The Urinary Levels of Some Essential and Toxic Metals in Type 2 Diabetic Patients and Non-diabetic Healthy Control Human Subjects

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ABSTRACT

Objective: To compare the levels of some selected essential and toxic metals in type 2 diabetic (T2D) patients and non-diabetic (ND) healthy controls. Furthermore, study aims to evaluate the possible interrelationship of metals in urine of type 2 diabetic (T2D) patients and non-diabetic (ND) healthy controls.

Methods: The study was conducted at Diabetes and Cardio-Metabolic Disorder (D&C-MD) Laboratory, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan, which comprised of T2D patients and an equal number of ND healthy controls. Two biofluids i.e. blood and urine samples of diabetes and control subjects were considered. Magnesium (Mg), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), manganese (Mn), iron (Fe) and zinc (Zn) levels were measured in urine samples of both groups. Metal concentrations were assessed by atomic absorption spectrophotometry, whereas biochemical parameters in sera of the study subjects were estimated using commercial kits on Microlab-300. Graph Pad Prism 5 was used for statistical analyses.

Results: There were 49 T2D patients with an average age of 53.5±10.4 years and 49 ND healthy controls with a mean age of 45.2±9.2 years. Fasting serum glucose and triglycerides were significantly ($p<0.05$) higher in diabetic relative to their non-diabetic control subjects. The levels of Mg, Zn and Pb were found to be significantly higher ($p<0.05$), while Fe was decreased more than 30 percent in urine samples of T2D patients compared to the ND control group. Cu, Cr, Mn and Ni manifested no change. A significant positive correlation of Pb with Zn ($r=0.3284$, $p=0.02$) and Mn ($r=0.3648$, $p=0.01$) was found in T2D patients.

Conclusions: An imbalance in the levels of selected metals was observed in urines of type 2 diabetes when compared with controls. Furthermore, statistical results revealed positive correlations between toxic metal Pb with essential trace elements i.e. Mn and Zn. Thus it could be concluded that toxic metals, especially Pb may have a role in renal dysfunction with successive loss of essential metals such as Zn and Mn through urination.

Keywords: Essential and toxic metals, type 2 diabetes, urine

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INTRODUCTION

Diabetes mellitus (DM) is one of the life style related diseases that is the most widespread worldwide. DM is usually classified as type 1 diabetes (T1D) and type 2 diabetes (T2D). In former case the hyperglycemia results from a severe insulin deficiency caused by the destruction of pancreatic beta cells (1). Over the time it can be regulated by using different insulin modalities (2,3). In latter case glucose elevation develops, because of insulin resistance (IR) and insufficient insulin secretion. Insulin is secreted from pancreatic beta cells in response to increased blood glucose level. Insulin binds to its receptor on the surface of target tissues and stimulates glucose uptake by the cells, result in blood glucose level is decreased. A decreased response to insulin may result in impaired glucose tolerance or T2D which is characterized by elevated blood glucose level and insulin resistance (4). Several factors such as heredity, age, smoking, obesity, diet, sex, sedentary life style, socio-economic status, hypertension etc. are involved in the disease development. Among all these parameters balanced diet and active lifestyle play vital roles in T2D prevention (5,6).

The quality and quantity of fat and carbohydrates based diet intake have significant impact on the risk of T2D (7). Imbalance in biologically active form of many metals such as iron (Fe), magnesium (Mg), zinc (Zn), chromium (Cr), manganese (Mn), lead (Pb), nickel (Ni), copper (Cu) etc. participate in the alteration of glucose metabolism of T2D patients (8). These metals are involved in the structural and catalytic activities of many metalloenzymes. Mg is needed as a cofactor for more than 300 enzymes, particularly involved in glucose homeostasis. Reduced plasma level of Mg has been reported in poorly controlled T2D patients (9,10). Prolonged hyperglycemia is also due to increased and decreased urinary and blood plasma levels of Zn. The decreased plasma level of Zn adversely affects the ability of pancreatic islet cells to

produce and secrete insulin (11). Zn plays key role in the storage and secretion of insulin. Previous studies show that people with type 2 diabetes have suboptimal Zn status due its low absorption and increased urinary excretion (8). Cu is needed for the catalytic activity of superoxide dismutase (SOD) that takes part in the protection of cells from superoxide radicals (8).

Whereas Zn protects sulfhyryl groups of proteins and enzymes by its antioxidant properties from free radical attack in the body. It is also reported that glycated proteins bind transition metals and their glycocholates play key role in the etiology of peripheral vascular dysfunction in T2D (12). Chromium (Cr) is involved in glucose metabolism by enhancing the effects of insulin. As certain low molecular weight Cr (LMWCr) compounds may enhance the response of insulin receptors to insulin. The ability of LMWCr compounds to activate the insulin receptors depend upon its Cr contents (13). Fe is a potential candidate in many cellular reactions that produce reactive oxygen species. Such reactions contribute to tissue damage and increased oxidative stress, thereby leading to the pathogenesis of T2D. Numerous evidences have also suggested the link between high body iron store and diabetes pathogenesis (14,15).

Similarly, lead (Pb) is dangerous to most of the human body organs and interferes with metabolism and cellular functions. Some of other toxic metals such as Cadmium (Cd), Arsenic (As) are implicated to disrupt the glucose uptake and alter the related molecular mechanism in glucose regulation (16,17). The concentration of these metals in various biofluids is adversely changed with disease state, particularly in serum and urine. Among these biospecimens, urine is good since it is freely obtainable and non-invasively tested (8). Therefore in the present study, urine samples of T2D patients were considered to assess various metals' levels in their samples

relative to the non-diabetic apparently healthy control subjects. Mutual interactions of toxic and essential metals were also calculated.

MATERIAL AND METHODS

Study subjects

This case-control study comprised of 98 Pakistani Punjabi adults including 49 non-diabetic (ND) control subjects and 49 well characterized type 2 diabetic (T2D) patients. The ND and T2D age (mean ± S.E.) was 45.2±9.2 years and 53.5±10.4 years respectively, with an age range of 35-80 years. A well-defined questionnaire was used to obtain information regarding occupation, physical activity, life style patterns and dietary habits, and their past and current illness as well as medication status. Their anthropometric measurements of body height, weight, waist and hip circumference were also measured. Type 2 diabetic patients with nephropathy and malignancy were not included in the present study. After measuring height and weight, BMI (Kg/m^2) was calculated by dividing weight (kg) to height in meter square (m^2). An informed consent was obtained from all subjects for their participation in this study. For the collection of urine and blood samples, camps were organized in Faisalabad, Pakistan. All subjects were informed to attend sample collection sites on a specific day in the morning after 12-14 hours overnight fast. During fasting period subjects were only allowed to drink plain water. The study received an approval from the institutional ethics committee.

Biological samples (Blood and Urine)

A venous blood sample was taken in gel containing vacutainer without anticoagulant between 8-10 AM, after an overnight fast of 12-14 hours duration. The blood taken in vacutainers was allowed to clot and subsequently centrifuged for 10 minutes at 900 rpm to separate the serum, which was stored at -20°C prior to downstream analysis. Sera were analyzed for biochemical tests such as fasting glucose, cholesterol, triglycerides, total protein etc. These biochemical parameters were estimated by colorimetric/ spectrophotometric methods using commercial kits (DiaSys, Germany) on a semi-automatic clinical chemistry analyzer (Microlab-300, Merck, Germany), as described by Khan *et al* (18).

The urine samples of the same ND control subjects and T2D patients were also collected for the analysis of various metals. Different aliquots were stored at -20ºC until carrying out further analyses.

Urinary metals analysis

Varian double beam flame atomic absorption spectrophotometer (FAAS) (AA240 FS, Australia), with manual background correction was employed for the determination of transition metals such as Zn, Cr, Cu, Mn, Pb, Ni, Fe and macronutrient such as Mg in urine samples of type 2 diabetes and control subjects according to the method described by Khan *et al* (19); Kazi *et al* (20), with few alterations. The analyses were carried out by using hollow cathode lamp of respective metal under standard instrumental operational conditions. Acetylene gas was used as fuel.

Analytical procedure

For minerals estimation, 5ml of each urine sample was digested in aqua regia and heated up to dryness. The residues were dissolved in 10ml conc. HNO_3 and heated carefully until the solution turned colorless, then transferred them into 25ml flasks and volumes were made up to the marks

by de-ionized water. These sample solutions along with standards were aspirated to FAAS. For Mg estimation, serum samples were diluted (1ml of urine in 25ml aqueous solution) with distilled water prior to their aspiration into atomic spectrophotometer for analysis. The amount of each element in the sample was determined from its respective calibration curve. The analysis of each sample was made in duplicate and averaged value is reported in results.

Statistical analysis

Statistical analyses were executed using MS Excel 2010 and Graph Pad Prism 5. All results were expressed as mean±standard errors (S.E.). Threshold for two-tailed p-value significance was set at $p<0.05$. Correlations were carried out using Pearson correlation coefficient(r).

RESULTS

General physical and biochemical characteristics of study subjects

General characteristics and laboratory findings of the studied human subjects are given in Table 1. A total of 49 ND control (males = 28 and females =21) subjects and 49 T2D (males = 20 and females = 28) patients were considered for selected metals analysis such as Cu, Mg, Cr, Fe, Zn, Ni and Mn using atomic absorption spectrophotometer (AAS). The mean age of the non-diabetic (ND) control and T2D subjects was 45.1±9.2 years and 53.5±10.4 years respectively. Mean BMI (28±0.7 kg/m^2) of T2D patients was significantly higher ($p=0.005$) than the BMI (25.5±0.34 kg/m^2) of ND control group. There was also a significant difference ($p=0.004$) in the mean value of WHR (0.97±0.08) of T2D patients when compared with the WHR (0.92±0.6) of ND control group. Fasting serum glucose and triglycerides were significantly ($p<0.05$) higher in diabetics relative to their non-diabetic control subjects. On the other hand, fasting serum cholesterol, total

proteins, creatinine were not significantly ($p<0.05$) different between T2D and ND subjects (Table 1).

Urinary metal levels in T2D patients versus non-diabetic (ND) control subjects

In urinary metal analysis, the levels of Mg and Zn were found to be significantly higher ($p<0.05$) in urine samples of T2D as compared to the ND control group. The mean values of Mg and Zn were 17.7±1.6 ppm and 0.46±0.03 ppm for T2D patients, while in case of ND control group; the levels of Mg and Zn were obtained as 11.48±1.3 ppm and 0.33±0.03 ppm respectively. The statistical analyses show that the levels of these two metals; Mg and Zn were 35% and 28% higher in urine samples of T2D patients relative to their respective ND control subjects. On the other hand, in urine samples, the Fe level (1.16 ppm) in T2D group was found to be lower (p: 0.005) than the value (1.77±0.2 ppm) obtained for Fe in urine samples of ND control human subjects. The Pb metal was found to be significantly higher ($p= 0.03$) in urine of diabetics relative to the ND control group. The comparative levels of Pb in T2D patients and ND control subjects were 0.047±0.003 ppm and 0.036±0.003 ppm respectively. The calculated percent fold increase of Pb in the urine samples of T2D patients was about 29% as compared to the ND control subjects. The comparative urinary levels of Cu, Cr, Mn and Ni were higher in T2D patients than in ND control subjects, but these differences were not statistically significant (Figure 1).

Figure 2 shows a significant positive correlation of Pb with Zn ($r=0.3284$, p: 0.02) and Mn ($r=0.3648$, p: 0.01) in the urine samples of T2D patients. On the other hand, no correlation was found between Pb and other studied metals such as Mg, Fe, Cu, Cr, Mn and Ni. Similarly no relationship was found among Mg, Mn, Zn, Fe, Cu, Cr, Mn and Ni in the same diabetic urine samples. ND control subjects and T2D patients were subdivided into ND control males versus

ND control females and T2D males versus T2D females for further analysis to investigate gender related changes in the metal concentrations.

Urinary metal levels of ND control male versus ND female subjects

The statistical analysis of the respective metal levels showed no significant difference in any of the above mentioned metals between ND control male versus ND control female sub-groups. However, a close look shows that all metals including Zn, Cu, Mg, Pb, Cr, Mn and Ni were found to be higher in the urine samples of ND males, except Fe metals which was 10 fold higher in urines of ND females.

Urinary metal levels between T2D male and female patients

Similarly, no significant difference was found between T2D male and female patients with respect to urinary Mg, Pb metals and other trace elements including Cu, Zn, Fe, Cr, Mn and Ni. In this case, urinary levels of Cu, Fe, Cr and Ni were observed non-significantly higher in T2D male versus T2D female patients. On the other hand, Zn, Mg, and Mn concentrations were marginally higher in T2D females while comparing with T2D male patients.

DISCUSSION

It has already been established that hyperglycemia in diabetic patients is linearly linked with the production of advanced glycation end products (AGEs) of several proteins and highly reactive oxygen species (ROS) such as H_2O_2, OH and O_2^- (21). Metals are central to hold the bioactivity of numerous enzymes and proteins on which life is based. Derangements in the metal (e.g. Zn, Mg, Cu, Mn, Cr, etc) metabolism could lead to the pathogenesis of diabetes. Imbalances in the status of essential metals have been reported as a worsening incidence in the progression of

diabetes (22). Several previously published studies manifested the functions of transition and trace elements Fe, Zn, Mg, Cu, Mn and Cr in insulin action and carbohydrate metabolism. The alterations in these elements attributed to hyperglycemia and enhanced magnitude of protein glycation in diabetic state. A case-control study revealed alterations in the metabolism of metallic elements such as Zn, Mg, Cu, Mn, Cr, etc in diabetic patients relative to their non-diabetic (ND) control subjects (23).

In the present study, an increased urinary level of Mg was observed in Type 2 Diabetic (T2D) patients compared to the ND control subjects. These findings are supported by a previously published report (24), in which negative correlation was reported between serum Mg and urinary Mg levels in diabetic patients. This correlation revealed a decrease of serum Mg with concomitant increase of urinary Mg level. Renal Mg excretion may be regulated by insulin. The mechanism of the urinary loss of Mg may result probably from the reduced capacity of tubular reabsorption of Mg due to osmotic action of glycosuria (9). Previous studies have also revealed an association between hypomagnesaemia and elevated glucose level. Yet, another study correlated Mg deficiency with fat accumulation in various body tissues which subsequently leads to obesity-related metabolic disorders (25). This provides a further explanation to high presence (loss) of Mg in the urine of T2D patients as more than 60% of them are obese.

Urinary Fe concentration was less in T2D patients than ND control subjects. This agrees with the previously published results (23), in which concurrent elevation of serum iron level with decrease urinary excretion of Fe was observed in diabetic patients. Several studies manifested possible association between high iron stores and various metabolic parameters such as blood serum insulin, glucose, dyslipidemia and obesity (26). Iron is a potential catalyst that produces

ROS, which subsequently develop oxidative stress and damages healthy tissues; thereby increasing the risk of T2D (27).

Copper concentrations in various biofluids including urine have been found to be altered in diabetic subjects. Higher urinary level of Cu was observed in T2D patients compared to the ND control subjects in the present study, though the difference was not significant. These results are consistent with the previously published results (28). It has also been manifested that Cu deficiency is an important cause of adiposity development (29).

Urine zinc (Zn) level was significantly higher in T2D patients compared to the ND control subjects. Previous clinical studies have reported excessive loss of Zn through urine with decreased values in the blood serum in diabetes as compared to ND control subjects (23,30). The decreased gastrointestinal absorption with increased urinary excretion of Zn in diabetes may lead to hypozincemia. It has been suggested that prolonged hyperglycemia may adversely affect the transport of Zn back into the renal tubules. In addition to this, mutation in the Znt8 transporter gene (*SLC30A8*) contributes to Zn deficiency. Zn deficiency in diabetes may be associated with increased oxidative stress and tissue damaging (23,31). Furthermore, Zn is required for the synthesis of insulin hexamer which is vital to keep the blood sugar level in normal range. It has been reported that mutation in *SLC30A8* gene is associated with type 2 diabetes (8,32).

In the present study, the level of Cr in the urine samples of T2D patients was higher than those of respective ND control subjects but the difference was not significant. It has been extensively studied that Cr takes part in increasing receptors number and binding of insulin to insulin-receptors in target organ (33). Trivalent chromium (Cr III) is effective for glucose uptake by the cells. Cr urinary excretion is expedited in hyperglycemic condition (33,34). Anderson *et al* reported antioxidant effect of the combined supplementation of Cr and Zn in T2D patients

(31). Mn urinary level was higher than their respective ND healthy control subjects. There is no data available in literature, which shows comparative urinary excretion of Mn in T2D patients relative to their ND control group. Nevertheless, studies report the possible involvement of Mn deficiency in the pathogenesis of diabetes mellitus (23). However, it has been reported that oral Mn administration improves the insulin sensitivity in diabetic patients (35).

In this study, mean levels of two toxic metals such as Pb and Ni was studied in T2D patients versus ND control subjects. The results of the present study revealed that the urinary level of Pb was significantly higher in T2D patients as compared to the ND control subjects. Pb is heavy transition metal which is harmful to most of the human tissues, and interferes with the body's physiological functions (20,36). The Pb is ubiquitous environmental toxin that is associated with wide range of biochemical and physiological dysfunctions (29). A recent study revealed that environmental exposure to Pb is contributing in the development of neuropathy related complications in T2D patients (37). Several studies have manifested a positive correlation between environmental Pb exposure and reduction in renal function efficiency, and Pb chelating therapies may be effective to improve renal function in ND subjects with chronic kidney disorders (38). Pb also imparts to various metabolic disorders including obesity by substituting vital trace elements (TE) e.g. Zn, Cr, Cu, etc. with sequential depletion of anti-oxidative potential of blood in individuals who were frequently exposed to toxic heavy metals including Pb (29).

In the present study, Pb shows positive associations with two essential trace elements (TEs) such as Zn and Mn in urine specimens of T2D patients. Both these TEs (Zn and Mn) act as cofactors for the activity of several enzymes. The increased interactive urinary excretion of TEs such as Zn with toxic Pb metal might adversely affect the levels of these two essential metals in blood sera of T2D patients. As interactions of toxic metals including Pb with essential trace

elements have been extensively studied (29). Continuous loss of Zn and Mn over time through urination causes deficiency in TEs levels that may demonstrates the decrease of Zn and Mn in blood of diabetic patients (39).

The urinary Ni level was not different between T2D patients and ND control subjects. There is scant information available in literature; some human and animal studies indicate that Ni deprivation lowers blood plasma glucose level which subsequently alters metallic elements (Zn, Fe, Cu, etc) tissue distribution and metabolism (40). An animal study shows that Ni induces oxidative stress in body organs particularly in kidney (41).

In present study, no association was found between the urinary levels of aforementioned metallic elements with age, gender, clinical and anthropometric characteristics. Kazi *et al* reported sex and age related impact on the bioavailability of TEs (23). The reason for the disparity in the findings is not yet clear and may be attributed to sample size. Simultaneously, the analysis of metals in various biological specimens including urine has been conducted in several previous studies. Among them blood plasma and urine may be more beneficial, since derangements of metals in blood seems to be reflected in urine of diabetic patients (10). Moreover, urine can be obtained easily and non-invasively compared to other biofluids such as blood plasma/serum, lacriminal fluid, synovial fluid etc. (8), therefore urine was used in this study.

CONCLUSION

In conclusion, our findings indicate that diabetic patients have distinct changes in their urinary metals status with excessive loss of essential trace elements, which might be due to renal dysfunction caused by increased accumulation of toxic metals.

LIMITATIONS

As primary goal of the study design was to assess urinary levels of some selected metals in humans, especially in T2D patients. Apart from reasonably good results, there are some limitations of this study. Firstly, this is a representative study of a small group of Punjabi population from Faisalabad, Pakistan. Thus, results cannot be generalized. Secondly, serum samples were not considered for said metals investigation. However, findings of this study are congruent with previously published studies in this area. Thus it is hoped that ongoing research efforts will not only elucidate the relationship among different metals in various biofluids including urine, but will also aid in better understanding the role of metals in diabetes mellitus development.

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AUTHORS' NOTE

Authors declare that they have no conflict of interest about this research publication.

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Table 1: Anthropometric and biochemical characteristics of ND and T2D subjects

Subjects Parameters	Non-diabetic controls (N=49)	T2D patients (N=48)	p-value
BMI (Kg/m^2)	25.5±0.34	28±0.7	0.005
WHR	0.92±.06	0.97±0.08	0.004
Glucose (mg/dl)	88.7±1.7	199.4±10.7	<0.0001
Cholesterol (mg/dl)	183.5±6.6	176.3±6.2	NS
Triglycerides (mg/dl)	127.9±9.9	179.3±15.07	0.005
Total proteins (g/dl)	7.7±0.5	7.6±0.13	NS
Creatinine (mg/dl)	0.73±0.05	0.78±0.04	NS

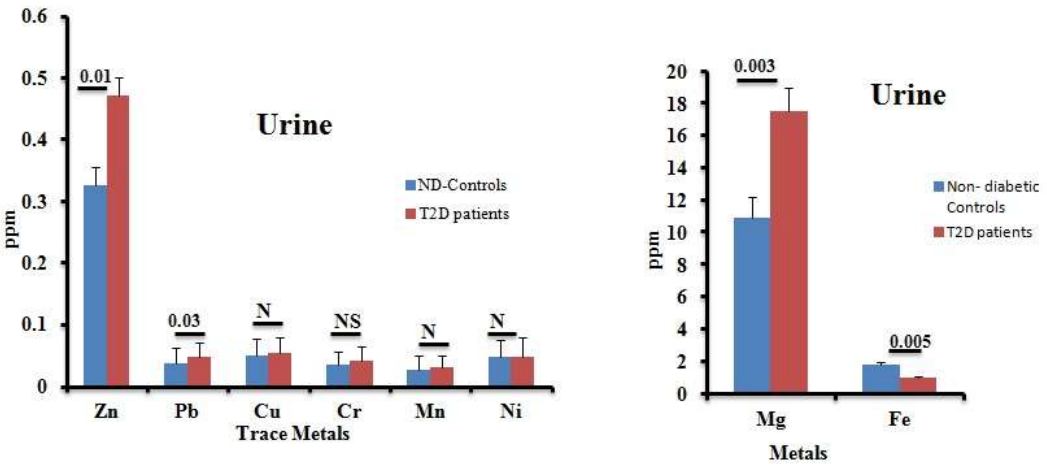

Fig. 1: Comparative urinary metals status of type 2 diabetics and ND control subjects.

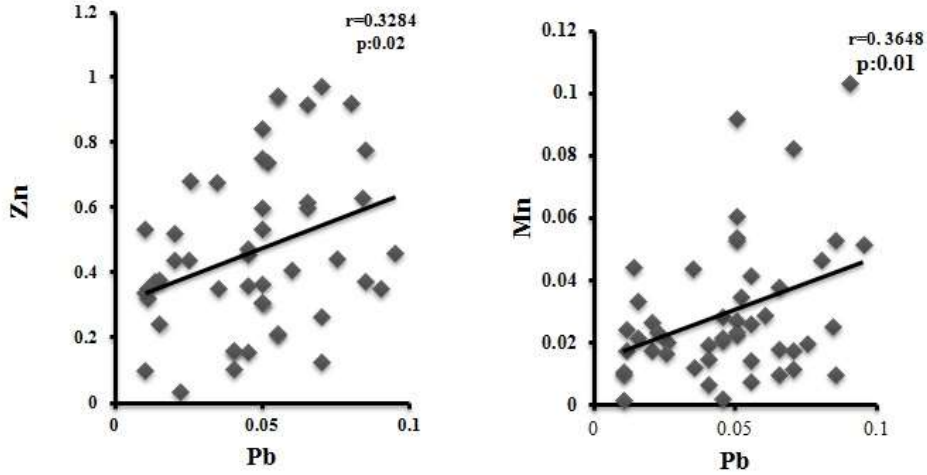

Fig. 2: Correlation analysis of Pb with Zn and Mn in the urine samples of T2D patients.