The Review of DIABETIC STUDIES

ORIGINAL DATA

Effect of Zinc Supplementation on Serum Homocysteine in Type 2 Diabetic Patients with Microalbuminuria

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■ Abstract

OBJECTIVES: Elevated homocysteine levels are considered to be an independent risk factor for cardiovascular complications in diabetic patients. The aim of this study was to find out if zinc supplementation improves homocysteine levels, which may exert vascular-protective effects in type 2 diabetes subjects with microalbuminuria. METHODS: In a randomized, double-blind, controlled, crossover study, 50 type 2 diabetic patients with microalbuminuria were subdivided into two groups and supplemented with 30 mg/d of zinc (group 1) or placebo (group 2) for three months with a four-week wash out period. Serum creatinine, vitamin B_{12}, folate, fasting plasma glucose, HbA1c, lipid profiles, zinc, homocysteine levels and random urine albumin were measured before and after the first and second phase of the study in all participants. RESULTS: Mean serum zinc was significantly increased after zinc supplementation (from 76 ± 16 μg/dl to 93 ± 20 μg/dl; $p < 0.05$), while there was no change in the placebo group (75 ± 16 μg/dl to 75 ± 15 μg/dl). With zinc supplementation, homocysteine levels reduced significantly (from 13.71 ± 3.84 μmol/l to 11.79 ± 3.06 μmol/l; $p < 0.05$), which did not occur on placebo (from 12.59 ± 2.13 μmol/l to 13.36 ± 2.03 μmol/l). Simple regression was used to show a positive correlation between urine albumin excretion and serum homocysteine ($r = 0.37$, $p = 0.023$). Vitamin B_{12} and folate levels increased significantly in patients who received zinc in comparison to those who received placebo. A negative correlation was observed between homocysteine and vitamin B_{12} concentration ($r = -0.36$, $p = 0.025$). CONCLUSION: Zinc supplementation reduced serum homocysteine and increased vitamin B_{12} and folate concentrations in type 2 diabetic patients with microalbuminuria.

Keywords: diabetes · microalbuminuria · cardiovascular · homocysteine · zinc · vitamin B12 · folate · trace element

Introduction

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia and disorders in the metabolism of carbohydrates, lipids and several essential trace elements [1]. The status of certain essential trace elements, such as Zn, Cu, Cr, Mn and Mg, has already been investigated in patients with type 2 DM [1-3].

Zinc (Zn) forms part of many protein domains, such as zinc metaloenzymes, and is involved in the synthesis, storage and secretion of insulin monomers [1, 4]. It is considered to be deficient if serum zinc is less than 60 μg/dl [5]. Diabetes is usually accompanied by zincuria and consumption of nutritional supplements can substantially increase zinc absorption by the gastrointestinal tract [6]. However, the effects of supplemental zinc, if dietary intake is adequate, are incompletely understood. More clinical data on diabetic patients who are at increased risk of zinc deficiency would be helpful as zinc has an insulinomimetic effect and protects against the oxidative damage associated

with the disease [7, 8]. In addition, in diabetic patients, oxidative damage may result in lower antioxidant micronutrient status, especially trace elements [9]. Abnormal serum Zn status, therefore, can promote the progression of diabetes [2, 10, 11].

In diabetic patients, microalbuminuria is a major factor which predicts the onset of overt renal disease and reflects glomerular dysfunction [12]. Zinc supplementation has beneficial effects on microalbuminuria and serum lipid profile in type 2 DM with microalbuminuria, as we have reported previously [13]. In the present study, we examined the effects of zinc on serum homocysteine levels in the same population. Homocysteine (hCys) is a sulfure-containing amino acid produced by demethylation of methionine (an essential amino acid) [14]. Glomerular filtration rate may be impaired in individuals with type 2 DM [15] and is a major factor in increasing plasma homocysteine concentration [16, 17]. Elevated homocysteine levels are toxic to the vascular endothelium [18]. They result in endothelial dysfunction, promote proliferation of vascular smooth muscle, increase low-density lipoprotein peroxidation and platelet activation and are considered to be an independent risk factor for cardiovascular disease in diabetic [19-23] and non-diabetic persons [24-26].

Two major vitamins, B_{12} and folate, are needed to convert hCys to methionine (Met) by the remethylation reaction [27, 28]. Therefore, folate and vitamin B_{12} deficiency can impair methionine synthesis. Accordingly, a reduction in methylene tetrahydrofolate reductase enzyme activity can cause subsequent homocysteine accumulation in the plasma [29, 30]. It has been shown that redox-reactive compounds can influence the activity of enzymes involved in hCys and Met metabolism, especially betaine-homocysteine methyltransferase (BHMT) and methionine synthase (MetH, remethylated hCys to methionine) [31, 32]. These enzymes are zinc metalloenzymes [33, 34]. So, changes in serum Zn levels, especially its deficiency, can influence the activity of the above-mentioned enzymes and subsequently increase serum hCys level.

To the best of our knowledge, there are no reports about the effects of Zn supplementation on serum hCys levels in type 2 DM with microalbuminuria. As both microalbuminuria and hCys are known risk factors for cardiovascular diseases in diabetic patients and hCys metabolism is related to zinc status, the aim of this study was to determine the effects of Zn supplementation on serum hCys level and to investigate the correlation between serum hCys level and albuminuria in type 2 DM with microalbuminuria.

Research design and methods

In a randomized, double-blind, crossover and placebo-controlled clinical trial, 50 type 2 diabetic patients with microalbuminuria were subdivided into two groups and supplemented with 30 mg/d zinc (group 1) or placebo (group 2) for three months with a four-week wash out period. The dosage of zinc was consistent with that administered in other studies [11, 14]. Patients and procedures have been described in detail in our previous report [13].

The patients were frequently contacted by telephone by one of our colleagues in the Isfahan Endocrine and Metabolism Research Center to monitor their compliance in taking medications. Forty five days after administering the medications (zinc or placebo), the patients were visited and interviewed to elicit possible side effects and to determine their degree of compliance [13]. The IEMRC Medical Ethics Committee approved the study protocol, and all participants provided written consent. The research complied with the current version of the Declaration of Helsinki.

Laboratory assessments

After a 12 h overnight fast, venous blood and urine samples were collected from all patients before any medication was taken. Serum and urine samples were

Table 1. Comparison of serum zinc, folate, vitamin B_{12} and homocysteine in type 2 diabetic patients with microalbuminuria before and after treatment with zinc (first phase) and placebo (second phase) in group 1

Parameter	Zinc (n = 21)		Placebo (n = 18)	
	Before treatment	After treatment	Before treatment	After treatment
Serum zinc (μg/dl)	76.00 ± 16.00	93.00 ± 20.00*	75.00 ± 16.00	75.00 ± 15.00
Folate (ng/ml)	9.71 ± 1.80	12.87 ± 3.01*	12.60 ± 1.59	10.50 ± 1.36
Vitamin B_{12} (pg/ml)	495.75 ± 163.33	549.89 ± 106.52*	510.77 ± 133.50	498.10 ± 119.18
Homocysteine (μmol/l)	13.71 ± 3.84	11.79 ± 3.06*	12.95 ± 2.13	13.36 ± 2.03

Legend: Data are mean ± SD. Group 1: first phase zinc (3 months), second phase placebo (3 months), 4 weeks wash-out in-between. * $p < 0.05$.

Table 2. Comparison of serum zinc, folate, vitamin B_{12} and homocysteine in type 2 diabetic patients with microalbuminuria before and after treatment with placebo (first phase) and zinc (second phase) in group 2

Parameter	Placebo (n = 21)		Zinc (n = 21)	
	Before treatment	After treatment	Before treatment	After treatment
Serum zinc (μg/dl)	73.00 ± 16.00	75.00 ± 14.00	75.50 ± 11.00	95.00 ± 22.00*
Folate (ng/ml)	9.32 ± 1.56	9.92 ± 1.87	10.57 ± 1.34	12.92 ± 1.58*
Vitamin B_{12} (pg/ml)	438.24 ± 102.70	462.00 ± 22.00	464.93 ± 26.00	591.48 ± 130.61*
Homocysteine (μmol/l)	13.46 ± 3.96	13.48 ± 3.68	14.86 ± 3.72	12.89 ± 2.49*

Legend: Data are mean ± SD. Group 2: first phase placebo (3 months), second phase zinc (3 months), 4 weeks wash-out in-between. * $p < 0.05$.

analyzed on the same day for all the variables except for serum zinc, vitamin B_{12}, folate and hCys levels. To measure these parameters, serum samples were stored at -70°C at the IEMRC laboratory and assayed at the end of each study phase. Vitamin B_{12} and folate were measured using a radioimmunoassay (RIA) kit (Diasorin, USA). An enzyme immunoassay (EIA) kit (Axis-Shield Diagnostic, UK) was used to measure homocysteine (free and protein bound). Other laboratory methods applied in the study have been explained in detail in our previously published report [13].

Statistical analysis

Kolmogorov and Smirnov tests were used for dependent and independent variables to assess the normality of the variables prior to further statistical analysis. A paired *t*-test was used to compare the mean of variables before and after zinc supplementation in each group. Student's *t*-test was used to compare the variables between two independent groups before and after each study phase. The Pearson correlation coefficient was used to determine the relationship between serum zinc or homocysteine with urinary albumin excretion and serum vitamin B_{12} with homocysteine concentration. It was decided to consider p-values less than 0.05 to be statistically significant. Statistical analysis of the data was performed using the Statistics Package for Social Science (SPSS version 13.0, SPSS Inc., Chicago, IL, USA).

Results

Demographic characteristics, systolic and diastolic blood pressure, serum lipids, HbA1c, fasting plasma glucose, urinary albumin excretion and serum zinc concentration, at the beginning and end of each phase have been reported in our previous paper [13]. We report here on additional effects of zinc supplementation on serum homocysteine levels in type 2 DM patients to verify possible protective effects on vasculature.

Effects of zinc supplementation on serum zinc level

At baseline in the first phase, serum Zn levels were similar in both groups before supplementation (Tables 1 and 2). In the first and second phase, serum Zn levels increased in both groups receiving supplemental Zn ($p < 0.05$). There was no significant change in Zn levels in the placebo-treated groups (Tables 1 and 2) at the first and second phase of the study. A negative significant correlation ($r = -0.34$, $p = 0.037$) was observed between urinary albumin excretion and serum Zn level (Figure 1A).

Effects of zinc supplementation on serum hCys levels

Serum hCys levels also reduced significantly at the end of the first phase in group 1 (zinc supplementation) compared to the second phase (hCys: 11.79 ± 3.06 μmol/l vs.13.36 ± 2.03 μmol/l) (Table 1). Figure 2A compares serum hCys levels in type 2 diabetic patients before and after administration of Zn or placebo in group 1 and 2. A positive significant correlation ($r = 0.37$, $p = 0.023$) was found between urinary albumin excretion and serum hCys level (Figure 1B).

Effects of zinc supplementation on serum folate and vitamin B_{12} levels

As shown in Table 1 and Figure 2B, folate and vitamin B_{12} significantly increased ($p < 0.05$) in the first phase in group 1 (Zn supplementation) in comparison to the second phase (folate: 12.87 ± 3.01 ng/ml vs. 10.50 ± 1.38 ng/ml and vitamin B_{12}: 549.89 ± 106.52 pg/ml vs. 498.10 ± 119.18 pg/ml). In group 2 (patients who received placebo in the first phase and zinc in the second phase), serum levels of folate and vitamin B_{12} did not significantly change at the end of the first phase (Table 2 and Figure 2B). In addition, an inverse correlation was found between serum hCys and vitamin B_{12} levels ($r = -0.36$, $p = 0.025$) (Figure 3), while

no significant relationship was observed between serum folate and hCys concentration.

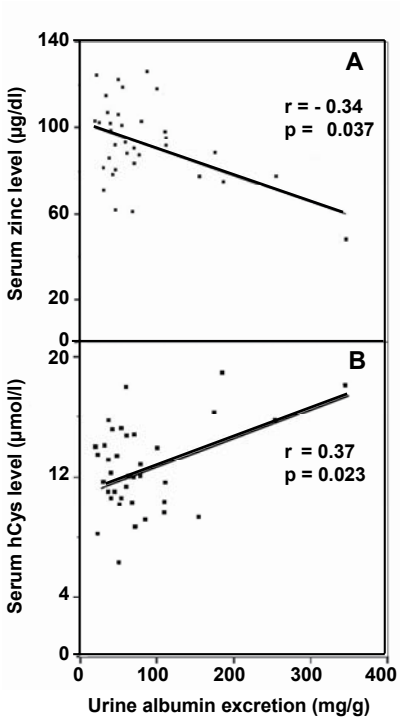

Figure 1. Correlation between serum zinc (**A**) and homocysteine (**B**) levels with urine albumin excretion in diabetic patients with microalbuminuria. Each individual value is represented by a dot. r = Pearson correlation coefficient.

Discussion

Clinical research suggests that the homeostasis of essential trace elements, especially zinc, can be disrupted by diabetes mellitus [1, 6, 35, 36]. Our data showed that zinc supplementation causes an increase in serum Zn level, as reported in other studies [4, 37]. This finding proved the good compliance shown by our patients in taking zinc supplementation.

Previous reports have demonstrated beneficial effects of zinc supplementation on the burden of oxidative stress [38, 39], retinopathy [40-42] and on the reduction in microalbuminuria in subjects with type 2 DM [13]. In type 2 DM with proteinuria or renal insufficiency, hCys usually increases [43, 44] and is an independent risk factor for arteriosclerosis in both diabetic and cardiac patients [38, 45]. Several studies have already investigated the correlation between serum hCys level and cardiovascular outcomes in type 2 DM [22, 38]. In normal subjects, but not in type 2 DM, insulin may play a role in hCys metabolism. Another finding is that hyperinsulinemia decreases serum hCys concentration [30].

It was found that insulin resistance in type 2 DM is associated with abnormalities in renal function and leads to an elevation in the hCys level that may finally cause subclinical renal dysfunction [46]. Moreover, it has been reported that hyperhomocysteinemia predicts the development of microalbuminuria in type 2 DM [47], and that supplemental Zn can improve the serum hCys level. It was therefore interesting to find out if Zn supplementation can reduce hCys levels in type 2 DM patients with microalbuminuria and thus positively affect albuminuria in these patients. We found that the consumption of Zn has a beneficial effect in that it improves serum hCys level and decreases both hCys concentration and albumin excretion in type 2 DM (Figure 2A, Table 1 and Table 2).

The effect of supplemental Zn on serum hCys concentration may be due to an influence on BHMT and methionine synthase (MetH) enzymes which convert hCys to Met in mammals and thereby exert important effects on the cellular and plasma levels of hCys [31, 33]. These enzymes are zinc metalloenzymes, which suggests that cysteine residues are involved in the zinc binding site of these enzymes. Zn is required for the binding of hCys to BHMT [33, 34]. It was shown that zinc ions activate hCys for its conversion to methionine [48]. BHMT is a key liver enzyme which is important for hCys homeostasis and accounts for approximately half of the methionine synthesized in the liver [33]. There is evidence that some of the zinc-dependent enzymes activating alkyl transfer to thiols, especially BHMT and MetH, are prone to oxidative inactivation and loss of zinc. Inactivation has been assumed to involve formation of a disulfide incorporating at least one of the cysteine ligands to zinc [48]. Castro *et al.* have shown that incubation of BHMT with oxidant agents can release Zn from the active site of the enzyme [32]. The presence of zinc as a component of the oxidant defense system is supported by findings obtained from studies conducted in animal and cell models, which indicate that zinc deficiency induces oxidative damage to cell components and alterations in antioxidant enzymes [39]. As zincuria is observed in diabetes mellitus, Zn deficiency could lead to inactivation of the above mentioned enzymes. Zn supplementation in diabetic patients increases plasma Zn level and probably promotes BHMT and MetH activity to decrease the plasma hCys level.

Homocysteine metabolism is dependent on the concentrations of B vitamins; that is, folate, serum vi-

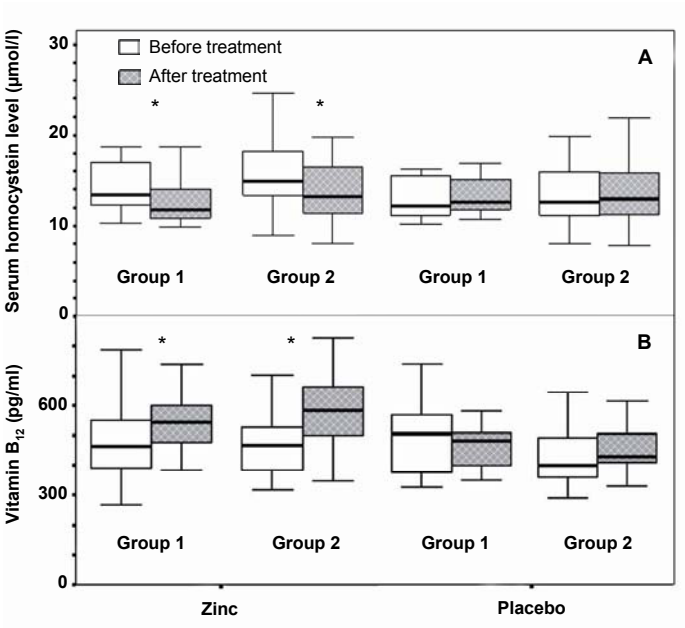

Figure 2. Serum homocysteine (**A**) and vitamin B_{12} (**B**) levels in type 2 diabetic patients with microalbuminuria before and after treatment with zinc or placebo in group 1 and group 2. Group 1 received zinc in the first and placebo in the second phase, while group 2 received placebo in the first and zinc in the second phase. * $p < 0.05$.

tamin B_{12} and vitamin B_6 status, which may play some part in the cardiovascular events mediated by hCys plasma concentrations [49]. Low circulating folate and vitamin B_{12} concentrations lead to high fasting total homocysteine concentrations in humans [50]. In turn, elevated fasting total homocysteine concentrations are usually normalized by administration of vitamin B_{12} and folic acid [50, 51]. In our study, vitamin B_{12} and folate levels were within the normal range at the beginning of the study. Zn supplementation, however, led to a significant rise in serum levels, which are important for hCys metabolism. Moreover, our results showed an inverse correlation between serum hCys and vitamin B_{12} in the groups given supplemental Zn (Figure 3), which has already been observed by other investigators [52-54]. The increased vitamin B_{12} and folate in our study is probably due to a reduction in serum hCys brought about by an elevated plasma Zn level, which promotes BHMT and MetH activity to decrease plasma hCys level.

It has also been demonstrated that hyperhomocysteinemia can be associated with albuminuria and renal failure [16, 17]. Therefore, urinary albumin excretion rates correlate with fasting plasma hCys concentration. In patients with diabetes and microalbuminuria, fasting hCys concentrations are higher than they are in those with normal albumin excretion. On the other hand, Zn supplementation resulted in the reduction of microalbuminuria in subjects with type 2 diabetes [13]. Therefore, we conclude that the increased levels of serum vitamin B_{12} and folate in the present study resulted from the decreased albumin excretion and promotion of BHMT and MetH activity brought about by Zn supplementation.

With the present study, we have shown that Zn supplementation improves the hCys and albumin profile of diabetic patients with microalbuminuria. However, we do not know if positive effects on vascular inflammation or insulin capacity could play critical roles in the underlying biological mechanisms. The measurement of inflammatory markers, such as highly sensitive C-reactive protein (HS-CRP), IL-6 and fibrinogen, could shed some light on this question. These measurements were not taken into consideration, which limits the explanatory power of our study in this regard. Nor did we perform IVGTT, which could supply information about the patients' insulin capacity. IVGTT could help to determine if Zn supplementation has an insulinomimetic effect, which may in part be due to decreased homocysteine levels. Therefore, we recommend to include IVGTT and inflammatory markers in future studies.

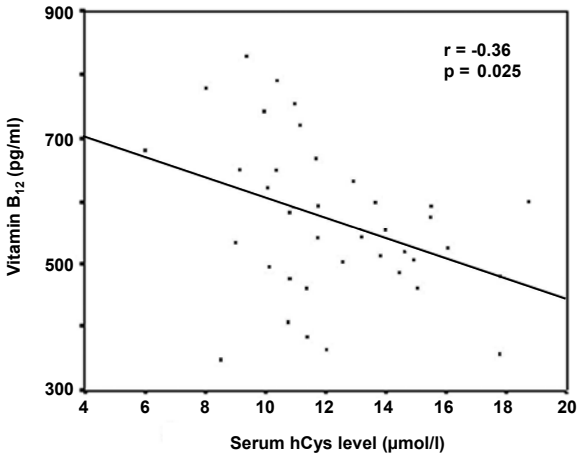

Figure 3. Correlation between serum homocysteine level and vitamin B_{12} in type 2 diabetic patients with microalbuminuria. Each individual value is represented by a dot. r = Pearson correlation coefficient.

Conclusion

Supplemental zinc decreases serum hCys and increases serum vitamin B_{12} and folate in type 2 diabetic patients with microalbuminuria. Therefore, zinc supplementation may be used to decrease hCys in type 2 DM with microalbuminuria.

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Conflict of interest statement: The authors declare that they have no competing conflict of interests with respect to financial or other issues.

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