Original Article

The Effect of Metformin Treatment on the Serum Levels of Homocysteine, Folic Acid, and Vitamin B12 in Patients with Polycystic Ovary Syndrome

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Abstract

Background and Objective: Hyperhomocysteinemia is a well-known risk factor for cardiovascular disease. Although metformin therapy can increase homocysteine (Hcy) levels, it frequently is used as an oral medicine in women with polycystic ovary syndrome (PCOS), who might be at risk of catching diabetes mellitus. The aim of this study was to investigate the effect of metformin on the levels of serum Hcy, vitamin B12 (vit B12), and folic acid in patients with PCOS. **Materials and Methods:** An interventional study was designed with 18 patients with PCOS at the Fatemehzahra infertility Hospital in Babol, Iran. Metformin treatment (500 mg twice daily) was initiated in all patients for a period of consecutive 6 months. The levels of serum Hcy, vit B12, and folic acid were measured in the participants before and after metformin treatment. **Results:** The mean vit B12 level showed a significant decrease in patients after 6 months of metformin treatment ($P = 0.002$). However, there was no significant difference in serum folic acid levels. The mean Hcy levels increased after treatment, but this difference not was statistically significant. When patients were stratified into four subgroups by their insulin sensitivity and body mass index (BMI), relatively similar results were obtained in the subgroups, except that Hcy levels in the overweight/obesity group (BMI > 25 kg/m^2) after treatment showed a significant increase ($P = 0.01$). **Conclusion:** These findings indicate that metformin increases the serum Hcy concentration in patients with PCOS especially in the women with BMI > 25 kg/m^2. The possible mechanism for this effect would be the obvious reduction in the levels of vit B12.

Keywords: *Folic acid, homocysteine, metformin therapy, polycystic ovary syndrome, vitamin B12*

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder (~15%) in women of reproductive age.[1] In PCOS, the ovaries are enlarged and contain several small follicles with one or more symptoms, including irregular menstrual cycles (oligomenorrhea or amenorrhea), hirsutism, and anovulation.[2] In addition, hypertension, dyslipidemia, hyperandrogenism, obesity, and insulin resistance have been frequently reported in patients with PCOS.[2,3] These findings suggest that women with PCOS may be at a higher risk for the early onset of cardiovascular disease (CVD).[2-4] Previous studies showed that there was a strong correlation between insulin resistance and elevated serum homocysteine (Hcy) levels in patients with PCOS.[2,3] Insulin resistance also involves patients of PCOS to a group of long-term metabolic disorders, including impaired glucose tolerance, type 2 diabetes mellitus, and CVD.[1,3] Metformin is an oral medicine that is frequently used in the treatment of type 2 diabetes mellitus (non-insulin-dependent diabetes). The major mechanisms of metformin, as an insulin sensitizer, are

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the following: inhibiting hepatic glucose production, increasing peripheral tissues sensitivity (i.e., muscle and fat) to insulin, and thus, decreasing insulin secretion and also reducing the absorption of blood glucose in the intestine.[3,5] Although metformin shows favorable effects on the clinical secondary changes such as hypertension, ovulatory rates, obesity, and hyperlipidemia,[5-7] there is much evidence that the long-term use of metformin may increase Hcy levels in patients with type 2 diabetes mellitus and coronary heart disease.[7,8] Hcy is a cytotoxic amino acid that is formed from an intermediate product of the methionine metabolism and is catalyzed by multiple enzymes along with the cofactors of B2, B6, and B12 vitamins and folic acid.[9] Many studies suggest that changes in the levels of Hcy metabolism may be an important factor for increased risk of CVD associated with PCOS.[10,11] High Hcy level acts on the cardiac–vascular endothelium and has a direct toxicity;[4] previous studies have proven that Hcy stimulates the proliferation of the vascular smooth muscle[12] and induces apoptosis in the vascular endothelial cells.[13] Hyperinsulinemic state has a key role in the metabolism of Hcy through effects on glomerular filtration and/or the activity of some important enzymes, such as methylenetetrahydrofolate reductase and hepatic cystathionine β synthase.[2,14] Many studies have reported metformin as a common drug in type 2 diabetes, with increased circulating Hcy concentration probably by decreasing plasma vitamin B12 (vit B12) and folic acid.[8,9,15] However, there is no detailed information on the long-term use of metformin and the risk of hyperhomocysteinemia in patients with PCOS. Therefore, this study aimed to estimate the effect of metformin treatment on the changes in Hcy, folic acid, and vit B12 levels in the serum of patients with PCOS according to body mass index (BMI) and insulin resistance.

Materials and Methods

Patients and PCOS diagnosis

This interventional study was conducted on 18 patients with PCOS aged between 18 and 35 years, who attended the PCOS clinic of Fatemehzahra infertility center in Babol, Iran between 2014 and 2015. PCOS was defined according to the 2003 Rotterdam consensus criterion.[16] The Ethics Committee of Babol University of Medical Sciences approved the study protocol. A written informed consent was obtained from all the patients. The diagnosis of PCOS was based on ultrasound, clinical, and biochemical criteria. Ultrasound criteria were used to detect polycystic ovaries, defined as the presence of at least 12 follicles of 2–9 mm diameter, and/or increased ovarian volume (>10 ml).[17] Clinical criteria included amenorrhea or oligomenorrhea (a cycle length >35 days or six periods in a year). The patients who were diagnosed with PCOS also had some trait symptoms of hirsutism with Ferriman and Gallwey score (≥8), acne, and alopecia, according to Ferriman and Gallwey.[18] Biochemical criteria included elevated serum testosterone (>2.8) nmol/L and/or biochemical signs of hyperandrogenism including increased circulating level of total or free testosterone or dehydroepiandrosterone sulfate (DHEAS); the other causes of hyperandrogenism had been excluded. The patients with the following conditions were also excluded from this study: pregnancy, hyperprolactinemia, thyroid dysfunction, hypertension, gastrectomy, medications for the treatment of cerebrovascular and coronary heart diseases, folate antagonist medication (i.e., methotrexate), phenytoin and carbamazepine medications, cigarette smoking, chronic alcohol consumption, contraceptive pills, and antiobesity, strenuous physical activity. All patients were treated with 500 mg metformin twice daily for a period of consecutive 6 months. Plasma Hcy, vit B12, and folic acid levels were assayed before and after metformin therapy. The patients who had used hormonal drugs or folic acid and vit B12 supplements within the previous 3 months before the start of the trial and during the 6 months of metformin treatment were excluded from the study. The patients also had no history of treatment with the drugs that affect the plasma Hcy levels.

Laboratory analysis

BMI was calculated by the formula (weight/height^2; [kg/m^2]). Waist-to-hip circumference ratio was reported as WHR (Waist/Hip Ratio) in Table 1. Insulin resistance was determined by static fasting glucose and serum insulin levels. Homeostatic assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) were calculated according to the following formula: HOMA-IR = [fasting insulin (μIU/ml) × fasting glucose (mg/dl)]/405 and QUICKI = [(1/(log fasting insulin (μIU/ml) + log fasting glucose (mg/dl)] indexes.[19] Insulin resistance was defined as HOMA-IR greater than 2.5, abnormal fasting insulin >20 mIU/L, and glucose/insulin ratio <0.25.[20] All hormonal and biochemical experiments were performed at the pathobiology lab of Pasteur Institute in Babol, Iran. Blood samples (~10 ml) were collected from each patient after an overnight fast for at least 12 h. The samples were immediately cooled, and the serum and the plasma were separated within 1 h and stored at −20°C until assayed.

Biochemical analysis

Biochemistry analysis including fasting blood sugar (FBS; mg/dl), cholesterol (mg/dl), triglycerides (mg/dl), high-density lipoprotein cholesterol (HDL-C; mg/dl), and low-density lipoprotein cholesterol (LDL-C; mg/dl) was performed with Azmoon kit (Teif Azmoon Pars Co.; Tehran, Iran) by the spectrophotometry method (Hitachi

911 Chemistry Analyzer, Germany). Serum Hcy level (μmol/L) was determined by Axis-Shield Homocysteine Enzyme Immunoassay. A Hcy level of 11 μmol/L or more was considered to be high. The serum vit B12 (pg/ml) and folic acid (ng/ml) were measured with Roche kit by electrochemiluminescence (ECL) assay. Serum insulin (μIU/ml) was analyzed by immunoradiometric assay (IRMA) using an IRMA kit (BI-Insulin IRMA; Bio-Rad, Marnes la Coquette, France).

Hormonal analysis

Hormonal analysis including serum 17-OH-progestrone level (ng/ml) was measured with the radioimmunoassay. Serum levels of prolactin (mIU/L), testosterone (ng/ml), DHEAS (μg/dl), follicle-stimulating hormone (FSH; mIU/ml), and luteinizing hormone (LH; mIU/ml) were determined with the chemiluminescence immunoassay kit. Thyroid stimulating hormone (TSH; mIU/ml) was determined with IRMA. Sex hormone binding globulin (SHBG; nmol/L) was measured using an ECL assay kit. Free testosterone (pg/ml) was analyzed using ELISA kit.

Statistical analysis

The results were expressed as the mean ± SD. Difference between mean plasma vit B12, folic acid, and Hcy levels before and after metformin treatment was assessed by paired *t*-test. All statistical tests were performed using the Statistical Package for the Social Sciences software (SPSS 16.0 for Windows; SPSS Inc., Chicago, IL). In all cases, $P < 0.05$ was considered statistically significant.

Results

After checking the inclusion and exclusion criteria, as previously described for the selection of patients, initially, 30 women with PCOS were included in this study. However, during metformin therapy for a 6-month period, 12 patients were excluded for the following reasons: five patients for the irregular use of metformin or a duration of less than 6 months, three patients were pregnant and refused to take metformin, three for antihypertensive medications, and one for as allergic reaction to metformin. Finally, 18 patients were maintained throughout the study. The mean age, BMI (kg/m^2), waist-to-hip (W/H) ratio, and the clinical and endocrinological parameters of patients with PCOS are shown in Table 1.

Hormonal and biochemical assay

On the basis of the results of hormonal and biochemical tests of the patients with PCOS in the study, of the 18 patients, four had LH/FSH ratio >2, five had a high level of testosterone, six had a high level of 17-OH-progesterone, one had a high level of LDL, two had a high level of DHEAS, seven had a low level of SHBG, five had a high level of fasting insulin, one had a high level of total cholesterol, and two had a high level of triglyceride in comparison with the normal range. Some patients had two or three of these disorders together [Table 1].

Specific biochemistry tests

The levels of serum Hcy, vit B12, and folic acid were measured and compared before and after metformin treatment. On the basis of the results, Hcy levels before treatment in three patients with PCOS were higher than the normal range (<11 μmol/L); however, after 6 months of treatment, Hcy levels showed an increase in 13 patients with PCOS. Thus, 10 patients had an abnormal range (≥11 μmol/L) of Hcy. The mean Hcy levels increased after 6 months on metformin treatment. However, this difference was not statistically significant ($P = 0.08$). The levels of vit B12 before and after metformin treatment were in the normal range (211–946 pg/ml) in all patients; however, after treatment, a decrease was observed in 14 patients and an increase in four patients with PCOS. The mean vit B12 levels showed a significant decrease in patients with PCOS after 6 months of metformin treatment ($P = 0.002$).

The levels of vit B12 before and after metformin treatment were in the normal range (211–946 pg/ml) in all patients, but a decrease was observed in 14 patients and an increase in four patients with PCOS after treatment. The levels of folic acid before treatment were higher than the normal

Table 1: Mean of clinical and biochemical parameters in patients with PCOS

Variables and normal range	PCOS (n = 18)
Ferriman–Gallwey Score	12.57 ± 8.44
Age (years)	23.22 ± 4.83
BMI (kg/m^2)	28.16 ± 6.33
Waist/hip ratio	0.79 ± 0.12
FSH (3.5–9.2 mIU/ml)	5.88 ± 2.56
LH (1.9–9.2 mIU/ml)	8.51 ± 4.77
LH/FSH ratio	1.42 ± 0.61
Fasting insulin (2.1–22 μU/dl)	15.95 ± 8.52
Fasting glucose (60–110 μU/dl)	90.16 ± 8.016
Free testosterone (0.04–4.1 pg/ml)	2.48 ± 0.84
Testosterone (0.05–0.81 ng/ml)	0.76 ± 0.16
17-OH-progesterone (0.15–1.1 ng/ml)	0.96 ± 0.45
DHEAS (58.7-227 μg/dl)	158.37 ± 50.08
SHBG (26.1–110 nmol/L)	31.24 ± 15.74
Prolactin (132–550 mIU/L)	474.38 ± 244.93
Triglyceride (50–200 mg/dl)	125.88 ± 57.30
TSH (0.2–5.2 mIU/ml)	2.51 ± 1.44
Total cholesterol (<200 mg/dl)	173.61 ± 23.51
LDL-C (<130 mg/dl)	98.11 ± 16.56
HDL-C (29–80 mg/dl)	50.00 ± 9.03
HOMA >2.5	3.58 ± 2.10
QUICKI <0.34	0.32 ± 0.03

The data were expressed as the mean ± SD.

range (4.6–18.7 ng/ml) in six patients, and 12 patients had normal ranges. After treatment, a decrease was observed in 10 patients and an increase in eight patients with PCOS; however, there was no significant difference in the mean serum folic acid levels of patients before and after treatment. The results are shown in Table 2.

The patients were divided into the following two subgroups based on their insulin sensitivity: insulin resistance (HOMA-IR $>$ 2.5), non-insulin resistance (HOMA-IR $\leq$ 2.5) [Table 3]. The patients were also classified into the following two subgroups according to their BMI: normal group (BMI $<$ 25 kg/m^2) and overweight/obesity group (BMI $>$ 25 kg/m^2) [Table 4]. Totally, there were seven patients with BMI 18.5–24.9 kg/m^2, four were overweight patients with BMI 25–29.9 kg/m^2, and seven were obese patients with BMI $\geq$ 30 kg/m^2. The results were investigated in all the subgroups before and after metformin treatment [Tables 3 and 4]. Hcy levels in the overweight/obesity group (BMI $>$ 25 kg/m^2), but no other groups, had significant increase after 6 months of metformin treatment ($P = 0.01$). In addition, the levels of folic acid did not show a significant difference in all the subgroups after treatment. But, comparison of vit B12 levels after metformin treatment revealed considerable decrease in all the subgroups; insulin resistance, non-insulin resistance, adequate weight (BMI $<$ 25 kg/m^2) and the overweight/obesity (BMI $>$ 25 kg/m^2) groups ($P = 0.02$, 0.05, 0.06 and 0.01, respectively) than before treatment [Tables 3 and 4].

Discussion

Several studies have demonstrated that women with PCOS are at increased risk for obesity, hypertension, insulin resistance or hyperinsulinemia, dyslipidemia, type 2 diabetes mellitus, and premature atherosclerosis; these types of complex traits seem to be responsible for the high levels of Hcy in patients with PCOS.[10,11] Studies showed that metformin therapy inhibited the absorption of vit B12 and folic acid, and, in fact, increased the Hcy levels in patients with type 2 diabetes mellitus.[8,21] This study was designed with 18 patients with PCOS to evaluate the effect of metformin treatment on the changes of serum Hcy, folic acid, and vit B12 levels in the patients. On the basis of our results, there were no significant differences in

Table 2: Comparison of serum Hcy, vit B12, and folic acid levels in PCOS before and after metformin treatment

Total PCOs = 18	Before metformin therapy	After metformin therapy	*P*-value
Hcy	10.22 ± 2.28	11.72 ± 3.36	0.08
Folic acid	18.55 ± 9.84	17.72 ± 7.72	0.78
Vitamin B12	456.88 ± 172.32	367.69 ± 128.100	0.002

The data were expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

Table 3: Comparison of serum Hcy, vit B12 and folic acid levels in PCOS according to insulin resistance before and after metformin treatment

Groups	IR$^-$ (n = 9)			IR$^+$ (n = 9)		
	Before metformin therapy	After metformin therapy	*P*-value	Before metformin therapy	After metformin therapy	*P*-value
Hcy	10.22 ± 2.86	12.11 ± 3.05	0.21	10.22 ± 1.71	11.33 ± 3.77	0.24
Folate	17.94 ± 9.52	18.17 ± 6.82	0.95	19.16 ± 10.71	17.26 ± 8.92	0.69
Vit B12	443.16 ± 180.01	361.24 ± 109.80	0.05	470.61 ± 173.99	374.14 ± 150.73	0.02

The data were expressed as the mean ± SD (IR$^-$: non-insulin resistant; IR$^+$: insulin resistant). $P < 0.05$ was considered statistically significant.

Table 4: Comparison of serum Hcy, vit B12 and folic acid levels in PCOS according to BMI before and after metformin treatment

Groups	BMI $<$ 25 (n = 7)			BMI $>$ 25 (n = 11)		
	Before metformin therapy	After metformin therapy	*P*-value	Before metformin therapy	After metformin therapy	*P*-value
Hcy	11.00 ± 2.76	10.85 ± 2.60	0.92	9.72 ± 1.90	12.27 ± 3.7	0.01
Folate	14.81 ± 4.66	15.48 ± 7.04	0.79	20.93 ± 11.65	19.14 ± 8.12	0.72
Vit B12	353.71 ± 103.95	283.95 ± 102.80	0.06	522.54 ± 178.33	420.98 ± 116.28	0.01

The data were expressed as the mean ± SD (BMI $<$ 25: adequate weight and BMI $>$ 25: overweight/obesity). $P < 0.05$ was considered statistically significant.

the serum Hcy levels and also folic acid in the patients with PCOS before and after metformin therapy; however, a significant decrease was observed in the serum vit B12 levels after 6 months of treatment.

Previous findings indicated that the administration of metformin could induce vit B12 deficiency in patients with type 2 diabetes and PCOS.[3,21-24] For example, there was a significant decrease of serum vit B12 levels after metformin treatment in patients with type 2 diabetes mellitus, but plasma Hcy levels did not show a significant difference between those in the metformin and the non-metformin groups.[22]

Hoogeveen *et al.*[25] showed that metformin therapy (500–2550 mg daily) for at least six months in Caucasian patients with non-insulin-dependent diabetes mellitus, have little effect on increasing the serum total Hcy levels. Similar results were reported in women with PCOS after 12 weeks of metformin therapy.[3] In addition, Carlsen *et al.*[26] reported reduction in serum folic acid and vit B12 levels, but no effect of metformin on the Hcy levels in women with PCOS. In contrast, a study has shown that in patients with type 2 diabetes, 16 weeks of treatment with metformin decreases the levels of folic acid and vit B12 leading to a modest increase in the plasma levels of Hcy.[8]

Different mechanisms have been proposed for vit B12 deficiency after metformin therapy in patients with diabetes mellitus: (I) alterations in small bowel motility, which stimulates bacterial overgrowth, (II) competitive inhibition or inactivation of vit B12 absorption, (III) depression of intrinsic factor (IF) secretion and interaction with the cubulinendocytic receptor, (IV) inhibition of the calcium-dependent absorption of the vit B12-IF complex in the terminal ileum by its receptor.[27-29]

Our findings are not in conformity with some studies, which reported that metformin treatment (1000 mg daily) for a short period of 4 weeks, significantly increased the serum Hcy levels in patients with PCOS.[30] Kilicdag *et al.*[31] showed a significant increase in plasma Hcy levels after metformin therapy for 3 months in women with PCOS, but significant changes were not observed in the parameters of insulin resistance, BMI, and in the basic levels of folic acid and vit B12 than after treatment.

In this study, when the patients with PCOS were classified based on their BMI and insulin sensitivity, relatively similar results were obtained in all the subgroups, except that Hcy levels in the overweight/obesity group (BMI $> 25\,kg/m^2$) after treatment showed a significant increase ($P = 0.01$). Contrarily, after 6 months of treatment with (500 mg twice daily) metformin, the vit B12 levels showed a significantly greater reduction in the insulin resistance (HOMA-IR > 2.5) groups, the non-insulin resistance (HOMA-IR ≤ 2.5) groups, and the overweight/obesity group (BMI $> 25\,kg/m^2$) than before treatment ($P = 0.02, 0.05, 0.01$, respectively) [Tables 3 and 4]. The results of this study showed that overweight or obese women with PCOS exposed to metformin treatment were more susceptible to hyperhomocysteinemia. Therefore, we suggest that dietary supplements rich in vit B12 with optimal dose and regular physical exercise may be beneficial in reducing plasma Hcy levels in patients with PCOS receiving metformin therapy.In agreement with the previous data, we did not find any association between insulin resistance and Hcy levels. Kilic-Okman *et al.*[32] suggested that high Hcy levels in patients with PCOS is independent of insulin resistance and may be due to other factors. The findings of Mohammadi *et al.*[33] showed that metformin increased plasma Hcy levels in type 2 diabetic patients, especially in men. However, if metformin is administered with folic acid, it can prevent this process. In contrast, there was no change in the level of Hcy in the patients who received folic acid plus metformin.[5] Another study reported that the administration of B group vitamins and folic acid reduced Hcy levels in patients with PCOS exposed to metformin treatment.[31] Some studies showed that inadequate folate intake first led to a decrease in serum folate concentration and thus increased the levels of Hcy.[34] The effects of supplementation with folic acid in patients with PCOS who were treated with metformin demonstrated a significant improvement in the structure and function of the vascular endothelium.[35] Overall, these
studies reported conflicting results regarding the effect of metformin therapy on Hcy levels in exposed patients by considering various doses and durations of the use of metformin. Consequently, to understand if metformin therapy is likely to be responsible for the increased Hcy levels and whether this association can be modified by dietary supplements of the B vitamin groups or folic acid, further research with a larger sample size is unavoidable.

Conclusion

According to our results, there is a strong association between metformin therapy and decreased vit B12 levels in patients with PCOS, particularly women with overweight/obesity and hyperinsulinemia. Since the increase in Hcy could be due to the decrease in its essential cofactors (folic acid and vit B12), it is concluded that metformin may effect hyper-homocysteinemia as an independent risk factor of CVD in the exposed patients with PCOS. Comprehensive studies with a larger sample size are needed to confirm the role of insulin-sensitizing drugs such as metformin in the increase of plasma Hcy levels.

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Conflicts of interest

There are no conflicts of interest.

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