

The Effect of an Indigenous Drug on Abnormal Folate Metabolism and Intellectual Disability: A Study of 5,10-Methylenetetrahydrofolate Reductase Activity

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ABSTRACT

Methylenetetrahydrofolate reductase, (MTHFR) is a key enzyme that is required to metabolize folate and is essential for normal development of central nervous system. Mutation in the MTHFR gene may result in elevated plasma homocysteine level. MTHFR C677T is most common polymorphism associated with impairment of cognitive function. In the present study, we examined the relationship between 677C>T MTHFR gene polymorphisms, homocysteine (tHcy) and the effect of test drug in the level of tHcy in the treated intellectual disability children (ID). Fifty patients of the both genders (male 31, female 19) with an age range of 8 to 12 years with ID (cases) were selected from Pediatric O.P.D of S.S. Hospital I.M.S, B.H.U. ID children were clinically diagnosed according to the DSM V screening system. MTHFR C677T polymorphism was detected in the normal control vs. ID children by PCR-RFLP using Hinf I enzyme. A total 30 out of 50 ID children were treated with the test formulation and remaining 20 ID children were placebo. Plasma homocysteine levels were measured by HPLC at initial, 3 & 6 months of study. CT of MTHFR C677T was higher in ID children than controls, although it did not show statistical significance ($p=0.1$) and the child T677T MTHFR genotype (TT) was found 4 % in ID children. In the treated group, level of homocysteine dropped while increased in the placebo after six months of treatment. In treated group ID children, CC with genotype the level of tHcy dropped from 9.06 to 8.21; CT 11.92 to 10.15 whereas in placebo no such change was observed. Therefore, the present study showed MTHFR C677T polymorphism associated with modifying level of tHcy. The result indicated that ID children with the MTHFR C677T gene showing the highest level of tHcy, when treated with test formulation exerted reduction in tHcy concentration suggesting improved cognitive function due to homocysteine re-methylation. The present study also suggested an association of high degree of genetic heterogeneity because of their mutation in ID children. It is concluded that the test formulation has the potentiality to reduce the risk of MTHFR C677T gene mutation because of its activity and thus, improved the cognitive ability of ID children.

Keywords: MTHFR, Intellectual disability, Test formulation, Heterozygous, Efficacy.

INTRODUCTION

Globally, intellectual disability (ID) is the most frequently encountered heterogeneous neurobehavioral disorder of childhood and adolescents, characterized by inattention, impaired cognitive function, linguistic, lack of social abilities and hyperactivity. Clinical symptoms of ID include muscular hypotonia, poor visual contact, diminished motor activity, abnormal play and late attainment of developmental milestones¹. ID is a complex neurodisorder associated with a strong genetic component as indicated by heritability. Several studies have shown that individual genetic variants are associated with “gene-gene” and “gene-environment” interaction; however epigenetic processes may play a significant role in the course of disease²⁻³. A number of factors, including different drugs modify the epigenetic process, chromatin

structure & DNA methylation, altering the gene expression, involved in the neural plasticity and also affect the synthesis of different neurotransmitter³. Modified epigenetic affects the chromosomal instability and concentration of homocysteine (tHcy) in ID children through re-methylation⁴⁻⁵. A number of studies have shown that folate is an essential constituent for the normal development of central nervous system during organogenesis whereas its deficiency leads to abnormal differentiation impaired neuronal activity resulting in neurodegenerative and psychiatric disorders. A mild increase in plasma homocysteine may be due to deficiency of folic acid⁶⁻⁷. A 5, 10-Methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism has been reported to be associated with ID and other neuropsychiatric disorder independently⁸. Frosst et al.

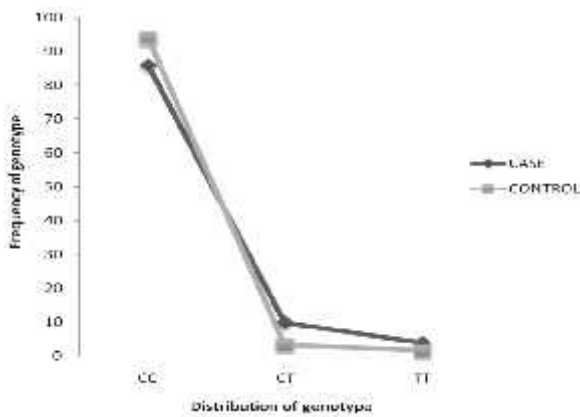


Fig 1: Represent the homozygous and heterozygous genotype of MTHFR C677T gene of cases via controls

(1995) described that MTHFR gene is located on chromosome 1p36.3, with two common single nucleotide polymorphism (SNPs) or alleles, C677T and A1298C to facilitate the enzymatic actions and involved in the metabolism of folate⁹⁻¹³. The studies have revealed the substitution mutation of Alanin with Valine (A222V) form C677T allele causes reduction in activity of enzyme including hyperhomocysteinemia¹⁴⁻¹⁵. MTHFR catalyses irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the methyl donor for methionine synthesis from homocysteine and participates in S-adenosyl methionine synthesis and DNA methylation¹⁰. It is already reported that MTHFR gene regulates the concentration of homocysteine level and consequently, improves the cognitive function like memory, attention and learning ability of ID patients⁹⁻¹¹. In light of the above facts, the present study was planned to investigate the association of MTHFR C677T gene polymorphism with ID children, level of tHcy in subjects (ID children) and subsequently to evaluate the role of the test formulation in reduction of tHcy level. Thus, a plant based formulation containing hydro-alcoholic extract of *Bacopa monnieri* (BM) and *Hippophae rhamnoides* (HR) in effective doses were prepared in the term of the syrup. The formulation was evaluated for its biological activity through reduction of tHcy concentration in plasma, resulting in improved cognitive ability among clinically diagnosed ID children.

MATERIALS AND METHODS

Under the study 50 clinically diagnosed ID children of both genders (31-male, female 19) with an age range of 8-12 years were recruited from Pediatrics Out Patients Department of S.S. Hospital I.M.S, B.H.U. were diagnosed on the basis of the DSM V screening system

which includes the following- language reading, writing skill, reasoning, knowledge, memory performance, social judgment, interpersonal communication, and inability in self-management. Fifty-nine normal children of the same age group were selected to serve as controls.

The study was divided into two groups-

Group I - ID children treated with test formulation (n=30)

Group II- ID children treated with placebo (n=20).

Treatment and Study Design: A double-blind, placebo, case and control study was designed for this clinical trial. Treated group received 10 ml per day, two times for six months continuously. The syrup contained 250 mg of BM and HR (150 mg) extract, remaining sugar candy powder (66%W/V I.P.) and the volume adjusts to the qs up to 10ml. Both extracts were prepared by hydro-alcoholic methods, from stems, leaves, fruits and roots. The placebo syrup was made up by sugar candy and given to per participant per day two times with the same dose. Before going for the clinical trial the safety and efficacy profile of test formulation was validated as per WHO norms. The study was ethically approved by the ethical committee of Institute of medical sciences Banaras Hindu University.

Biochemical and Genotyping Analyses: Blood samples were collected between 9 to 11:30 AM into a vacuum tube containing potassium EDTA from patients before & after administration of test drug and also from controls. Total plasma homocysteine was measured using HPLC, developed by method of Fiske strand T et al., (1993). For genetic determination, DNA was isolated from nucleated blood cells of patients and controls, using Bioner kit (Korea). MTHFR 677C T mutation was determined by polymerase chain reaction and primers C677T (F-5'TGAAGGAGAAGGTGTCTGCGGA3') & (R-5'AGGACGGTGCGGTGAGAGTG3'). Restriction fragment length polymorphism (RFLP) analysis was carried out to determine missense mutation in the presence of HinfI as reported by Frost et al (1995) (9). The PCR product (6 µl) were digested at 37°C for 3 hr. in reaction volume of 25 µl containing 1U of Hinf-I restriction enzyme (New England, Biolabs) and NEB buffer (2.5 µl). Digested product of RFLP was separated on 3% agarose gel electrophoresis stained with ethidium bromide and visualized on the Gel Doc system.

STATISTICAL ANALYSIS

Genotype and allele frequencies in ID patients and controls were determined by Hardy - Weinberg equilibrium equation. Statistical analysis was carried out using chi test (X² - test) and comparison was made between the groups.. The odds ratio at 95% confidence interval was calculated

Table 1-MTHFR C677T polymorphism in ID patients and controls.

Groups	MTHFR C677T			Genotype	O.R (95% C.I) p value cases vs. controls	Allele frequency (%)	
	CC	CT	TT			C	T
ID patients (n=50)	86.0	10.0	4.0	CC	0.32(0.06-1.53) 0.1	0.91	0.09
Control (n=59)	94.9	3.38	1.69	CT	3.16(0.5-2.48)0.1	0.96	0.03
				TT	2.4(0.16-6.90) 0.04		

CC; wild homozygous genotype, CT; mutated genotype, TT; rare mutated homozygous genotype

Table 2: The level of tHcy in the ID children whose have a different genotype of both placebo and treated at initial, after 3 months and six months of treatment with the p-value within the group (P/P and T/T).

Group	Initial			After 3months			After 6months			Initial			After 3 months			After 6 months		
	CC	CT	TT	CC	CT	TT	CC	CT	TT	CC/CT	CT/T	T	CC/CT	CT/T	T	CC/CT	CT/T	T
P (n=20)	N=16 8.90	N=3 11.89±	N=1 13.2	N=16 9.70±	N=3 12.59	N=1 13.9	N=16 10.36	N=3 13.24	N=1 14.3	0.70	0.49	-	0.87	>0.05	-	>0.05	0.13	-
tHcy	±0.7	0.87	1	0.76	±0.62	8	±1.07	±0.76	5									
T (n=30)	N=27	N=2 11.92±	N=1 13.5	N=27 11.00	N=2 13.6	N=1 8.21±	N=27 10.15	N=2 12.0	N=1									
tHcy	9.06±0.75	1.23	1	8.69±	±1.47	1	0.72	±1.62	0									

0.74

to determine the risk factor between ID and their respective control. Statistical significance was defined as $p < 0.05$ or < 0.001 by SPSS package (paired and unpaired students t-test), paired t-test was performed within the group and unpaired t-test between the group.

RESULT AND DISCUSSION

Frequency of MTHFR gene polymorphism in the controls vs. Cases: A total number 109 children from an Indian population were recruited (controls n=59 and 50 cases) for the present study. The study did not reveal how much statistical difference existed between the control & ID patients due to small sample size. The genotypic distributions of CC, CT, and TT for patients with ID patients were evaluated to be 86.0 %, 10.0 %, and 4.0% while for controls 94.9%, 3.38% and 1.69% respectively. The Relative Risks and Odds Ratios with 95% Confidence Intervals (CI 95%) of MTHFR C677T polymorphism in ID patients and controls is illustrated in Table 1 & figure-1.

Test Drug Treated Outcome by MTHFR Genotype And Group: The level of homocysteine was indirectly linked to MTHFR polymorphism-CC, CT and TT genotype. Following six months of test drug treatment decreased the homocysteine level in the plasma of ID treated children whereas no such changes were observed in placebo treated group (table-2)

Out of 50 patients, 30 patients had been randomized for treatment (treated group) and 20 with placebo treatment group. Out of 30 children, 27 ID children had C allele (CC, n=27) and rest of the patients had T allele carrier group, CT (n=2) & TT (n=1) MTHFR genotypes. In placebo group 80% individuals showed CC genotype whereas CT/TT was 15/5 %. The present results revealed that individuals with CC genotype in the placebo group had significant association with increased level of tHcy ($p < 0.001$) and CT ($p < 0.001$) within 6 months. In the treated group T allele carrier individual including both CT/TT MTHFR genotype, showed better response (11.92 to 10.51µmol/l - CT;13.51 to 12.00µmol/l -TT) of test drug, but showed non-significant difference when compared within the treated group ($p = 0.086$ & 0.098 ; $df = 1$ with 3 & 6 months respectively). Treated group with CC genotype showed significant difference ($p < 0.001$; $df = 26$) with better response in reference to

dropped the level of homocysteine 9.06 to 8.21 µmol (table-2). When the data was compared between treated and placebo from initial to 6 months of treatment, the level of tHcy dropped significantly. In the three and six months of treatment CT and CC genotype individual showed- $F = 7.5$ & 12.0 ; $p > 0.05$ at $df = 41$ & 3 , that the drug significantly reduced the level of homocysteine in the blood plasma of ID children (table-2 & 3).

ID is recognized as a complex neuropsychiatric disorder and has a complicated etiology, influenced by interaction of “gene-gene” & “gene-environment”. Several genes have been associated with folate regulation (homocysteine metabolism) and with risk of development of ID including other neuropsychiatric disorders. Although enzyme involved in the folate dependent homocysteine metabolism pathway are strongly concerned to ID, as several candidate gene, wide ethnic variation & population diversity have different allele frequency of their polymorphic variation. Therefore, apart from genetic, environmental factors are also responsible for development of ID.

One of gene 5’-10 MTHFR is specifically involved in the folate metabolism and it is strongly associated with increased risk for ID. MTHFR C677T is a common polymorphism, susceptible to development of mental illness. Numerous studies have also suggested that mutation in the MTHFR gene, partially have a protective effect against the risk of mental disability. Low level of folate (vitamin-B9) and cobalamin (B12) are associated with risk of ID. Both vitamins are required in transmethylation reaction and essential for the configuration of neurotransmitter. Interruption in transmethylation, lead to accumulation of tHcy in the neuron cell which may further lead to cerebral dysfunction. When tHcy is not converted in methionine through re-methylation reaction and is present in the neuronal cell in free form (homocysteine) it becomes injurious to brain cell and destructs the mental activity.

Insufficient folate and cobalamin can also lead to ID in the foetus because its deficiency impairs the activity of an enzyme involved in the folate metabolism and simultaneously, increases the level of homocysteine, decreases the vitamin B12 as comparisons of normal children 16. Thus, elevated homocysteine & cobalamin are independent indicators of ID. These conditions are able to

Table 3: Level of significance in relation to different genotype.

Group	Initial Vs after 3months (CCi/CC3)	Initial Vs after 6months CCi Vs CC6	Initial Vs after 3months CTi Vs CT3	Initial Vs after 6months CTi Vs CT6	Initial Vs after 3months TTi Vs TT3	Initial Vs after 6months TTi Vs TT6
P (n=20)	<0.001	<0.001	>0.05	<0.001	--	--
T(n=30)	<0.001	<0.001	0.086	0.098	--	--

Normal range 3.2 to 7.6 $\mu\text{mol/l}$; >0.05, <0.001

CCi = CC initial, CC3 = CC after 3 months, CC6 = CC after 6 months, CTi = CT initial, CT3 = CT after 3 months, CT6 = CT after 6 months, TTi = TT initial, TT3 = TT after 3 months, TT6 = TT after 6 months

cause mutation in the MTHFR C677T gene. However, MTHFR gene polymorphism is also affected by level of RBC folate and plasma homocysteine. Therefore, ID have the multifactor origin of CNS disorders with the common variant in more than one gene involved in folate and homocysteine metabolism.

In the present study, we have examined the effect of 677C>T MTHFR polymorphisms on the treatment outcome of ID patients treated with the test formulation.

There was statistically significant association of the MTHFR C677T genotypes with response to treatment. In the multivariate analysis additionally adjusted for patient age and gender, the MTHFR C677T allele was associated with increased frequency, reduce the tHcy concentration in plasma of blood.

The test formulation contains two ingredient- BM and HR. BM is known as a nerve, brain tonic that enhances the efficiency of transmission of nerve impulses, thereby strengthening memory, cognition, improve academic performance. Thus act as a neuroprotective agent over the neuronal cell because of its active component-Bacoside A & B^{17,19-22}. During mechanism BM have action on the special excitatory and inhibitory (cholinergic, glutamergic, serotonergic, nor-adrenergic, dopaminergic GABA and monoamines) neurotransmitter for the neuronal endurance. These components repress the neuronal oxidative stress and acetyl cholinesterase inhibitory activities by increase in the levels of protein kinase and then get involved in the synthesis of new proteins and various neurotransmitters. These proteins show the neuroplasticity properties and help to restore the synaptic activity of neuron 3. The signal passed from the sensory organ to the receptor of the hippocampus that further activates the electric pulse and these pulses are mediated through a customized structure of the translational product and are able to transit signal to the next neuron through synapse.

However, the chronic intake of BM creates a bond between neuron and memory and influences the neural activity, which leads to improve, store and generate a new memory¹⁷⁻¹⁸. Therefore, BM extract are helpful to protect the patients from neurodegenerative disorders linked to oxidative stress. BM is also known as "Mind power booster" or "Brain tonic" as it possess a great property to improve and maintain acuity of intellectual and memory¹⁷. As BM Hippophae rhamnoides has also shown to increase the neural activity and is a rich source of folic acid. It has a key role in the development of the central nervous system²²⁻²³, if there are any errors in the folate metabolism it causes numerous neurodegenerative disorders. Numerous studies have shown that HR act as an antioxidant agent and is involved in the biosynthesis of

dopamine. Dopamine is a clinical messenger responsible for transmitting signals between the substantial nigra and the next 'relay station' of the brain to produce smooth, controlled movement and protection of nerve 18. Therefore, the increased frequency of remission of disorder when the patients treated with test formulation. We observed increased in enzymatic activity and decrease in the MTHFR expression as a result of test drug might be associated with a better response to treatment. This effect may involve more efficient down-regulation of 5-methyl-THF synthesis through MTHFR, and subsequently reduced methionine production from homocysteine and 5-methyl-THF through methionine synthase and S-adenosylmethionine.

S-adenosylmethionine is the main donor of a methyl group in several biochemical pathways and reactions of DNA methylation⁶⁻¹¹. A limited availability of S-adenosylmethionine may affect the expression of genes involved in the development of ID. The study also focused on the effect of the test formulation in the expression of MTHFR gene and supported the study conducted by Haagsma et al 1999²⁴. This is the first study of its kind conducted on Indian population. Thus, according to our findings, we hypothesize that the test drug may affect epigenetic either at the DNA or histone level and decrease the concentration of homocysteine through re-methylation process via up regulating DNA methyltransferases in the specific region of the brain. It may affect the level of heterochromatinization in the genomic part of neuronal cells and increase the level of neurotransmitter for the mental activity and decline the cognitive problem. The present study revealed that the methylation is an important process for MTHFR gene expression in the treated group of ID patients and also for analysis of genetic base treatment.

CONCLUSION

The present results indicated and confirmed the traditional use of these plant based test formulation for the management and treatment of ID children. Test formulation showed preventive property of neurobehavioral problem thereby reducing the level of plasma tHcy. It can be proposed as one of the suitable remedial measures for the management of ID. The drug is safe and can be given for a long period. Due to involvement of high cost and safety constraints, the result could not be revalidated.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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