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RESEARCH ARTICLE

# Thiamine deficiency perturbed energy metabolism enzymes in brain mitochondrial fraction of *Swiss* mice

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**BACKGROUND:** Thiamine is an essential cofactor associated with several enzymes in energy metabolism and its deficiency may lead to neurological deficits. Current research evaluated the biochemical and molecular changes in TCA cycle enzymes using the mitochondrial fraction of the brain following thiamine deficiency (TD) in mice.
**METHODS:** The investigation was carried out on Swiss mice (6-8 week old) allocated into three groups. First group was control; second and third group were made thiamine deficient for 8 and 10 days.
**RESULTS:** Current study showed that alpha-ketoglutarate dehydrogenase (KGDHC) (thiamine-dependent enzyme) level found to be significantly reduced in experimental groups as compared to control group. In comparison to control group, a significant decrease in the succinate dehydrogenase (SDH) activity was calculated in group II and group III ($p < 0.0001$) mice. Diminished enzymatic activity of fumarase and MDH enzyme in thiamine deficient groups exposed for 8 and 10 days was calculated as compared to control group. The expression analysis of different genes governing TCA cycle enzymes in different experimental groups showed that there was a negotiable change in the expression of fumarase and DLD (dihydrolipoyl dehydrogenase- E3 subunit of KGDHC) whereas a declined in the expression of SDH and two subunits of KGDHC i.e. OGDH (2-oxoglutarate dehydrogenase- E1 subunit of KGDHC) and DLST (dihydrolipoyllysine-residue succinyltransferase- E2 subunit of KGDHC) was observed as compared to control group.
**CONCLUSIONS:** Hence, current findings strongly entail that TD promotes alteration in energy metabolism in brain mitochondria which will decline the neuronal progression which may lead to neurodegenerative diseases such as Alzheimer's diseases.

**Keywords** thiamine, brain, TCA cycle enzymes, mitochondrial dysfunction

## Introduction

An incessant dietary intake of thiamine supports carbohydrate metabolism in the body and removes toxicity inside the cells (Zastre et al., 2013). Glucose is the prime fuel for any energy challenging activity in the body that together with oxygen is delivered by the circulation for the metabolic errands that keep neurons healthy (Mergenthaler et al., 2013). Reduced glucose metabolism is an invariant feature of Alzheimer's diseases (AD) and an outstanding biomarker of disease progression (Gibson et al., 2013). Glucose metabolism may be acting as an attractive therapeutic target, whether the decline initiates AD pathophysiology or is a critical component of a cascade (Gibson et al., 2013). The anomaly in cerebral glucose utilization includes a diminished activity of key enzymes, which are involved in intermediary metabolism notably the activity of glutamine synthetase, creatine kinase, aconitase, pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase (KGDHC) (Sorbi et al., 1983; Gibson et al., 2000). The tricarboxylic acid (TCA) cycle enzymes except SDH are soluble proteins positioned in the mitochondrial matrix. SDH is an integral membrane protein that is firmly attached to the inner mitochondrial membrane, where it communicates directly with components of the respiratory chain (Rutter et al., 2010). In addition, mitochondria have the common pathway to oxidize fuel molecules completely, primarily after their conversion to acetyl CoA, which is the product of oxidative decarboxylation of pyruvate by the

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pyruvate dehydrogenase (PDHC). ATP is generated by the oxidation of reducing equivalents (e.g. NADH) produced during the cycle. The close proximity of enzymes responsible for catalyzing consecutive steps of the TCA cycle increases the chances of protein–protein interactions among them (Lyubarev and Kurganov 1989; Vélot et al., 1997). These enzymes are highly susceptible to oxidative modification and are altered by exposure to a range of pro-oxidants. KGDHC impairment increases the production of amyloid precursor protein and amyloid β peptide formation. An abnormality in TCA cycle enzymes in the brain was observed in several diseases like Alzheimer's disease (AD) and Huntington diseases. Thiamine deficiency (TD) provides a pertinent experimental system to understand the neurodegenerative disorder in which mitochondrial dysfunction attributes to the failure of TCA cycle enzymes (Sheu et al., 1998; Sharma et al., 2013). For inducing complete TD experimentally in animals (Kruse et al., 2004), pyrithiamine is used. It is a potent antagonist of thiamine in the body (Nose et al., 1976). In rats, TD is induced by a thiamine-deficient diet combined with daily injections of pyrithiamine (Eliash et al., 2009; Dror et a.l 2010). Pyrithiamine administration for 10 days to animals produces neurological disturbances and depleted levels of thiamine (Troncoso et al., 1981). The TCA cycle enzymes in mitochondria can act as an aim to cure neurodegenerative diseases such as AD. Keeping above facts in consideration, current study was designed to find out possible effects of TD on mice brain energetics *via* studying TCA cycle enzymes in mitochondrial fractions.

## Materials and methods

### Reagents

Pyrithiamine hydrobromide (P0256) and oxaloacetic acid (O4126) were purchased from Sigma Aldrich (St-Louis, MO, USA). Trizol (15596-018) was purchased from Invitrogen (USA). Coenzyme A (0348300), tris buffer (204982) and α-ketoglutaratic acid (114914) were purchased from Sisco research laboratories (Mumbai, India). Agarose (105192), Taq DNA polymerase (105924) and dNTPs (105407) were purchased from Genei (Banglore, India). RT-PCR kit was purchased from Fermentas (Germany). Gene-specific primers were designed from Imperial Life Sciences (Haryana, India). All other chemicals and reagents used were of analytical grade.

### Animal care

Healthy Swiss mice, 6-8-week old were procured from C.C.S. Haryana Agricultural University, Hissar. Prior to the experimentation, animals were acclimatized to the conditions provided in the animal house for one week. For acclimatization, mice were housed in cages and fed with pelleted diet (Ashirwad rat pelleted diet, India) with water *ad libitum* under the standard conditions of light, temperature and relative humidity. Maintenance and treatment of animals were done in accordance with the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) with Study Ref no. 253 (Institutional CPCSEA no.- 574/GO/REBI/5/2002/CPCSEA).

### Induction of thiamine deficiency

Mice were allocated into 3 groups with the minimum of 12 mice in each group. Experimental mice were made thiamine deficient by injecting the pyrithiamine hydrobromide intraperitoneally (5μg/10 g b.w.) and feeding with a thiamine deficient pelleted diet. Control animals were fed with normal diet and injected with normal saline (0.1mL/10 g b.w.) intraperitoneally.

### Biochemical Estimation

*Isolation of mitochondrial fraction of mice brain*

Mitochondria were isolated from the brain by differential centrifugation using a specific buffer (0.32 M sucrose, 1 mM EDTA and 10 mM tris-HCl, pH 7.4) (Vermulst et al., 2008). The homogenate was centrifuged at 3000 rpm for 10 min at 4°C to pellet nuclei and cell debris. The supernatants of the samples were collected and re-centrifuged at 3000 rpm for 10 min at 4°C. Again, the supernatants were collected and used for mitochondrial isolation. The supernatants were centrifuged at 12000 r/min for 20 min at 4°C. The supernatants were removed and resuspended the pellet in homogenizing medium and centrifuged at 12000 r/min for 10 min at 4°C. The resulting mitochondrial pellet was further used for biochemical estimation and stored at −80°C. Resulting mitochondrial pellet was re-suspended in the specific buffer as mentioned below:

1. 50 mM tris-HCI buffer, 0.1 M EGTA (ethylene glycol tetraacetic acid), 1 M DTT (dithiotheritol), 20% triton X-100 (For the estimation of KGDHC)
2. 50 mM tris-HCl,1 mM EDTA,10% glycerol, pH 7.6 (For the estimation of SDH, Fumarase, MDH)

*Estimation of enzyme activities*

**KGDHC (EC 1.2.4.2; EC 2.3.1.61; EC 1.8.1.4)** KGDHC activity was measured by monitoring the conversion of NAD to NADH with the spectrophotometer by the method given by Gibson et al., (1988). To the 100 μL sample, 100 μL of reaction mixture, 50 μL of assay mix were added and content was mixed properly. Absorbance was taken at 340 nm. Afterward, 25 μL of ketoglutaric acid was added and rate of change of absorption was taken at 340 nm for 30 min.

**SDH (EC 1.3.99.1)** The activity of SDH was measured

spectrophotometrically by following the rate of reduction of the artificial electron acceptor, dichloroindophenol dye (DCIP). A blue color solution of DCIP becomes colorless when SDH enzyme reduces DCIP (Singer et al., 1969). The reaction mixture contains the mitochondrial sample, 1200 μL of the buffer, 200 μL of 0.4 M succinate, 200 μL of sodium azide and 200 μL of DCIP. The samples were mixed properly and read at 600 nm for 15 min with the time scan of 1 min each.

**Fumarase (EC 4.2.1.2)** Hill and Bradshaw (1969) method was used for biochemical estimation of fumarase activity. The reaction mixture contains 1700 μL of malate, 200 μL of BSA and 200 μL of sample. All the contents in the reaction mixture were mixed and read at 250 nm for 1 h with the time interval of 5 min.

**MDH (EC 1.1.1.37)** The activity of MDH was measured by the rate of oxidation of NADH in the presence of oxaloacetate (Kitto, 1969). The reaction mixture of MDH contains 1180 μL of sodium phosphate buffer, 600 μL of oxaloacetic acid and 20 μL of NADH and rate of change of absorbance was read at 340 nm for 5 min with the time interval of 1 min.

*Statistical evaluation*

Data of present investigation was statistically analyzed by one-way analysis of variance (ANOVA) using statistical package for social science program (SPSS 16.0). Significance levels of samples were calculated at 95% and 99% interval. Graphical expression of the biochemical data of the various results were made by using Sigma Plot (8.0 version). Intergroup comparison was made by using Tukey's test.

### Molecular expression

*RNA isolation and expression of different enzymes*

Total RNA was isolated from mice brains by using Trizol reagent. The tissues were homogenized in trizol reagent (100mg/mL) with RNase-free homogenizer. 100 μL of chloroform was added and incubated on ice for 15 min. Samples were centrifuged at 12000 r/min at 4°C for 15 min. The aqueous phase was transferred to RNAse-free tube and an equal volume of isopropanol was added for RNA precipitation. The tube was incubated at −80°C for 15 min/ overnight. The samples were centrifuged at 12000 rpm at 4°C for 15 min. The pellet was washed with 70% ethanol. RNA pellet was dried and resuspended in mili q water. The RNA integrity was analyzed on 1.5% agarose gel electrophoresis. Genomic DNA contamination in RNA sample was removed by using DNA free kit (Genei, Banglore) according to manufacturer's protocol. 1μg of RNA (DNA-free) was used for first strand synthesis for each sample. Primers were designed by using the gene runner (Version 3.05) software according to cDNA sequence of enzymes collected from NCBI (Table 1). RNA of brain tissue was used for the synthesis of the first strand cDNA synthesis using Revert aid first strand cDNA synthesis kit (Fermentas, USA) by using reverse transcription. The reaction mixture contains Revert Aid M-Mulv reverse transcriptase (200U/μL), 10mM dNTP mix (1μL), oligo dT primers in 5X reaction buffer. After first strand cDNA synthesis, gene specific amplification was performed by gradient PCR. The reaction mixture contains RNase-free water (39 μL), 10X PCR buffer (5 μL), dNTP (10mM) (1μL), Forward and reverse primer (1μl), 2 μL of first-strand cDNA synthesized from RT-PCR kit (Fermentas), Pfu HF DNA polymerase (1 μL). The PCR program which was run on the cycler was- Denaturation- 95°C for 2 min, Denaturation-95°C for 30 s, Annealing- 51°C for 30 s (Oxoglutarate dehydrogenase and Succinate dehydrogenase)/52°C for 30 s (DLST)/ 53°C for 30 s (DLD and fumarase)/54°C for 30 s (MDH)/ 60°C for 30 s (GADPH-housekeeping gene), Extension- 72°C for 1 min for 35 cycles and final extension was 72°C for 10 min. Further, the results were analyzed by agarose gel electrophoresis.

## Results

### Biochemical

The biochemical levels of different enzymes in control and thiamine deficient mice showed variable results. KGDHC is a thiamine-dependent enzyme which shows a significant reduction in brains of thiamine deficient groups exposed for 8 days ($p < 0.05$) and 10 days ($p < 0.0001$) as compared to

**Table 1** Primer sequences of different TCA cycle enzymes

| Enzyme | Subunit of enzymes | Forward primer | Reverse primer | Annealing temperature (°C) | Amplicon size |
|---|---|---|---|---|---|
| KGDHC | OGDH | 5′TGCTAAGCCATCTTGCCAGAC 3′ | 5′CAACCTAACGTCGACAAACTCG 3′ | 51 | ~6400bps |
| | DLST | 5′AATGGTGTAGATGTAGTAGCAG 3′ | 5′CAGCGTTTGCAGAGTCTG 3′ | 52 | ~2400bps |
| | DLD | 5′GCTGACGTGACAGTGATAG 3′ | 5′TTTGGTGTCTTCATTCCC 3′ | 53 | ~2000bps |
| SDH | | 5′GAGAACAAGAAGGCATCAGCT 3′ | 5′TCAAGGAAGTCAGGGCATGA 3′ | 51 | ~5400bps |
| Fumarase | | 5′ATCTGACGTATTAGGGGGC 3′ | 5′AACTGCTCTGCTGTGAGATAG 3′ | 53 | ~1500bps |
| MDH | | 5′GTCGTTGGAGTCACTCGTCTT 3′ | 5′TTGGAAGAGATGCTGATGCT 3′ | 54 | ~5000bps |

control group (Fig. 1). Among all the groups, maximum deterioration in enzymatic activity was significantly depicted in the thiamine deficient group for 10 days. In Fig. 2, alteration in SDH activity following TD was observed in different experimental groups. Data clearly illustrates a significant decrease in the SDH activity in group II and group III ($p < 0.0001$) mice, compared to control group. Furthermore, it was also observed that there was a maximum decline in enzymatic activity in group III ($p < 0.001$) mice as compared to group II mice. The diminished enzymatic activity of fumarase enzyme was found in group II as compared to control group. A significant decrease was observed in group III, compared to control mice ($p < 0.0001$). Thus, it can be said that maximum decrease in fumarase activity was pragmatic in TD 10 days group at the significant level among all the experimental groups (Fig. 3). Figure 4 clearly indicates a decline in MDH activity in treated groups (Group II and Group III) as compared to control

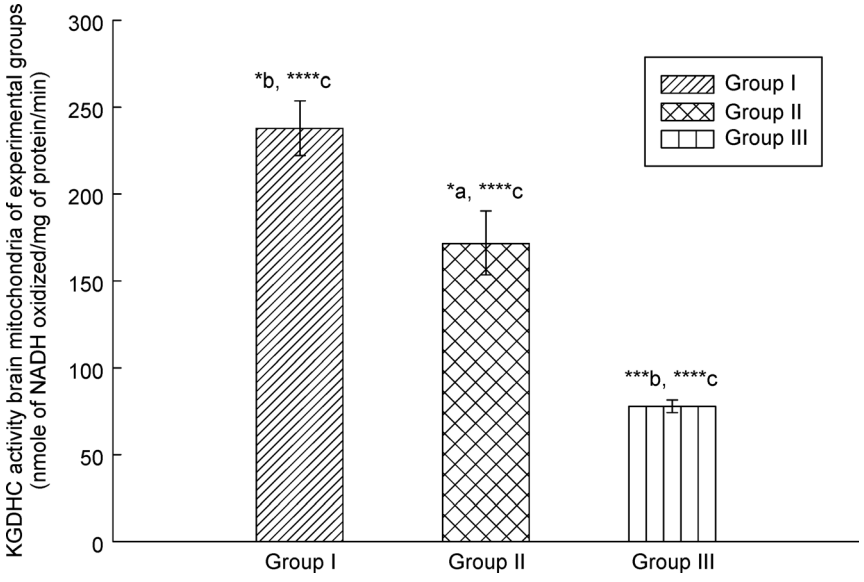


**Figure 1** KGDHC activity in brain mitochondria of different experimental groups. Results were expressed as mean±SE. a-compared to group I; b-compared to group II; c-compared to group III. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

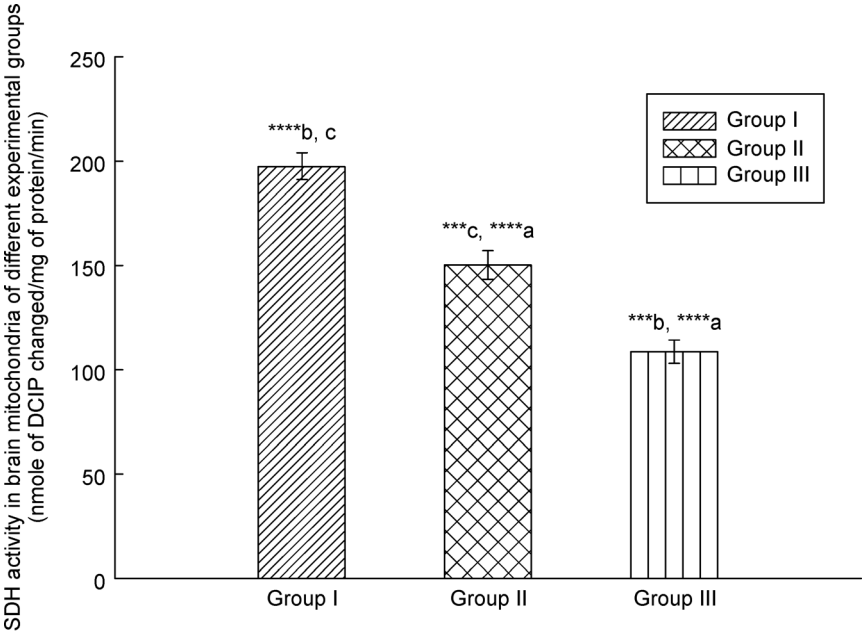


**Figure 2** SDH activity in brain mitochondria of different experimental groups. Results were expressed as mean±SE. a-compared to group I; b-compared to group II; c-compared to group III. ***$p < 0.001$, ****$p < 0.0001$.

(Group I) and significant ($p < 0.0001$) maximum reduction was recorded in group III mice.

## Molecular Results

RNA was isolated from the all the experimental groups ($n = 3$), using the standard Trizol method as described previously. The integrity of total extracted RNA was checked by standard agarose gel (1.5%) electrophoresis (Fig. 5). Expression results of different genes encoding enzymes i.e., fumarase and DLD (subunit of KGDHC) show that there was a negotiable change in the expression, whereas a decline in the expression of MDH, SDH, OGDH, and DLST. The molecular result showed that subunits of KGDHC (OGDH and DLST) as well as MDH and SDH (thiamine independent enzymes) were highly affected due to thiamine deficiency (Fig. 6). As the deficiency of thiamine increases in the group, gene expression of enzymes in different experimental group decreases. This showed a dose and time dependent relationship with expression of genes of respective enzymes.

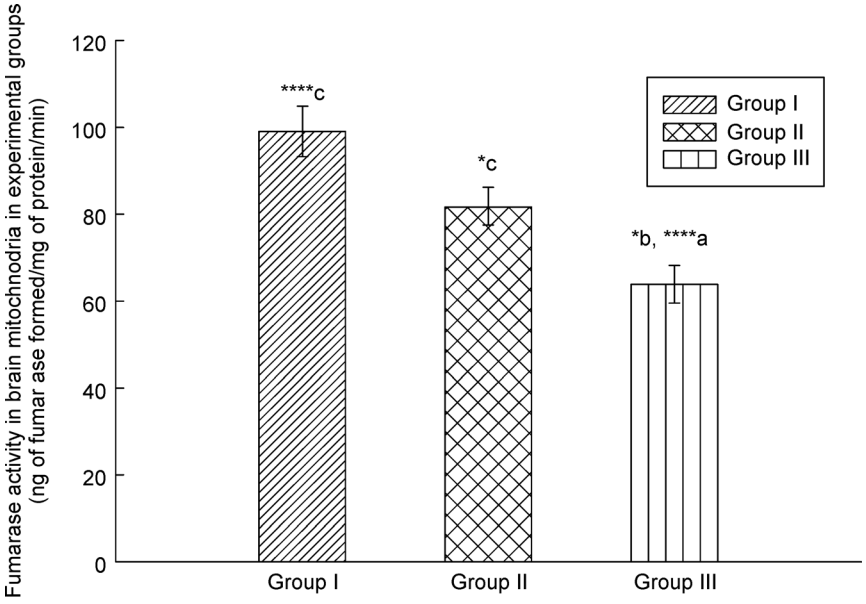


**Figure 3** Fumarase activity in brain mitochondria of different experimental groups. Results were expressed as mean±SE. a-compared to group I; b-compared to group II; c-compared to group III. *$p < 0.05$, ****$p < 0.0001$.

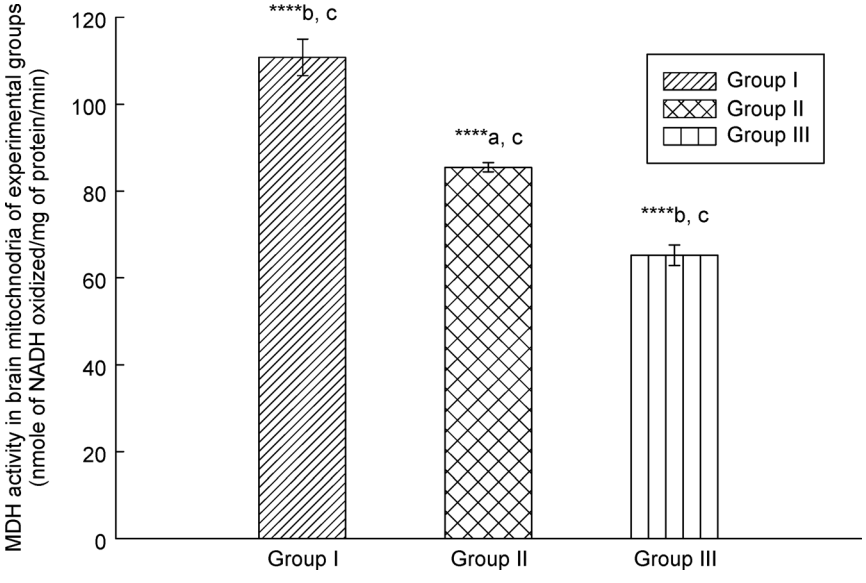


**Figure 4** MDH activity in brain mitochondria of different experimental groups. Results were expressed as mean±SE. a-compared to group I; b-compared to group II; c-compared to group III. ****$p < 0.0001$.

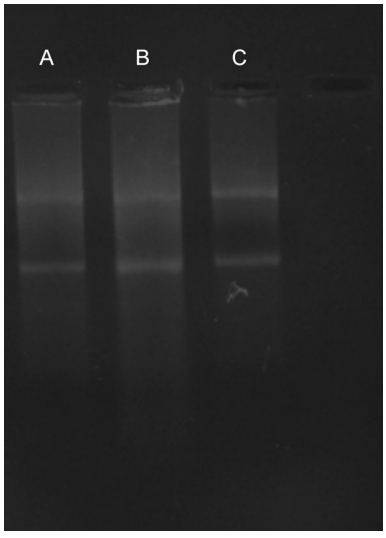


**Figure 5** RNA of different experimental groups. A- Group I; B- Group II; C-Group III.

Hence, the results of biochemical studies are in agreement with the molecular expression of same enzymes after thiamine deficiency which deteriorates the enzymatic activity of TCA cycle enzymes in brain mitochondria.

## Discussion

Thiamine is an essential water-soluble vitamin and its availability is a prerequisite for normal cellular metabolism of the brain (Liu et al., 2016). It serves as a specific cofactor of certain enzymes involved in energy metabolism of cells and its deficiency may affect the enzymes of TCA cycle (Sharma and Bist, 2014; Sharma et al., 2013). TCA cycle carries sequential reactions catalyzed by thiamine-dependent and thiamine-independent enzymes. KGDHC (thiamine-dependent enzyme), SDH, fumarase and MDH (thiamine-independent enzymes) are evaluated for any change by TD in the present investigation because it creates interest to know the mechanism of brain thiamine homeostasis. Previously it was already reported by several studies that TD causes abnormalities in thiamine-dependent enzymes, creates oxidative stress which diminishes the energy metabolism in various neurodegenerative diseases such as AD (Ke et al., 2003; Gibson et al., 2010). Gibson and Blass (2007) observed that KGDHC is positioned at the vital and arguably rate-limiting steps in the brain metabolism. In the current study, KGDHC activity was found to be significantly reduced in brain mitochondria of thiamine deficient groups. This reduction in activity of KGDHC was one of the earliest biochemical alterations in TD (Gibson et al., 1984; Bubber et al., 2004). The maximum reduction in KGDHC was recorded in group III, which was experimentally completely devoid of thiamine. Tretter and Adam-Vizi (2000) stated that enzymatic activity of KGDHC gets inactivated in TD mice because of generation of peroxynitrite, a product of LPO. LPO is elevated in oxidative stress conditions induced due to TD (Tretter and Adam-Vizi, 2000**;**Bist et al., 2010; Sharma and Bist, 2014). Molecular results of current research stated that decrease in the expression of subunits of KGDHC i.e., OGDH (E1) and DLST (E2). E1 and E2 are unique to KGDHC whereas DLD (E3) is also a component of the PDHC and branched-chain α-ketoacid dehydrogenase complex. Subunits of KGDHC show a selective response to oxidative stress. E1 and E2 are highly

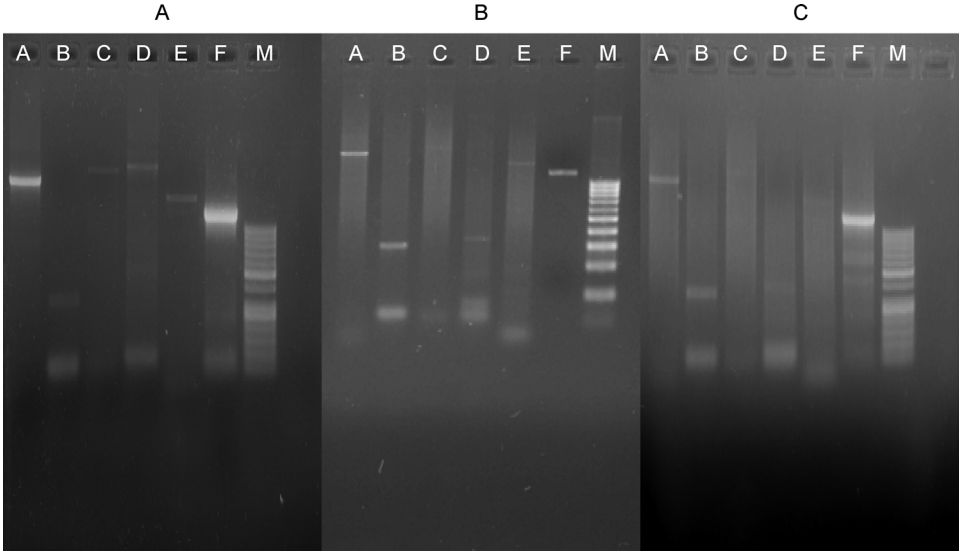


**Figure 6** (A) Group I- Gene expression of different enzymes in control group. Lane A- MDH, B- Fumarase, C- SDH, D- OGDH, E- DLST, F- DLD and M- marker. (B) Group II- Gene expression of different enzymes in thiamine deficient group for 08 days. Lane A- MDH, B- Fumarase, C- SDH, D- OGDH, E- DLST, F- DLD and M- marker. (C) Group III- Gene expression of different enzymes in thiamine deficient group for 10 days. Lane A- MDH, B- Fumarase, C- SDH, D- OGDH, E- DLST, F- DLD and M- marker.

sensitive to oxidative stress in compared to E3 (Park et al., 1999; Shi et al., 2005). The decline may be due to the presence of lipoate residue which makes the subunit highly vulnerable to oxidative stress created due to TD (Nakano et al., 1991). Free lipoic and dihydrolipoic acid are antioxidants which react readily with hydroxyl radicals and induce oxidative damage.

The decline in the activities of SDH, fumarase and MDH following TD may be due to close propinquity and direction of enzymes in the TCA cycle for maintaining the metabolic flux. The reductions in the expression of SDH gene in experimental groups were recorded. In 2008, Capettini and his coworkers explained that the dehydrogenases activity of TCA cycle enzymes was affected by free radicals resulting in inhibition of mitochondrial substrate oxidation leading to the depletion of cellular energy metabolism. Under oxidative stress, the decrease in SDH activity was also accounted by Mailloux et al. (2007). Further, Bogdan (1983) related reduced thiamine level with a decline in the activity of SDH in liver. Thus, the results of the present study are in consistent with earlier observations.

Diminution in the activity of fumarase enzyme in thiamine deficient groups exposed for 8 and 10 days. This may be due to loss of mitochondrial integrity in brains of thiamine deficient mice. However, contradictory results were reported by others, where no significant change in the activity of fumarase was observed in frontal cortex area of AD brain (Sorbi et al., 1983; Gibson and others, 2010) and brain of thiamine deficient mice (Bubber et al., 2004). No significant changes where observed in expression study of fumarase. This shows that fumarase levels do not seem to be unduly influenced by other enzymes of the TCA cycle of thiamine deficient brain of mice.

Additionally, our studies also showed a decrease in MDH activity of treated mice as compared to the control group. Gene expression of MDH enzyme found to be reduced in treated groups (group II and group III) as compared to control group. The decline in MDH activity attributed to low succinate oxidation in TCA cycle, due to TD (Shaikh et al., 2012). The maximum reduction in MDH activity was found in the group III, among all the groups. The earlier report explained that TD promotes the reduction in the activity of MDH in brain homogenate (Bubber et al., 2004). The trivial decline in MDH and Fumarase transcript level were observed in the current study, it may that thiamine deficiency was not severely affecting the thiamine independent enzymes at the transcript level.

Hence, above results of current research conclude that TD leads to a reduction in the activities of TCA cycle enzymes in mitochondrial fraction of the brain thus endorses the disturbances in energy metabolism. This, in turn promotes oxidative stress in the brain mitochondria. Mitochondrial dysfunction in the brain leads to the neurological disorder such as AD. It is always important to develop a better understanding of the role of mitochondrial energy metabolism, since it may lead to finding more effective treatment strategies for AD.

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## Compliance with ethics guidelines

Anupama Sharma received a research grant from ICMR, New Delhi. Anupama Sharma, Renu Bist and Surender Singh declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed in the current study.

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