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Full Length Research Paper

Effect of high dose thiamine therapy on activity and molecular aspects of transketolase in Type 2 diabetic patients

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Commonest form of diabetes mellitus is Type 2, treated with oral hypoglycemic agents, which often carry potential adverse effects and do not address the intracellular metabolism of glucose. Thiamine is an essential co-factor for vital subcellular enzymes and has potential to benefit Type 2 diabetics. This study was therefore designed to investigate the effect of high dose thiamine therapy on the activity and molecular aspects of transketolase in Type 2 diabetic patients. Over 100 Type 2 microalbuminuric diabetics were enrolled in a randomized, double blind placebo controlled clinical trial for 6 months. Patients were divided into two groups, one treated with 300 mg/day thiamine and the other group was administered placebo for a period of 3 months followed by a 2 month washout period. 50 normal healthy controls participated for baseline estimations only. Transketolase activity of mononuclear cells and erythrocytes were determined. Also q-polymerase chain reaction (PCR) was used to determine expression levels of transketolase gene in mononuclear cells. All enrolled Type 2 diabetics had > 40% lower mononuclear transketolase activity as compared to healthy individuals. Thiamine therapy for three months resulted in a 65% significant increase in transketolase activity which persisted into washout period. Mononuclear transketolase gene expression was significantly reduced in Type 2 diabetics as compared to normal controls (0.66 fold thiamine group) and (0.89 fold) placebo group). High dose thiamine therapy resulted in highly significant increase (2.86 fold) in expression of transketolase gene in mononuclear cells which was sustained at 2.91 fold after washout period. These results indicate that high dose thiamine therapy improves both transketolase activity and expression in Type 2 diabetic patients with incipient nephropathy.

Key words: Diabetes mellitus Type 2, thiamine, transketolase, mononuclear cells, erythrocytes, qRT-PCR.

INTRODUCTION

Diabetes mellitus is a wide occurring disease. Pakistan with a diabetic population of 5.2 million, 90% of whom are Type 2, ranked 6th in the year 2000 in a list developed by World Health Organization (WHO) showing the countries with the highest number of diabetics (Wild et al., 2004). Thiamine as TPP is a cofactor for some of the key enzymes involved in glucose utilization such as transketolase (TKT) (Christopher et al., 1999). Hence, it plays a key role in carbohydrate metabolism and an indirect role even in lipid metabolism (Christopher et al., 1999). TKT is an important cytosolic enzyme of the reductive pentose phosphate pathway (RPPP). Three genes; TKT, transketolase like TKTLI and transketolase like TKTL2 encode for proteins with TKT activity which participates in the

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Abbreviations: TKT, Transketolase; **RPPP,** reductive pentose phosphate pathway; **PBMCs,** peripheral blood mononuclear cells; TPI, triose phosphate isomerase; GDH, glycero-3-phosphate dehydrogenase; **RBC,** red blood cells; **BLAT,** basic local alignment tool; **PCR,** polymerase chain reaction.

RPPP reactions at two sites, transferring two carbon groups from xylulose 5 phosphates, thus also reorganizing the carbon atoms that enter this pathway and generating three carbon molecule glyceraldehyde-3-phosphates, which can be shunted to glycolysis and oxidized to pyruvate (Kochetov and Sevpstyanova, 2005). TKT enzyme genetic variants and depreciated enzyme activities were noted in neurodegenerative diseases like Wernickes Korsakoff syndrome and Alzheimers disease (Coy et al., 2005). While upregulation of the TKT L1 gene has been found in a number of malignancies, resulting in enhanced total transketolase activity and cellular proliferation in human colon cancer (Hu et al., 2008). TKT has begun to emerge as a target in the cellular immune response in multiple sclerosis (Lovato et al., 2008).

In diabetes mellitus Type 2 experimental model, the role of transketolase in the RPPP and its activation by administration of lipid soluble thiamine derivative benfotiamine is well documented (Hammes et al., 2003). No currently available medication for Type 2 diabetes targets TKT as a prospect for enhancement of intracellular glucose metabolism which could probably add a new dimension to diabetes management. This study aimed to determine the effect of thiamine HCl, administered at a high dose of 300 mg per day, on TKT activity and expression level of its gene, in Type 2 diabetic microalbuminuric patients in a double blind placebo controlled clinical trial setting.

MATERIALS AND METHODS

Chemicals and reagents

Purple and red topped vaccutainers, and 10 cc syringes were purchased from Beckton Dickinson Company. Ficoll-Paque PLUS and B1 tablets were supplied by GE healthcare (Waukesha, WI, USA) and Roche (Basel, Switzerland) respectively. B1 Trizma base (T3253)-HCl, phenylmethylsulfonyl fluoride (P7626), potassium ethylenediamine tetraacetic acid, (ED2P were purchased from Sigma, Germany. TritonX-100(93426), dithiothreitol DTT (43819), triton X-100 (93426) were procured from Fluka (Germany) and sodium deoxycholate (43035) was purchased from BDH, UK. Haemoglobin estimation kit was supplied by Crumlin UK. For transketolase assay, D-xylulose 5 phosphate sodium salt (XO754), D-ribose5-phosphate disodium dehydrate (R-7750), glycerol 3 phosphate dehydrogenase from rabbit muscle, triose phosphate isomerase from baker yeast. NADH disodium salt was obtained from Calzyme Laboratories (USA). Primers were synthesized by Eurofins MWG/ Operon (USA). Cell lysis solution and Tri Reagent LS (TS 120) were purchased from Gentra (USA) and Molecular Research Institute (USA), respectively. DNA marker ladder mix, cDNA synthesis Kit and Maxima SYBRGreen/ROXq polymerase chain reaction (PCR) MasterMix were obtained from Fermentas (Germany). Microplate reader and 96 well Quartz plate were purchased from Biotek (USA) and Hellma (Germany), respectively. Real time PCR analyzer (ABI 75000) and 96 well plates were supplied by Applied Biosystems (USA).

Selection of patients

Over 100 Type 2 diabetic patients with microalbuminuria and 50 normal healthy individuals (35 to 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 (Rabbani et al., 2009; Riaz et al., 2010). 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. Half of the inducted diabetic individuals were administered thiamine 3 x 100 mg tablets/day for three months, while the other half was 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 months thiamine/placebo administration and 2 months follow up period for this study (Rabbani et al., 2009; Riaz et al., 2010).

Ethical approval of the study

Ethical approval for the study was taken from the Ethical/Protocol/Synopsis Committee of FPGMI (Sheikh Zayed Federal Post Graduate 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 (WHO); international clinical trials registry platform search portal http://www.ctri.in/Clinicaltrials/ViewTrial.jsp?trialno=203trialid=CTRI/2008/091/000112apps.who.int/trialsearch/trial.aspx?trialid=CTRI/2008/091/000112)

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 for biochemical, erythrocyte and peripheral blood mononuclear cell isolation for transketolase activity and gene expression analyses. 24 h urine collections were also made for determination of micro albuminuria (Rabbani et al., 2009).

Cell preparation

The peripheral blood mononuclear cells (PBMCs) were isolated from the KEDTA treated blood samples at room temperature on a ficoll-paque gradient as described by Boyum (1968). These PBMCs were recovered from the interface and subjected to short washing steps with Hanks balanced salt solution to remove any platelets, Ficoll-Paque PLUS and plasma. The purity of the mononuclear cells 97% was confirmed using Trypan blue exclusion test and microscopy (Philips, 1973). The obtained pellet was lysed by resuspending and sonicating in a cold lysis buffer containing 20 mmol/L Tris-Cl (pH 8.0), 1 mmol/L dithiothreitol, 1 mmol/L potassium EDTA, 0.2 g/L Triton X-100, 0.2 g/L sodium deoxycholate and 0.2 mmol/L phenylmethylsulfonyl fluoride (Pekovich SR, 1996). (Chamberlain et al., 1996). The protein concentration was determined using the Brad-ford protein assay and the appropriate amount was added to the assay mixes as described below.

Packed red blood cells (100 µl) were put in a 1 ml micro centrifuge tube. 400 µl ice-water were added and mixed on a vortex mixer and left on ice for 10 min. Red blood cells (RBC) membranes by centrifuging at 6000 g for 10 min, 400 µl of the supernatant was removed and stored at – 80ºC until used further in the assay. The absorbance of the cyanomethhemoglobin was monitored at 540 nm

for hemoglobin estimation. Hemoglobin was estimated to be approximately 60 mg/dl in each sample.

Transketolase assay in mononuclear cells

TKT activity was measured by using the enzyme-linked method (Smeets et al., 1971), under conditions in which coupling enzymes were not limiting (Tate and Nixon, 1987). Reactions were initiated by the addition of 0.1 mg total protein/mL of reaction mix to an otherwise complete reaction mix of 100 mmol/L Tris-Cl (pH 7.6), 10 mmol/L ribose 5-phosphate, 2 mmol/L xylulose 5-phosphate, 1.2 mmol/L $MgCl_2$, 0.1 mmol/L NADH, 2000 U/L glycerol-3-phosphate dehydrogenase and triose phosphate isomerase, making a final volume of 300 µl. All test samples were run in duplicate with (sample minus substrate) serving as controls. Reactions were conducted at 37°C. The oxidation of NADH, which is directly proportional to transketolase activity, was followed by monitoring the decrease in absorbance at 340 nm for 80 mins using a Biotek 808IU microplate reader. Activity was expressed as nmol/mg protein/min).

Transketolase assay in erythrocytes

The activity of the TPP-dependent enzyme TKT was measured by the kinetic method of Chamberlain[14] which determines TKT activity by coupling the formation of GA3P from R-5-P and xylulose-5-phosphate to the oxidation of NADH using triose phosphate isomerase (TPI) and glycero-3-phosphate dehydrogenase (GDH). 200 µl of aliquots of 10 ml assay cocktail containing 5 ml of 500 mM Tris/HCL, pH 7.8, 3.65 ml water, 1 ml of ribose-5-phosphate stock solution (148 mM), and 253 µl of stock NADH solution (10 mM), 37 ul of triose phosphate isomerase stock solution (50,000 U/ml), 60 µl of glycero-3-phosphate dehydrogenase stock solution (1000 U/ml) were added to the wells of a 96-well microplate. The hemolysate test samples were further diluted 6-fold in water and 20 µl of this was added to the assay well in the microplate. Final volume was 230 µl and all test samples were run in duplicate with (sample minus substrate) serving as controls. The solution was mixed in the thermostatic, shaker equipped Biotek 808IU microplate reader using a 96 well Helma quartz plate at 37ºC and the absorbance was read at 340 nm at 20 min intervals for 80 min. From the assay of haemoglobin in the haemolysate, the activity of TKT was deduced in mU/mg Hb/min from the standard curve of NADH.

RT-PCR for transketolase expression

For total RNA isolation from the whole blood, PBMC (white blood cells) pellet was isolated first. KEDTA treated blood was aliquoted in 1.5 ml eppendorf tubes and 900 µl of cell lyses solution was added to each aliquot, vortex and left at room temperature for 10 min, then centrifuged at 1000 rpm for 1 min at 4°C. PBMC formed a tight pellet at the base of the tube. The whole process was performed once again if RBCs were not completely lysed and pellet appeared red. The PBMC pellet was dissolved in 50 µl of the lyses solution and then further processed to RNA extraction. To isolate RNA from PBMC, 750 µl tri reagent (TS120) were added according to manufacturer's protocol. Sequences of TKT and GAPDH human genes under study were retrieved from NCBI (National Center for Biotechnology Information), and final sequences were then obtained using BLAST (Basic Local Alignment Search Tool) (www.genome.ucsc.edu). Primer designing was done using (www.primer3) software and a final reconfirmation completed using In SilicoPCR (www.genome.ucsc.edu).

Reverse transcription was done using Revertaid minus strand cDNA synthesis kit and the instruction manual. DNase-treated total RNA (2 µg) was converted to cDNA and its semiquantitative PCR was performed to verify the amplification of the thiamine dependant enzyme genes. Amplification was conducted in a Biocycler (ABI) using temperatures of 94ºC (5 min)/94ºC (30s)/62ºC (30 s)/72ºC (40 s)/72ºC (7 min) for 35 cycles using gene specific primers TKT 5'TACCACAAGCCTGACCAGCAGA,TKT3'CTTGGAGAGCACAAAGCGGTCA(216 bp)GAPDH5'-GGTCACCAGGGCTGCTTTTAAC,GAPDH-3'CACTTGATTTTGGAGGGATCTCG(210 bp).

GAPDH was used as internal standard. Real time PCR analysis was also performed using the SYBR green kit and its provided instruction manual utilizing ABI 7500 instrument in a 20ul reaction volume. Quantitative PCR was conducted using the following parameters 50ºC (2.00 min)/95ºC (10 min)/95ºC (15 s)/62ºC (45 s)/72ºC (45 s)/72ºC (45 s) for 40 cycles. The relative gene expression analysis was done by using SDS 3.1 software provided by ABI. Each real time PCR assay was performed in duplicate. The GAPDH gene was used as a control for normalization. After each stage, whether qualitative or quantitative PCR products were gel separated to verify the required sized bands.

Statistical analysis

All significance testing and correlation analysis were performed using the statistics package for social scientists (SPSS version 16.0). Data are presented as mean ± SD. , In placebo and thiamine treated group, all measurements at baseline, therapy and washout out period or post therapy, groups were compared and ANOVA(Parametric) or Kruskall wallis (Non Parametric) tests were applied. ANOVA was used when groups are found to be normal and Kruskall-wallis test was used when non-normality was found. For normality check, Kolmogorove smirnov test was used. Significance of difference of proportions was made by reference to Finney's 2×2 contingency tables. $p < 0.05$ was consideredstatistically significant.

RESULTS

Detailed biochemical profiles were maintained for the enrolled Type 2 micro albuminuric patients throughout the trial duration, including the 3 months post therapy and 2 month washout period which have already been published in our paper (Rabbani et al., 2009). These revealed certain beneficial effects of high dose 300 mg/day thiamine therapy and absence of adverse effects (Rabbani et al., 2009).

Kinetic absorbance profile of transketolase in mononuclear cells

In order to ascertain activity of transketolase kinetic assays were carried out using a Biotek ELX 808IU microplate reader. Duplicate samples of normal controls and Type 2 diabetics (thiamine and placebo groups) were assayed at baseline; 3 months post therapy and washout. Each sample activity was monitored against its own control well where the reaction mix was identical but lacking in substrate only. Kinetic absorbance profiles were generated for each well by the reader.

Normal healthy individuals mononuclear transketolase had a kinetic absorbance profile at baseline which showed

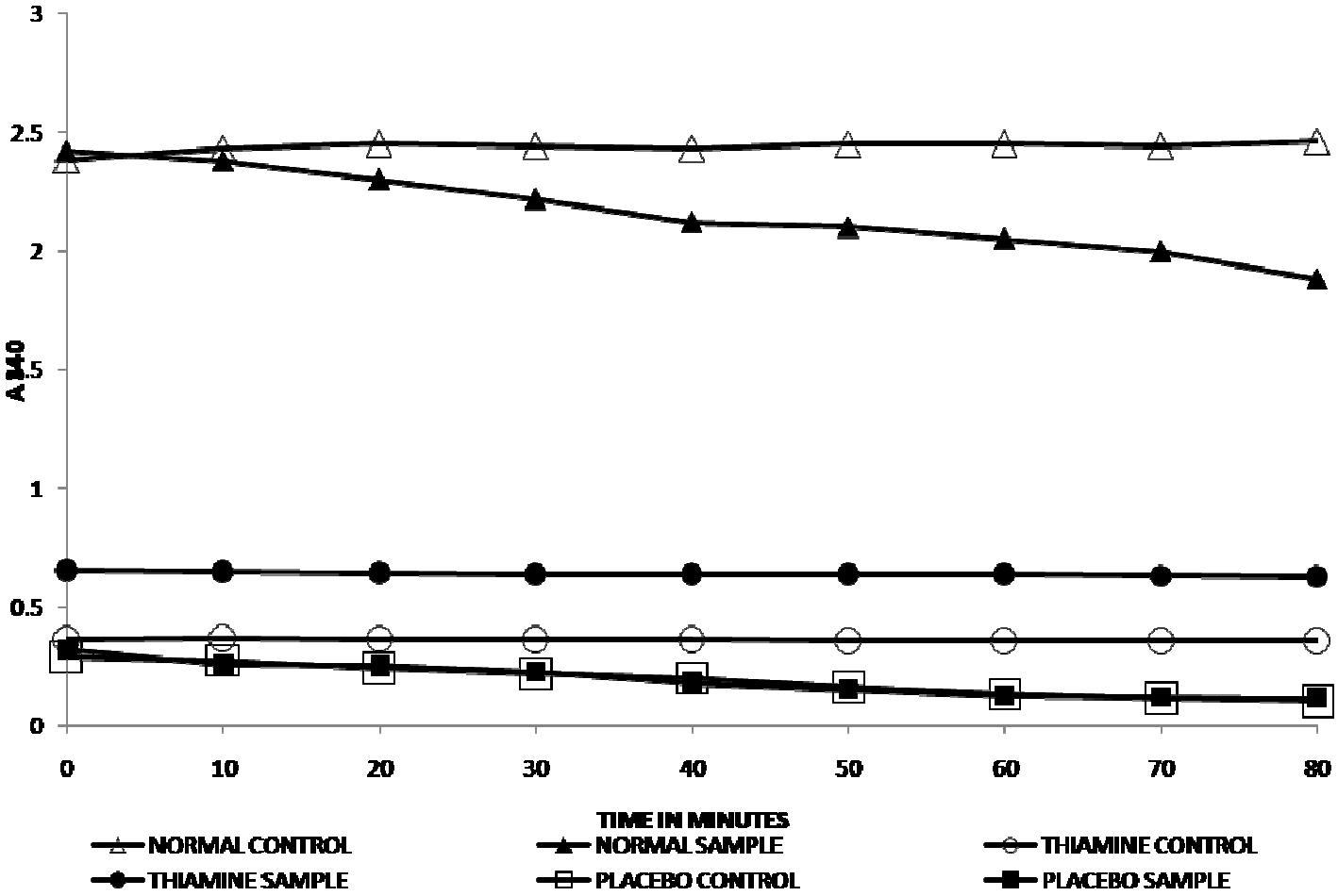

Figure 1. The mean baseline kinetic absorbance profile of transketolase in samples of human mononuclear cells of healthy individuals, thiamine and placebo administered Type 2 diabetics, versus their control samples.

an absorbance difference of 0.46 nm compared to their control samples, which was noticeably higher than transketolase of thiamine treated Type 2 diabetics which revealed an absorbance difference (0.02 nm) and that of the placebo group (0.01) baselines when analyzed against their own control samples (Figure 1). As kinetic absorbance is an essential component in measuring enzyme activity, mononuclear transketolase in normal healthy controls can be inferred to be more functional than Type 2 diabetics at baseline (Figure 1). Thiamine treated mononuclear transketolase absorbance profile rose to 0.182 nm after 3 months of therapy and further to 0.25 nm after 2 months washout in comparison to their control samples (Figure 2). While placebo treated diabetics mononuclear transketolase absorbance profile remained unchanged after 3 months post therapy (0.011 nm) and the same (0.01 nm) at washout when compared to their control samples over similar time period (Figure 3).

Thus thiamine therapy improved kinetic absorbance profile of mononuclear transketolase in contrast to an indistinguishable placebo effect.

Kinetic absorbance profile of transketolase in erythrocytes

Normal healthy individuals erythrocyte transketolase had a kinetic absorbance profile at baseline which showed an absorbance difference of 0.113 nm, which was exactly similar to that of erythrocyte TKT of thiamine treated Type 2 diabetics which revealed an absorbance difference (0.113 nm) but higher than placebo group (0.06) baselines (Figure 4). In thiamine treated group absorbance increased to 0.13 nm after 3 months therapy and regressed to 0.06 nm at washout. In comparison the erythrocyte transketolase absorbance profile of placebo treated Type 2 diabetics, after 3 months post therapy declined to 0.007 nm and at washout returned to 0.07 nm in comparison to their baseline levels (Figure 5). As kinetic absorbance is an essential component in measuring enzyme activity, erythrocyte transketolase in normal healthy controls appeared to be equally functional to Type 2 diabetics thiamine allocated diabetics and only marginally better than the placebo group (Figure 5). Therefore, no remark-

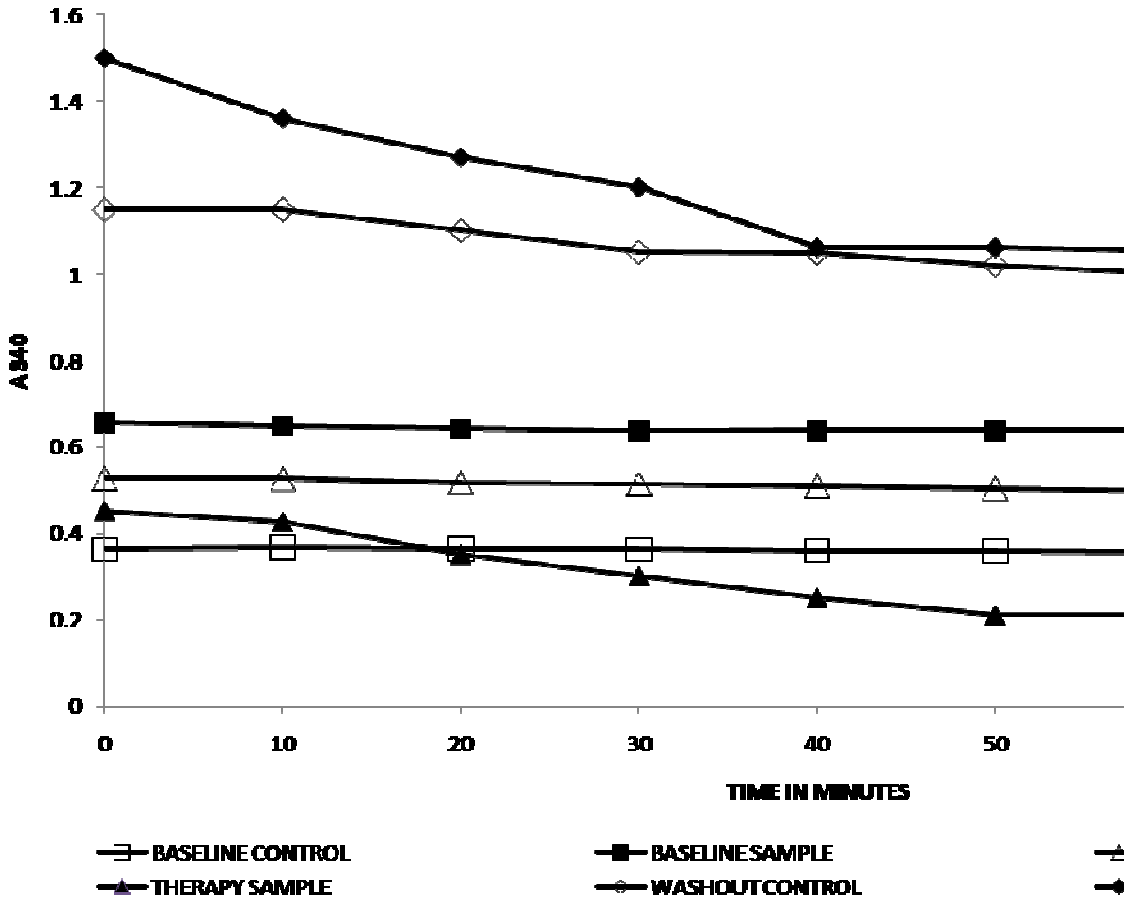

Figure 2. The mean kinetic absorbance profile of mononuclear transketolase in samples of thiamine administered human Type 2 diabetic versus control at baseline; 3 months post therapy and washout).

able difference in kinetic absorbance profile was noted with either thiamine or placebo therapy.

Transketolase activity in mononuclear cells

The mean values of transketolase activity at baseline of the thiamine allocated diabetics were 34.00 ± 31.15 nmoles/mg protein/min and placebo group values 35.98 ± 30.43 nmoles/mg protein/min and were both statistically significant $p < 0.01$ from baseline values 62.83 ± 41.16 nmoles/mg protein/min of healthy controls (Figure 6, Table 2).Thiamine therapy group had an incremental highly significant rise in transketolase activity to mean 56.10 ± 32.13 nmoles/mg protein/min (p value < 0.001) after 3 months thiamine therapy. This increase in activity also persisted after 2 months washout period as mean 57.12 ± 34.13 nmoles/mg protein/min $p < 0.05$). Placebo treated patients had no significant change in activity between mean baseline activity of 35.83 ± 30.43 nmoles/mg protein/min, mean 3 month post-treatment activity of 27.13 ± 41.83 nmoles/mg protein/min and 2 month washout levels of 24.19 ± 25.57 nmoles/mg protein/min. Intergroup analysis between thiamine and placebo groups showed significant increase of transketolase activity in favour of the thiamine administered patients at therapy and washout compared to their baseline activity ($p < 0.01$) (Figure 6, Table 2)

Transketolase activity in erythrocytes

The mean values of activity TKT at baseline of the thiamine allocated diabetics 0.98 ± 0.17 mU/mg protein/min were statistically non significant from baseline values of 1.08 ± 0.10 mU/mg protein/min observed in controls while the placebo randomized also 0.95 ± 0.17 mU/mg protein/min were statistically significant $p < 0.05$ (Figure 7, Table 1).

Mean transketolase activity in erythrocytes in thiamine treated group rose to 1.00 ± 0.16 mU/mg Hb/min at the 3 month post therapy period and at washout was 1.00 ± 0.18 mU/mg Hb/min which at no point was recorded as a significant change. In the placebo group as well the mean

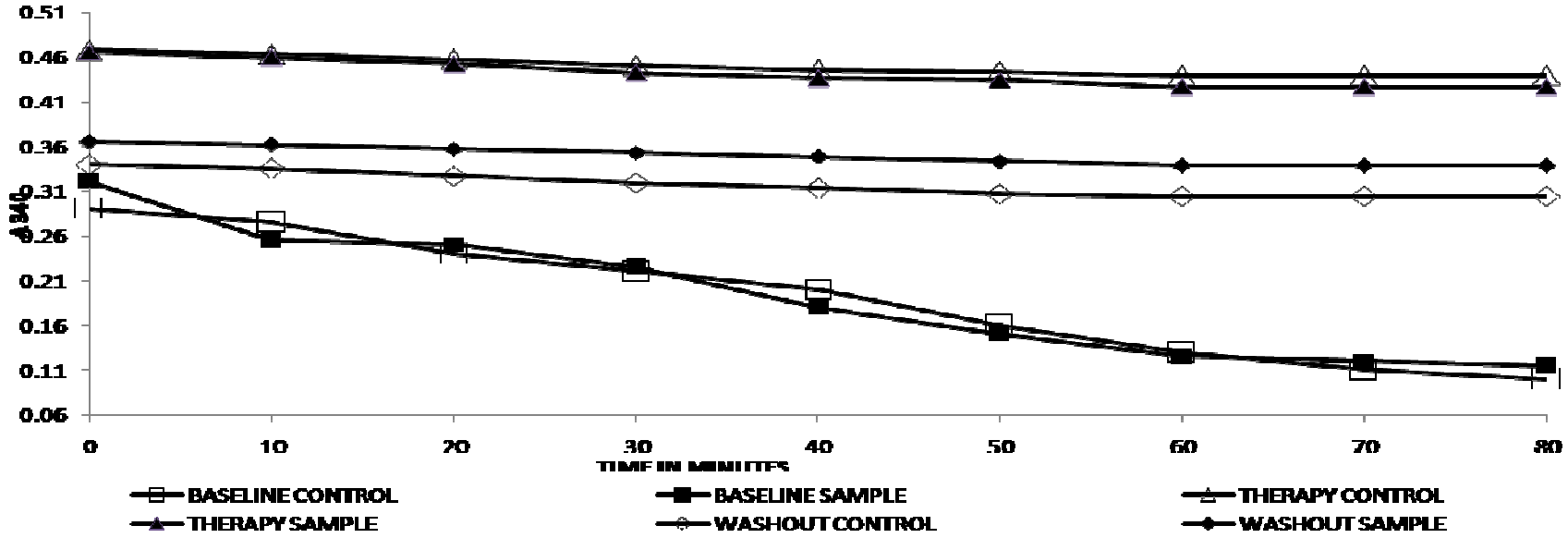

Figure 3. The mean kinetic absorbance profile of monononuclear transketolase in samples of placebo administered human Type 2 diabetic versus control at baseline, 3 months post therapy and washout).

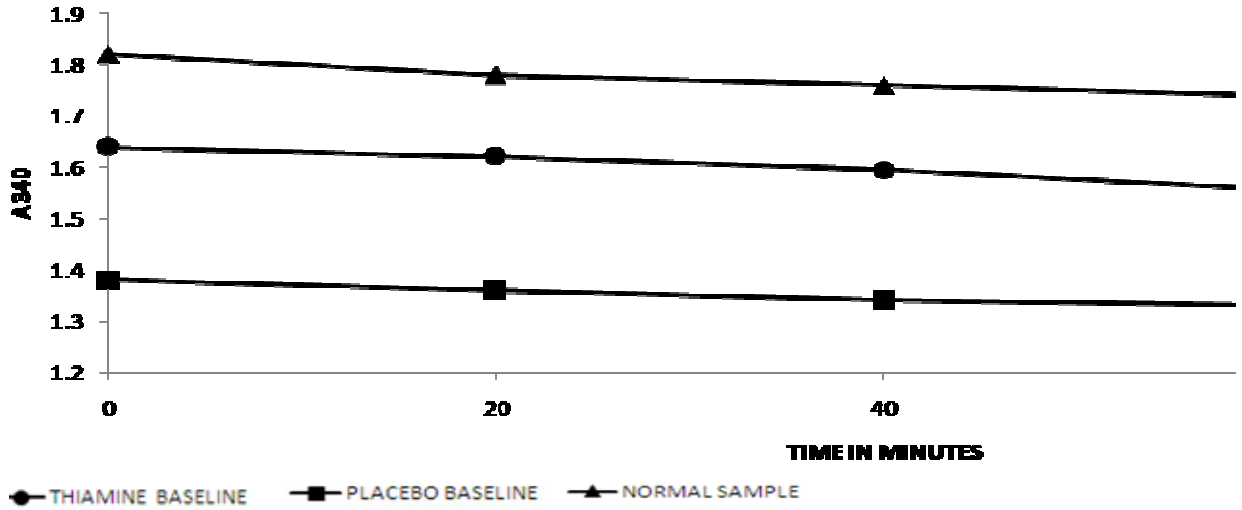

Figure 4. The mean baseline kinetic absorbance profile of transketolase in erythrocytes of healthy individuals, thiamine and placebo administered Type 2 diabetic, versus their control samples.

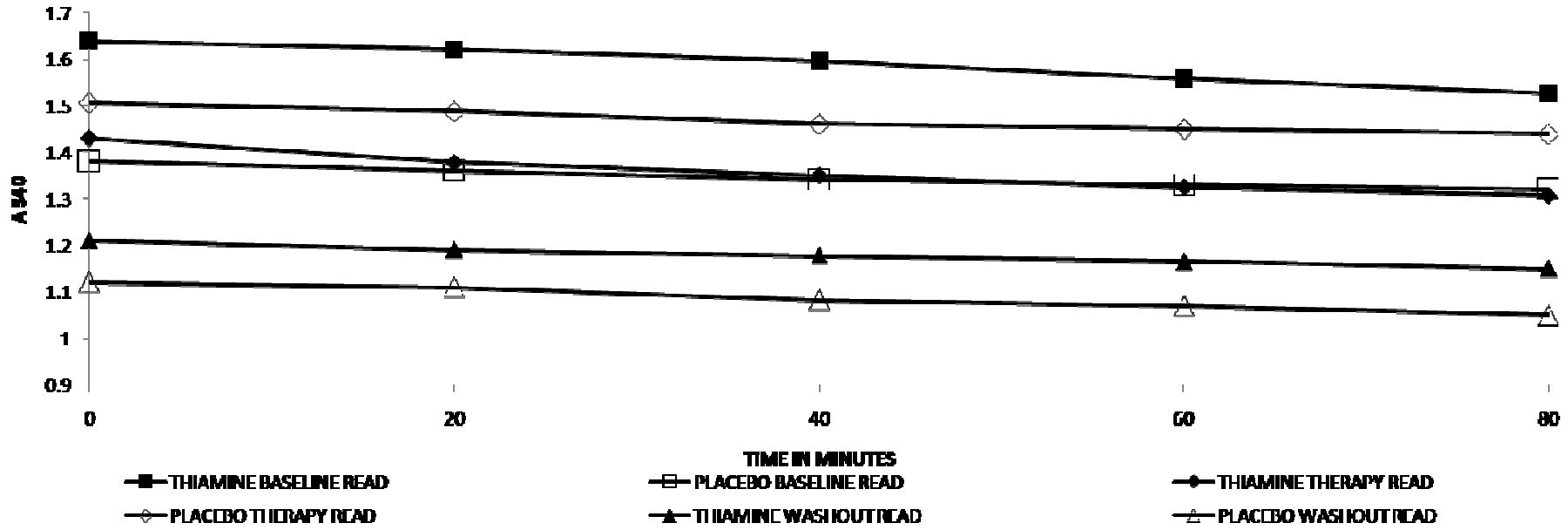

Figure 5. Mean kinetic absorbance profiles of erythrocyte transketolase in thiamine administered Type 2 diabetics versus placebo treated Type 2 diabetics is shown at pre-treatment, 3 months post therapy and washout.

TKT activity levels at baseline were 0.95 ± 0.17 mU/mg Hb/min, 3 month post treatment 0.96 ± 0.69 mU/mg Hb/min and at washout were 1.00 ± 0.21 mU/mg Hb/min and similarly non significant at all points ($p > 0.05$) (Figure 7, Table 1).

Expression analysis of transketolase

Analysis of the effect of high dose thiamine or placebo on expression of TKT gene of the reductive pentose pathway in diabetic patient's PBMCs was carried out. Total cellular RNA was isolated from normal, Type 2 diabetic blood and 2 µg of the RNA was reverse transcribed. The baseline expression of TKT gene in thiamine and placebo treated diabetics was (0.66 fold) and (0.89 fold) respectively which was significantly lower (p 0.017) than normal controls. The expression levels of TKT gene in thiamine and placebo treated diabetics blood were further quantified against their own baselines maintaining GAPDH as internal control to observe any change after 3 months post therapy and following washout. Following therapy, the thiamine treated patients recorded mean TKT expression (2.86 folds) registering a highly significant fold change (1.86 fold) from baseline or 186% increase in expression $p < 0.001$ and even after 2 months washout they had TKT expression levels of (2.91 folds) was a further marginal increase of expression from post treatment levels and therefore remained significantly raised from baseline $p < 0.001$. While placebo treated patients after therapy had TKT expression of (0.88 fold) and during washout (0.86 fold) which was not significantly different from baseline $p > 0.05$. Therefore, increased expression TKT occurred in PBMCs of thiamine treated Type 2 diabetics in comparison to placebo (Figure 8).

DISCUSSION

There are varying reports on the levels of thiamine and thiamine-dependent enzymes encountered in diabetes mellitus, chronic alcoholism, Wernickes encephalopathy and HIV AIDS (Des Jardins et al., 2005). A UK study on diabetics both types 1 and 2 revealed considerably decreased blood plasma thiamine concentrations and surprisingly increased thiamine clearance in types 1 and 2 diabetics

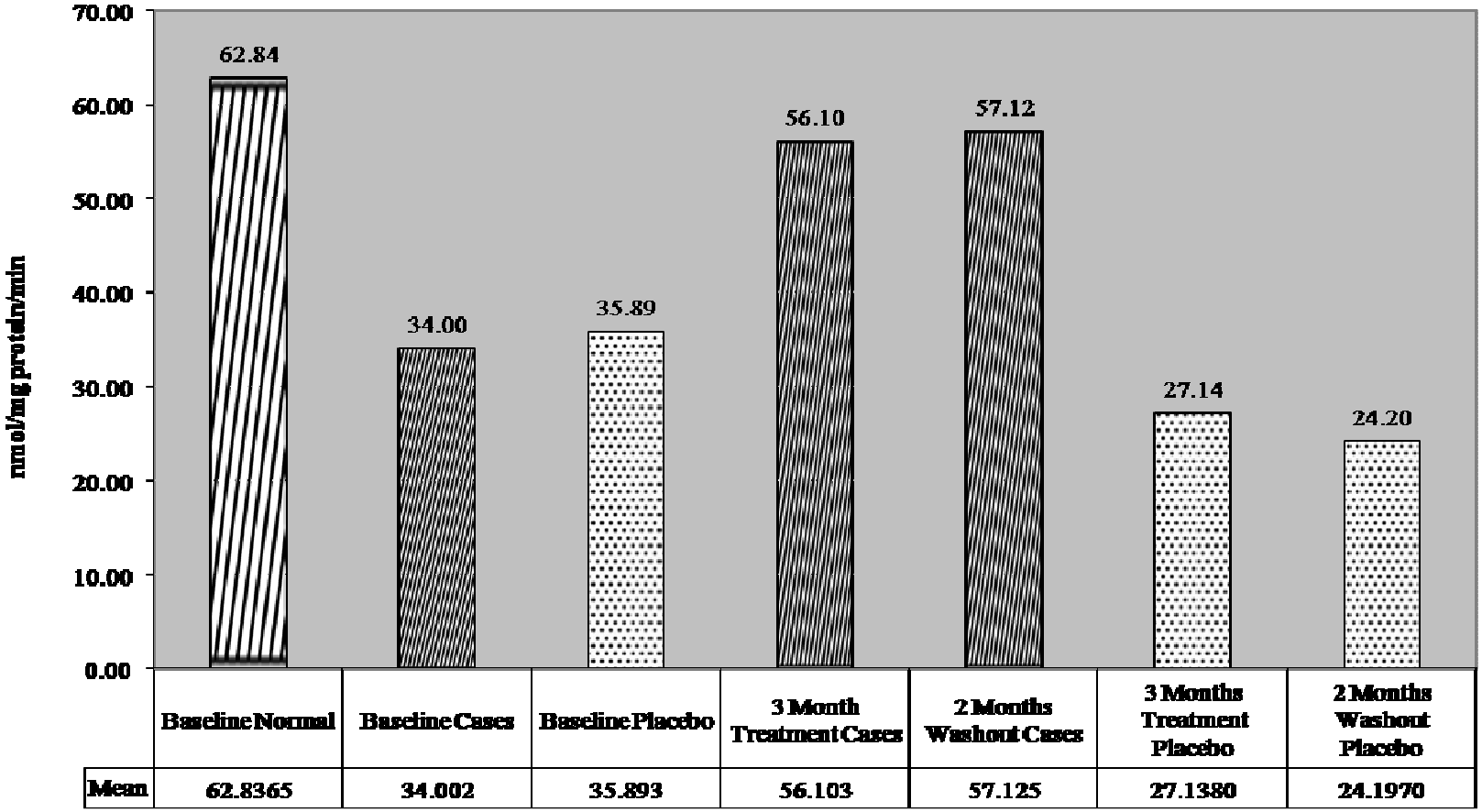

Figure 6. Mean transketolase activity in mononuclear cells of thiamine and placebo treated Type 2 diabetic patients at baseline, therapy and washout as compared to healthy controls.

Table 1. Erythrocyte activity of thiamine dependant enzyme transketolase in normal healthy controls, thiamine and placebo treated Type 2 diabetics.

Control group (n = 20)	Thiamine treatment (n = 20)			Placebo treatment (n = 20)		
Baseline	Baseline	Three months post therapy	Two months post washout	Baseline	Three months post therapy	Two months post washout
1.08 ± 0.10	0.98 ± 0.17	1.00 ± 0.16	1.00 ± 0.184	0.95 ± 0.17 [b]	0.95 ± 0.17	1.00 ± 0.21

Data are means ± SD values, Values in munits/mg Hb/min, [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.

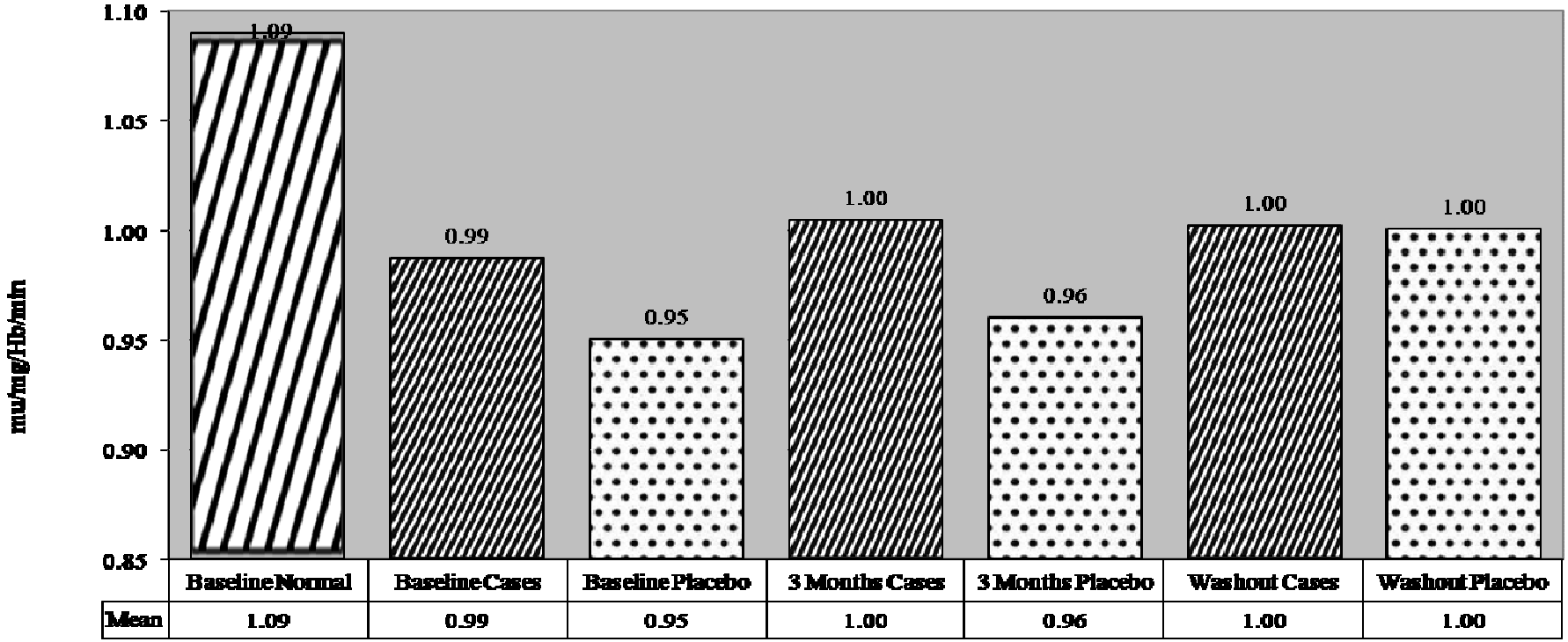

Figure 7. Mean erythrocyte transketolase activity in thiamine/placebo treated Type 2 diabetics at baseline compared to normal controls, post therapy and washout.

Table 2. Mononuclear activity of the thiamine dependant enzyme transketolase in normal healthy controls, thiamine and placebo treated Type 2 diabetics.

Control group (n = 20)	Thiamine treatment (n = 20)			Placebo treatment (n = 20)		
Baseline	Baseline	Three months post therapy	Two months post washout	Baseline	Three months post therapy	Two months post washout
62.83 ± 41.16	34.00 ± 31.01[a]	56.10 ± 32.13[f,b]	57.12 ± 34.13[b],	35.98 ± 30.43[a]	27.13 ± 41.83	24.19 ± 25.57

Data are means ± SD values, (Values in nmoles/mg protein/min), [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.

compared to healthy volunteers (Thornalley et al., 2007). *In vitro* activity of a key thiamine dependent enzyme TKT, of the pentose phosphate pathway, was investigated in cultured human lymphoblasts. These when exposed to pyrithiamine generated varying levels of thiamine and TKT deficiency which could only recover by 25% with thiamine therapy suggesting decrease in the synthesis rate of the protein and thiamine's direct effect on TKT gene or

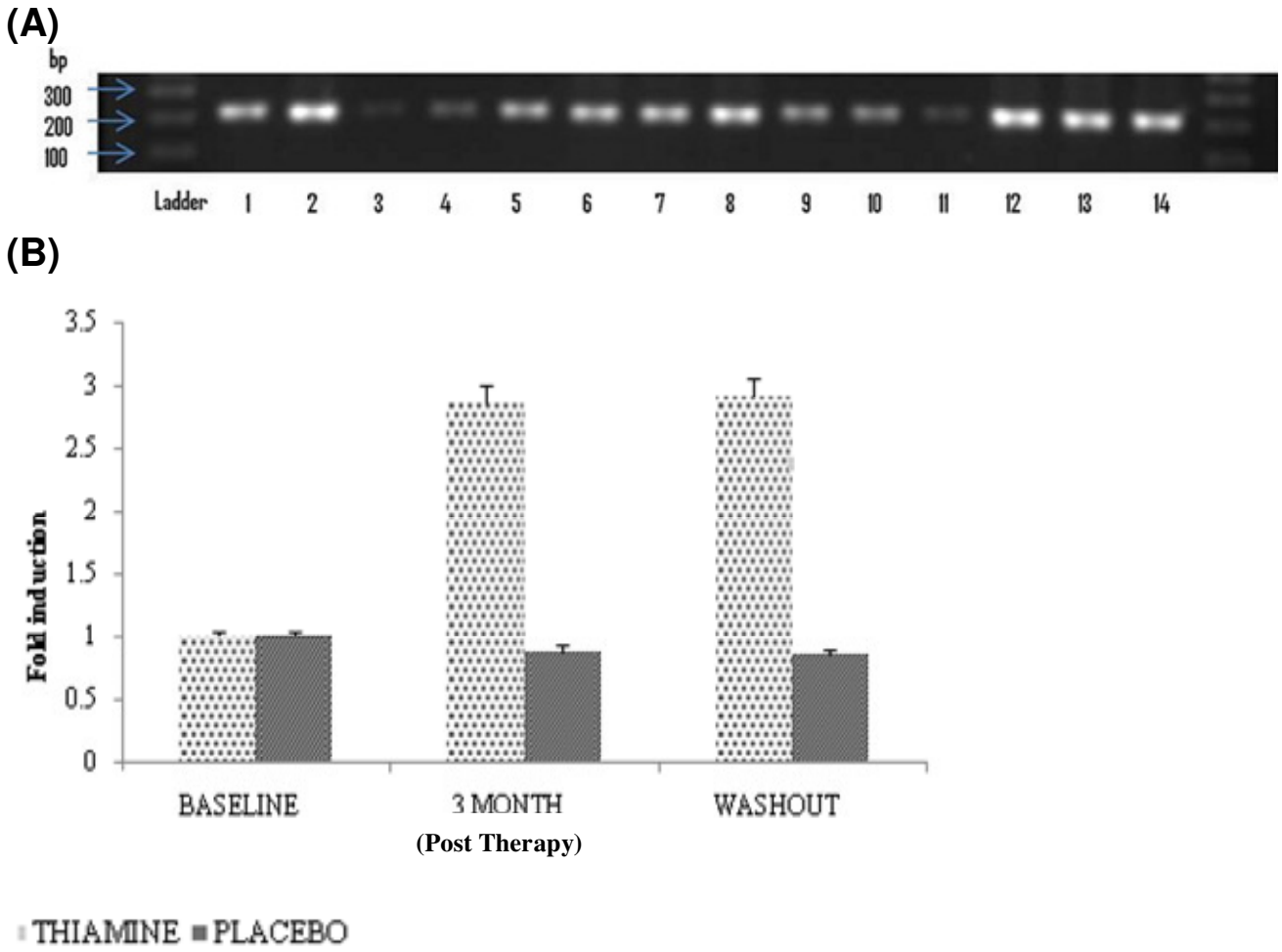

Figure 8. Comparison of expression of transketolase gene in diabetic samples of thiamine versus placebo samples at baseline, treatment and washout. **A)** RNA was extracted from Type 2 diabetic PBMC samples of thiamine verses placebo patients. Relative RNA determinations were carried out using semi-quantitative RT-PCR. The expression of transketolase gene was significantly lower (band 3 (baseline) of thiamine treated Type 2 diabetics and (band 9 (baseline) of placebo treated diabetics as compared to (band 1*—normal controls). Following thiamine therapy expression of transketolase gene increased significantly (band 4) and even more in washout (band 5) as compared to its baseline (band 3).In comparison there was slight decrease in transketolase expression seen in placebo treated patients at post therapy(band 10) as compared to baseline (band 9) and slightly decreased expression at post washout (band 11). GAPDH was maintained as internal control in bands (2, 6-8, 12-14). **B)** Comparison of expression of transketolase gene expressed as relative fold induction in Type 2 thiamine treated versus placebo treated patients' quantified using real time PCR with GAPDH as internal control. Fold induction viewed at baseline, post therapy and post washout. All experiments were performed having duplicate samples in each with GAPDH as internal control. Error bars indicate, mean S.D, *$p < 0.01$ versus baseline.

its expression (Pekovich et al., 1996). A group study of Type 2 diabetics in Japan reported that TKT activity of RBCs, another cell line used for measuring and thiamine concentration in plasma were below the normal permissible range in majority of patients (Saito et al., 1991). Results of a study on a larger group of Type 2 diabetic patients in Israel showed TKT activity in RBCs to be lower than the minimum normal range in 18% of the patients (Havivi et al., 1991).

Reduction in the activities of one or more thiamine dependant enzymes TKT, α ketoglutarate dehydrogenase and pyruvate dehydrogenase are thought to be responsible for the tissue damage and impaired cell function that accompany thiamine deficiency (Martin et al., 1993). Also, a decrease in TKT activity diminishes the production of reducing equivalents and pentoses vital for cellular function (Butterworth, 1986). An enhanced degradation of apoenzymes generated during thiamine deficiency may explain the loss of activity of the TPP requiring enzymes (Singleton et al., 1995). Thiamine may regulate the expression of genes that encode the enzymes that utilize ThDP.

Thus pathologically micro vascular and macro vascular damage experienced as chronic renal failure, cerebrovascular accident, myocardial infarction, angina, visual impairment and a myriad of other diabetes related patho-

logies appear to originate from dysfunctional thiamine depleted enzymes of the vital intracellular Krebs cycle and reductive pentose phosphate pathway (RPPP).

Oral hypoglycemic or a combination of insulin and oral hypoglycemic are required for Type 2 diabetic patients. There are currently 5 groups of oral hypoglycemic agents which are sulphonylureas, insulin secretagogues like meglitinide, diphenylalanines, biguanides, thiazoladinediones and alpha glucosidase inhibitors. All major pharmacologic approaches to treating Type 2 diabetes rest on either the ability of insulin or insulin secretagogues to assist the entry of glucose into the cell and of other ones which reduce hepatic glucose production, renal gluconeogenesis, gastrointestinal absorption, enhance glycolysis in enterocytes and tissues, increase removal of glucose from blood, reduce plasma glucagon levels, act as competitive inhibitors of alpha intestinal glucosidases, modulators of expression of genes associated with lipid and glucose metabolism, delay gastric emptying, have central nervous system mediated anorectic effects or latest as potentiators of glucose mediated insulin release (Katzung et al., 2009). Additionally, all of these pharmacologic interventions have substantial adverse effects like gastrointestinal, haematological, cutaneous, hepatic and nephrotoxic. They are also fetotoxic and teratogenic (Goodman, 2006) and are contraindicated in patients with renal disease, hepatic disease and chronic cardiopulmonary dysfunction which are usually the results of micro vascular and macro vascular pathology arising from Type 2 diabetes thus the irony of the situation. Bearing this scenario in mind as yet no therapeutic strategy for Type 2 diabetes is being researched dealing with the metabolism of glucose intracellularly, based on the role of vitamin B1 as a cofactor in the activity of thiamine dependant enzymes, in particular TKT and to determine its impact on TKT activity and TKT gene expression in Type 2 diabetics. High dose vitamin B1 administration thus appears to be as plausible intervention based on its previous preclinical studies in diabetic animal models and its excellent safety profile data recorded in its use as a vitamin supplement in normal individuals. Due to mounting international evidence regarding the molecular mechanisms underlying the activities of TPP dependant enzymes and the absence of such a characterization in the normal population and in Type 2 diabetics with incipient nephropathy in Pakistan and the impact of high dose thiamine therapy on them needed to be investigated in a clinical trial.

In this clinical trial, TKT activity in erythrocytes in placebo group was significantly decreased at baseline (p0.003) and non significantly (p0.062) in thiamine treated diabetics compared to normal controls. While no significant change in activity could be found during placebo and thiamine therapy during the treatment and washout periods. This finding differs from the Dutch benfotiamine trial which showed highly significant ($p < 0.001$) increase in TKT activity in erythrocytes of benfotiamine treated patients versus the placebo group and no baseline comparison to normal controls was given (Alkhalaf et al., 2010). Our results also differed from the UK study where erythrocyte TKT activity was not changed significantly in both types 2 and 1 diabetic patients at baseline with regard to normal volunteers TKT activity in RBCs has often been measured to evaluate thiamine status in the human body (Brady et al., 1995), masking of human clinical thiamine deficiency in erythrocytes occurs due to upregulation of thiamine transporter genes THTR1 and RFC1 and thus increased levels of THTR1 and RFC1 transporter proteins as reported in the UK study (Thornalley et al., 2007). The reason for these differences in our study could fall in a slightly different thiamine transporter expression in the Pakistani population (Asian), which is not upregulated in plasma thiamine deficiency, which is pharmacogenetically possible. However lack of improvement with thiamine therapy also needs to be explored further. Therefore at any rate RBC TKT activity did not appear to be a good source of judging thiamine levels in our population.

While TKT activity measured in mononuclear cells at baseline in thiamine and placebo groups was significantly decreased by 45 and 42.73% ($p < 0.05$), respectively as compared to normal controls. There was highly significant increase in TKT activity during thiamine therapy of 65% (p0.000) which persisted into the washout period ($p < 0.05$) while non significant decrease in activity of 24.5% and a further 3% was observed in the placebo group.

The baseline fold expression of TKT gene in thiamine and placebo treated diabetics compared to normal controls was significantly lower (p0.017) than normal controls. During thiamine therapy, there was a highly significant increase in expression of TKT gene in mononuclear cells (p, 0.000) which was sustained into washout period and slightly increased at washout (p,0.000). The placebo treated diabetics showed a slight non significant decrease in TKT gene expression during therapy and washout. Our finding is supported by Hammes et al. (2003), where TKT deficiency was found in experimental diabetes model and benfotiamine administration improved its activity. This trial compares positively to a recent trial where, improvement in TKT activity was noticed in mononuclear cells of type 1 diabetic patients administered benfotiamine and lipoic acid for 2 weeks (Du et al., 2008). TKT has a short half life of approximately 25 min when unsaturated with TPP (Soh et al., 1998), decreased tissue concentrations of TPP are expected to lead to a prompt decrease in TKT expression. And finally the prolonged enhancement of TKT expression into the washout period in thiamine treated diabetics could be that even though the half-life of thiamine is about 2 days being relatively short (Weber and Kewitz, 1985), its biological half-life (9 to 18 days) is much greater (Nejad et al., 1970). So, even though the plasma concentration and urinary excretion of thiamine in the thiamine treatment arm returned to baseline after 2 months washout period (Rabbani et al.,

2009), it is likely that increased tissue levels of TPP, activities of thiamine dependant enzymes and related pharmacological responses remained above baseline for more than one biological half life of thiamine into the washout period of the patients in the thiamine treatment group (Thornalley et al., 2007).

Thus further substantiating the benefits of thiamine therapy is its supportive role in augmenting intracellular glucose metabolism in Type 2 diabetes through enhanced TKT activation of the reductive pentose pathway.

Conclusion

In a nutshell, baseline TKT activity and expression levels in Type 2 diabetics are significantly lower in Type 2 diabetics as compared to healthy individuals. High dose thiamine therapy of 300 mg/day significantly improved TKT activity and gene expression in Type 2 diabetic patients having micro albuminuria and may prove to be a valuable adjunct in treatment of Type 2 diabetes and its complications.

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