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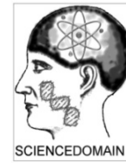

Induction and Activities of Pyruvate Dehydrogenase and α-ketoglutarate Dehydrogenase in Type 2 Diabetic Patients and Therapy with Vitamin B1

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Authors' contributions

This work was carried out in collaboration between both authors. Author SSA designed the study, wrote the protocol, enrolled the patients, arranged for the placebo and B1, conducted the research and wrote the first draft of the manuscript. Author SR played a supportive role throughout the research, assisted in the patient enrollment, sampling, the experimental process and manuscript revision. Both authors read and approved the final manuscript.

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ABSTRACT

Aims/Introduction: Thiamine deficiency in diabetes mellitus may impair the function of thiamine pyrophosphate dependent enzymes pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase complexes (α KGD) resulting in renal dysfunction. This study was designed to investigate the effect of high dose thiamine therapy on the expression and activities of PDH and α KGH in such patients.
Materials and Methods: 125 patients with type 2 diabetes and microalbuminuria were assessed for enrollment in a randomized, double blind placebo controlled clinical trial for 5 months. 40 Patients fulfilling the requisite criteria were divided into two groups, one treated with 300mg/day thiamine and the other group was administered placebo. Fifty normal healthy controls were included in the study

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only for baseline estimation of the parameters.
Results: The enrolled patients with type 2 diabetes showed decreased activities of mononuclear enzymes as compared to the healthy controls. Q-PCR study showed that the expression levels of the genes encoding PDE1β and α KGDE1k were significantly reduced in the patients with type 2 diabetes as compared to the healthy controls. Thiamine therapy resulted in significant increases in the expression of PD*E1β* and α KGD*E1* genes, which persisted even 2 months after the washout. Thiamine therapy therefore resulted in significant increase in activities of these enzymes and incremental activity persisted into the washout period.
Conclusion: These results indicate that the thiamine acts as an inducer in the expression of mononuclear PDH and α KGD thus enhancing their activities in the type 2 diabetes patients with incipient nephropathy.
It was internationally registered with the South Asian Clinical Trials Registry based in India as CTRI/2008/091/000112 and with the World Health Organization's (WHO) International clinical trials registry Platform search portal http://www.ctri.in/Clinicaltrials/ViewTrial.jsp?trialno=203

Keywords: Pyruvate dehydrogenase; α-ketoglutarate dehydrogenase; diabetes mellitus type 2.

ABBREVIATIONS

TTP- (thiamine pyrophosphate); PDH- (pyruvate dehydrogenase); α-KGD- (α-ketoglutarate dehydrogenase); α KGDE1 k- (oxoglutarate dehydrogenase lipoamide E1subunit); q-PCR- (quantitative polymerase chain reaction).

1. INTRODUCTION

A variety of micronutrient approaches have been suggested as therapeutic interventions in patients with type 2 diabetes [1-3]. Thiamine (vitamin B1) is a water-soluble vitamin and an essential normal dietary component [4]. Thiamine deficiency exists in both type 1 and type 2 diabetes patients [5] and in patients with type 2 diabetes and microalbuminuria [6-9]. Thiamine and benfotiamine therapy prevents the development of microvascular complications in experimental diabetes [10-11] and could possibly also have a beneficial role to play in the treatment of type 2 diabetes mellitus [5].

The pyruvate dehydrogenase multienzyme complex consists of PDE1 (pyruvate dehydrogenase: PDH), PDE2 (dihydrolipoyl transacetylase) and PDE3 (dihydrolipoyl dehydrogenase) subunits. TPP on binding to PDH initiates a series of reactions resulting in oxidative decarboxylation of pyruvate to yield acetyl-CoA and NADH [12]. PDH serves as the gate keeper anzyme that strategically links glycolysis, Krebs cycle and lipogenic pathways [13].

The α-KGDE1k (OGDH: oxoglutarate dehydrogenase) also functions as a complex in association with E2k (dihydrolipoyl transsuccinylase) and E3k (dihydrolipoyl dehydrogenase) subunits. Binding of thiamine to OGDH is required for the complex to convert oxoglutarate, a key intermediate in the krebs cycle, to succinyl co A and generating NADH and CO_2 in an irreversible reaction [14].

A dysfunctional PDH complex, with an unchanged total amount, is found in animal tissues in experimental diabetes, obesity and in skeletal muscles of diabetic patients [15-18]. In diabetes, thiamine dependent megaloblastic anaemia and sensorineural deafness associated with deficient α-KGD activity has also been reported [19]. Reduction in activities of thiamine dependant enzymes transketolase, α-KGD and PDH are thought to be responsible for the tissue damage and impaired cell function that accompany thiamine deficiency [20]. Specially, depreciation in oxidative decarboxylation of the α-keto acids through loss of activities of the thiamine dependent enzymes would reduce ATP synthesis, leading to cellular acidosis [21]. Thiamine deficiency in vitro leads to enhanced degradation of apoenzymes leading to loss of activity of the TPP requiring enzyme. [22] Thiamine may also have a role in the expression of genes that encode the TPP requiring enzymes [23-24] and its replenishment by thiamine therapy improved both activity and expression [25].

Experimental diabetes is associated with thiamine deficiency characterised by a marked decrease in plasma thiamine concentration and decreased activity and expression of the thiamine-dependent enzyme transketolase in renal glomeruli [6]. In this model correction of decreased plasma thiamine concentration countered the adverse effects of hyperglycaemia by activation of the reduced pentosephosphate pathway, providing an alternative route for disposal of accumulating glycolytic intermediate (fructose-6-phosphate and triosephosphates) preventing metabolic dysfunction in renal glomeruli and development of early stage nephropathy [26].

Diabetic nephropathy develops progressively over 5–40 years of diabetes and research shows patients to have low thiamine levels [6]. Correction of low levels of plasma thiamine in diabetic patients and related metabolic responses may regress the microalbuminuria and prevent early decline in GFR [26]. Based on the above evidence a novel strategy to counter biochemical dysfunction linked to the development of diabetic nephropathy is high-dose thiamine therapy. In our previous published pilot study, we evaluated the effect of oral high dose supplements of thiamine on urinary albumin excretion (UAE), a marker of early-stage diabetic nephropathy, in type 2 diabetic patients with microalbuminuria and an improvement in microalbuminuria and transketolase levels [6,9].

AKDH and PDH are both thiamine dependent enzymes as well, required for effective utilization of the glucose intracellularly in all tissues including the kidney and in generation of ATP for all processes including renal function. Their activities and expression could be reduced in type 2 diabetics based on the evidence stated previously leading to dysfunction of glomerular epithelial cells, podocytes and tubular epithelial cells causing loss of thiamine resulting in incipient nephropathy and microalbuminuria. [27] This in turn would further compound the loss of thiamine from the kidneys and a vicious cycle of lowering puruvate dehydrogenase and alphaketoglutarate dehydrogenase function and expression associated with worsening diabetic nephropathy would ensue.

This study, based on a double blinded placebo controlled clinical trial reports the effects of high dose thiamine therapy on the activities and expression of PDE1 and α-KGDE1 in patients having type 2 diabetes with microalbuminuria.

2. MATERIALS AND METHODS

2.1 Ethical Approval

Ethical approval for the study was taken from the Ethical Committee of FPGMI (Sheikh Zayed Federal Post Graduate Medical Institute, Lahore, Pakistan). The study was assigned the number Eth/P 609/FPGMI 2006. It was internationally registered with the South Asian Clinical Trials Registry based in India as CTRI/2008/091/000112 and with the World Health Organization's (WHO) International clinical trials registry Platform search portal http://www.ctri.in/Clinicaltrials/ViewTrial.jsp?trialno=203

2.2 Selection of Patients and Controls

Diabetic patients were recruited from patients attending the Diabetes Clinic at the Sheikh Zayed Hospital, Lahore, Pakistan between October and December 2006. The main inclusion criteria were: age 35–65 years, type 2 diabetes and persistent microalbuminuria (AER 30-299 mg/24 h), diabetes duration ≥5 years, HbA_{1c}≤12.5%, and BMI 19–40 $kg/m^{2.}$ 125 patients with diabetes type 2 and 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, between October 1, 2006 and December 1, 2006. Stringent inclusion and exclusion, and randomization and treatment procedure of the patients and controls was done as described previously [6]. The trial duration was three months therapy and two months washout period. The medicine was given for three months only. Out of the enrolled diabetic individuals and controls, 40 patients and 20 controls completed the 3 month thiamine/placebo administration and 2 month follow up period for this study. Baseline data of the recruited patients and controls has been presented in our previously published data. [6] At baseline and throughout the study, nine patients in the thiamine-treatment group and three in the placebo group were receiving insulin therapy ($p<0.05$). There were no other significant differences in the proportions of patients receiving therapeutic agents in the thiamine-treatment and placebo groups. One patient achieved glycaemic control by diet only; all others received therapy with hypoglycaemic agents (sulfonylureas, metformin and

thiazolidinediones). The age and gender of healthy controls was comparable to the diabetic patients.

2.3 Sampling

Fasting Blood samples were obtained from the patients and healthy individuals at baseline, after 3 months therapy and 2 months washout. These were processed for the biochemical analysis as well as isolation of erythrocytes and peripheral blood mononuclear cells (PBCMs), which were used for pyruvate dehydrogenase (PDE1) and α-ketoglutarate dehydrogenase (α -KGD) assays and gene expression studies. 24 hr urine collections were also made for determination of microalbuminuria.

2.4 Mononuclear Cell Separation

The PBMCs were isolated from EDTA treated blood samples, which were equally distributed into 2 sets of 15 ml falcon tubes at room temperature on a ficoll-paque(GE Healthcare) gradient [24]. The cells were recovered from the interface and subjected to short washing steps with Hanks balanced salt solution to remove any platelets or plasma [28]. All samples were processed fresh within 2-3 hours after collection and maintained on ice until the enzyme assays the same day.

2.5 Enzyme Assays in Mononuclear Cells

All routine reagents used in the assay were of analytical grade and were either procured from Sigma (Germany), Fluka or Reidel-de-Haen(Germany) . Each assay was conducted in a microplate reader (Biotek 808IU) using a 96 well quartz plate (Hellma). All test samples were run in duplicate with (sample minus substrate) serving as controls.

2.5.1 PDE1 assay

The method used for the PDE1 assay was the same reported previously [29]. However, in order to adapt this method for the microplate reader the reaction mixture volumes were reduced as mentioned below. This protocol describes a coupled assay to measure the pyruvate dehydrogenase activity. In this citrate synthase reaction was applied for the assay of two acetyl CoA producing enzymes pyruvate dehydrogenase and acetyl–CoA synthetase. Reaction were initiated by the addition of 0.1 mg total protein to an otherwise complete reaction mix of 12.5 µl 0.2 M sodium pyruvate, 12.5 µl 4 mM sodium coenzyme A, 12.5 µl 40 mM NAD, 25 µl 10 mM $MgCl_2$,50 µl 0.25 M Tris-HCl Buffer(pH 8.0), 69 µl deionized water, 12.5 µl 200 mM DTT, 25 µl 25 mM oxaloacetate,12.5 µl 0.05 g 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) in 10 mL 100% ethanol and 5 µl Citrate synthase (250 U/mL). Final volume was 300 µl.This method had a run time of 100 s at 30℃ at a wavelength of 415 nm. Activity was measured in U/mg protein. One unit (U) of pyruvate dehydrogenase E1 activity is defined as the amount of enzyme required to produce 1.0 µmole of acetyl CoA in one minute.

2.5.2 α-KGD assay

In this assay as well the methodology remained the same as previously reported [22]. However adaptation was made for the microplate reader by reducing the reaction volume as given below. Reactions were initiated by the addition of 0.1 mg total protein to an otherwise complete reaction mix of 43 µl 50 mmol/L MOPS (pH 8.0), 72 µl 1.2 mmol/L $MgCl_2$, 72 µl 1.2 mmol/L $CaCl_2$, 10 µl 0.16 mmol/L coenzyme A, 37.5 µl 6 mmol/L α-ketoglutarate, 33 µl 0.1 mmol/L NAD, 1.6 µl 0.5 g/L Triton X-100 and 9.5 µl 0.04 mmol/L rotenone. Final volume was 300 µl. The formation of NADH, which is directly proportional to α-KGDH activity, was measured at 340 nm wavelength at 30℃. One unit of alphaketoglutarate dehydrogenase activity was defined as that which converts 1.0µmole of β NAD to βNADH per minute at pH 7.4 at 30℃ in the presence of saturating levels of coenzyme A. Protein concentration was determined using the Bradford method [23].

The intra-CV of PDH assays was 1.42% and inter-CV assay was 2.5%. Intra-CV for AKDH assay was 1.05% and inter-CV was 2.0%. The lowest detection limit for assay of aKDH was 2 nmol/mg protein per minute and for assay of PDH was 39 nmol/mg protein per minute.

2.6 Quantitative RT-PCR Analysis

Total RNA was isolated from the whole blood using Tri reagent LS (TS 120) Gentra (USA) following standard protocol. DNase-treated total RNA (2 µg) was reverse transcribed with the Revertaid minus Strand cDNA synthesis Kit (Fermentas) and semiquantitative PCR was performed to verify the amplification of the thiamine dependent enzyme genes. Primers were designed using Primer 3 [30] and UCSC

Genome Bioinformatics [31] as shown in Table 1. These were synthesized by Eurofins MWG/ Operon (USA). Amplification was done in ABI Biocycler using the conditions: initial denaturation at 94℃ (5min), annealing at 62℃ (30 s) and extension at72℃(40 s) for 35 cycles followed by final extension at 72℃ for 7 min. 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 with glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene as a control for normalization. These procedures were performed as per standard referred protocols and methods mentioned in the kits of Fermantas company.

2.7 Statistical Analysis

2.7.1 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 noncompliance to therapy.

2.7.2 Statistical analysis

The data collected was analysed for statistical significance and correlation testing using statistical package SPSS15 Chicago, (IL, U.S.A). A normality check was performed for the entire data using the Kolmogorov Smirnov test. Following that Anova'(Analysis of Variance) statistical package was used for Parametric data when groups are found to be normal. While Kruskall-wallis test was used when non-normality was found. 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. Non-parametric correlation analysis of study groups were calculated by Spearman's rho statistic. While Real Time PCR results were also analyzed for significance using Anova' Analysis of Variance test.

3. RESULTS

In the present research work, only two patients reported gastrointestinal distress and three complained of tachycardia in the thiamine treated group and had to discontinue intervention. Tachycardia has also been previously been reported and might also have a reason due to sensitivity to thiamine in these patients and lesser dose could have been more acceptable. However due to requirements of 300 mg daily dose of thiamine in this clinical trial these patients were omitted from the trial completely. Two patients infact reported improvement in skin, hair and nail strength.

The baseline urinary albumin excretion levels in mg /24 hrs was significantly higher in the type 2 diabetic patients in both thiamine and placebo assigned groups. The thiamine group showed 53.86±22.62 mg/24 hrs and placebo group showed 55.74±23.71 mg/24 hrs as compared to controls (7.96±5.07 mg/24 hrs). The baseline HbA1C levels of the thiamine allocated diabetics was 9.2±1.3%, while the patients randomized to the placebo arm had mean HbA1C levels of 8.82±1.8% as compared to control (5.6±0.39%).

Detailed biochemical profiles were maintained for the enrolled type 2 diabetic patients with microalbuminuria at baseline, after 3 months post therapy and at 2 month washout period and have been published previously [6]. These revealed certain beneficial effects of high dose 300 mg/day thiamine therapy and absence of adverse effects. Markedly lower baseline median plasma thiamine concentration of diabetic patients (7.5 nM) was present compared to normal range of normal healthy human subjects (944.6 – 93.7 nM). Thiamine treatment for 3 months increased median plasma thiamine concentration 10 fold and urinary thiamine excretion 29 fold. It importantly caused a regression of microalbuminuria to normal albumin levels in 35% of the patients .A decrease in mean glycated haemoglobin levels by 1.4% was also observed two months after washout of thiamine.

Table 1. Forward and reverse primers used for amplification of the enzymes PDE1 and α-KGD

Primer name	Sequence (5' → 3')
PDE1F	GTCTGGCTTGGTGCGGAGAC
PDE1R	ATTTCTTCCACAGCCCTCGACTAAC (218bp)
α-KGDF	AAGGACTTGTGCTGCTAAGTTGAGG
α-KGDR	TGTCCCATGACTTATGTACACTTTTGG (221bp)
GAPDHF	GGTCACCAGGGCTGCTTTTAAC
GAPDHR	CACTTGATTTTGGAGGGATCTCG (210bp)

Table 2. Pyruvate dehydrogenase-1 and α-ketoglutarate dehydrogenase activities in mononuclear cells before and after 3 months thiamine treatment and after 2 months washout period

Enzyme activities	Healthy controls	Thiamine treatment			Placebo treatment		
	Baseline	Baseline	Therapy	Washout	Baseline	Therapy	Washout
Pyruvate dehydrogenase-1	375.08±86.32	172.80±39.56a	353.05±77.85d,b	372.90±88.39a	168.36±40.89	177.10±57.91	165.45±34.7
α-ketogutarate dehydrogenase	19.29±5.03	3.23±0.88b	16.23±4.16 e,b	24.85±5.36h	3.86±0.82 b	4.70±1.6	9.30±1.62

Data are means ± SEM values, (Values in U/mg protein), a p<0.05, b p<0.01, c p<0.001 compared with placebo;
d p<0.05, e p<0.01, f<0.001 compared with baseline;
g p<0.05,. h p<0.01, i p<0.001 compared with post-therapy

3.1 PDE1 and α-KGDE1 Subunit Activities

Normal individuals showed mean PDE1 activity levels of 375.08±86.3 U/mg protein. While the thiamine treated diabetics had baseline mean activity values of 172.80±39.56 which increased to mean 353.05±77.85, a 104% increase during therapy ($p=0.04$) and 372.90±88.39 U/mg protein ($p=0.045$) at washout. During the same time the placebo group registered non significant change in activity from 168.36±40.89 Units / mg protein at baseline to 177.10±57.91 at 3 months post therapy and during the washout period activity decreased to 165.43±34.7 Units/ mg protein. Therefore no significant decrease of activity recorded in the placebo group (see Table 2).

Normal individuals showed mean activity levels of α KGD as 19.29±5.03 units/ mg protein. In the thiamine treated group there was a significant change in activity from mean baseline level of 3.23±0.88 Units / mg protein to 16.23±4.16 following 3 months of therapy ($p<0.01$) which increased further by 51% to reach a mean value of 24.58±5.36 units/ mg protein at 2 months washout ($p<0.01$). In contrast the placebo having a mean baseline value of 3.86±0.82 Units / mg protein registered a nonsignificant increase in their mean therapy values 4.70±1.64 ($p=0.067$) and a similar nonsignificant increase between therapy and mean washout levels of 9.30±1.62 units/ mg protein, ($p=0.055$) (Table 2).

3.2 Expression Analysis of PDE1 and α-KGDE1

The mean baseline expression levels of mononuclear PDE1β gene in the thiamine group was 0.65 fold and 0.85 fold in the placebo group. While baseline α-KGDE1 gene expression levels in thiamine treated group was 0.60 fold and 0.71 fold in the placebo treated diabetics as compared to normal healthy controls. ($p=0.007$) (Fig. 1).

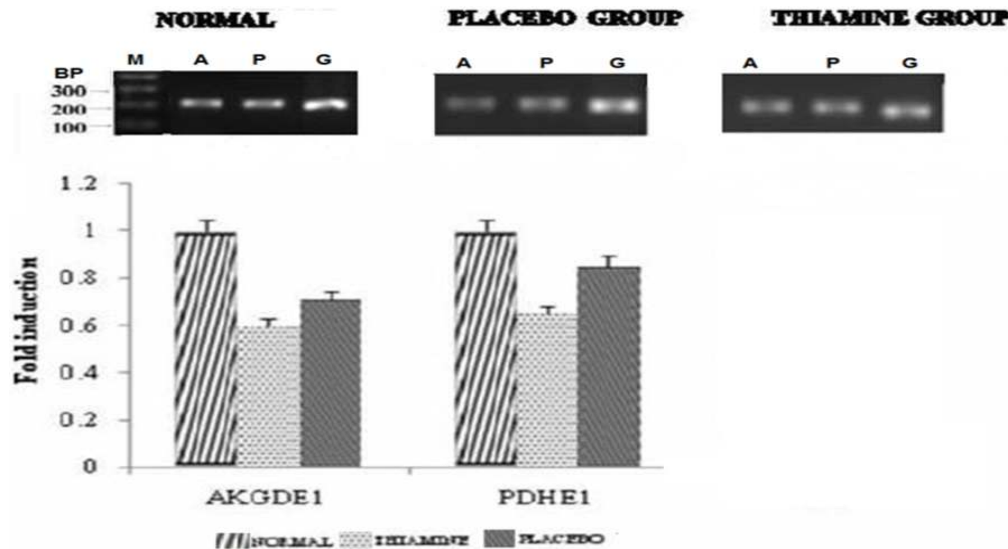

Fig. 1. Comparison of baseline expression of PDE1β, AKGDE1 genes in PBMCs of normal versus thiamine and placebo treated type 2 diabetic patients relative RNA determinations were carried out using semi- quantitative RTPCR

Band: Each band represents the semiquantitative PCR products which have been visualized using agarose gel at baseline of the clinical trial.

Agarose gel image of thiamine dependant enzymes in thiamine and placebo treated type 2 diabetics reveal significantly less baseline expression of AKGDE1, and PDE1β as less band intensities in both enzymes compared to normal healthy individuals.

GAPDH was used as internal control. (M=MARKER) (A=AKG) (P=PDH) (G=GAPDH)

Comparison of baseline expression expressed as relative fold induction of PDE1β (PDE1), (AKGDE1), in PBMCs of healthy versus thiamine & placebo treated patients.

Band: Each band represents the semiquantitative PCR products which have been visualized using agarose gel at different points of the clinical trial: baseline, posttherapy and washout.

Statistically the expression levels of mononuclear PDE1 and α-KGDE1 genes were significantly reduced in thiamine and placebo treated diabetics as compared to normal healthy controls. High dose thiamine therapy for 3 months resulted in significantly higher PDE1 expression of 2.9 fold, $p<0.001$ than baseline, an increase of about 200%, which was maintained after washout at 3.02 fold, $p<0.001$. The PDE1 gene, in the placebo group showed non significant changes in expression of 1.15 fold, $p=0.05$ after 3 months therapy and similarly after 2 months washout to 0.96 fold, $p=0.055$ (Fig. 2). The results indicated that the expression levels of PDE1β gene were significantly high in thiamine treated diabetics as compared to placebo treated diabetics patients. High dose thiamine therapy for 3 months also resulted in a statistically significant increase in expression levels of the alphaketoglutarate dehydrogenase gene by 2.65 fold ($p<0.001$), which remained statistically significant, at 2.72 fold ($p<0.01$) even after washout. While in the placebo group statistically non significant changes in fold expression of the oxoglutarate dehydrogenase gene were observed at 0.91 fold after therapy, and 1.09 fold ($p=0.065$) after washout (Fig. 3).

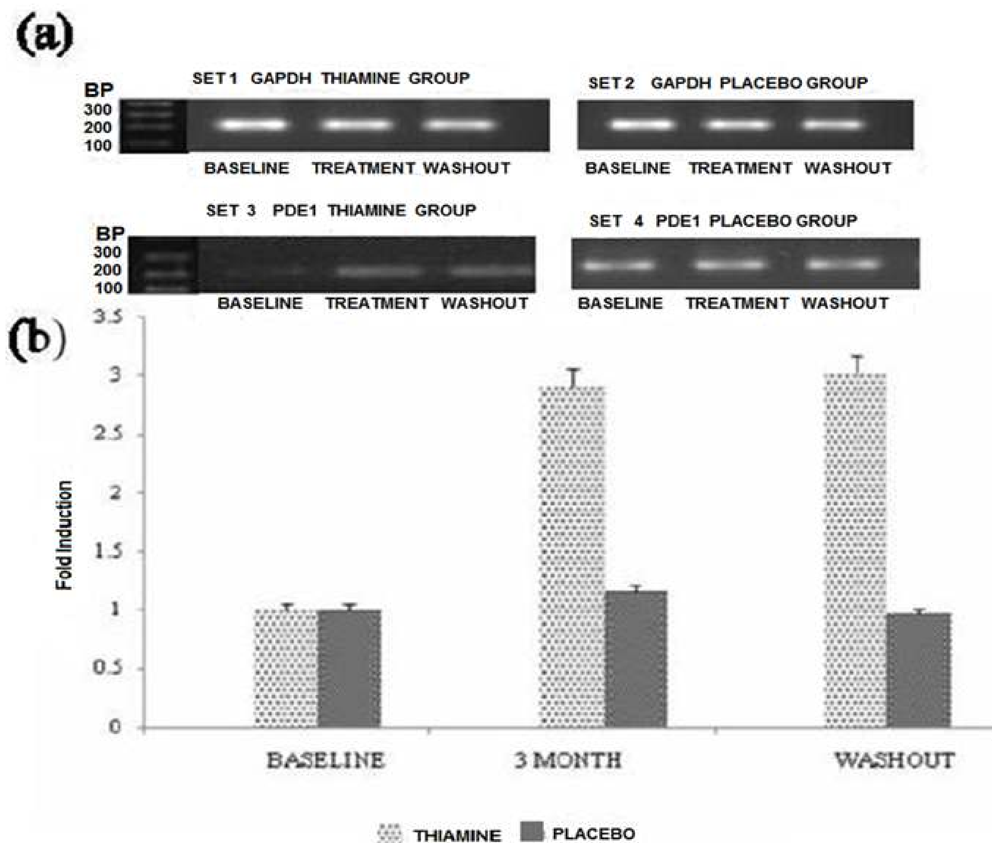

Fig. 2. Changes in the expression of PDE1β gene following thiamine and placebo administration. relative RNA determinations were carried out using semi-quantitativ e RT-PCR

Band: Each band represents the semiquantitative PCR products which have been visualized using agarose gel at different points of the clinical trial: baseline,posttherapy and washout.

The expression of PDE1β gene was significantly lower (SET 3 band1 (baseline) of thiamine treated type 2 diabetics and (SET 4 BAND 1 (baseline) of placebo treated type 2 diabetic patients.

Following thiamine therapy expression of PDE1β increased significantly (SET 3 BAND2) and slightly more after washout (SET 3 BAND3) as compared to its baseline (band 1). In comparison there was no change in PDE1β expression seen in placebo treated patients at post therapy (SET 4 band3) as compared to baseline (SET 4 band 2) and remained unchanged at post washout (SET 4 band 3) .GAPDH was maintained as internal control in bands SETS 1& 2 (BANDS 1, 2&3).

Comparison of expression of PDE1β gene expressed as relative fold induction in type 2 thiamine treated versus placebo treated patient's quantified using Real time PCR with GAPDH as internal control

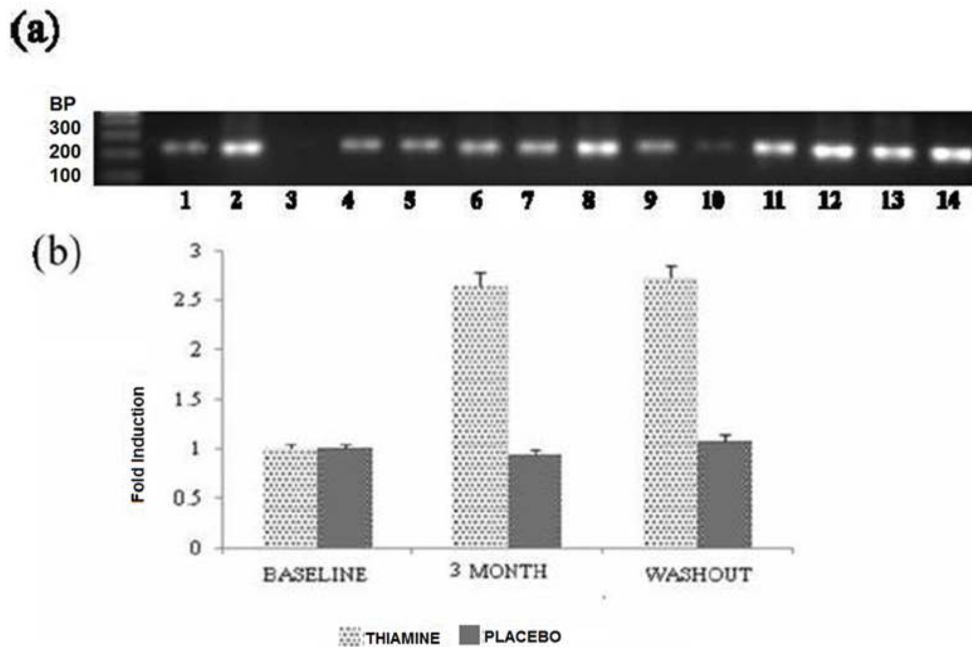

Fig. 3. Expression of AKGDE1 gene was pictured using gel electrophoresis

Band: Each band represents the semiquantitative PCR products which have been visualized using agarose gel at different points of the clinical trial: baseline, posttherapy and washout.

Comparison of expression of OGDHE1 gene in diabetic samples of thiamine versus placebo samples at baseline, treatment and washout. Relative RNA determinations were carried out using semi-quantitative RT-PCR. The expression of AKGDE1 gene was significantly lower, almost absent (band3 (baseline) of thiamine treated type 2 diabetic patients and less but visible (band9) (baseline) of placebo treated type 2 diabetics as compared to (band1 – normal controls).

Following thiamine therapy expression of AKGDE1 increased significantly (band4) and even more in washout (band5) as compared to its baseline (band 3). In comparison there was slight decrease in AKGDE1 expression seen in placebo treated patients at post therapy (band10) as compared to baseline (band 9) and slightly increased expression at post washout (band 11) GAPDH was maintained as internal control in bands (2,6-8,12-14)

Comparison of expression of AKGDE1 gene expressed as relative fold induction in type 2 thiamine treated versus placebo treated patient's quantified using Real time PCR with GAPDH as internal control.

4. DISCUSSION

In Type 2 diabetes, plasma thiamine levels are markedly reduced and there is enhanced washout of thiamine as well. The aKDH and PDH are both thiamine dependent enzymes and therefore their activities were found to be correspondingly reduced in type 2 diabetics because of lowered thiamine levels. Our study shows that thiamine reduction impacts both activities and expression of the enzymes adversely. These enzymes are located in all the cells and a part of energy cycles. Therefore, in diabetes dysfunction of a varying range of cells is observed such as diabetic retinopathy, neuropathy and nephropathy.

Our previous study also showed that thiamine therapy led to the reduction in HbA1C which was representative of the improvement in overall long term glucose regulation, as improved functioning of the thiamine dependent enzymes aKDE1 and PDH was observed. These enzymes are obviously vital for glucose handling.

Additionally thiamine intervention also resulted in decreased microalbuminuria. This might have been a consequence of increasing plasma concentration of thiamine reversing dysfunction of glomerular endothelial cells, podocytes and tubular epithelial cells improving glomerular and tubular structure and function and reducing low grade vascular inflammation observed as decreased urinary albumin excretion in type 2 diabetic patients of our study.

4.1 Thiamine and its Effect on PDE1

Thiamine may regulate the expression of genes that encode the enzymes that utilize ThDP [20].

As previously reported by us these diabetics with microalbuminuria were also thiamine depleted [7] and a possibility of a change in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase activities and the expression levels of their genes also needed to be investigated. Activity of the mononuclear pyruvate dehydrogenase (PDE1) subunit in diabetic patient was <54% than that of normal healthy individuals. Corresponding expression analysis of the mononuclear PDHE1β gene in diabetic patients revealed it to be significantly lowered to 75% of that in normal controls. The result thus indicated that the activity of pyruvate dehydrogenase enzyme and expression levels of PDE1β gene was significantly increased in mononuclear cells of thiamine treated diabetic patients. The result is also supported by the previous data which showed an enhanced degradation of apoenzymes generated during thiamine deficiency may explain the loss of activity of the TPP requiring enzymes [23]. As suggested earlier thiamine deficiency in vitro decreased activities of TK and PDH and its replenishment by thiamine therapy improved both activity and expression [22]. Our previously published results indicated that high dose thiamine therapy for 3 months significantly enhanced both mononuclear transketolase activity and gene expression in type 2 diabetic patients with incipient nephropathy [20].

4.2 Thiamine and Its Effect on α-KGDE1

In our present study, alphaketoglutarate dehydrogenase activity was reduced by nearly 82% in diabetics respectively as compared to normal controls. While α-KGD E1 gene expression in diabetics was significantly reduced to 65% of that in normal controls. The impact of thiamine therapy on α-KGD E1 gene expression was also evident as a highly significant increase 2.65 fold increase in the mRNA levels was observed persisting into washout at levels of 2.72 fold. Therefore thiamine administration increased both enzyme activity and expression of α-KGD E1, in PBMCs of diabetic patients.

The activities of PDE1, and α-KGDE1 Subunit were even higher in wash period due to reason as thiamine has a long biological half life (9-18 days) so even though the plasma concentrations and urinary excretion of thiamine in the thiamine treatment arm returns to baseline after two months washout period [32]. It is likely that increased tissues levels of TPP (thiamine pyrophosphate), activities of thiamine dependent enzymes and related pharmacological responses remained above baseline for at least one biological half life of thiamine in to the washout period of the patients in the thiamine treatment group. No adverse effects of high activities of both PDE1, and α-KGDE1 were noted [33].

This result was different from that of the cultured thiamine responsive megaloblastic anaemia patients (TRMA) lymphoblasts cells and in the α-KGD E1 enzyme activity and mRNA levels of these cells, where no correlation between total protein level/or activity and mRNA could be found [22]. The reason for this difference could have been in vitro limiting conditions and that being a tissue culture study [22]. The current study was obviously different in that the mononuclear cells were extracted from the diabetic patients who were orally administered thiamine or placebo and an in vivo analysis of enzyme activity and expression conducted in freshly extracted samples.

Our research on activity and expression analysis of PDE1 and alphaketoglutarate dehydrogenase in mononuclear cells revealed continued increment of both activity and expression levels of these enzymes after 3 months of high dose thiamine therapy and its persistence even 2 months after stoppage of therapy. The mean baseline plasma thiamine levels of the diabetic patients were found to be directly proportional to their enzyme activities and expression levels i.e both showed improvement with thiamine therapy [6-9].

5. CONCLUSION

This clinical intervention trial on 300 mg/day B1 therapy was the first randomized, double blinded, placebo controlled and pilot scale project for a period of 5 months to study the effect of high dose thiamine therapy on biochemical profile and activities of thiamine dependent enzymes on diabetics in the Pakistani population. The trial was also pioneering on the subject of diabetic nephropathy and the effect of thiamine supplementation on it. Baseline activity and expression levels of mononuclear PDE1 and α-KGDE1 in diabetic patients are significantly lower in diabetics as compared to healthy individuals. High dose thiamine therapy of 300 mg/ day significantly improved PDE1 and α-KGDE1 activity and gene expression in diabetic patients and caused a regression of microalbuminuria to normal albumin levels in 35% of the patients. Thus, further exemplifying the advantages of

thiamine therapy in its beneficial and cooperative role in enhancing Krebs cycle glucose metabolism through PDE1 and α-KGDE1. These findings however deserve further examination and extension in larger clinical intervention studies of diabetic patients. Thiamine therapy may prove to be a valuable adjunct in treatment of diabetes and its complications.

CONSENT

It is not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Sahin K, Onderci M, Tuzcu M, Ustundag B, Cikim G, OzercanI, Sriramoju V, Juturu V, Komorowski J. Effect of chromium on carbohydrate and lipid metabolism in a rat model of type 2 diabetes mellitus: The fat-fed, streptozotocin-treated rat. Metabolism. 2007;56(9):1233-40.
2. Bursell SE, Clermont AC, Aiello LP, Aiello LM, Schlossman DK, et al. High dose vitamin E supplementation normalizes retinal blood flow and creatinine clearance in patients with type 1 diabetes. Diabetes Care. 1999;22:1245-51.
3. Cuningham JJ. The glucose/insulin system and vitamin C: Implications in insulin dependent diabetes mellitus. J. Am Coll Nutr. 1998;17:105-8.
4. David L Nelson, Michael M Cox. The citric acid cycle in: Lehninger Principles of Biochemistry 5th edition: 620-35.
5. Thornalley PJ, Babaei-Jadidi R, Al Ali H, et al. High prevalence of low plasma thiamine concentration in diabetes linked to marker of vascular disease. Diabetologia. 2007; 50:2164-170.
6. Rabbani N, Alam SS, Riaz S, Larkin JR, Akhtar MW, Shafi T, Thornalley PJ. High dose thiamine therapy for people with type 2 diabetes and microalbuminuria: A randomised, double-blind, placebo-controlled study. Diabetologia. 2009;52(2): 208-212.
7. Riaz S, Alam SS, Akhtar MW. Proteomic Identification of human serum biomarkers in diabetes mellitus type-2. Journal of Pharmaceutical and Biomedical Analysis. 2010;51(5):1103-07.
8. Alam SS, Riaz S, Waheed Akhtar M. Effect of high dose thiamine therapy on risk factors in type 2 diabetics. J Diabetes Metab. 2012;3:233.
   DOI: 10.4172/2155-6156.1000233.
9. Saadia Shahzad Alam, Samreen Riaz, Waheed Akhtar M. Effect of high dose thiamine therapy on activity and molecular aspects of transketolase in type 2 diabetic patients. African Journal of Biotechnology. 2011;10(75):17305-17316.
10. Hammes HP, Du X, Edelstein D. Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. Nat Med. 2003;9(3):294-299.
11. Thornalley PJ. The potential role of thiamine (vitamin B1) in diabetic complications. Curr Diabetes Res. 2005; 1:287-98.
12. Ciszak EM, Makal A, Hong YS, et al. How dihydrolipoamide dehydrogenase –binding protein bindsdehydrolipoamide in the human pyruvate dehydrogenase complex. J. Biol. Chem. 2006;281(1):648-55.
13. Patel MS, Roche TE. Molecular biology and biochemistry of pyruvate dehydrogenase complexes). FASEB J. 1990;4:3224-233.
14. McCartney RG, Rice JE, Sanderson SJ, Bunik V, Lindsay H, et al. Subunit interactions in the mammalian α-ketoglutarate dehydrogenase complex. Evidence for direct association of the α-ketoglutarate dehydrogenase and dihydrolipoamide dehydrogenase components. J. Biol. Chem. 1998;273(37): 24158–64.
15. CatersonI D, Kerbey AL, Cooney GJ, Frankland R, Denyer GS, Nicks J, Williams PF. Inactivation of pyruvate dehydrogenase complex in heart muscle mitochondria of gold- thioglucose-induced obese mice is not due to a stable increase in activity of pyruvate dehydrogenase kinase. Biochem. J. 1988; 253:291–4.
16. Bajato G, Murakami T, Nagasaki M, Tamura N, Harris RA, et al. Downregulation of the skeletal muscle

pyruvate dehydrogenase complex in the Otsuka Long-Evans Tokushima fatty rat both before and after the onset of diabetes mellitus. Life Sci. 2004;75(17):2117-30.

17. Kelley DE, Simoneau JA. Impaired free fatty acid utilization by skeletal muscle in non- insulin-dependent diabetes mellitus. Journal of Clinical Investigation. 1994;94: 2349–56.
18. Marco Piccinini, Michael Mostert, Gianfrancesco Alberto, Cristina Ramondetti. Down-regulation of pyruvate dehydrogenase phosphate in obese subjects is a defect signals insulin resistance. Obesity Research. 2005;13: 678-86.
19. Abboud M, Alexander D, Najjar S. Diabetes mellitus, thiamine-dependent megaloblastic anemia, and sensorineural deafness associated with deficient α-ketoglutarate dehydrogenase activity. J Paeds. 1985;107(4):1A-28A.
20. Butterworth RF. Cerebral thiamine dependent enzyme changes in experimental Wernicke's encephalopathy. Metab. Brain, Dis. 1986;1:165-175.
21. Aikawa H, Watanabe IS, Furuse T. Low energy level in thiamine deficient encephalopathy. J. neuropath. Exp Neurol. 1984;43:276-87.
22. Pekovich SR, Martin PR, Singleton CK. Thiamine pyrophosphate-requiring enzymes are altered during pyrithiamine-induced thiamine deficiency in cultured human lymphoblasts. J. Nutr. 1996;126: 1791-98.
23. Pekovich SR, Martin PR, Singleton CK. Thiamine deficiency decreases steady-state transketolase and pyruvate dehydrogenase but not a-ketoglutarate dehydrogenase mRNA levels in three human cell types. J Nutr. 1998;128:683-87.
24. GE Healthcare Ficoll-Paque Instructions 71-7167-00 AG for in vitro isolation of lymphocytes protocol.
25. Terbukhina RV, Ostrovsky YM, Petushok VG, et al. Effect of thiamine deprivation on thiamine metabolism in mice. J. Nutr. 1981;111:505-513.
26. Toyoda M, Najafian B, Kim Y, Caramori ML. Podocyte detachment and reduced glomerular capillary endothelial fenestration in human type I diabetic nephropathy. Diabetes. 2007;56:2155-2160.
27. Rabbani N, Shahzad Alam S, Riaz S, Larkin JR, Akhtar MW, Shafi T, Thornalley PJ. Response to comment on Rabbani et al. High dose thiamine therapy for patients with type 2 diabetes and microalbuminuria: a pilot randomised, double-blind, placebo-controlled study. Diabetologia. 2009;52(6): 1214–16.
28. Boyum A. Isolation of mononuclear cells and granulocytes fron human blood. Scand J. Clin. Lab. Invest. 1968;97(4):77-89.
29. Szutowicz A, Stepien M, Piec G. Anal. Bioch. 1981;115:81-87. Available:atcmbe.engr.uga.edu/assays/pyruvatedehydrogenase.pdf CMBE.Driftmier. University of Georgia.
30. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ. 2000;5–386.
31. UCSC Genome Bioinformatics. UCSC Genome Bioinformatics Group:Center for Biomolecular Science &Engineering CBSE/ITI, 501D Engineering II Building University of California, Santa Cruz.1156 High St., Santa Cruz, CA 95064. Available:www.genome.ucsc.edu.
32. Sasso FC, Carbonara O, Persico M. Irbesartan reduces the albumin excretion rate in microalbuminuric type 2 diabetic patients independently of hypertension: a randomized double-blind placebo-controlled crossover study. Diabetes Care. 2002;25:1909–13.
33. Andersen S, Brochner-Mortensen J, Parving HH. Kidney function during and after withdrawal of long-term irbesartan treatment in patients with type 2 diabetes and microalbuminuria. Diabetes Care. 2003;26:3296–302.

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