

Changes in Serum Levels of Vitamin B₁₂, Folic acid and Homocysteine in Patients with Type 2 Diabetes before and after Treatment with Metformin

Fariba Karimi¹✉ and Gholamhossein Ranjbar Omrani²

¹Associate professor, Endocrinology and metabolism research center, School of medicine, Shiraz University of medical Sciences, Zand Street, Namazee Square, Namazee Hospital, Department of Internal medicine, Shiraz, Iran

²Professor, Endocrinology and metabolism research center, School of medicine, Shiraz University of medical Sciences, Zand Street, Namazee Square, Namazee Hospital, Department of Internal medicine, Shiraz, Iran

✉ Corresponding author's E-mail: karimif2002@yahoo.com

ABSTRACT: Diabetes mellitus is one of the most common chronic disorders in the world. Previous investigations have suggested that metformin, as a key treatment option in type 2 diabetes, may lead to hyperhomocysteinemia through decreasing the levels of vitamin B₁₂ and folic acid. In this clinical trial, 55 patients with type 2 diabetes who referred to two clinics of Shiraz University of Medical Sciences participated. At first, the participants followed diet regimen alone for 3 months and then in the next 3 months they were treated by metformin. Serum levels of vitamin B₁₂, folic acid and homocysteine were checked before and after the diet alone and metformin consumption, then they were compared. After 3 months of diet regimen alone the levels of vitamin B₁₂, folic acid and homocysteine did not show statistically ($P \leq 0.05$) significant differences. However, three months of treatment with metformin resulted in significantly higher levels of homocysteine ($P=0.001$) although serum levels of folic acid and vitamin B₁₂ did not change significantly again ($P=0.1$ and $P=0.4$, respectively). Our data provided no support for this hypothesis that metformin consumption increases homocysteine secondary to folate and/or B₁₂ deficiency.

Keywords: Type 2 diabetes, Metformin, Folic acid, Vitamin B₁₂, Homocysteine

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INTRODUCTION

Type 2 diabetes, as a chronic disorder which exacerbates atherosclerosis, is associated with a 2-6 fold greater risk of cardiovascular disease in diabetics compared with their non-diabetic counterparts. This high incidence of vascular complications is multifactorial and may not be explained by hyperglycemia alone (van Guldener et al., 2002; Shargorodsky et al., 2009; Hwang et al., 2013). In this regard, other presumably unrelated risk factors, such as hyperhomocysteinemia may be involved in the atherothrombotic process (Lippi et al., 2012; Schaffer et al., 2014; Akhabue et al., 2014). Interestingly, there is evidence that hyperhomocysteinemia is a strong risk factor in patients with type 2 diabetes and several studies have demonstrated that elevated Homocysteine (Hcy) levels

predict the risk of death or coronary events in these patients (Hoogeveen et al., 2000 and Wijekoon et al., 2007). However, conflicting results regarding the Hcy level in patients with diabetes have been reported. For example, Bussychaert et al. (2000) in their study found a significant association between fasting hyperhomocysteinemia and peripheral as well as coronary artery disease in diabetic subjects. The underlying mechanism of this increased vascular risk may relate to the synergistic worsening of endothelial dysfunction and oxidative damage (Wijekoon et al., 2007 and Schaffer et al., 2016). Since 1970, some investigators reported an increased risk of diabetic macrovascular complications as a side effect of oral hypoglycemic agents and later studies have considered similar suspicion for insulin therapy as well (Schramm et al., 2011 and Rahmi et al., 2013). In this regard, the United

Kingdom Prospective Diabetes Study (UKPDS) also failed to show that any of the specific treatments for type 2 diabetes compared with diet alone could increase or decrease the risk of diabetic cardiovascular complications (Stratton et al., 2000). Metformin, as the first line oral hypoglycemic agent, has been used for several years and vitamin B₁₂ or folate deficiency were mentioned as its well-known side effects (De-Jager et al., 2010; Liu et al., 2014; Haeusler et al., 2014).

Indeed in some of the previous reports the degree of B₁₂ deficiency in metformin-treated subjects was comparable to that of the patients with gastrectomy (Sakuta et al., 2005). Moreover, folate and vitamin B₁₂ are needed as cofactors in homocysteine metabolism. Therefore, their deficient levels can lead to hyperhomocysteinemia (Langan et al., 2011 and Kibirige et al., 2013). Carlsen et al. (1998) in their study showed that metformin consumption resulted in an elevated serum Hcy but reduced the levels of vitamin B₁₂ and folate in non-diabetic males with coronary heart disease.

However, there are conflicting results regarding metformin-induced changes of Hcy level and its mechanism in diabetic patients. Indeed positive as well as no relationship have been observed between Hcy level and metformin consumption (Wulfele et al., 2003; Palomba et al., 2010; Rajagopal et al., 2012; Osama et al., 2016). To provide an answer to these contradictory results and elucidate the possible mechanism, we conducted this study to evaluate the effect of metformin on the serum levels of Hcy, vitamin B₁₂ and folate in metformin-treated diabetic patients.

MATERIALS AND METHODS

This study was conducted during the years 2010-2012 in Motahari and Faghihi Clinics of Shiraz University of Medical Sciences, Shiraz, Iran. In this clinical trial, 55 patients with type 2 diabetes mellitus participated. The participants were selected by convenience sampling method. Inclusion criteria were: age range of 30-75 years old, a negative history of previous diabetic ketoacidosis, recent diagnosis of type 2 diabetes mellitus with mild to moderate hyperglycemia, Fasting Blood Sugar (FBS) of 135-200mg/dl and 2 hours postprandial blood sugar 180-250mg/dl. The exclusion criteria were: pregnancy or planning for pregnancy, renal function defect (serum creatinine 1.3 mg/dl or higher), a previous history of diabetic ketoacidosis, and patients with congestive heart failure, New York Heart Association class III/IV or higher, other serious medical or psychiatric diseases, consumption of medications or supplements with effect on the serum levels of measured parameters, a history of previous operation on alimentary tract, history of hypothyroidism, and cancerous disorders. For each patient, a questionnaire containing demographic and anthropometric characteristics, drug history and a history of cardiovascular disease was filled. From the beginning of the survey, all

the subjects were given advice for a healthy, moderately calorie-restricted diet, and an active lifestyle. After 12 weeks, treatment with metformin from a minimum dose of 500 milligrams per day to maximum dose of 2.5 grams daily was started. Metformin dose was gradually increased according to monthly-checked plasma glucose levels. The treatment goals were fasting and postprandial plasma glucose levels between 80-130mg/dl and 140-180mg/dl, respectively (American Diabetes Association, 2016).

Body Mass Index (BMI) was calculated as body weight in kilogram divided by the square of height in meter. At the beginning and then 3 months after diet regimen alone and again 4 weeks and 12 weeks after metformin consumption, the venous blood samples were drawn for measurement of homocysteine, folate, vitamin B₁₂, and HbA1c levels. Also, the participants whose blood glucose could not be controlled adequately with diet and metformin were excluded and replaced by other patients. Finally, the obtained data were statistically analyzed. Serum levels of vitamin B₁₂ and folic acid were measured by Radioimmunoassay (RIA) kits simulTRAC-SNB radioimmunoassay (DRG instruments. GmbH, Germany). Homocysteine level was determined by enzyme immunoassay (EIA) (Axis-shield diagnostic. UK). The glucose-oxidase method was used for plasma glucose assay (BioSystems S.A. Costa Brava 30, Barcelona, Spain).

Ethical approval

The review board and ethics committee of Shiraz University of Medical Sciences approved the study protocol and informed consents were taken from all the participants. The article's Iranian Registry of Clinical Trials (IRCT) code was: IRCT2015011614717N2.

Statistical analysis

Statistical analysis was performed using paired T test and repeated measurement with SPSS software version 16 and $P \leq 0.05$ was considered statistically significant.

RESULTS

In this clinical trial, 55 patients (41 females and 14 males) with recent diagnosis of type 2 diabetes, and age range of 30-75 years (mean age of 53.21 ± 9.75) were enrolled. After 3 months of diet regimen, mean levels of FBS, 2 hours postprandial blood glucose and HbA1c levels decreased significantly. But BMI of the participants showed no statistically significant changes. Also, folate, B₁₂ and Hcy levels did not change significantly (Tables 1 and 2).

Following the 3 months of metformin consumption, the mean values of FBS, postprandial blood glucose, HbA1c, and BMI changes were comparable with diet-alone period. Folate and vitamin

B₁₂ levels did not change significantly again, but serum Hcy level showed higher values after metformin treatment (Tables 3 and 4). We did not find any

relationship between age, sex or plasma glucose level and Hcy concentration. Also, Hcy level showed no relationship with B₁₂ or folate levels after diet regimen and metformin treatment.

Table 1. FBS, 2hpp BS, HbA1c and BMI means before and after diet regimen alone among patients with type 2 diabetes

Variables	Pre diet, Mean(±SD)	Post diet, Mean(±SD)	P value
FBS(mg/dl)	159.21±30.87	134.55±27.85	0.001
2hpp BS(mg/dl)	226.95±146.96	186.46±55.80	0.036
HbA1c (%)	7.61±1.35	7.12±1.05	0.001
BMI (kg/m ²)	28.09±4.06	27.85±3.8	1.000

Measurements are expressed as mean± SD (P value ≤ 0.05 is significant); FBS: Fasting Blood Sugar; 2hpp BS: 2 hours postprandial blood sugar; BMI: Body Mass Index

Table 2. Mean of Homocysteine, vitamin B₁₂ and folic acid levels before and after diet regimen alone among patients with type 2 diabetes

Variables	Before diet Mean(±SD)	After diet Mean(±SD)	P value
Homocysteine (µmol/L)	9.17±3.27	10.35±2.70	0.055
Vitamin B ₁₂ (pg/ml)	260.73±165.28	283.78±185.15	0.147
Folic acid(ng/ml)	4.04±1.97	4.47±1.55	0.060

Measurements are expressed as mean± SD (P value ≤ 0.05 is significant)

Table 3. FBS, 2hpp BS, HbA1c and BMI before and after metformin treatment among patients with type 2 diabetes

Variables	Before metformin, Mean (±SD)	After metformin, Mean (±SD)	P value
FBS (mg/dl)	159.21±30.87	129.25±31.06	0.001
2hpp BS (mg/dl)	226.95±146.96	173.43±51.37	0.008
HbA1c (%)	7.61±1.35	6.9±1.07	0.001
BMI (kg/m ²)	28.09±4.06	28.00±3.87	0.598

Measurements are expressed as mean± SD (P value ≤ 0.05 is significant); FBS: Fasting Blood Sugar; 2hpp BS: 2 hours postprandial blood sugar; BMI: Body Mass Index

Table 4. Homocysteine, vitamin B₁₂ and folic acid means before and after metformin treatment among patients with type 2 diabetes

Variables	Before Metformin Mean(±SD)	After Metformin Mean(±SD)	P value
Homocysteine (µmol/L)	11.90±4.27	13.44±4.93	0.001
Vitamin B ₁₂ (pg/ml)	279.87±160.20	293.28±178.60	0.485
Folic acid (ng/ml)	4.67±1.49	4.95±1.46	0.112

Measurements are expressed as mean± SD (P value ≤ 0.05 is significant)

DISCUSSION

This clinical trial in patients with type 2 diabetes showed statistically significant (P≤0.05) higher values of serum Hcy, but unchanged folate and B₁₂ levels after 3 months of metformin treatment. However, diet alone did not cause significant changes in these variables. Homocysteine, as an independent coronary risk factor, may be more important in diabetic patients. In this regard, previous studies yielded conflicting results (Buysschaert et al., 2000; Pongchaidecha et al., 2004; Masuda et al., 2008). Although its significance is still controversial, in patients with type 2 diabetes, plasma Hcy concentration has been shown to be related to macrovascular disease and death (Palomba et al., 2010; Ramachandran et al., 2012). Meanwhile, some other reports revealed an independent relationship between hyperhomocysteinemia and diabetic microvascular complications (Wile et al., 2010; Lim et al., 2012; Xu et al., 2014). It is noteworthy that Hcy by promoting oxidative stress, inflammation and endothelial damage, like those secondary to hyperglycemia, exerts its atherogenic effects (Aghamohammadi et al., 2011; Huang et al., 2013; Schaffer et al., 2016).

Metformin, as a cornerstone in the treatment of type 2 diabetes for several years, and vitamin B₁₂ or folate deficiency, as its side effects, have raised some questions about its effect on Hcy. Wulffele et al. (2003) in their study as the first placebo-controlled survey in this field suggested the hypothesis that decreased folic acid and vitamin B₁₂ after metformin consumption could result in Hcy elevation. They showed lower B₁₂ and folate but higher Hcy values after 16 weeks of the addition of metformin to insulin. Kilicdag et al. (2005) also in their study on patients with Polycystic Ovary Syndrome (PCOS) found increased Hcy levels in those who received metformin. But at the same time, Hcy level reduced in patients who consumed folic acid and B₁₂ simultaneously with metformin. Moreover, some other studies reported no changes in Hcy concentration after metformin treatment (Pongchaidecha et al., 2004; Carlsen et al., 2007; Rajagopal et al., 2012). Our

survey demonstrated higher Hcy levels after metformin treatment, despite no significant ($P \leq 0.05$) differences in folate and B₁₂ concentrations. Thus based on these findings, one should speculate that metformin exerts its effect on Hcy through different pathways, unrelated to the vitamins. In this regard, Insulin Resistance (IR), as one of the important characteristics of type 2 diabetes, and metformin, as a drug which improves insulin sensitivity, led the researchers to consider a possible relationship between Hcy level and features of IR (Buysschaert et al., 2000; Bar-On et al., 2000; Meigs et al., 2001; Mahalle et al., 2013). Indeed, the metabolic syndrome of IR is a well described cluster of risk factors associated with elevated coronary risk and, also, a risk for subsequent type 2 diabetes mellitus (Meigs et al., 1997; Ji Yeon Kang et al., 2012; Sreckovic et al., 2016).

Several studies on the relationship between Hcy and IR have been published, revealing conflicting results. Some investigations concluded that marked hyperhomocysteinemia could be a feature of IR (Meigs et al., 2001; Rekha et al., 2012; Mahalle et al., 2013). Moreover, some other studies have demonstrated an inverse relationship between these two well-known risk factors of atherosclerotic vascular disease, even in non-diabetic subjects (Bar-On et al., 2000; Gallistl et al., 2000; Fonseca et al., 2003). In fact, their inverse association could be an explanation for our finding of higher Hcy level after metformin treatment. This drug exerts its hypoglycemic effect mainly through the inhibition of gluconeogenesis and reducing IR.

Therefore it can be speculated that in the presence of elevated insulin level, as an anabolic hormone, protein synthesis should be augmented. As a result there may be an increased incorporation of amino acids, including precursors of Hcy into the cells and this may cause the subsequent depletion of Hcy from the plasma. Accordingly it can be concluded that after metformin consumption and improved IR, the reduced insulin level will attenuate its anabolic effect on protein synthesis, leading to higher Hcy levels. Further support for this effect is the fact that insulin causes an increase in translation of mRNA into protein in the liver and muscles (White et al., 1994). Jacobs et al. (1998) conducted studies on streptozotocin-induced diabetic rats to examine the possible role of insulin in regulating plasma Hcy. They found the plasma Hcy level to be approximately 40% lower in the diabetic rats than in the controls. Meanwhile the insulin-treated diabetic rats, maintained a plasma Hcy level comparable with the control rats. Also it is noteworthy that insulin-treated type 1 diabetic subjects, who had normal renal function, were found to have plasma Hcy levels lower than in healthy people (Robillon et al., 1994 and Matteucci et al., 2002). Another study on patients with type 2 diabetes and normal kidney function also found that in patients, basal level of Hcy was 35% lower compared with healthy subjects (Mazza et al., 2005).

These studies provide a metabolic explanation for the reduced plasma Hcy levels seen in both the insulin-resistant and diabetic stages before the onset of renal complications. This reduction seems to be achieved by increases in the activities of two major enzymes involved in the removal of plasma Hcy through trans-sulfuration as well as the remethylation pathways (White et al., 1994; Wijekoon et al., 2007). Another reason for the observed inverse association between IR and Hcy level might be an increased rate of renal excretion of Hcy or its metabolites due to hyperfiltration. This possibility is supported by previous studies which showed a higher glomerular filtration rate and microalbuminuria already in healthy subjects with IR (Wollesen et al., 1999; Meigs et al., 2001; Matteucci et al., 2002; Wijekoon et al., 2007). However diabetes provides an interesting situation with changes in kidney function being superimposed on the already existing metabolic alterations. Therefore it can be assumed that the lower serum Hcy concentrations in insulin-resistant subjects seems to be influenced by hyperinsulinemia and, also, probably by glomerular hyperfiltration. Furthermore, it should be noted that Hcy metabolism varies among different races. For example, blacks metabolize Hcy more effectively than whites. Also, Hcy level can be affected by genetic background, physical activity and different life styles in various populations (Saw et al., 2001; Husemoen et al., 2004; Fakhrzadeh et al., 2006; Daset et al., 2010). Thus it will be necessary that the determinants of Hcy be concurrently analyzed by multi-factors.

One of the limitations of this study was that we did not have any information on nutrition status of vitamin B₆, B₁₂ and folic acid in our subjects. Although we did not measure IR in our subjects which is the other limitation of this study, we can speculate on a metabolic interference of IR and Hcy. Accordingly it seems that there are some differences in the factors associated with Hcy between diabetics and non-diabetics. In this regard, further research on the specific role of these differences is recommended.

CONCLUSION

Our study demonstrated that the increase in plasma Hcy concentrations after metformin treatment was not associated with any significant effect on the level of serum folate or vitamin B₁₂. Subsequently these findings do not conform to the common view that the effect of metformin on Hcy levels is mediated by reduced levels of these vitamins. As the further research, it will be necessary to clarify the other possible mechanisms and clinical implications of Hcy changes secondary to metformin treatment in diabetic patients, e.g. whether Hcy lowering interventions could reduce type 2 diabetic mortality or mortality related to its long term complications.

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Competing interests

The authors have declared that no competing interest exists.

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