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Serum Homocysteine and Paraoxonase1 Levels in Women with Polycystic Ovary Syndrome Treated with Metformin Versus Metformin and Folic Acid

By Dr. Tarek H. Abed Tawfik Al-Khyat, Dr. Melal Mohammed Al-Jeborry & Qusay Mohsin Kadium

University of Babylon, Iraq

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Patients and Methods: Fifty patients with PCOS were enrolled in this study that subdivided into two groups according to type of treatment received i.e. G1 (comprising 25 patients) treated with Metformin only, G2 (comprisin25 patients) with Metformin and Folic acid. Serum Homocysteine (Hcy), Paraoxonase 1(PON1) Apolipoprrptein A-1(Apo A-1) and Apolipoprotein B(Apo B) were determind by using enzyme-linked immunosorbent assay (ELISA). Reduced Glutathion (GSH) and Malondialdehyde (MDA) were determined by using colorimetric method.

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Results: The results indicated a significant increase in the serum levels of Hcy($p<0.01$), PON1 and GSH($p<0.05$) anda significantdecreasein thelevels of MDA and Apo B/Apo A-1 ratio($p<0.01$)in PCOS group after Metformin only(G1-A).But, after Metformin with Folic acid treatment (G2-A)Hcy, Apo B/Apo A-1 ratio and MDA levels were significantly lower ($p<0.01$) and PON1 as well as GSH levels were significantly higher ($p<0.01$) in comparison with those before treatment. Whereas, a significant decreasein level ofHcy, MDA ($p<0.01$) and Apo B/Apo A-1 ratio($p<0.05$) anda significantincrease in PON1($p<0.01$) and GSH levels ($p<0.05$) in (G2-A)as compared with (G1-A). The results revealed a significant positive correlation between Hcywith MDA and Apo B/Apo A-1 ratio as well as between PON1 and GSH. And a significant negative correlation between HcywithGSH, PON1and also, between PON1 with MDA and Apo B/Apo A-1 ratio in patients groups.

Conclusion: Folic acid administration to PCOS patients tends to lower homocysteine levels in the serumof the corresponding patients and alleviate the oxidative stress in those patients. Besides, increase of PON1 prevents atherogenic effects by changing Apo B/Apo A-1 ratio.

Authors α ρ : Dept. of Clinical Biochemistry, College of Medicine, University of Babylon, Hilla, Iraq.

Author σ : Dept. of Obstetrics and Gynecology, College of Medicine, University of Babylon, Hilla, Iraq. E-mail : qusaym64@yahoo.com

I. Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common female endocrine disorders and is a complex, heterogeneous disorder of uncertain aetiology, but it can be classified as a genetic disease [1]. Itis one of the most common causes of anovulatory infertility with a prevalence between 6% and 10% based on the U.S. National Institute of Health (NIH) criteria and as high as 15% when the broader Rotterdam criteria are applied [2]. Typically, PCOS is first identified during the early reproductive years. The clinical expression varies but commonly includes oligo-ovulation or anovulation, hyperandrogenism (either clinical or biochemical) and the presence of polycystic ovaries [3]. Theaetiology ofPCOS is not fully known, but many environmental and genetic factors may cause this disease, such as diet, pollution and sedentary lifestyle. Stress may contribute to its development [4].

Homocysteine (Hcy) is an intermediate product formed during the breakdown of the amino acid methionine, and may undergo remethylation to methionine (folate and cobalamin is involved in Hcyremethylation) or trans-sulphuration to cystathionine then to cysteine (vitamin B6 is involved in Hcy trans-sulphuration) [5]. Excess Hcy in the blood stream may cause injures to arterial vessels due to its irritant nature, and result in inflammation and plaque formation by impaired endothelial function, increased oxidative stress, alterations of lipid metabolism, increasing platelet adhesiveness, activation of the coagulation system and stimulating vascular smooth muscle cell proliferation [6].

PON1 is a member of family of proteins that also includes PON2 and PON3. that share considerable structural homology and are located adjacently on chromosome 7 in humans. All the three proteins prevent oxidative stress and fight inflammation [7]. PON1 has different anti-atherogenic features such as protection from free radical induced oxidation of cholesterol in arterial wall and protection against harmful effects of oxidized LDL. PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is

associated with apoA-I incorporation in the high-density lipoprotein (HDL) particles [8].

The ApoB/ApoA-I ratio represents the balance of pro-atherogenic and anti-atherogenic lipoproteins, which is the better parameter than routine lipid measurements in predicting risk of cardiovascular diseases(CVD), might also be potential biomarkers in predicting risk of CVD in patients with PCOS[9]. Various studies had demonstrated that this ratio was associated with the incidence metabolic syndrome (MS), insulin resistance (IR) and increased free androgen index and visceral adiposity [10].

Oxidative stress, defined as the rate of Reactive Oxygen Species (ROS) (and/or Reactive Nitrogen Species (RNS) production) and the rate of their removal by cellular defense mechanisms [11].In humans, oxidative stress is thought to be involved in the development of an environment unsuitable for normal female physiological reactions [12]. This, in turn, can lead to a number of reproductive diseases including endometriosis, PCOS, and unexplained infertility. It can also cause complications during pregnancy, such spontaneous abortion, recurrent pregnancy loss, preeclampsia, and intrauterine growth restriction [13].

Metformin is an insulin-sensitizing drug from the biguanide class. It is orally administered drug used to lower blood glucose concentrations in patients with non-insulin dependent diabetes mellitus (NIDDM), and is now also used in the treatment of polycystic ovary syndrome [14]. Metformin had many benefits such as, decrease in body mass index (BMI), androgens in both lean and obese women activity for around 20% andimprovement of menstrual cycles (ovulation rates). Normal menstrual cycles achieved within 3 months of starting treatment in some groups of patients, reduce early pregnancy losses and reduce risk of miscarriage,a 10-fold reduction in gestational diabetes (31% to 3%) and prevention or delay of onset of diabetes, lowering the blood insulin levels, increasing insulin sensitivity and lowering glucose level[15]. However, serum vitamin B12 and Folic acid levels are known to decrease during Metformin therapy probably due to malabsorption. Hence, Hcy levels might increase [16]. Folic acid may help reduce certain complications of PCOS as Folic acid supplements are "likely effective" for the treatment of high Hcy levels. Folate is involved in Hcy remethylation to methionine.

Women with PCOS may be able to improve their chances of getting pregnant by taking Folic acid every day [17] and besides possibly reducing infertility associated with PCOS, Folic acid may also improve pregnancy outcomes with PCOS. As with all pregnant women, pregnant women with PCOS need to get sufficient Folic acid to prevent neural tube defects. Both low Folic acid levels and PCOS are linked to miscarriage. Pregnant women with PCOS may thus help reduce their risk of miscarriage by taking Folic acid [18].

In the present study, the relationship between homocysteine, paraoxonase 1 and oxidative stress levels was elucidated in different regimes of treatment with Metformin and Folic acid in Iraqi patients inflicted with polycystic ovarian syndrome and the effect of those treatments regime on ApoB/Apo A-1 ratios in the corresponding patients.

II. Patients and Methods

This study was conducted in Babylon Maternity and Pediatric Teaching Hospital and in the laboratory of Biochemistry Department, College of Medicine, University of Babylon in the period starting from November 2012 to June 2013. Fifty patients with PCOS were enrolled in this stady. The patients were divided into two groups according to type of treatments received. The first group (G1) included 25 patients with PCOS received Metformin only, their age ranged between (18 - 30) years. The second group (G2) included 30 patients who received Metformin with Folic acid, their age ranged between (19 - 30) years. Full history was taken for all the patients which includes: age, address, length, weight, past history of diseases, obstetrical history, smoking, family history of disease, medical history. No drugs were prescribed to those patients that may interfere with the measured parameters (fertility drugs, oral anti-diabetic agents and oral contraceptive pills).

Seven to ten milliliters of blood were obtained from those patients. Blood samples were collected in tubes without anticoagulants and were left for 15 minutes at room temperature to clot. After that, the blood samples were centrifuged at 1500 xg for approximately 10 minutes. The serum was isolated and divided into five aliquots using eppendrof tubes and stored at (-20°C) until time of use.

Serum Hcy and PON1, were determined using ELISA kit provided by CUSABIO, China. Whereas, Apolipoprotein A-1 and B were determined using ELISA kit provided byEAGLE Biosciences, USA. The determination of Serum Hcy, PON1, Apo A-1 and Apo B is based on sandwich principle., using ELISA technique .Serum GSH concentration was determined by using a modified procedure utilizing Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNBwhen Sulfhydryl group of (GSH) reduces disulfide chromogen DTNB and change it to an intensely yellow compound which is proportional to total GSH and measured spectro-photometrically at 412 nm. The method of determination of serum MDA was based on the reaction with thiobarbituric acid (TBA) at 90-100°C and pH 2-3 for 15 minutes to form pink color product; forming an MDA-TBA_2 adduct that absorbs strongly at 532 nm.

All statistical analysis was performed by using SPSS version 18 for windows. Data were expressed as Mean ± SD. The normality of the distribution of all

variables was assessed by the ANOVA, LSD and Pearson correlation analysisthat have been used to determine the significant difference between PCOS patients groups. P values less than (0.05) is considered significant while valuesless than (0.01) is considered highlysignificant.

III. Results and Discussion

The results in table (1) show non-significant differences in the age among different groups. This is due to the selection of subjects who are nearly within the same age. In fact, this is an important aspect for the comparison of other parameters especially those parameters which vary with age.

The body mass index (BMI) is a measure for human body shape based on an individual's mass and height. This anthropometric parameter is an important measure of obesity. Elevated BMI was associated with insulin resistance [19]. In our study, neither Metformin therapy nor Metformin and Folic acid therapy changed the BMI significantly. Our results were in agreement with at least two studies that failed to demonstrate the non-significant BMI reduction during the treatment with Metformin only [15,20] and also, during the treatment with Metformin plus Folic acid [15,21]. The small number of patients in the studies and differences in the treatment periods may explain these discrepancies.

In this study, A significant increase in Hcy ($p<0.01$), a significantly low in Apo B/Apo A-1 ratio and MDA levels ($p<0.01$) were observed in PCOS patients treated with Metformin only while there were significant increase in PON1 and GSH ($p<0.05$) in the corresponding patients. The results revealed a high significant decrease in Hcy, Apo B/Apo A-1 ratio and MDA levels ($p<0.01$)in patients on Metformin plus Folic acid but, there weresignificant increase in PON1 and GSH($p<0.01$)in the corresponding patients. Table (1).

Some investigators found that Metformin tends to decrease Folic acid which in turn leads to increase of Hcy in the PCOS patients [22, 23]. in spite of the benefits of such treatment in PCOS patients to improve fertility, normalization of menstrual cycles and reduction of thecal androgen production, there were some harms in using this drug alone[15]. For this reason Folic acid was administrated for PCOS patients with Metformin to improve antioxidant status and to lower Hcy levels. Previous studies that are done by Palomba [21] and Kilicdag [20] revealed a significant decrease in Hcy levels after adding Folic acid to Metformin in PCOS patients.

Elevated Hcy levels can damage endothelial cells, impairing the release of nitric oxide (NO) and leading to a net increase in the production of superoxide ($O_2^{\bullet}$-) which lead the formation of atherosclerosis plagues and myocardial infarction (MI). It also leads to structural changes in lipoproteins especially LDL molecules [6]. This may give the idea about the correlation between Hcy, oxidative stress and Apo B/ApoA-I ratio as shown in table (2).

The significant increase in PON1 associated with Folic acid administration to Metformin indicate the essential role of this vitamin in the improving lipid status since the latter is involved in the incorporating of Apo A-1 in HDL-particles[8]. This was confirmed by the negative correlation between PON1 and Apo B/ApoA-I ratio in PCOS patients after treatment with Metformin and Folic acid in the present study.

PON1 may acts synergistically with Folic acid to lower Hcy levels since PON1 can hydrolyze Hcy-thiolactone back to Hcy and Hcy may be then converted either back to methionine (this reaction which needs folate and vitamin B12 as co-factors), or condensed with serine to form cystathionine in a reaction that is dependent on vitamin B6 as reported by Yilmaz [24]. This conclusion can be attributed to negative correlation between PON1 and Hcy in PCOS patients treated with Metformin and Folic acid as reported in table (2).

The results revealed a significant decrease inApoB/ApoA-I ratio after treatment with of Metformin plus Folic acid in PCOS patients because Metformin therapy improves glycemic control and may decrease oxidative stress related oxidation of LDL [25] as well as the protective effects of Folic acid supplementation on endothelial dysfunction by direct effect on free-radical oxidation of LDL lipids and prevent the structural and functional modification of Apo A-1in HDL due to nitration [9,26]. In fact, the decrease in Apo B/ApoA-I ratio levels in this study is more important because it is a potential biomarkers in predicting risk of CVD in patients with PCOS [9].

In the present study, the addition Folic acid to Metformin had further effect on oxidative stress parameters (decrease MDA and increase GSH) due to the fact that Metformin was able to regulate ovarian oxidative stress by decrease androgen level via reducing pituitary gonadotropin secretion [23, 27]. And Folic acidhad direct antioxidant role and free radical scavenging activity[28].

The unique idea in our work is the relationship among Hcy, PON1, oxidative stress and Apo B/Apo A-1 ratio since previous studies lack the correlations among those parameters and the results in table 2 confirmed such relations among different parameters.

Finally administration of a combined treatment of Metformin and Folic acid is quite essential for the lowering of serum levels of Hcy, oxidative stress and improving apolipoprotein status in PCOS patients to exclude further complications associated with this disease.

Table 1 : The anthropometric and clinical characteristicsof PCOS groups before and after treatment

Variable	Group	N	Mean ± SD	Range	*P* Value
Age (year)	G1-B	25	22.84 ± 3.85	18 – 30	
	G2-B	25	23.92 ± 3.54	19 – 30	P_1 >0.05
BMI (kg/m^2)	G1-B	25	29.25 ± 5.99	21.77– 44.96	P_1> 0.05
	G1-A		29.01 ± 6.01	21.91 –45.54	P_2> 0.05
	G2-B	25	30.56 ± 5.83	22.14– 47.38	P_3> 0.05
	G2-A		28.73 ± 5.67	19.56– 46.09	P_4> 0.05
Hcy (nmol/ml)	G1-B	25	13.96 ± 0.68	12.69 – 15.50	P_1>0.05
	G1-A		16.12 ± 1.03	14.69 -19.93	P_2<0.01
	G2-B	25	14.30±0.79	12.78 – 16.03	P_3<0.01
	G2-A		12.75±0.77	11.50 – 14.21	P_4<0.01
PON1 (mlU/ml)	G1-B	25	163.44±16.54	125.42 – 191.44	P_1>0.05
	G1-A		170.51±7.71	155.49- 183.13	P_2<0.05
	G2-B	25	165.12± 10.62	143.36- 182.06	P_3<0.01
	G2-A		188.15±12.2	167.00 – 211.92	P_4<0.01
GSH (μM)	G1-B	25	22.23±5.18	11.99 – 31.26	P_1>0.05
	G1-A		25.22±5.25	16.12 – 34.01	P_2<0.05
	G2-B	25	20.95±4.91	12.26 – 30.15	P_3<0.01
	G2-A		28.05±4.60	18.97 – 36.86	P_4<0.05
MDA (μM)	G1-B	25	5.89±0.91	4.23 – 8.09	P_1>0.05
	G1-A		5.03±0.85	3.87 – 6.79	P_2<0.01
	G2-B	25	6.10±0.57	5.15 – 7.73	P_3<0.01
	G2-A		4.39±0.69	3.65 – 6.87	P_4<0.01
Apo B/Apo A-1 ratio	G1-B	25	11.16±2.72	5.59– 16.81	P_1>0.05
	G1-A		9.25±1.52	5.47 – 12.20	P_2<0.01
	G2-B	25	10.31±1.96	6.39 – 14.06	P_3<0.01
	G2-A		7.94±1.61	5.19 – 10.67	P_4<0.05

G1-B= patients group before treated with Metformin only, G1-A= patients group after treated with Metformin only, G2-B= patients group before treated with Metformin and Folic acid, G2-A= patients group after treated with Metformin and Folic acid, SD= standard deviation, N= number of patient, BMI= body mass index, HCY= Homocysteine, PON1= Paraoxonase1, CAT= Catalase, GSH= Glutathione, MDA= Malondialdehyde, Apo B/Apo A-1 ratio=Apolipoprotein B/ Apolipoprotein A-1 ratio, P_1= between G1-B and G2-B, P_2= between G1-B and G1-A, P_3= between G2-B and G2-A, P_4= between G1-A and G2-A.

Table 2 : The Pearson correlations between different parameters in PCOS groups before and after treatment

		G1-B		G1-A		G2-B		G2-A	
		Hcy nmol/l	PON1 mlU/ml	Hcy nmol/l	PON1 mlU/ml	Hcy nmol/l	PON1 mlU/ml	Hcy nmol/l	PON1 mlU/ml
Age (year)	r	- 0.106	- 0.040			0.275	- 0.249		
	P-value	0.615	0.850			0.182	0.230		
BMI	r	0.156	- 0.388	0.141	- 0.315	0.0.157	- 0.140	0.371	- 0.184
(kg/m^2)	P-value	0.457	0.055	0.501	0.125	0.454	0.504	0.068	0.378

MDA (μM)	r	0.517	- 0.751	0.424	- 0.703	0.788	- 0.697	0.506	- 0.611
	P-value	0.008	0.000	0.034	0.000	0.000	0.000	0.010	0.001
GSH (μM)	r	- 0.682	0.751	- 0.529	0.688	- 0.701	0.765	- 0.850	0.890
	P-value	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000
Apo B/ Apo A-1 ratio	r	0.553	- 0.554	0.413	- 0.554	0.634	- 0.752	0.752	- 0.680
	P-value	0.004	0.004	0.040	0.004	0.001	0.000	0.000	0.000
Hcy nmol/l	r		- 0.463		- 0.418		- 0.537		- 0.843
	P-value		0.020		0.038		0.006		0.000

G1-B= patients group before treated with Metformin only, G1-A= patients group after treated with Metformin only, G2-B= patients group before treated with Metformin and Folic acid, G2-A= patients group after treated with Metformin and Folic acid, BMI= body mass index, HCY= Homocysteine, PON1= Paraoxonase1, GSH= Glutathione, MDA= Malondialdehyde, Apo B/Apo A-1 ratio=Apolipoprotein B/ Apolipoprotein A-1 ratio and r= pearson correlations coefficient

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