this study were 55 PCOS women which were selected from the endocrinology and gynecology services of Iran University of Medical sciences (IUMS) from July 2016 to August 2016 at summer season. Diagnosis of PCOS was performed according to the Rotterdam criteria [17]: those with the 2 of the following criteria were considered as having PCOS: oligo- and/or anovulation (defined as delayed menses > 35 days or < 8 spontaneous hemorrhagic episodes/year), clinical (hirsutism using modified Ferriman-Gallwey score of > 8) and/or biochemical signs of hyperandrogenism and polycystic ovaries (12 or more follicles in each ovary measuring 2–9 mm in diameter, and/or increased ovarian volume > 10 ml³).

Ethics statements
This research was done in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, and written informed consent was obtained from all subjects prior to the intervention. The current study was approved by the ethics committee of IUMS and was registered in the Iranian website for registration of clinical trials (http://www.irct.ir: IRCT201608115623N87).

Inclusion and exclusion criteria
Vitamin D-deficient (< 20 ng/mL) subjects with PCOS aged 18–40 years with phenotypes A (oligo-anovulation + hyperandrogenism + polycystic ovary morphology) and D (oligo-anovulation + polycystic ovary morphology) were included in the study. Exclusion criteria were pregnancy, adrenal gland disorders and/or other endocrine diseases and hormonal treatments in the previous 6 months.

Study design
At first, persons were randomly divided into 2 groups by random permuted blocks to intake either 500 mg calcium, 200 IU vitamin D plus 90 µg vitamin K supplements (n = 28) or placebo (n = 27) twice a day for 8 weeks. The randomized allocation sequence, enrolling subjects and assigning patients to the groups were conducted as blindness by a trained midwife. Calcium, vitamins D and K capsules and its placebos (cellulose) were provided by Arian Salamt Sina (Tehran, Iran) and Barij Essence Pharmaceutical Company (Kashan, Iran), respectively. At baseline, subjects were asked not to alter their routine physical activity or usual dietary intakes throughout the study and not to take any antioxidants supplements, anti-inflammatory medications and other medications that might affect their reproductive physiology during the 8-wk intervention [18]. All persons were recorded 3-day dietary records and 3 physical activity records at baseline, weeks 2, 4, 6 and 8 of intervention. To compute daily macro- and micro-nutrient intakes of persons according to 3-day food diaries, we used Nutritionist IV software (First Databank, San Bruno, CA) adjusted for Iranian foods.

Treatment adherence
Every 2 weeks, subjects were taken enough supplements and placebos and were instructed to return all unused supplements and placebos at each visit. Compliance to the take of calcium, vitamins D and K supplements and placebos was checked through asking participants to bring the medication containers and receiving short messages on their cell phones each day.

Assessment of anthropometric measures
All patients were evaluated at the study start on the third day of a spontaneous or progesterone-induced menstrual cycle [18]. Anthropometric measures including height, weight (Seca, Hamburg, Germany) and body mass index (BMI) were quantified in the onset and the end of the study.

Assessment of outcomes
The primary outcome measurements were HOMA-IR in the current study. The secondary outcome measurements were lipid profiles.

Biochemical assessment
12-h fasting blood samples were taken by venipuncture at weeks 0 and 8 at the IUMS reference laboratory. Blood samples were taken based on a standard protocol and immediately centrifuged (Hettich D-78532, Tutlingen, Germany). Then, the samples were stored at ~ 80°C until analysis at the IUMS reference laboratory. To evaluate fasting plasma glucose (FPG), serum triglycerides, VLDL-, total-, LDL- and HDL-cholesterol concentrations, we used available kits (Pars Azmun, Tehran, Iran) with enzymatic method. All inter- and intra-assay CVs for FPG and lipid fractions were lower than 5 %. Circulating concentrations of serum insulin were assessed using ELISA kit (Monobind, California, USA) with the intra- and inter-assay CVs 3.0 and 4.8 %, respectively. HOMA-IR, homeostatic model assessment for B-cell function (HOMA-B) and the quantitative insulin sensitivity check index (QUICKI) were calculated according to suggested formulas [19].
Sample size
To determine the sample size, we used a randomized clinical trial sample size formula where type one (α) and type 2 errors (β) were 0.05 and 0.20 (power = 80%), respectively. Based on a previous study [10], we used 1.2 as SD and 1.0 as the difference in mean (d) of HOMA-IR as primary variable. Based on this, we needed 25 subjects in per group. Assuming a dropout of 5 persons in each group, we calculated to have 30 persons per group.

Statistical methods
To assess normal distribution of variables, we conducted the Kolmogrov-Smirnov test. To detect differences in the general characteristics, and daily macro- and micro-nutrient intakes between the 2 groups, we used independent samples Student’s t test. To demonstrate the effects of calcium, vitamins D and K co-supplementation on glucose metabolism and lipid fractions, one-way repeated-measures ANOVA was used. To control the effect of confounders including baseline values of biochemical parameters, age and BMI at baseline, we applied ANCOVA. A P-value <0.05 was considered statistically significant. All statistical analyses were conducted using the Statistical Package for Social Science version 18 (SPSS Inc., Chicago, Illinois, USA).

Results
At the screening visit, 850 participants were screened for PCOS. 760 participants of the 850 screened participants were excluded from the first visit due to not having PCOS. Thus, at baseline, we invited 90 women with PCOS according to Rotterdam criteria; however, 30 participants were excluded from the study because of not living in Tehran (n = 12) and not having inclusion criteria (n = 18) (Fig. 1). Among participants in the calcium, vitamins D and K group, 2 participants (withdrawn due to personal reasons in the second month) and in the placebo group, 3 participants (withdrawn due to personal reasons (2 participants in the second month and 1 participant in the third month) did not complete the trial. Finally, 55 participants [vitamins D and K co-supplements (n = 28) and placebo (n = 27)] completed the trial. On average, the rate of compliance in this trial was high, such that more than 90% of tablets were taken throughout the study in both groups. No adverse events were reported following consumption of calcium, vitamins D and K co-supplements in participants with PCOS throughout the study.

Mean age, baseline weight and BMI, and end-of-trial weight and BMI were not significantly different between the 2 groups (Table 1). Based on the 3-day dietary records taken throughout the intervention, no significant change was observed between the 2 groups in terms of macro- and micro-nutrient intakes (Data not shown).

After the 8-week intervention, compared with the placebo, joint calcium, vitamins D and K supplementation resulted in significant decreases in serum insulin concentrations (−1.9 ± 3.5 vs. +1.8 ± 6.6 µIU/mL, P = 0.01), HOMA-IR (−0.4 ± 0.7 vs. +0.4 ± 1.4, P = 0.01), HOMA-B (−7.9 ± 14.7 vs. +7.0 ± 30.3, P = 0.02) and a significant increase in QUICKI (+0.01 ± 0.01 vs. −0.008 ± 0.03, P = 0.01) (Table 2). In addition, significant decreases in serum triglycerides (−23.4 ± 71.3 vs. +9.9 ± 39.5 mg/dL, P = 0.03) and VLDL-cholesterol levels (−4.7 ± 14.3 vs. +2.0 ± 7.9 mg/dL, P = 0.03) was observed following supplementation with combined calcium, vitamins D and K compared with the placebo. We did not see any significant change in FPG and other lipid profiles.

When we adjusted the analysis for baseline values of biochemical values, age and baseline BMI, QUICKI (P = 0.06) became non-significant, and other findings did not alter (Table 3).
Discussion

We found that calcium, vitamins D and K co-supplementation among vitamin D-deficient subjects with PCOS for 8 weeks improved markers of insulin metabolism, serum triglycerides and VLDL-cholesterol levels compared with the placebo, but did not affect FPG and other lipid profiles.

There have been many studies indicating a significant relation between vitamin D concentration and beta cell function [20] and plasma glucose levels [21]. In addition, there is few evidence that between vitamin D concentration and beta cell function [20] and improved markers of insulin metabolism, serum triglycerides and VLDL-cholesterol levels compared with the placebo, but did not affect FPG and other lipid profiles.

Table 1 General characteristics of study participants.

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 27)</th>
<th>Calcium, vitamins D and K group (n = 28)</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>23.3 ± 3.4</td>
<td>23.5 ± 4.2</td>
<td>0.88</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.0 ± 6.0</td>
<td>162.2 ± 4.2</td>
<td>0.57</td>
</tr>
<tr>
<td>Weight at study baseline (kg)</td>
<td>64.3 ± 8.5</td>
<td>63.8 ± 12.5</td>
<td>0.85</td>
</tr>
<tr>
<td>Weight at end-of-trial (kg)</td>
<td>64.1 ± 8.1</td>
<td>63.7 ± 12.3</td>
<td>0.89</td>
</tr>
<tr>
<td>Weight change (kg)</td>
<td>−0.2 ± 1.6</td>
<td>−0.1 ± 1.0</td>
<td>0.63</td>
</tr>
<tr>
<td>BMI at study baseline (kg/m²)</td>
<td>24.3 ± 3.9</td>
<td>24.2 ± 4.8</td>
<td>0.95</td>
</tr>
<tr>
<td>BMI at end-of-trial (kg/m²)</td>
<td>24.2 ± 3.7</td>
<td>24.2 ± 4.7</td>
<td>0.99</td>
</tr>
<tr>
<td>BMI change (kg/m²)</td>
<td>−0.1 ± 0.6</td>
<td>−0.03 ± 0.4</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Data are means ± SDs; 1 Obtained from independent t test

Table 2 The effect of calcium, vitamins D and K and calcium co-supplementation on markers of insulin metabolism and lipid profiles in PCOS patients.

<table>
<thead>
<tr>
<th></th>
<th>Placebo group (n = 27)</th>
<th>Calcium, vitamins D and K group (n = 28)</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D (ng/mL)</td>
<td>14.8 ± 3.9</td>
<td>14.5 ± 5.0</td>
<td>−0.3 ± 4.3</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.4 ± 0.6</td>
<td>9.3 ± 0.7</td>
<td>−0.1 ± 0.3</td>
</tr>
<tr>
<td>FPG (mg/dL)</td>
<td>83.6 ± 13.0</td>
<td>88.6 ± 20.5</td>
<td>5.0 ± 19.7</td>
</tr>
<tr>
<td>Insulin (μIU/mL)</td>
<td>10.4 ± 5.3</td>
<td>12.2 ± 6.1</td>
<td>1.8 ± 6.6</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.1 ± 1.0</td>
<td>2.5 ± 1.3</td>
<td>0.4 ± 1.4</td>
</tr>
<tr>
<td>HOMA-B</td>
<td>44.7 ± 30.9</td>
<td>51.7 ± 30.0</td>
<td>7.0 ± 30.3</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.34 ± 0.02</td>
<td>0.34 ± 0.02</td>
<td>−0.008 ± 0.03</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>108.5 ± 49.6</td>
<td>118.4 ± 43.1</td>
<td>9.9 ± 39.5</td>
</tr>
<tr>
<td>VLDL-cholesterol (mg/dL)</td>
<td>21.7 ± 9.9</td>
<td>23.7 ± 8.6</td>
<td>2.0 ± 7.9</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>159.0 ± 36.9</td>
<td>159.1 ± 30.5</td>
<td>0.1 ± 31.2</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>82.4 ± 29.4</td>
<td>77.2 ± 28.3</td>
<td>−5.2 ± 32.9</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>55.0 ± 11.9</td>
<td>58.3 ± 18.4</td>
<td>3.3 ± 14.3</td>
</tr>
</tbody>
</table>

1 All values are means ± SDs; 2 Obtained from repeated measures ANOVA test; FPG, fasting plasma glucose; HOMA-IR, homeostasis model of assessment-estimated insulin resistance; HOMA-B, homeostasis model of assessment-estimated b cell function; QUICKI, quantitative insulin sensitivity check index
in increased risk of CVD [37]. Several mechanisms can explain the beneficial effects of combined calcium-vitamin D supplementation on serum triglycerides and VLDL-cholesterol levels. Calcium intake might result in reduced absorption of fatty acids and increased fecal fatty acid content through formation of insoluble calcium-fatty soaps in the gut [38], which in turn decrease serum triglycerides and VLDL-cholesterol levels. In addition, increased intracellular calcium in liver results in stimulating microsomal triglycerides transfer protein (MTP), and then causes decreased serum triglycerides and VLDL-cholesterol levels [39]. Moreover, vitamin D suppresses apo A1 gene expression at the transcriptional levels [40], which may result in decreased levels of triglycerides and VLDL-cholesterol.

This trial had some limitations. Due to limited funding, we did not evaluate the effects of calcium, vitamins D and K co-supplementation on serum vitamin K concentrations. Another limitation was that we did not assess gene expression related to insulin and lipid. It must be kept in mind that in the present study, we used the dosages of 400 IU vitamin D, 1 000 mg calcium plus 180 µg vitamin K per day based on observed beneficial effects of combined vitamin D, K and calcium supplementation on metabolic profiles in a previous study in overweight diabetic patients with CHD [10]. However, several studies have used higher doses of vitamin D in PCOS women [41, 42], we agree that future studies are needed to confirm our findings. In addition, our study was relatively of short duration of intervention for sustainable changes in the predefined outcome parameters. Long-term interventions might result in greater changes in circulating levels of metabolic profiles. It must be considered that we had not data about the time of sunlight exposure at baseline, end-of-trial and throughout the intervention. Although we believe that this status can affect our findings, this should be taken into account in the interpretation of our findings.

Overall, calcium, vitamins D and K co-supplementation for 8 weeks among vitamin D-deficient women with PCOS had beneficial effects on markers of insulin metabolism, serum triglycerides and VLDL-cholesterol levels, but it did not affect fasting plasma glucose and other lipid profiles.

Authors’ Contributions
ZA contributed in conception, design, statistical analysis and draft of the manuscript. MK, MA, M-SR, MJ, MK and MA contributed in data collection and manuscript drafting. ZA supervised the study.

Acknowledgements
The current study was supported by a grant from the Vice-chancellor for Research, IUMS, and Iran.

Clinical trial registration number

Conflicts of Interest
No conflicted.

References

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Adjusted changes in markers of insulin metabolism and lipid profiles in PCOS patients1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D (ng/mL)</td>
<td>Placebo group (n = 27)</td>
</tr>
<tr>
<td></td>
<td>−0.3 ± 0.6</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>−0.1 ± 0.1</td>
</tr>
<tr>
<td>FPG (mg/dL)</td>
<td>5.4 ± 2.8</td>
</tr>
<tr>
<td>Insulin (µIU/mL)</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>HOMA-B</td>
<td>6.3 ± 3.9</td>
</tr>
<tr>
<td>QUICKI</td>
<td>−0.006 ± 0.004</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>10.3 ± 6.5</td>
</tr>
<tr>
<td>VLDL-cholesterol (mg/dL)</td>
<td>2.1 ± 1.3</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>2.0 ± 4.6</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>−2.9 ± 4.4</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>3.3 ± 2.1</td>
</tr>
</tbody>
</table>

1 All values are means ± SEs; 2 Obtained from ANCOVA test adjusted for baseline values, age and BMI at baseline; FPG, fasting plasma glucose; HOMA-IR, homeostasis model of assessment-estimated insulin resistance; HOMA-B, homeostasis model of assessment-estimated b cell function; QUICKI, quantitative insulin sensitivity check index


[34] Rajpathak SN, Xue X, Wasseith-Smoller S et al. Effect of 5 y of calcium plus vitamin D supplementation on change in circulating lipids: Results from the Women’s Health Initiative. Am J Clin Nutr 2010; 91: 894–899


[37] Ozler S, Oztas E, Tokmak A et al. The association of thiol/disulphide homeostasis and lipid accumulation index with cardiovascular risk factors in overweight adolescents with polycystic ovary syndrome. Clin Endocrinol (Oxf) 2016; 84: 516–523

[38] Reid IR. Effects of calcium supplementation on circulating lipids: potential pharmacoeconomic implications. Drugs Aging 2004; 21: 7–17


