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High Dose Thiamine Intervention for Non Traditional Risk Factors in Type 2 Diabetic Angiopathies

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ABSTRACT

Background: Impaired fibrinolysis, prothrombotic states, altered second messenger signaling mechanisms and enhanced oxidative stress levels have all been termed as the nontraditional risk factors of micro and macrovascular disease in diabetes mellitus. These are significant promoters of the pathogenesis of diabetes in general and of type 2 diabetes in particular .High dose Vitamin B1 (thiamine) has been found at preclinical level to play an ameliorative role through a number of intracellular metabolic pathways. In order to demonstrate whether this was translatable to a clinical therapeutic level in type 2 diabetics, pioneering research on the effect of high dose thiamine on associated markers of incipient diabetic nephropathy, hemostasis (plasminogen activation inhibitor 1PAI1), oxidative stress (plasma thiols) and second messenger signaling (protein kinase C) was conducted. **Methodology**: Over 100 Type 2 microalbuminuric type 2 diabetics were enrolled in a randomized, double blind placebo controlled clinical trial for 6 months. Patients were divided into two groups, one was orally administered 300mg/day thiamine, while the other group was provided placebo for a period of 3 months followed by a 2 month washout period .50 normal healthy controls participated for baseline estimations only. Plasma and urinary thiamine levels, microalbuminuria, metabolic, plasminogen activation inhibitor 1(PAI1), oxidative stress marker plasma thiols and second messenger signaling protein kinase C profile was determined in normal controls at exclusively baseline. While in type 2 diabetics the same profiling was performed at baseline, 3 months post therapy and 2 months post washout, using HPLC flourimetric detection for thiamine, estimation kits for protein kinase C, plasminogen activation inhibitor, glycated hemoglobin, microalbuminuria and specialized assay protocol for plasma thiol determination. **Results**: Markedly lower median plasma thiamine concentration of diabetic patients (7.5 nM) was present compared to normal range of normal healthy human subjects (44.6–93.7 nM.). Thiamine treatment for 3 months increased median plasma thiamine concentration 10 fold and urinary thiamine excretion 29 fold .It reduced significantly, microalbuminuria by 33% and glycated haemoglobin by 1.4%. Type 2 diabetics had significantly higher plasminogen activation inhibitor type 1 and protein kinase C levels at baseline, +82.5%($p<0.000$) and 3.56 fold ($p<0.001$) respectively as compared to normal controls. Following 3 months thiamine therapy, plasminogen activation inhibitor type 1 levels remained unchanged, while levels of protein kinase C were reduced significantly by 55% in thiamine treated diabetics and this decrease was maintained at a lowered rate of 44% even 2 months after discontinuing the drug ($p<0.01$). While the placebo group in comparison registered a significant increase of 26.09% in protein kinase C levels ($p<0.05$) which persisted during the washout period. Oxidative stress marker , plasma thiols was reduced significantly by 21% in diabetics at baseline versus normal controls with no change occurring following thiamine or placebo therapy as well as after washout. **Conclusion**: Type 2 diabetics were microalbuminuric, thiamine depleted with deranged metabolic, fibrinolysis, second messenger signalling and oxidative stress profile as compared to the normal population .High dose thiamine therapy significantly improved micoalbuminuria, glycated haemoglobin, thiamine status and decreased protein kinase C levels with no significant impact on oxidative stress and fibrinolysis profile.

INTRODUCTION

International morbidity and mortality statistics, elucidate that some 3.2 million people die of diabetes each year and 6 people die of diabetes related ailments each minute[1]. Individuals with type 2 diabetes have 2-4 times more heart disease at an earlier age and a two times higher risk of stroke than

non-diabetics. Diabetes ranked second in causation of retinopathy leading to loss of vision and incipient and overt nephropathy worldwide[2].Thus a disproportionate increase in tendency towards life threatening conditions is observed in diabetics emenating from microvascular and macrovascular angiopathies which appear to be linked to certain risk factors and altered intracellular signaling mechanisms[3] .

It is believed that hyperglycemia is one of the most important metabolic factors in the development of both micro- and macrovascular complications in diabetic patients. Several prominent hypotheses exist to explain the adverse effect of hyperglycemia[4]. One of them is the chronic activation by hyperglycemia of protein kinase (PKC)[5] a family of enzymes that are involved in controlling the function of other proteins. PKC has been associated with vascular alterations such as increases in permeability, contractility, extracellular matrix synthesis, cell growth and apoptosis, angiogenesis, leukocyte adhesion, and cytokine activation and inhibition.[6] These perturbations in vascular cell homeostasis caused by different PKC isoforms (PKC-α, -β1/2, and PKC-δ) are linked to the development of pathologies affecting large vessel (atherosclerosis, cardiomyopathy) and small vessel (retinopathy, nephropathy and neuropathy) complications.[7-9]

An important determinant of hemostasis , the endogenous fibrinolytic system represents a balance between activators of plasminogen (primarily tissue plasminogen activator) and inhibitors of these activators(such as plasminogen inhibitor type (PAI-1[10]. Impaired fibrinolytic function in diabetes correlates with the severity of vascular disease and is a risk factor for myocardial infarction in both diabetic and non diabetic subjects[11-15].

Coagulation disorders have also been reported in type 2 diabetics ranging from alterations in vwf antigen and, factors 5,2,7 with decreased antithrombin3[16]. The importance of the endothelium in maintaining vascular health has also been widely recognized and assessment of the biochemical parameters of this reveal vascular endothelial dysfunction as raised svcam levels and vwf levels in type 2 diabetics as compared to normal controls [3].Our own previously published work in Diabetologia on endothelial dysfunction svcam and vwf revealed increased levels of both in type 2 diabetics[17,18] and a decrease in their levels following high dose thiamine therapy.

Oxidative stress additionally plays a pivotal role in the development of diabetes complications. The metabolic abnormalities of diabetes predominantly hyperglycemia cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, as well as in the myocardium. This increased superoxide production causes the activation of 5 major pathways involved in the pathogenesis of complications[19]. These being increased activation of the polyol pathway[20] nonenzymatic glycation, and AGEs (advanced glycation end products) pathway[21], enhanced reactive oxygen production and actions[19] and activation of the diacylglycerol (DAG)–protein kinase (PK) C pathway[5]. It also directly inactivates 2 critical antiatherosclerotic enzymes, endothelial nitric oxide synthase and prostacyclin synthase.[22] Through these pathways, increased intracellular reactive oxygen species (ROS) cause defective angiogenesis in response to ischemia, activate a number of proinflammatory pathways, and cause long-lasting epigenetic changes that drive persistent expression of proinflammatory genes after glycemia is normalized (hyperglycemic memory). Atherosclerosis and cardiomyopathy in type 2 diabetes are caused in part by pathway-selective insulin resistance, which increases mitochondrial ROS production from free fatty acids and by inactivation of antiatherosclerosis enzymes by ROS. Over expression of superoxide dismutase in transgenic diabetic mice prevents diabetic retinopathy, nephropathy, and cardiomyopathy[23] Altered plasma thiol levels which are also representative of oxidative stress have also been observed in type 2 diabetics[24].

Thus these proposed underlying and unifying multiple biochemical pathways lead to the hyperglycemia-induced intra- and extracellular changes and to alterations of signal transduction pathways, affecting gene expression and protein function, ultimately causing the cellular dysfunction and damage noticed in diabetes mellitus.

Thiamine has been shown reduce these through its effect on interconnecting pathways.

High dose thiamine and Benfotiamine may counter the development of microvascular complications by activation of the reductive pentosephosphate pathway[25,26]. The mechanism appeared to be normalizing transketolase expression and activity in the non oxidative pentose pathway causing increased conversion of triosephosphates and fructose 6 phosphate to ribose-5-phosphate[25,26]

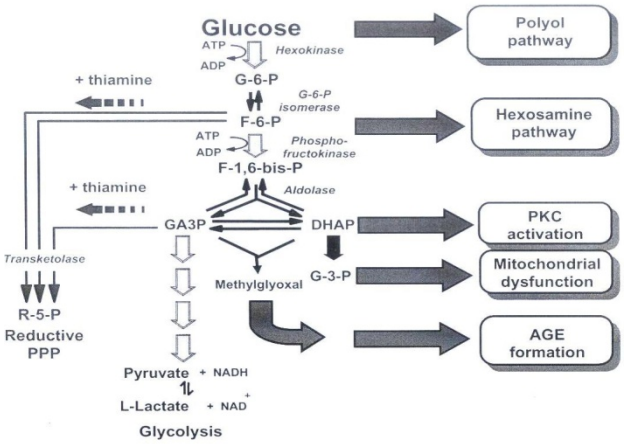

Fig. 1: Proposed mechanism of high dose thiamine therapy in countering deleterious activation of the polyol, hexosamine, PKC, mitochondrial dysfunction & AGE formation (Adapted from PJ Thornalley06)

Bearing the above data in mind a pioneering clinical trial was conducted at Sheikh Zayed Hospital to study the effect of high dose thiamine therapy on its own levels, metabolic profile, microalbuminuria, plasminogen activation inhibitor 1(PAI1), oxidative stress plasma thiols and second messenger signaling protein kinase C profile in type 2 diabetic patients and to ascertain a therapeutic effect.

EXPERIMENTAL

Chemicals, reagents, kits and instrumentation

Purple topped and red topped vaccutainers, 10cc syringes were purchased from Beckton Dickinson Company. Biochemical analyses kits for blood glucose, total cholesterol, triglycerides, LDL, total bilirubin, ALP, AST, AST, uric acid, serum/ urine creatinine analysis, CPK, blood urea nitrogen, were supplied by Dade Behring clinical chemistry system for dimension autoanalyser. The micro-albuminuria kit, glycated hemoglobin estimation kit and Diastat autoanalyzer was supplied by Randox, while the spectrophotometer was provided by Shimadzu.

Plasma thiol assay

DTNB (D8130), DETAPAC (32320), reduced glutathione (K5278690) were purchased from Sigma, Fluka, and Merck respectively.

Thiamine analysis

TMP chloride dihydrate (T8637), TPP (C8754), THTP were supplied by Sigma. Columns for HPLC were 4µm ODS column (3.9x150 mm, NOVOPAK) with a pre column Sentry guard (3.9x20mm). Potassium hexacyanoferrate, (12643), potassium dihydrogen phosphate, (PO0259), vitamin B1 (95160) were purchased from Reidel-de-Haen, Scharlau and Biochemica respectively.

Immunoassay kits

Plasminogen activation inhibitor1 immunoassay kit was purchased from Technclone, USA while PKC Activity kit EKS 420A was provided by Stressgen Bio Reagents.

Selection of patients and controls

Over 100 type 2 diabetic patients with microalbuminuria and 50 normal healthy individuals (35–65 years old) were initially inducted at the Diabetes Clinic of Sheikh Zayed Hospital in a double blind randomized placebo controlled clinical trial applying stringent inclusion and exclusion criteria recommended by the American Diabetes Association for type 2 diabetes between October 1, 2006 and December 1, 2006[17,18]. The principal inclusion criteria for diabetic patients included type 2 diabetes defined by WHO (1999) criteria with age 36-65, duration (5 or more years), HbA1c (≤12.5%), body mass index (19-40kg/m^2), persistent microalbuminuria defined as albumin excretion between 30 and 300 µg/min in 3 overnight urine collections. The principal exclusion criteria were Hepatitis, allergy to thiamine, taking thiamine supplements, severe excess alcohol consumption consume head and visceral organs of raw fish (source of thiaminase activity), pregnant women or breast feeding, women of child bearing not taking contraceptive medicines or precautions, untreated or

unsuccessfully treated psychiatric disease. Patients with persistent uncontrolled or untreated severe hypertension, planning to undertake surgery within the next 6 months, any abnormalities during recruitment screening with abnormal LFTs, RFTs and deranged haematological profile were also excluded.

While the principal inclusion criteria for normal healthy controls included good health, age 35-65 years, matching for age and gender to the recruited diabetic patients. [17,18]

Randomization and treatment

The patients and controls were divided on the basis of the randomization table into three groups A (normal healthy controls), B and C (diabetic type 2) having equal number of individuals in each group. Randomization was done by central office in sequentially numbered opaque and sealed envelopes. Participants, caregivers and those assessing the outcomes were blinded to this group assignment. Half of the inducted diabetic individuals were administered thiamine 3x100 mg tablets/day for three months, while the other half were treated with placebo. The 3 month treatment period was followed by two month washout period. Out of enrolled diabetic individuals and controls, 20 controls and 40 patients completed the 3 month thiamine/placebo administration and 2 month follow-up period for this study[17,18]. Adverse and side effects of thiamine and placebo were determined by renal and liver function tests . Out of the 40 patients who completed the trial, in the placebo group, there were 7 male and 13 female patients of age 52.4±8.7 yrs and BMI 28.3±4.4 kg/m^2. In the thiamine treatment group, there were 9 male and 11 female patients of age 52.7±8.4 years and BMI 28.1±4.6 kg/m^2. At baseline and throughout the study, 9 patients in the thiamine treatment group and 3 in the placebo group were receiving insulin therapy ($P<0.05$). There was no other significant difference in proportions of patients receiving therapeutic agents in thiamine treatment and placebo groups. One patient achieved glycaemic control by diet only all others received therapy with hypoglycaemic agents (sulfonylureas, metformin and thiazolidinediones). Nine patients in the placebo group and 8 patients in the thiamine treatment group received therapy with anti-hypertensive agents. Some patients in thiamine and placebo treatment arms were receiving cholesterol-lowering statin therapy: 3 and 1 patient at baseline, 7 and 2 patients after the 3 month treatment period and 7 and 3 patients after the 2-month washout period, respectively. One patient in the thiamine treatment group was receiving anti-hyperlipidaemic fibrate therapy[17,18].

Ethical approval of the study

Ethical approval for the study was taken from the Ethical/Protocol/Synopsis Committee of FPGMI (Sheikh Zayed Postgraduate Medical Institute, Lahore, Pakistan). The study was assigned the number as Eth/P 609/FPGMI 2006. It was internationally registered with the South Asian Clinical Trials Registry based in India as CTRI/ 2008/091/000112. Also registered with the World Health Organization's (WHO) International clinical trials registry platform searchportal http://www. ctri.in/Clinicaltrials/ViewTrial.jsp?trialno=203 trialid=CTRI/2008/091/000112apps.who.int/ trialsearch/trial.aspx?trialid=CTRI/2008/091/00011 2) [17,18]

Power calculation

The primary endpoint was urinary albumin excretion. Group CV values and intervention effects of 30% were assumed, similar to previous studies . For power =0.8 and $\alpha<0.05$, patient group size was 17. Patient groups of 20 were employed to allow for non-compliance to therapy[17,18].

Sampling

Fasting Blood samples were obtained from the enrolled diabetic patients and normal healthy controls at baseline and subsequently after 3 months therapy and 2 months washout. Plasma, serum, RBCS, mononuclear cells and RNA were removed from blood by centrifugation and specialized techniques. These were analyzed for biochemical parameters, transketolase, pyruvate dehydrogenase and alphaketoglutarate dehydrogenase activity and gene expression analyses. 24 hr urine collections were also made for determination of micro-albuminuria[17,18] .

Methodologies

Routine biochemical parameters and specialized metabolic and renal profile parameters such as glycated hemoglobin and microalbuminuria were determined by standard referred protocols.[27-37]and protocols provided by the kit manufacturers respectively.[38,39]

Measurement of glycosolated hemoglobins (HbA1C)

The concentration of HbA1C in whole blood was measured by low pressure cation exchange chromatography in a Diastat automated analyzer. The DiaSTAT instrument and reagent kit were used for in vitro estimation of human glycated hemoglobin in samples from normal and diabetic patients. The ratio of glycated hemoglobin to total hemoglobin A was estimated in the presence of hemoglobins S and C[38] in hemolyzed whole blood[39]. The separated hemoglobin fractions were monitored by means of absorption of light at 415 nm. The chromatogram obtained was recorded on the operating system disk. The software program performed an analysis of the chromatogram and generated a results report on the thermal printer.

Sample preparation

The patient sample test tubes of anti-coagulated whole blood were placed on a platform mixer and mixed until samples were homogenous.

While patient samples were mixing, new sample vials for controls and patient samples were labelled. 20 uL of control material or well-mixed whole blood were pipetted into the properly labeled sample vials.1 ml of Hemolysis Reagent was added to each of the controls and patient sample vials. Each sample was capped and vortexed. The samples were incubated for 30 minutes at 37°C and loaded onto the Diastat for glycated hemoglobin estimation.

Estimation of the microalbuminuria based on the immunoturbidimetric method

Undiluted sample was added to a buffer containing antibody specific for human serum albumin. The absorbance at 340nm of the resulting turbid solution was proportional to the concentration of albumin in the sample urine[40]. By constructing a standard curve from the standards, the albumin concentration of sample was determined. The assay was carried out manually at room temperature.

Sample collection and storage

For 24 hours urine sample of diabetic patient's type 2 were collected at morning in fasting and stored at between +2 to +8ºC prior to testing.

Procedure

All reagents and urine samples were brought to room temperature prior to use. All samples were run in duplicate with appropriate controls at 340nm, 1cm path length cuvette at 25°C. Measurements were taken against distilled water blank.

Standard curve and calculations of results

ΔA sample/standard= A2-A1 (of sample/standard)
Plotted ΔA standard against albumin concentration, stated on the side of each vial, using semi-logarithmic graph paper. Sample concentrations were determined from the standard curve.

Thiamine analysis

Assay of Plasma and Urinary Thiamine and Phosphorylated Metabolites

Thiamine, TMP and TDP were determined by HPLC with flourimetric detection (precolumn derivitization to thiochromes) because of the increased specificity, sensitivity and reduced analysis time of this method[41]. Plasma thiamine concentration, urinary thiamine excretion and renal clearance of thiamine were also deduced.

Preparation of samples

50µl aliquots of plasma and urine were deproteinized using (20µl) of trichloracetic acid (TCA 10% for urine and 20% for plasma and 10µl of (0.5µM) IS was added. The samples were mixed, placed on ice for (10 min urine samples and 30 min plasma samples) and centrifuged [(6000g, 10 min and 4°C) for urine samples and (10,000g, 10 min and 4°C.) for plasma samples]. The supernatants (40µl) were removed and the pH was adjusted to 4.5 by addition of 2M sodium acetate with urine samples and aliquots of 1M sodium acetate. Microspin filter tubes were used to centrifuge the neutralized samples (4000g, 10 minutes, 4°C). Aliquots of these solutions (40µl) were applied to

the HPLC for thiochromes. Samples were stable over the time of analyzing the storage time. Samples were stable at -80°C during the storage time.

Pre-column derivitization procedure

Alkaline oxidation of thiamine and its phosphate phosphate esters to highly fluorescent thiochromes has frequently been combined with high performance liquid chromatography (HPLC used) to separate the thiochromes from other fluorescent compounds in the sample[41]. Pre column derivitization of samples was done using alkaline potassium hexacyanoferrate as derivitizing agent.10 µl of dervatising agent [(prepared by mixing 15% (w/v) NaOH (9µl) with 1% (w/v) K3 Fe (CN) 6 (1µl)]. 10µl of dervatising agent was freshly prepared and applied automatically by the autosampler transfer routine to 40µl of sample immediately prior to sample analysis. 20µl was injected into the column[42]. Agilent 1100 HPLC was used for thiamine estimation.

HPLC elution profile

The mobile phases were solvent A and B. Solvent A: 25mM potassium phosphate buffer KH2PO4 (ph 8.4). Solvent B: 25mM potassium phosphate with 50% methanol (pH 8.4).

HPLC conditions using precolumn derivitization technique

The thiochrome derivatives were separated on a 4µm ODS column (3.9x150 mm, NOVOPAK) with a pre column Sentry guard (3.9x20mm). The flow rate was 1ml/min.

Analytes detection

The analyte adducts of thiamine monophosphate ThMP, thiamine diphosphateThDP and thiamine triphosphateThTP and chloroethylthiamine internal standard were detected by fluorescence (excitation wavelength 365nm, emission 439nm) with an Agilent 1100 flourescence detector.

Marker of oxidative stress

Plasma thiol assay

The concentration of plasma thiols (mostly Cys-34 of serum albumin) was determined as originally described by Ellman[43] and modified by Hu[44]. Here, thiols interact with 5,5'-dithiobis-(2-nitrobenzoic acid) to form a highly coloured anion with maximum peak at 412 nm.

Plasma thiols absorbance was measured at 415 nm produced by the reaction of plasma protein with DTNB. DTNB solution was made with 1mM DTNB and 0.2mM DETAPAC in 100mM sodium phosphate buffer at pH 7.4. 125µL of DTNB solution was added to the well of a 96 well microplate and was diluted to 225 µL by the addition of 100 µL water. A 25 µL aliquot of plasma was then added and the absorbance at 415nm recorded for until a steady maximum state was attained (~20 minutes). The increase in absorbance recorded by adding the plasma was used to deduce the concentration of plasma thiols. The assay was calibrated in the range 0-30 nmoles using reduced glutathione (GSH) as the standard sulphhydryl group analyte and building a standard curve from which the plasma thiols were calculated. Two blanks are used for each sample: 125 µL DTNB solution + 125 µL water and 225 µL plasma. Together, these compensated for absorbance at 405 nm of both the DTNB and the plasma alone.

Estimation of human PAI-1 antigen ELISA test procedure

The ELISA based method was used to determine PAI-1 antigen levels in type 2 diabetic patients and controls.

Preparation of samples

EDTA plasmas were used. Centrifuged for 15 minutes at a minimum of 2500g and stored at -80°C immediately after centrifugation for testing within 6 months.

Reagents

1. 5 standards were provided having concentrations 0ng/ml, 4ng/ml, 34.6ng/ml and 124.8ng/ml. Two control plasmas were also provided having concentrations 3.4-9.0ng/ml and 36.3-60.4ng/ml respectively. Stable after reconstitution for 6 months at -20°C
2. An ELISA 96 well microplate (strip form) antibody coated was provided in an

aluminium bag with a dessicant.

3. Washing buffer concentrate having stability of 6 months at 2-8°C from which the washing buffer in 1+9 dilution was reconstituted with stability of 3 weeks at 2-8°C
4. Incubation buffer with stability of 2 months at 2-8°C
5. Conjugate which in original form had stability of 6 months at 2-8°C and in working solution form could maintain activity for 60 mins at room temperature
6. Chromogen TMB which was stable after opening at 2-8°C till its expiry date.

Preparation of reagents

1. Before starting the test, all the required components were brought to room temperature.
2. The washing buffer. was diluted 1 part by volume washing buffer concentrate with 9 parts by volume distilled water (1+9) and mixed well to allow for crystalline precipitations to dissolve at 37°C within 10 minutes.
3. A detailed 96 well plate layout was written to ensure accurate pipetting and referencing of the standards, control plasmas, patient plasmas and the normal healthy plasmas to be assayed in duplicate.Labeling/number strips with a water resistant pen was done.
4. Reconstituting calibrators and control plasmas: Calibrators and control plasmas were reconstituted with 500 µl distilled water and mixed for 10 seconds after a reconstitution time of 15 minutes (vortex mixer). Reconstituted components were slightly turbid.
5. The conjugate working solution (1+50) was prepared by diluting 1 part by volume conjugate with 50 parts by volume incubation buffer.

Test procedure

1. A detailed 96 well plate layout was written to ensure accurate pipetting and referencing of the standards, control plasmas, patient plasmas and the normal healthy plasmas to be assayed in duplicate. 1.25µl of calibrators, control plasmas and samples were pipetted into assigned wells.
2. 0.75 µl of incubation was added to all the wells and they were covered with a provided plastic film.
3. The plate was incubated for 60 mins at 37°C
4. Discarded the contents of the microplate by decantation or aspiration after decanting the plate was tapped dry on a blotting paper.
5. 100 µl of conjugate working solution was pipetted into wells., The plate was covered with film.
6. The plate was incubated for 60 mins at 37°C.
7. Washed the plate thrice each time with 200µl of the washing buffer using the automated ELISA plate washer. After the last washing the plate was inverted on a blotting paper and dried thoroughly by gentle tapping to remove the last traces of buffer present.
8. 100µl of the substrate solution was pipette into the each of the test wells and covered with plastic film.
9. Incubated at room temperature (20-25ºC) for 10 minutes
10. Finally 100 µl of stopping solution was added into each well and mixed gently for 10 seconds.
11. Read the absorbance in each well at 450nm within 10 minutes

Calculation of results

A standard reference curve was extrapolated for the given standard calibrators and the values of the unknown samples were ascertained from them standard curve.

Mononuclear PKC kinase activity

The PKC kinase Activity Assay is based on a solid phase enzyme linked immunoabsorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay is designed for analysis of PKC activity in the solution phase.

1. Test mononuclear cells were transferred to a 15 ml conical tube and spun at 1200 rpm for 5-10 min to pellet.
2. Homogenized with 0.5 ml of lysis buffer A

[20mM Tris/HCL ph7.5, 0.5mM EGTA, 2mM EDTA, 1mM phenylmethylsulphonyl-fluoride (PMSF) and 25 µg/mL leupeptin and 0.1 mg aprotinin, and 0.33 M sucrose] at 4°C.

3. The homogenate was centrifuged at 20,000 rpm for 30 min and 4°C. The supernatant fraction was retained as the cytosolic pool and stored at -80°C until analysis.
4. The pellet was washed once with buffer B (buffer A without sucrose) and solubilized in 0.5 ml of buffer B WITH 1% Triton X.
5. After incubation on ice for 45 minutes, the solubilized membrane fraction was obtained by centrifugation at 20,000g for 30 minutes; the supernatant was maintained as the membranous pool.
6. For optimal results, it was recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.
7. Protein concentration was determined using BCA method.

Assay procedure summary

1. Brought to room temperature: PKC Substrate Microtiter Plate, Antibody Dilution Buffer, Kinase Assay Dilution Buffer, TMB Substrate and Acid Stop Solution.
2. Acive PKC control (stored at -80ºC was used as positive control and serially diluted in kinase assay dilution buffer to a final volume of 30µl (kept on ice) while performing assay.
3. 30µl of kinase assay dilution buffer was used as the assay blank.
4. A detailed 96 well plate layout was written to ensure accurate pipetting and referencing of the standards, blanks, patient samples and the normal healthy samples to be assayed in duplicate
5. Soaked wells of the PKC Substrate Microtiter Plate with 50uL Kinase assay dilution buffer at room temperature for 10 minutes. Carefully aspirated liquid from each well.
6. Added samples to appropriate wells of the PKC Substrate Microtiter Plate.
7. Initiated reaction by adding 10uL of diluted ATP (reconstituted with 2 ml kinase assay dilution buffer) to each well.
8. Incubated for up to 90 minutes at 30°C.
9. Stopped reaction by emptying contents of each well and dabbing on a paper towel
10. Added 40uL of Phosphospecific Substrate Antibody to each well.
11. Incubated at room temperature for 60 minutes.
12. Washed wells 4 times with 100uL IX Wash Buffer with an automated plate washer
13. Added 40uL of diluted Anti-Rabbit IgG: HRP Conjugate (prediluted with antibody dilution buffer to 1µg/ml concentration and kept on ice) to each well.
14. Incubated at room temperature for 30 minutes.
15. Washed wells 4 times with 100uL IX Wash Buffer with plate washer.
16. Added 60uL of TMB Substrate to each well.
17. Incubated at room temperature for 30-60 minutes. Incubation time should be determined by the investigator based on color development.
18. Added 20uL of acid stop solution to each well.
19. Measured absorbance at 450 nm.

Formula for calculating PKC activity in Cell Lysates

Relative kinase activity = (Average absorbance (sample) - Average absorbance (blank) / Quantity of crude protein used per assay (2µg)

Statistical analysis

The data collected was analysed using statistical package SPSS15 (Chicago IL, U.S.A), Significance of difference between mean, median analytes of thiamine and placebo groups were determined by using students t-test and Mann Whitney U test respectively. Significance of differences from baseline and post therapy and post washout mean and median analytes were determined by paired sample T test and Wilcoxin signed rank test.[17,18]

RESULTS

Detailed biochemical profiles were maintained for the enrolled type 2 micro-albuminuric patients throughout the trial duration, at baseline, the 3 months post therapy and 2 month

washout period and have already been published in our paper.[17,18] These revealed certain beneficial effects of high dose 300mg/day thiamine therapy and absence of adverse effects[17,18]

Estimation of physical and biochemical characteristics:

A clinical profile of physical, biochemical and physiological characteristics was developed. For physical parameters, age, sex, BMI, weight, height and blood pressure as diastolic and systolic levels were measured. (Table 1)

Baseline urinary albumin excretion

Urinary albumin excretion in mg/24hrs was significantly higher in the type 2 diabetics of both thiamine (53.86±22.62) mg/24hrs and placebo groups (55.74±23.71) mg/24hrs as compared to controls (7.968±5.07) mg/24hrs. This reflected the presence of microalbuminuria (30-300mg/24hrs) in the patients as compared to controls which was an inclusion criteria requirement (Table 1).

Thiamine profile at baseline

The median plasma thiamine concentration of thiamine and placebo treated diabetic patients was 10.6 (0.8-84.5) nmol/l and 7.1(1.1-31.3) nmol/l respectively (Table 1) which was markedly lower than the normal range of normal healthy human subjects (44.6 – 93.7 nmol/l). The median urinary thiamine excretion was 1.63 (0.45-7.66) μmol/24 h and thiamine clearance was 112 (8-819) ml/min in the thiamine group. While in the placebo group the median thiamine excretion was 1.53 (0.67-4.46)μmol/24 h and thiamine clearance was 189 (26-995) ml/min in the placebo group. One patient had a plasma thiamine concentration above the normal range and a urinary thiamine output 30-fold greater than the normal mean, consistent with prior thiamine supplementation and was excluded from the data analysis. At baseline, there was a negative correlation of plasma thiamine concentration with thiamine clearance ($r = -0.52$; $P<0.01$) and a positive correlation of urinary thiamine excretion and thiamine clearance ($r = 0.52$; $P<0.01$). There was no significant difference in the plasma thiamine concentration, urinary thiamine excretion and renal clearance of thiamine for patients as shown in Table 1.

Metabolic profile

The characteristics of normal healthy controls and type 2 diabetic patients recruited for this study and randomly placed in the placebo and thiamine treatment arms at baseline are given in (Table 1). The mean metabolic profile of glucose control and lipids in normal healthy controls was mean fasting serum glucose concentration 89.3±19.64mg /dl, glycated haemoglobin 5.6±0.39%, lipid profile LDL 115.89±29.37 mg/dl, VLDL 26.62±20.22 mg/dl, HDL 44.15±120.05 mg/dl, cholesterol 188.3± 33.93mg/dl and triglycerides 133.10 ±108.45 mg/dl.(Table 1)

Type 2 thiamine allocated diabetics baseline biochemical parameters profile registered plasma glucose concentration at 181.8±57.6 mg/dl , mean glycated HbA1c 9.2±1.3 (%), LDL106.34±47.14 mg/dl, VLDL46.33±31.79 mg/dl, HDL 49.88±13.53 mg/dl, cholesterol203.40±50.27 mg/dl and triglycerides 240.10±168.55 mg/dl. (Table 1)

Those randomized to the placebo arm had fasting plasma glucose concentration 171±55.8 mg/dl, mean glycated haemoglobin 8.82±1.8%, LDL 106.34±35.96 mg/dl, 185.22±40.98l mg/dl, VLDL 42.67±7.10 mg/dl, HDL 47.95±10.44, cholesterol, 185.22±40.9 mg/dl and triglycerides 156.15±81.74mg/dl (Table 1).

Thus there was a statistically highly significant difference ($p<0.001$) in clinical chemical variables of plasma glucose, triglycerides (thiamine group) and less significantly so in the placebo group ($p<0.01$for TG Only) at baseline between the normal controls and the type 2 diabetics. While statistically significant differences ($p<0.01$) were found between normal controls and both thiamine and placebo diabetics in glycated haemoglobin and VLDL levels and least significantly ($p<0.05$) in the serum HDL levels. Total cholesterol levels were significantly raised in the thiamine group only, whereas statistically non significant results were found for LDL in both thiamine diabetics and placebo groups (Table 1).

Baseline hemostasis, oxidative stress and cellular signalling mediator profile

The mean and standard deviation values of oxidative stress marker plasma thiol of thiamine allocated diabetics and placebo group did not

Table 1: Average values of the various physical and biochemical parameters of the different groups at baseline (n=20).

S. No.	Variables*(Normal range)	Control group	Thiamine allocated group	Placebo allocated group
1	Age (years)	49.13±8.79	52.7± 8.4	52.40± 8.7
2	Sex	11F,9M	9M, 11F	7M, 13F
3	Height (m)	1.59±0.7	1.57± 28.4	1.58± 52.9
4	Weight (kg)	68.56± 16.99	69.12± 18.4	71.27± 22.7
5	Diastolic BP (mmHg)(<90)	78± 7	87 ± 8	84 ± 6
6	Systolic BP(mmHg)(<140)	118.2±9	126 ± 14	131 ± 10
7	BMI(kg/m2)(19-40)	26.93± 4.92	28.1 ± 4.6	28.3 ± 4.4
8	FBS (mg/dl) (<100)	89.3±19.64	181.8.±57.6[c]	171±55.8[c]
9	HbA1c (%)(4.3-6.1)	5.6±0.39	9.2±1.3 [b]	8.82±.1.8[b]
10	LDL (mg/dl)(70-130)	115.89±29.37	106.34±47.14 [a]	106.34±35.96[6a]
11	VLDL (mg/dl)(5-41)	26.62±20.22	46.33±31.79 [a]	42.67±7.103 [a]
12	HDL (mg/dl)(35-60)	44.15±120.05	49.88±13.53 [a]	47.95±10.44 [a]
13	Cholesterol (mg/dl)(0-200)	187.21±33.19	203.40±50.27 [b]	185.22±40.98
14	TG (mg/dl)(30-150)	133.10±108.45	240.10±168.55[c]	156.15±81.740 [b]
25	Urine Albumin (mg/24h)(0-30)	7.968±5.07	53.86±22.62 [c]	55.74±23.71 [c]
15	GFR (ml/min)(90-120)	102.43±38.60	85±19	93±23
16	Uric acid mg/dl(3.5-8.5)	4.65±1.00	4.65±.10.05	3.99±1.09
17	BUNmg/dl(7-21)	10.45±2.350	11.37±4.87	12.25±2.245 [a]
18	Serum Creatinine mg/dl(0.8-1.4)	0.820±.132	0.915±0.195.	0.84±0.24 [a]
19	Urine Creatininemg/dl(30-125)	47.63±36.117	42.39±25.635 [a]	42.67±31.76 [a]
20	Bilirubin (mg/dl) (0.1-1)	0.59±0.403	0.55±.244	0.52±0.279
21	ALP (U/L) (20-160)	95.90±34.20	103.30±33.4847 [b]	88.20±29.848 [a]
22	AST (U/L) (15-37)	29.15±11.123	22.60±9.703	21.65 ±7.700[a]
23	ALT (U/L) (30-65)	40.90±12.191	360.05±10.49 [a]	36.10±10.40
24	CK(U/L)(M:38-174, F:96-140)	108.50±70.04	81.85±52.30	69.95±55.35
5	Plasma thiamine (nmol/L)	44.63-93.7	10.6(0.8-84.5) [c]	7.1(1.1-31.3) [C]
26	Urine thiamine (µmol/24 h)		1.63(0.45-7.66) [c]	1.53(0.67-4.46) [c]
27	PAI1(ng/ml) (7-43)	3.094±1.83	5.40±10.01c	5.91±1.68 c
28	Protein kinase C(pg/ug)	20.57±7.7	68.00±68.45c	76.65±56.22c
29	Plasma thiols(nmol/µl)	5.13±0.85	3.91±.72b	4.15±0.86b

Data are mean ± SD values. For comparison of control with diabetes samples, [a]P <0.05 (statistically significant), [b]P < 0.01 (statistically significant), [c]P < 0.001 (statistically highly significant).Normal ranges given in brackets next to each variable.

significantly differ at baseline between themselves (Table 1). These were 3.91±0.72µmol/ul in the thiamine group. While in the placebo group the plasma thiol levels were 4.15±0.86 µmol/µl. A fibrinolysis marker plasminogen activation inhibitor PAI1 values at baseline for normal controls were 3.09±1.83 (ng/ml) which were significantly lower than thiamine group PAI1 values of 5.40±10.01 (ng/ml) and placebo group levels of 5.91±1.68 (p,<0.001). (Table 1).

The mean protein kinase c levels at baseline of both thiamine 68.00±68.45 pg/µg and placebo group 76.65±56.22 pg/µg were significantly different from normal control values of 20.57±7.7pg/µg (p:0.008) and (p0.000 highly significant) (Table 1).

Effect of therapy with high dose thiamine: microalbuminuria

The effect of high dose thiamine therapy or placebo on urinary albumin excretion changes were a noticeable endpoint of this study. At baseline the thiamine group median MAU values were 43.7 (33.0–120.9mg/24hrs) versus 50.9 (32.9-121.7mg/24hrs). After the treatment period, patients receiving thiamine therapy had decreased median urinary albumin excretion, with respect to patients receiving the placebo (30.1 (12.0-38.2) versus 35.5 (6.4-82) mg/24 h; P<0.01). At washout the MAU values for thiamine versus placebo group

were 20.9 (70-35.00mg/24h) versus 30.0 (3.5-80.4 mg/24h). Urinary albumin excretion was decreased significantly with respect to baseline in the patients treated for 3 months with thiamine (17.7 mg/24 h; P'<0.001) but not in patients treated with placebo. The decrease in MAU was maintained after the washout period when the decrease in the urinary albumin in the patients treated with placebo also became significant (Table 2; Fig 2). There was a noticeable progressively declining pattern of urinary albumin excretion over time in patients treated with thiamine and placebo. However linear regression of urinary albumin excretion on treatment time indicated the rate of decrease in urinary albumin excretion was increased 4-fold in patients treated with thiamine with respect to placebo (5.62 versus 1.52 mg/24 h) (Table 2, Fig 2).

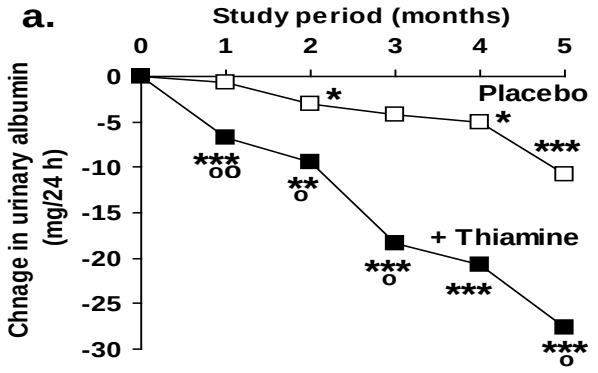

Fig. 2: Effect of high-dose thiamine on UAE: a) Change from baseline of UAE in type 2 diabetic patients receiving thiamine (black squares) or placebo (white squares). Median values are shown: *p<0.05, **p<0.01 and ***p<0.001 compared with baseline; †p<0.05 and ††p<0.01 compared with baseline change for the placebo(.Reprinted from Rabbani, Alam. SS 2009)

Glycemic profile

There was no significant effect on daily glycaemic control during the treatment period. However after the washout period, fasting plasma glucose 144mg/dl was found significantly decreased (37.8mg/dl) p<0.05 with respect to baseline levels 181.8 mg/dl in thiamine group while the placebo treatment group also showed a similar decrease of 33.58mg/dl from 177.58mg/dl at baseline to 144mg/dl at washout which were significantly low p <0.05) (Table 2).

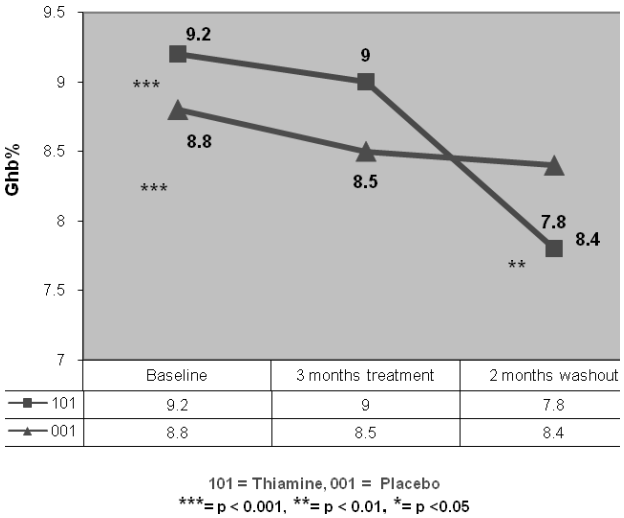

Fig. 3: Mean glycated hemoglobin levels in thiamine/ placebo treated type 2 diabetics from baseline, 3 months post therapy to washout period.

However long term glycemic regulation monitored by HbA_{1c} levels was significantly decreased by 1.4% (p<0.01) to an intermediary therapy level of 9.0% and a washout level of 7.8% in the thiamine treatment group with respect to baseline value 9.2%. No such significant decrease occurred in the placebo group where the HbA1c levels remained relatively steady between 8.8% at baseline, 8.5% during therapy and 8.4% during washout (Table 2, Fig. 3).

Plasma and urinary thiamine profile

The plasma thiamine levels of the thiamine treated group rose from (median value) 10.6(0.8-84.5) nmoles/l) to (median value 98.2 (2.6-294.5) nmoles/l after 3 months treatment which was almost a tenfold increase, significantly higher than baseline (p<0.01) while placebo group plasma thiamine levels at baseline were 7.1 (2.4-16.6 nmoles/l) (p<0.001). During washout these levels returned to 10.9 (3.6-22.7nmoles/l) in the thiamine group which were significantly lower than during therapy (p<0.001). The plasma thiamine levels in the placebo group remained significantly unaltered ranging from start of therapy at 7.1 (1.1-31.3 nmoles/l) median value to 9.1 (4.2-16.3nmoles/l) at 2 months washout (Table 2, Fig. 4).

Table 2: The Thiamine, metabolic and liver function profile before and after three month thiamine treatment and after two month washout period.

Variable (Diabetic Range)	Thiamine Treatment			Placebo		
	Baseline	Therapy	Washout	Baseline	Therapy	Washout
Plasma thiamine (nM/l)	10.6 (0.8–84.5)	98.2 (2.6–294.5) c, f	10.9 (3.6–22.7) i	7.1 (1.1–31.3)	7.1 (2.4–16.6)	9.1 (4.2–16.3)
Urinary thiamine (µmol/24 h)	1.63 (0.45–7.66)	47.69 (0.40–182.79) c,f	1.42 (0.10–9.72) i	1.53 (0.67–4.46)	1.71 (0.15–9.73)	1.16 (0.00–5.44)
Urinary albumin Excretion (mg/24 h)(30-300 MAU)	43.7 (33.0–120.9)	30.1 (12.0–38.2)b,f	20.9 (7.0–35.0)f,h	50.9 (33–122)	35.5 (6.4–82.0)	30 (3.5–80.4)f
Thiamine clearance(ml/min)	112 (8-819)	273(3-789)d	92(18-237)h	189(26-955)	149 (12-663)	102(12-334)e
Plasma glucose (mg/dl)(>126)	181.8±57.6	181.8±70.2	144±6.3d	177.58±55.8	158.4±43.24	144±55.8d
Glycated HbAic% (>7 diabetes)	9.2±1.3	9.0±1.8	7.8±1.6 e,h	8.8±1.8	8.5±1.7	8.4±1.7
Total cholesterol (mg/dl) 200-299)(high)	203.40±50.27	188.32 (100.15-374.90)	178.26 (127.22–278.42	185.22±40.98	171.30±64.19	215.39±76.7h
LDL-cholesterol (mg/dl) (130-159(borderline high)	106.34±47.14	98.996±66.12	93.19±32.09a	106.34±35.96	920.05±460.01	123.35±47.95g
VLDL(mg/dl)(40-100(high)	46.330±31.792	55.62±36.46b	48.260± 30.62	31.30 ±16.339	36.425±17.24d	38.760 ± 13.9 e
HDL-cholesterol (mg/dl)(F<50,M<40)increased risk of coronary artery disease	49.88±13.53	41.76±14.30d	44.47±9.28	47.95±10.44	37.50±8.89f	52.97±21.65h
Triacylglycerol (mg/dl) (200-499 high),	198.47 (53.4-739.59)	2020.03 (81.66-620.73)	231.4(79.21-7360.03)	134.39(72.98-377.3)	159.31 (56.07-357.78)d	175.33 (96.12-396.94)d
BILIRUBIN(0.1-1mg/dl)	0.550±0.244	0.470±0.194 e	0.503±0.24	0.514±0.283	0.382±0.185g	
ALP(U/L)20-160	103.30±33.48	102.85±33.25	91.10±24. 83	88.20±29.84	80.65±44.75	84.1±34.15
ALT(U/L)30-65	360.05±10.49	31.15± 10.36	32.10±10.59	36.10±10.40	31.85±11.41	36.20±15.81
AST(U/L)15-37	22.60±9.70	19.00 ± 7.21	22.15±9.36	21.65±7.70	15.95±8.80 d	20.55±5.68

Data are means ± SD or medians (minimum–maximum). UAE was measured by immunoturbidimetry (Randox, Crumlin, UK,) Fasting plasma glucose, total cholesterol, HDL-cholesterol and triacylglycerol were determined by the Dimension Clinical Chemistry Analyzer method,. Serum LDL-cholesterol was deduced from the Friedewald equation. Plasma and urinary thiamine was determined by pre-column derivatisation to thiochrome and HPLC with fluorimetric detection. [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ compared with placebo; [d] $p<0.05$, [e] $p<0.01$, [f] $p<0.001$ compared with baseline;[g] $p<0.05$, [h] $p<0.01$, [i] $p<0.001$ compared with post-therapy

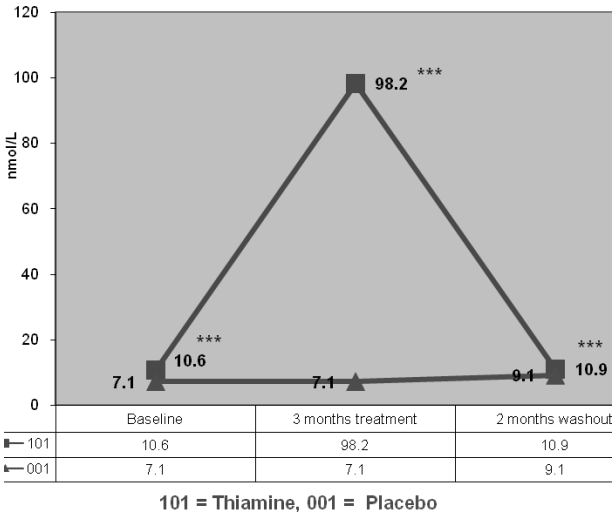

Fig. 4: Mean values of plasma thiamine in thiamine/placebo treated type 2 diabetics from baseline, 3 months post therapy to washout period.

The urinary thiamine values of the thiamine treated group increased significantly from baseline (median value) 1.63 (0.45-7.66 µmoles/24hrs) to 47.69 (0.40-182.79 µmoles/24hrs) (median value) post therapy, an increase in thiamine excretion by 29 fold which was significantly higher than its own baseline ($p<0.001$) and placebo group 3 months post treatment urinary thiamine excretion values of 1.71 (0.15-9.73 µmoles/24hrs, $p<0.001$). During the washout period urinary thiamine median values 1.42 (0.10-9.72 µmoles/24hrs) in the thiamine group reduced by 46.27 µmoles/24hrs (33.58 folds) to significantly lower than treatment values $p<0.001$) returning to its near baseline levels. These values were still higher than the washout urinary thiamine median levels of 1.16 (0.00-5.44 µmoles/24hrs) of the placebo treated patients (Table 2, Fig 5).

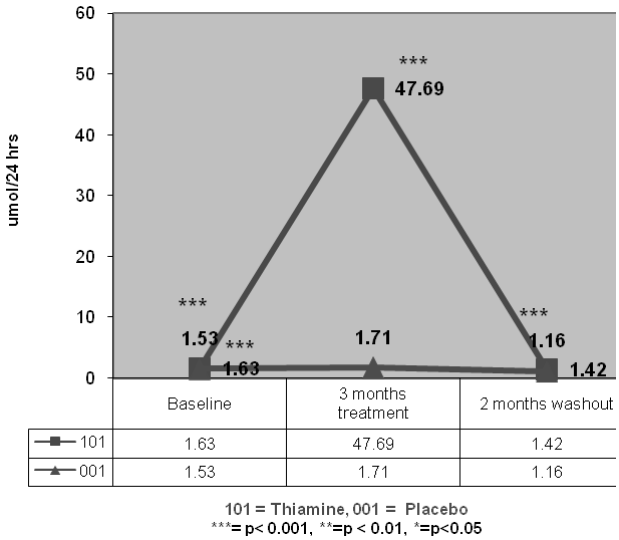

Fig. 5: Mean values of urinary thiamine in thiamine/ placebo treated type 2 diabetics from baseline, 3 months post therapy to washout period.

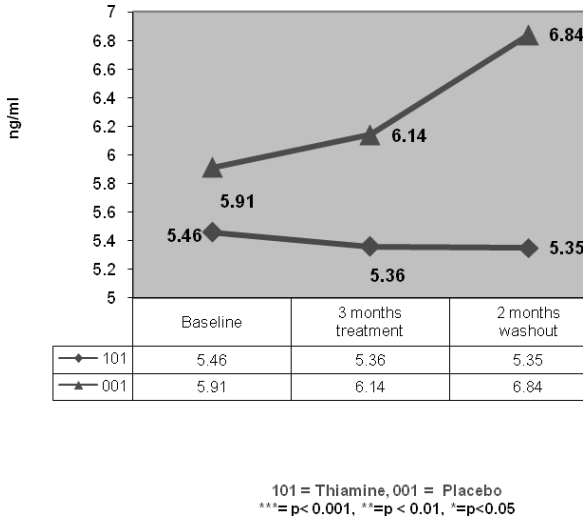

Fig. 6: Mean levels of plasminogen activation inhibitor 1 in thiamine/placebo treated type 2 diabetics from baseline, 3 3 months post therapy to washout period.

Mean plasminogen activation inhibitor 1

Mean Plasminogen Activation Inhibitor 1 levels of the thiamine group at baseline were 5.46±10.01 ng/ml remained steady at 5.36±0.99 ng/ml during therapy and during washout 5.35±1.26 ng/ml and did not register any significant change. However during the same period the placebo group registered a significant steady rise in its PA1 values from 5.91±1.68 ng/ml (baseline) to 6.14±1.44 ng/ml post treatment and 6.84±1.36 (washout value). Thus a significant difference (0.006) was noted between the thiamine and placebo PAI1 washout values (Table 4, Fig. 6).

Plasma thiols

Plasma thiols levels were 3.91±0.72 nmol/ul at baseline, 3.71±0.72 nmol/ul (3 months post therapy) and 3.69±0.64 nmol/µl (washout stages) in the thiamine treated group which did not significantly differ from the placebo group values of 4.15±0.86 nmol/ul (baseline), 4.01±0.89 nmol/ul (therapy) and 3.97±0.85 nmol/ul. Thus no significant impact of thiamine therapy was noted in type 2 diabetics (Table 4, Fig 7).

Protein kinase c

Protein kinase c levels after 3 month thiamine treatment were significantly reduced from mean

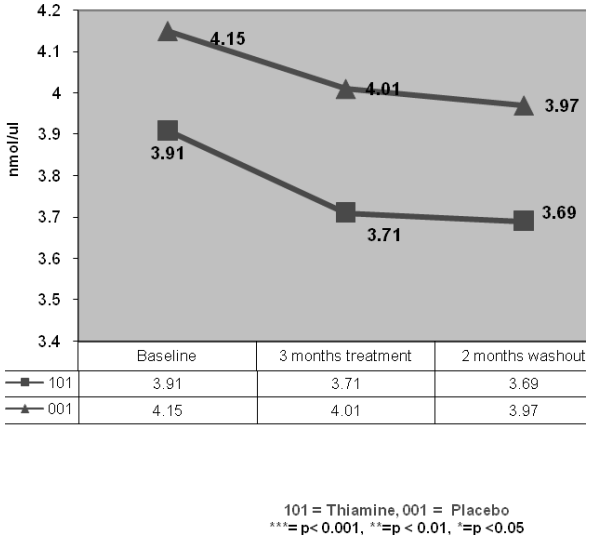

Fig. 7: Mean levels of plasma thiols in thiamine/placebo treated type 2 diabetics from baseline, 3 3 months post therapy to washout period.

baseline levels 60±68.45 pg/ug to 30.26±19.37 pg/ug 55.5% (-37.74 ng: p<0.05) which remained statistically significant during the washout period 37.98±18.66 pg/ug compared to placebo (p0.003). Whereas in the placebo group baseline PKC levels were 76.65±56.22 pg/µg which showed no significant change during therapy 77.75±58.29 pg/µg

Table 3: Renal function profile and physiological data before and after three month thiamine treatment and after two month washout period.

Variable	Thiamine Treatment			Placebo		
	Baseline	Therapy	Washout	Baseline	Therapy	Washout
Creatine Kinase U/L(M:38-174; F:96-140)	81.85±52.30	86.20±62.04g	141.90±114.20id	69.95 ± 55.35	58.30 ± 37.19g	75.85±42.78
Uric acid mg/dl(3.5-8.5)	4.65±10.05	3.75±1.49h	4.82±1.47e	3.99±1.09	40.03±1.70	4.45±0.93
Blood Urea Nitrogen mg/dl(7-21)	11.70 ± 4.87	12.45 ± 5.24	140.05 ±4.76d	12.25±2.24	9.9 ±4.59d,g	14.8 ±5.92d
GFR (ml/min) (<60 chronic renal disease)	85 ±19	90±30	77 ±20	93 ± 23	97± 18	87± 21 P'<0.05, P"<0.01
Serum creatinine mg/dl) (0.8-1.4)	0.915±0.195	0.84±0.28g	0.95±0.25	0.84±0.24	0.77 ± 0.20i	0.94±0.21
Urine creatinine (mg/dl) (30-125)	42.4±25.63	45.86±31.14	43.73±37.37	42.65±31.76	59.1±41.37d	66.59±34.78e
Diastolic BP (mmHg)(<90)	87 ± 7	85 ± 9	87 ± 7	84 ± 6	84 ± 8	83 ± 7
Systolic BP (mmHg)(<140)	126 ± 14	133 ± 20	135 ± 14 P'<0.05	131 ± 10	130 ± 17	130 ± 13

Table 4: Endothelial Dysfunction, Hemostasis, Oxidative Stress and Cellular Signalling Mediator Profile before and after three month thiamine treatment and after two month washout period.

Variable	Thiamine Treatment			Placebo		
	Baseline	Therapy	Washout	Baseline	Therapy	Washout
S VCAM1 (ng/ml)	588±267	554 ± 224	481± 192d, g P'<0.05; P"<0.05	648±255	608 ± 178	600 ± 232
VWF (U/ml)	0.67 (0.07 – 5.18)	0.48 (0.01 – 2.95)	0.51 (0.071 – 1.97)	0.48 (0.01 – 4.35)	0.48 (0.01 – 4.41)	0.59 (0.01 – 1.61)
P AI1 (ng/ml)	5.40±10.017	5.36±0.99fn	5.35±1.26 h	5.91±1.688	6.14 ± 1.44	6.84±1.36
Plasma thiols nmol/µl	3.91±0.723	3.71±0 .727 fn	3.69±0.64	4.15±0.86	40.012± .892	3.97±0.854
Protein kinase C pg/µg	60.00±68.45	30.26 ±19.37b,d	37.89 ±18.86 g	76.65±56.22	77.75 ±58.29 d g	98.67±76.48

Table 3 & 4: Data are means ± SD or medians (minimum–maximum). Renal function parameters, Creatine Kinase ,uric acid,BUN serum and urinary creatinine were measured bystandard referred protocols using the Dimension Clinical Chemistry Analyzer . GFR levels were calculated using the Cockroft Gault formula. [a] p<0.05, [b] p<0.01, [c] p<0.001 compared with placebo; [d] p<0.05, [e] p<0.01, [f] p<0.001 compared with baseline; [g] p<0.05, [h] p<0.01, [i] p<0.001 compared with post-therapy,fn baseline normal<0.001

and increased significantly by mean (+20.92pg/ug) during the washout period to 98.67±76.48 pg/ug (p.020) (Table 4, Fig 8).

DISCUSSION

Diabetes mellitus and specially type 2 continues to impact the lives of millions of people world wide and especially a substantial number of the Asian population.Pakistan has the 6th largest diabetic population and ever increasing in number, 90% of whom are type 2 and stand to be adversely impacted by diabetes in terms of both morbidity and mortality arising from both micro and macro-vasculopathies. These are experienced as chronic renal failure stemming from incipient to overt diabetic nephropathy, cerebrovascular accident, myocardial infarction, angina, visual impairment and a myriad other diabetes related pathologies. In Pakistan diabetes is more a disease of the poor than the privileged with reduced health care budget, rising cost of treatment and growing poverty and reduced standards of living impacting the largest segment of society. In this scenario, the first international WHO registered double-blind, placebo-controlled randomized trial to assess the effect of high dose thiamine therapy in type 2 diabetic microalbuminuric patients was conducted

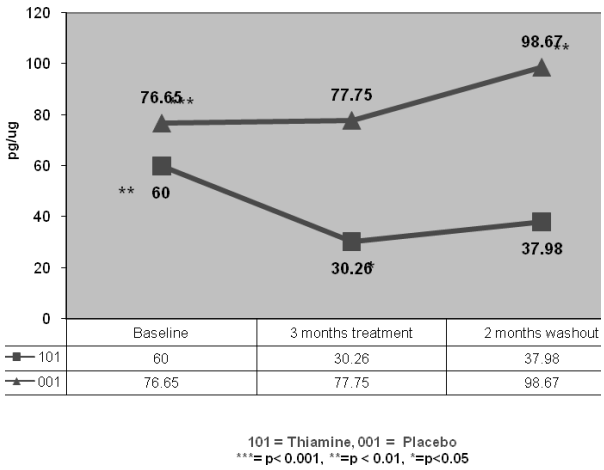

Fig. 8: Mean values of protein kinase C in thiamine/placebo treated type 2 diabetics at baseline, 3 months post therapy to washout period.

here. In its entirety, an indepth analysis was undertaken to determine the effect of high dose thiamine therapy a relatively low cost, safe and accessible vitamin supplement on traditional and non traditional parameters of the biochemical profile. This involved a wide range of, metabolic, vascular dysfunction, hemostasis, oxidative stress and cellular signalling parameters analyses and a molecular study of thiamine dependant enzymes in a particular subset of type 2 diabetic patients with incipient nephropathy who were at increased risk of chronic renal failure. Proper functioning of these enzymes pyruvate dehydrogenase, alphaketo-glutarate dehydrogenase of the Krebs cycle and transketolase of the reductive pentose pathway is imperative for intracellular glucose metabolism once it enters the cells. Their dysfunction could therefore be the basis and possibly be implicated in diabetes pathogenesis and its complications.

Effect of thiamine vs placebo therapy on biochemical profile

Impact of B1 therapy on its own plasma levels-urinary excretion

The baseline characteristics of 40 type 2 diabetic patients recruited for this study confirmed previous findings that plasma thiamine concentration is abnormally low in type 2 diabetic patients with microalbuminuria. The median plasma concentration was 7.5 nmole/l. This was 83.18-92% lower than the range determined for normal healthy controls (44.6-93.7nM/l)[45]. When determined independently for the two groups the mean plasma concentration was 66.5-97.5% lower in the placebo group and 9.8-98.25% lower in the thiamine group. These findings corroborated with previous ones[46,47] as seen in a UK study plasma thiamine levels where type 2 diabetics had (16.3nmol/l), 75% lower mean plasma thiamine levels than in normal volunteers (64.1nmol/l)[45]. In a more recent Dutch study, the lipid soluble congener of thiamine named benfotiamine in a dose of 900mg/day /placebo was administered to 38 out of 82 type 2 diabetics for 12 weeks in a double blind randomized placebo control trial. The baseline mean plasma and whole blood thiamine concentrations of benfotiamine treated patients was 31.8 and 126 nmoles /l and 31.6 and 122nmoles /l in the placebo group were higher than both the UK study and our study but less as compared to mean thiamine concentrations of 64.1 nmoles/l reported for healthy subjects in the UK study. These differences could be possibly due to both dietary and genetic differences related to the handling of drugs by the cytochrome P450 of the liver[48]. It was also evident in our study that following 3 months thiamine/placebo therapy median plasma and urinary thiamine levels both rose significantly 10 fold and 29 fold respectively in the thiamine treated patients while remaining non significant in the placebo group. This was understandable and a proof that the placebo group patients had not been taking thiamine from any other source during this period. In the benfotiamine trial no comparable plasma thiamine levels were found, but whole blood plasma thiamine status improved significantly with benfotiamine therapy ($p<0.001$) from 129 nmol/l to 290 nmol/l during 6 weeks of therapy and a further to 300 nmol/l by the end of 12 weeks of therapy as compared to placebo. No washout study was conducted[49]. However on the basis of our own findings there appeared to be a significant loss of thiamine re-absorptive capacity in the proximal tubules of the kidney as thiamine reserves were depleted in the body at baseline. It appears that relative lack of B1 at baseline was responsible for increased fractional thiamine excretion seen at that time and washout in type 2

diabetics and it's during therapy reversal with 300mg/day thiamine replenishment in thiamine treated diabetics.

Another important point was that urinary excretion of thiamine >0.20 μmol/24 hrs indicates adequate dietary thiamine sufficiency for adult human subjects[50]. On this basis, all diabetic patients in this study had an adequate dietary intake of thiamine. Therefore the low levels of plasma thiamine were because of increased washout and not inadequate ingestion of vitamin B1.

An explanation for the decrease in thiamine re-absorption by the kidney upon encountering thiamine depletion could lie in the transporter protein and in the expression of the genes encoding them. Physiologically, thiamine re-uptake occurs in the proximal convoluted tubules by THTR1 and THTR2 transporters via a sodium independent proton antiport mechanism regulated by CA/calmodulin[51].Evidence suggests the expression of the genes encoding these transporters to be regulated by SP1 promoter elements[52,53]. SP1 signalling in the tubular epithelium is impaired in hyperglycemia associated with diabetes by increased O-glycosylation of the SP1 via enhanced hexosamine pathway activity[54]. Thus re-uptake of thiamine in the current study could have reduced by the hexosamine linked pathway or further due dicarbonyl glycation of thiamine transporters[55] or even by acidification of tubular lumen[56]. The improvement in thiamine reuptake observed with the 3 month thiamine versus placebo therapy and its return to baseline behaviour after 2 months washout could also have been due to the increased activity of thiamine dependant enzymes PDH, OGDH and TKT activity within the tubular epithelial cells and improved cellular metabolism and functioning of these cells reflected as decreased thiamine clearance versus plasma levels. However eventually at the end of 2 months washout plasma thiamine levels had declined but intracellular enzyme activity was maintained specially in the mononuclear cells and less so in the renal tubular cells which are reported to suffer quick depletion and have low TK expression[45].

Thiamine therapy effect on glycemic control

The patients enrolled in this study exhibited poor glycemic control with significantly raised mean fasting blood sugar levels 103% and 98.76% ($p<0.001$) in both the thiamine and placebo as compared to normal controls at baseline. Their corresponding glycated hemoglobin levels were also significantly elevated 9.2% and 8.82% as compared to 5.6% glycation in normal controls ($p<0.001$). This was comparable to a previous study in our population where the fasting blood glucose levels were glycated hemoglobin values were 9.4% in type 2 diabetics and 6.4% in normal healthy controls[57] and also to studies conducted[58]. Mean fasting plasma glucose was 5.6 mmol/l in normal controls and higher 9.1 mmol/l in type 2 diabetics. Mean glycated HbA1c levels in 24 type 2 diabetics with microalbuminuria in the UK were 8.5%[45] and also comparable to our baseline levels. In the benfotiamine trial the fasting blood sugar levels of the benfotiamine and placebo treated patients were not published. Thiamine 300 mg/day/placebo therapy for 3 months both had no impact on fasting blood initially during this period but during 2 months washout period there were comparable decreases in fasting blood sugar in both groups which was 20.83% in the thiamine group and 18.90% in the placebo group ($p<0.05$). This finding is different from authored by workers from the University of Guadaljara in Mexico and reported by the European Journal of Nutrition, in a study involving thiamine supplementation in a randomized double blind placebo controlled trial in drug naïve type 2 diabetics. Twenty-four participants with Type 2 diabetes were included in this study. Twelve received 150 mg of thiamine orally once a day for one month. The other twelve received placebo. At the end of a month, the thiamine treated group showed significant decreases (6.7mmol/l) in blood sugar levels[59]. Our results differed as both thiamine and placebo groups were equally balanced for hypoglycemic drugs other than thiamine during the trial and the reduction in blood sugar levels in both groups at washout reflected the effect of these agents and an absence of any thiamine effect on this parameter.

However fasting plasma blood sugar level is only representative of that day's fasting glucose record and is not representative of the overall glycemic control. Glycated hemoglobin is thus

more representative of the holistic glycemic scenario. Interestingly during this trial glycated hemoglobin levels in the thiamine treated type 2 diabetics registered a significant decrease from 15.2% ($p<0.01$) in glycated hemoglobin from the start of therapy to the washout period, while no significant change was recorded in the placebo group with mean glycated hemoglobin levels ranging between 8.8 and 8.4%[17]. This decrease in thiamine group glycated hemoglobin at washout was different from the finding of the most recently concluded Dutch benfotiamine study where the mean glycated hemoglobin levels ranged between 7.1 to 7.4 during the trial duration and no washout levels were determined. As is known buildup of glycated hemoglobin within the red cell reflects the average level of glucose to which the cell has been exposed during its life cycle (120 days). Measuring glycated hemoglobin thus assesses the effectiveness of therapy by monitoring long term serum glucose regulation. The HbA1c level is generally considered to be proportional to the average blood glucose concentration over the previous 8-12 weeks [60]. Some researchers state that the major portion of its value is related to a rather shorter period of two to four weeks[61]. Therefore the reason for the gradual decrease in hemoglobin in the thiamine treated group during the trial period is probably actual first representation of reduced glycation of the previous 3 months and the significant decrease noticed in the washout period was actually a more apparent manifestation of decrease in HbA1c during the last month of therapy.

Impact of B1 therapy on diabetic nephropathy

In this study, the baseline median values of microalbuminuria were significantly raised in thiamine treated group 43.79mg/24hrs (6.7 fold) in the thiamine group and 50.91mg/24 hrs (7 fold) in the placebo group as compared to normal controls ($p<0.001$). The UK microalbuminuric patient's median 46mg/24 hrs[45] was comparable to our study. Whereas in the Dutch benfotiamine trial the median baseline values of microalbuminuria were 90mg/24hrs) in the benfotiamine group and (97mg/24hrs) in the placebo group[49] which were approximately 2 times higher than in our study. We found that high dose thiamine therapy (3 x 100 mg per day) significantly decreased urinary excretion of albumin in type 2 diabetic patients with microalbuminuria from baseline values after 1–3 months therapy ($p<0.001$). After therapy for 3 months, regression of microalbuminuria to normal urinary albumin had occurred in 35% of the patients. This decrease in urinary albumin excretion even during the washout period showed continued benefit from thiamine supplementation in the previous 3 months. No significant decrease in urinary albumin excretion (UAE) was observed with those who had received placebo. UAE continued to decrease in the 2 months washout period in both groups but not significantly. Linear regression of UAE in relation to treatment time indicated that the rate of decrease in UAE was increased about fourfold in patients treated with thiamine with respect to placebo (5.62 vs 1.52mg/24h) (Fig. 3.)[17,18]. In the benfotiamine trial for 12 weeks did not significantly decrease 24 hr (UAE)[49]. The possible explanations for this observed difference noted in our study with thiamine therapy could possibly be visible in enrolment criteria as our enrolled patients had diabetes of more than 5 years duration and more middle aged age group 35-65 years (in relatively early stage diabetic nephropathy) versus median 12 years diabetes duration and median 65 years age in the benfotiamine trial (more advanced diabetic nephropathy). Moreover, there were geographical, life style, food differences and probably even pharmacogenetic differences between the two groups, ours being Pakistani and theirs Caucasian. However it is possible that increasing the plasma concentration of thiamine in diabetes may have reversed dysfunction of glomerular endothelial cells, podocytes and tubular epithelial cells and thereby improved glomerular and tubular structure and function, low-grade vascular inflammation and decreased urinary albumin excretion in our patients[17,18]. And finally the prolonged regression of micro albuminuria into the washout period could be that even though the half-life of thiamine is about 2 days being relatively short [62], its biological half-life (9-18 days) is much greater [63]. So even though the plasma concentration and urinary excretion of thiamine in the thiamine treatment arm returned to baseline after 2 months washout period, it is likely that increased tissue

levels of TPP, activities of thiamine dependant enzymes and related pharmacological responses remained above baseline for at least one biological half life of thiamine into the washout period of the patients in the thiamine treatment group [45].

Thiamines effect on oxidative stress

Oxidative stress plays a crucial role in atherogenesis in diabetes [19a] and[3]. Measurements of plasma thiols a (marker for oxidative stress) at baseline revealed significant difference between baseline levels of thiamine treated -23% (p 0.001) and placebo group -19.10% (p 0.002) from normal controls with no significant change during treatment or washout stage in both thiamine and placebo groups. Thus thiamine displayed no effect on oxidative stress by reducing or enhancing the level of antioxidant plasma thiols in type 2 diabetics in this study. This result corroborates the findings of Telci in 2000 and Wittenstein in 2002 who found decreased plasma thiols in diabetic patients. A significant decrease in plasma thiols was also reported in diabetic patients on hemodialysis, which improved after a single dialysis session but remained low as compared to control participants and patients with DM[64].The decreased thiol concentration could be the result of enhanced oxidative stress in diabetic patients on hemodialysis [65]. However the absence of thiamine effect on plasma thiols could be neither corroborated nor refuted with other studies due to absence of such data. There being no effect of thiamine on plasma thiols is probably because the oxidative stress pathway involves many chemically important mediators some of which remained unaffected by thiamine therapy.

Effect of B1 therapy on protein kinase C

Protein kinase c a phospholipid and calcium sensitive protein kinase linked to the second messenger signaling mechanism of some hormones, neurotransmitters and growth factors is G protein linked. It can regulate a number of cellular events by activating MAP kinases which depending on cell type including extracellular signal related kinases ERKS, cJun N terminal kinases JNKs and p38 MAPKs[66]. In diabetes, increased hyperglycemia induced superoxide causes glycolytic intermediates to be shunted into the major pathways of hyperglycemic damage. These intermediates activate intracellular AGE formation,the hexosamine pathway and protein kinase C[67]. In this study protein kinase c levels at baseline were significantly 3.4 fold higher (p.008) in thiamine treated group and 3.72 fold higher (p.000) in the placebo treated group versus normal controls. While during treatment, level of PKC in monocytes of thiamine treated diabetics were reduced significantly by 55% (p0.027) and was (-37.74 pg/ug: $p<0.05$) which was not sustained during the washout period. Placebo group in comparison registered a significant increase in protein kinase C levels of 27.22% (p value 0.020) during the washout period. During this 2 month washout period, the placebo treated group had a significant increase in their protein kinase C levels of 26.09% (20.92 pg/ug: $p<0.05$) which persisted during the washout period. Comparative analysis of the pkc levels between the two groups at 3 months post treatment showed this change to be even more significant during treatment (p0.002) and (p0.003) during washout. Comparative analysis with another trial was not possible as protein kinase C activity was not be measured in circulating monocytes in both benfotiamine trial on type 2 diabetics[49] and the benfotiamine and alpha lipoic acid trial in type 1 diabetics[68]. Thiamine's therapeutic benefit of reversal of hyperglycemia induced protein kinase C expression and decreased extracellular matrix deposition during therapy with prolongation of this effect has been reported [69].

Effect of B1 therapy on hemostatasis parameter PAI 1 & vWF

Plasminogen activation inhibitor type 1(PAI1) is another non traditional risk factor for cardiovascular disease in diabetes[3]. This is part of the endogenous fibrinolytic system and its increase signals impaired fibrinolysis and prothrombotic state[10]. At baseline, measurements revealed 74% and 91% significantly higher levels of PAI1 in thiamine and placebo treated patients respectively as compared to normal controls (p0.000). In this study an increase of 15% was noted from baseline to washout PAI1 values of the placebo group was significant in comparison to the thiamine group at

washout (p0.005). There are reports of drugs such as ACE inhibitors significantly decreasing PAI1 without altering tissue plasminogen activator and no such effect being seen with ARB[70]. Better glycemic control in the thiamine group displayed as reduced glycated hemoglobin levels in the 3 months treatment and washout period may have prevented increase in the PAI-1 as compared to the placebo group. Some studies have suggested that blood thrombogenicity is related to chronic glycemic control and that improved diabetic control leads to a less thrombogenic state.[71].

CONCLUSION

In this internationally pioneering study thiamine levels of type 2 microalbuminuric diabetics as compared to normal controls were determined. Both thiamine and placebo groups were significantly thiamine deficieny as compared to normal controls.We found that at baseline the intracellular signalling mediator, protein kinase C, and hemostasis marker PAI1 in type 2 diabetics were raised significantly as compared to normal controls. While the oxidative stress marker plasma thiols levels were significantly lower than in normal controls. Following 3 months thiamine therapy there was significant improvement of urinary albumin excretion in 35% of patients[17,18] and preservation of glomerular filtration rate suggesting that the decline in renal function occurs in background of thiamine deficiency as significantly decreased plasma levels were encountered in our patients .A simultaneous reduction in plasma levels of sVCAM-1,vWF [17,18],protein kinase C, was also observed involving high dose thiamine therapy involving 300mg/day B1 therapy for a period of 3 months . These results were encouraging and suggests that the microvascular dysfunctions especially incipient diabetic nephropathy in type 2 diabetics occurs in the background of thiamine depletion leading to, impaired second messenger signaling mechanisms, endothelial malfunction and altered hemostasis through enhanced thrombogenicity which appear to respond well to thiamine therapy.

Our study recommends daily oral thiamine administration in type 2 diabetics to prevent depreceation of thiamine levels and maintenance of normal thiamine status in addition to conventional therapy of diabetes, as thiamine supplementation added a novel, viable, feasible, and secure dimension to the current diabetic pharmacotherapy as no hepatic renal or other significant adverse effects were noted.(Tables 2 & 3)

However the duration of therapy was relatively short and hence further larger and longer-term clinical intervention trials are required to confirm, assess and advance the potential of thiamine supplements and thiamine derivatives in patients with diabetic nephropathy, neuropathy, retinopathy and cardiovascular disease in the patients of both type 1 and type 2 diabetes.

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