THE EFFECT OF PLASMA THIAMINE DEFICIENCY ON THE ANTIOXIDANT STATUS OF TYPE 2 DIABETICS HAVING DIABETIC NEPHROPATHY

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ABSTRACT

Objective: To see the effects of plasma thiamine on the antioxidant status of the body by measuring plasma thiol levels in type 2 diabetic patients with diabetic nephropathy.

Study design: Cross sectional comparative study.

Place and duration of study: Department of Biochemistry and Molecular Biology, Army Medical College, Rawalpindi, from January 2009 to December 2010.

Methodology: The study included 80 participants, 20 were control and 60 were known diabetics who were inducted from various diabetic clinics of urban areas of Rawalpindi. The participants were divided into three groups based on their albuminuria status. Group I (n=20) consisted of healthy volunteers having blood glucose level <6 mmol/L, group II (n=20) consisted of normoalbuminurics type 2 diabetics, groups III (n=20) consisted of microalbuminuric type 2 diabetics and group IV (n=20) consisted of macroalbuminuric type 2 diabetics. Fasting blood samples of diabetic and control groups were analyzed for glucose, glycosylated hemoglobin (HbA1c) for the assessment of glycemic status, thiol for antioxidant status, thiamine chloride and thiamine monophosphate for assessment of thiamine status. Twenty four hour urine samples were analyzed for microalbuminuria, thiamine chloride and thiamine monophosphate.

Results: Plasma thiol levels were significantly reduced in diabetics as compared to normal controls. Moreover, the thiol levels decreased with increasing urinary albumin excretion, being lowest in the macroalbuminuric group. Furthermore, a significant positive correlation was found between plasma thiamine and plasma thiol levels in all the groups.

Conclusion: Thiamine levels are reduced in the diabetic population and this thiamine deficiency is positively correlated with plasma thiol levels in diabetics. Furthermore this progressive decline in the plasma thiol level corresponds to the increase in urinary albumin excretion.

Keywords: Antioxidant status, Diabetes mellitus, Macroalbuminuria, Microalbuminuria, Thiamine chloride, Thiamine monophosphate, Thiol

INTRODUCTION

Diabetes mellitus is growing like an epidemic in the entire world but more so in the developing countries. A study published in 2011 revealed that 15.41% men and 12.31% women in Pakistan had diabetes, making a total prevalence of 13.41%. Impaired glucose tolerance (IGT) was found in 5.14% men and 5.78% women, a total prevalence of 5.61%. The overall frequency of IGT was 20.55% in men and 18.09% in women of Pakistan[1]. Vascular complications of diabetes develop progressively after 5 to 40 years of onset of diabetes or IGT despite strict control of plasma glucose level. This leads us to consider other factors which may affect or improve the vascular status in diabetes.

Thiamine and thiamine pyrophosphate act as vital coenzymes for several steps of metabolic pathways of the body including the hexose monophosphate shunt. It is believed that diabetes is a thiamine deficient state, if not in absolute terms then at least in relative terms, due to amplified glucose metabolism[2]. Thiamine and transketolase levels are reduced in diabetics as compared to healthy controls[3].

Hyperglycemia is known to initiate a series of cellular events that lead to the production of reactive oxygen species (ROS). Some of these mechanisms include: glucose auto-oxidation, nonenzymatic protein glycation and activation of polyol pathway [4]. The enhanced production of ROS is induced by the release of superoxide anion via the mitochondrial electron transport chain in response to hyperglycemia[5].

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Superoxide anion then promotes a cascade of endothelial processes that engage increasing numbers of cellular elements to produce oxygen derived free radicals. The intracellular and the extracellular reduced thiol (–SH) groups either in free form (reduced glutathione) or bound to proteins (protein bound thiols) play a major role in maintaining the antioxidant status of the body[6] by reducing the reactive free radicals. Studies indicate low thiol levels in diabetes mellitus[7]. Thiamine and its dependent enzymes, transketolase (TK), pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and the branched chain α-ketoglutarate dehydrogenase complex are involved in the maintenance of NADPH levels and carbohydrate metabolism in the cell[8]. High dose thiamine and benfotiamine both have been found to reduce oxidative stress, PKC activation and hexosamine levels and reducing the risk of diabetic vascular disease[9].

The objective of our study was to determine the levels of thiamine and thiol in diabetics and to find a relation between plasma thiamine and plasma thiol levels to see if thiamine deficiency can compromise the body's antioxidant status.

METHODOLOGY

This quasi experimental study was conducted at the Department of Biochemistry and Molecular Biology, Army Medical College, Rawalpindi from January 2009 to December 2010. A total of 80 participants were included in the study through non-probability convenience sampling. Diabetic patients between the ages of 18 to 65 years, with a body mass index between 19-40 kg/m2 and a history of 5 or more years were included in the study. All these patients were on treatment with oral hypoglycemics (n=37) or insulin (n=23). Age and gender matched healthy volunteers having fasting blood glucose level below 6 mmol/L were inducted in the control group. Group I (n=20) comprised healthy volunteers inducted from colleagues and family of the researches. Known diabetics were enrolled from the diabetic clinics of Military Hospital and Holy Family Hospital, Rawalpindi and were divided into three more groups based on albumin excretion rate (AER). Group II (n=20) comprised normoalbuminuric (AER <30mg/24h) type 2 diabetics, group III (n=20) had microalbuminuric (AER between 30-300 mg/24h) type 2 diabetics, and group IV (n=20) consisted of macroalbuminuric (AER >300 mg/24h) type 2 diabetic subjects.

Patients with significant co-morbidities like chronic liver disease, ischemic heart disease, undergone major transplant surgery etc., patients with end stage renal disease (creatinine clearance <10 ml/min), patients taking B-complex supplements and pregnant or lactating women were excluded from the study.

Samples of 10ml fasting venous blood and 24 hour urine were collected and transferred to labeled plain tubes and tubes containing EDTA after getting informed written consent from the patients. Whole blood, plasma, serum and urine were separated and stored at -80^{0}C for estimation. Plasma glucose was estimated by enzymatic colorimetric method (Linear Chemicals, Spain) using glucose oxidase enzyme (levels < 6.0 mmol/L were considered within reference range)[10,11]. Glycosylated haemoglobin was determined by column chromatography with cation exchange resins (Globe diagnostics S.r.I, Italy, values <6.5 % were considered within reference range)[12]. The concentration of plasma thiols (mostly Cys-34 of serum albumin) was determined by measuring the absorbance at 405nm produced by the reaction of plasma protein with 5,5'-Dithio-bis(2-nitrobenzoic acid) DTNB (Uptima France, Catalog number: UP01566I) using reduced glutathione (Merck KGaA, Germany) as quality control (reference range 279 ± 12umol/L)[13]. Albuminuria was determined using Microlab semi-analyser (Randox, UK). Thiamine chloride and thiamine monophosphate (TMP) in the plasma and urine were determined by high performance liquid chromatography (HPLC) with fluorimetric detection and precolumn derivatisation to thiochromes[14]. Chloroethylthiamine (CET) (0.5µM) was used as the internal standard as it has great structural similarity to thiamine and its derivatives. Stock solutions of standard analytes (100µM) were prepared using 10mM

sodium acetate buffer. Thiamine metabolites stock solution (0.1µM) was prepared by mixing equal volumes of 100µM thiamine (thiamine hydrochloride LOT no. G9307A, CAS no. 67-03-8, EEC no. 200-641-6, Alfa Aesar GmbH and Co KG, A Johnson Mathey Company) and TMP (thiamine monophosphate chloride dehydrate, catalogue number 2685369, Fluka Sigma Aldrich Chemie GmbH Germany) solutions. Calibration standards were analyzed in the range (1-5 pmol) analytes with each sample containing (5pmol) of internal standard and 20µL of TCA (10% TCA with urine samples and 20% TCA with plasma samples). Aliquots of these solutions (40µL) were applied to HPLC for analysis of thiochromes[15]. Samples (50µL) were deproteinized by the addition of 20 µL of TCA (10% TCA with urine samples and 20% TCA with plasma), and 10 µL of (0.5 µL) internal standard was added. Aliquots of these solutions (40 µL) were applied to HPLC. Precolumn derivatisation was done using alkaline potassium hexacyanoferrate solution as a derivatising agent.. The concentrations of thiamine and its phosphorylated metabolites were calculated after measuring the spectroscopical absorbance at λmax 247nm wavelength for all analytes, and pH 5.5 for thiamine and 2 for TMP. The analyte adducts and internal standard were detected by fluorescence (excitation wavelength 365 nm, emission wavelength 439 nm) with a Perkin Elmer fluorescence detector (series 200). All substances were completely separated with in 35 minutes. The oxidized thiamine derivatives were quantified using a fluorometric spectrometer.

For the purpose of statistical analysis SPSS version 17 was used. Mean and standard error of mean (SEM) were used to describe numeric variables. Analysis of variance (ANOVA) was applied to find out significant differences among groups. ANOVA was followed by Post Hoc Tuckey's test for multiple comparisons among groups. A p value<0.05 was considered significant and p value <0.01 was considered highly significant.

RESULTS

There were 12 males and 8 females in group I while group II had 7 males and 13 females. In groups III and IV there were 10 males and 10 females and 7 males and 13 females respectively. The gender difference between groups was found to be non-significant.

The mean ± SEM value for blood glucose in groups I, II, III and IV was 78.4 ± 1.75 mg/dL, 162.8 ± 6.68 mg/dL, 242.3 ± 8.63 mg/dL (range 184 – 296 mg/dL) and 262.95 ± 5.23 mg/dL respectively. The mean ± SEM value for HbA1c in groups I, II, III and IV were 3.14 ± 1.75 %, 5.0 ± 0.18 %, 7.54 ± 0.27 % and 8.29 ± 0.30 % respectively. The plasma thiamine chloride concentration (mean ± SEM) in group I was 7.33 ± 0.36 nmol/L, in group II it was 6.83 ± 0.31 nmol/L, in group III it was 5.52 ± 0.28 nmol/L while in group IV it was 4.68 ± 0.21 nmol/L. The urinary thiamine chloride concentration (mean ± SEM) in groups I, II, III and IV was 42.85 ± 3.49 µg/day, 59.99 ± 4.89 µg/day, 71.99 ± 5.87 µg/day, 79.19 ± 6.46 µg/day respectively. In group I the mean ± SEM value of plasma thiamine monophosphate was 8.37±0.36 nM/L, while in groups II, III and IV it was 6.12±0.28 nM/L, 4.38 ± 0.28 nM/L and 2.71 ± 0.25 nM/L respectively. The mean ± SEM value of thiol in group I was 254.15 ± 12.17 µmol/L (range 121.39 to 340.21 µmol/L) in group I. In groups II, III and IV it was 195.08 ± 8.26 µmol/L (110.81 to 266.89 µmol/L), 160.70 ± 8.09 µmol/L (92.34 to 241.39 µmol/L), 68.26 ± 3.18 µmol/L (45.98 to 106.2 µmol/L) respectively.

Comparison of mean values of plasma thiol among different groups is shown in figure whereas post hoc comparisons in the mean values of various parameters of glycemic status and thiol levels are shown in table 1. There was a significant difference in the plasma thiol level in different groups except groups II and III where though plasma thiol level was higher in groups II as compared to groups III but the difference was not significant. The mean difference in various parameters of thiamine status in different groups is given in table 2.

Pearsons's correlation coefficient was applied to see the relation between plasma thiamine and plasma thiol levels. A significant positive correlation was found between plasma thiamine and the plasma thiol in all groups (Table 2).

Table-1: Mean differece in plasma levels of various parameters of glycemic status and thiol in different groups.

Group comparison of healthy and diabetic participants	Glycosylated haemoglobin		Plasma glucose		Thiol	
	Mean difference	p-value	Mean difference	p-value	Mean difference	p-value
Groups I and II	-1.88**	<0.001	-84.40**	<0.001	59.07**	<0.001
Groups I and III	-4.40**	<0.001	-163.90**	<0.001	93.45**	<0.001
Groups I and IV	-5.15**	<0.001	-184.55**	<0.001	185.89**	<0.001
Groups II and III	-2.51**	<0.001	-79.50**	<0.001	34.38NS	0.028
Groups II and IV	-3.26**	<0.001	-100.15**	<0.001	126.82**	<0.001
Groups III and IV	-0.75NS	0.101	-20.65NS	0.088	92.44**	<0.001

NS = p > 0.05
** = p< 0.001

Table- 2: Table showing mean difference in levels of plasma and urinary thiamine in different groups

Group comparison of healthy and diabetic participants	Plasma thiamine chloride		Urinary thiamine chloride		Plasma thiamine monophosphate		Transketolase	
	Mean difference	p-value	Mean difference	p-value	Mean difference	p-value	Mean difference	p-value
Groups I and II	0.50NS	0.633	-17.14NS	0.110	2.24***	<0.001	0.039NS	0.655
Groups I and III	1.80***	<0.001	-29.14***	0.001	3.99***	<0.001	0.10**	0.015
Groups I and IV	2.64***	<0.001	-36.34***	<0.001	5.66***	<0.001	0.19***	<0.001
Groups II and III	1.30**	0.013	-12.00NS	0.385	1.744***	<0.001	0.06NS	0.224
Groups II and IV	2.15***	<0.001	-19.20NS	0.059	3.41***	<0.001	0.16***	<0.001
Groups III and IV	0.83NS	0.191	-7.20NS	0.772	1.67***	0.001	0.09**	0.036

NS not significant
** significant
*** highly significant.

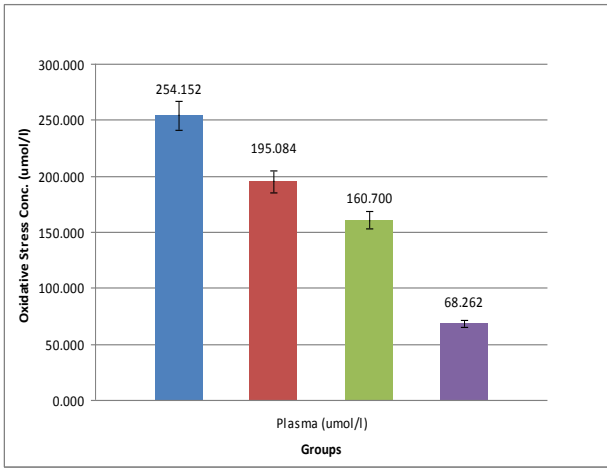

Figure: Comparison of plasma thiol levels in different groups. Data are means ± SE. (p<0.001)

Table-3: Table showing correlation coefficients between thiamine and oxidative stress (thiol) in different groups

Groups	Correlation coefficient (r)	p-value
I	0.82	<0.001**
II	0.88	<0.001**
III	0.52	0.019*
IV	0.71	<0.001**

* = < 0.05
** = < 0.01

DISCUSSION

We found reduced levels of plasma thiamine in the diabetics which showed progressive decline with advancing microalbuminuria. This was similar to a study conducted by Alkhalaf which showed decreased levels of plasma thiamine and transketolase in Dutch diabetic population[16]. In a Britain based study a comparison of thiamine status was made between the normal control group and diabetics with and without microalbuminuria. The level of thiamine in blood were found to be reduced while urinary thiamine was raised in the diabetic population as compared to normal control[17]. We also found a decrease in the plasma thiol levels of diabetics which increased with worsening of urinary albumin excretion. It was found in a study that as hyperglycemia developed in placebo-treated animals there was an elevation in the levels of markers of oxidative stress in the blood[18]. Artenie et al found decreased activity of superoxide dismutase and reduced levels of GSH with increased microalbuminuria in diabetic patients[19]. A study by Parham et al showed a positive role of zinc in reducing microalbuminuria in type 2 diabetics which is attributed to its antioxidant effects[20].

Furthermore, a strong positive correlation was established between plasma thiamine and plasma thiol levels in all groups of our study. The highest levels of thiol were seen in control group in which the levels of plasma thiamine were also highest while lowest levels were seen in group IV (macroalbuminuric diabetics) in which the level of plasma thiamine was also found to be lowest. There is evidence of the fact that thiamine can improve the anti oxidative status of the body. Thiamine inhibits lipid peroxidation[21] thus reducing the oxidative stress on the body. Our findings are supported by Balakumar et al and Verma et al who found reduction in the oxidative stress induced in rats by various means through the administration of thiamine analogue benfotiamine[22,23]. Deficiency of thiamine leads to decreased activity of the enzyme transketolase in the absence of which there is reduced activity of the pentose phosphate pathway leading to the accumulation of deleterious products of glucose metabolism like ROS, AGEs, PKC etc. Reversal of these changes can be seen by the administration of thiamine which activates transketolase which in-turn enhances the activity of pentose phosphate thus removing these harmful metabolites from the circulation[15]. Schmid et al found a direct antioxidant effect of benfotiamine on cells exposed to oxidative stress inducing compounds and also found increased activity of transketolase in human cultured cells[24]. However, there is no data available, to the best of our knowledge, which shows a relation between oxidative stress and thiamine in humans. We have now established a link between the two parameters which should prove to be useful in treating diabetic patients and preventing the onset of related complications.

CONCLUSION

The reduction in plasma thiamine level in diabetics leads to decreased ability of the body to respond to the oxidative stress as seen by the reduction in the level of thiol which represents the antioxidant status of the body. Moreover, as the albumin excretion rate increases in the diabetics there is a further decline in the plasma thiamine as well as plasma thiol level. Therefore in patients suffering from diabetic nephropathy there is increased oxidative stress which corresponds to decreased plasma thiamine level. Replenishment of thiamine may restore the body's protective antioxidative system and prevent the progression of diabetic complications including diabetic nephropathy.

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