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Abstract

Homocysteine (Hcy) is a sulfur containing metabolite produced by de-methylation of methionine in one-carbon metabolism of pathway where methionine is converted to S-adenosyl methionine (SAM) through activation of ATP. SAM works as a major methyl donor in the cell and after donating the methyl group it is converted to S-adenosyl homocysteine (SAH) which in turn hydrolysed to homocysteine. In humans, plasma Hcy level is regulated by its remethylation to methionine and by transulfuration to cystein. There are numerous clinical and epidemiological data showing the direct correlation of abnormal Hcy levels with generation of different types of diseases like cardiovascular diseases, central nervous system disorders and others. Still, it is not clear whether abnormal level of homocysteine is the cause or the impact of the disease. Hyperhomocysteinemia has been considered as an indirect marker of vitamin B deficiency and is a neurotoxic agent since the metabolic pathways involved in Hcy breakdown are missing in the brain. The relation of hyperhomocysteinemia with Parkinson Disease (PD) arouse after the observation of increased plasma Hcy level in PD patients treated chronically with L-DOPA (3,4-dihydroxyphenylalanine), the precursor of dopamine, the most effective drug in the symptomatic management of PD. The Present study is aimed to establish the correlation of elevated plasma Hcy level, measured by High performance liquid chromatography (HPLC) using fluorescence detector, with PD, in north-Indian PD patient cohort and also to give an account of overview of literatures known so far to understand the mechanisms underlying the enhanced homocysteine levels and its related pathophysiological cascades.
Key words: Hyperhomocysteinemia; Parkinson Disease; L-DOPA; Dementia; Homocysteine

1. Introduction

Hcy is a non-essential amino acid, biosynthesized by demethylation of an essential amino acid methionine [1]. It is a homologue of the amino acid cysteine, differing by an additional methylene bridge (-CH₂-) [Figure 1]. Methionine is a proteinogenic amino acid taken by the body through diet and is being used to synthesize other important proteins in the body. It is the precursor of Hcy. Methionine and Hcy work together to maintain the levels of several other proteins in the body. In mammals, Hcy is required for cysteine biosynthesis. It also helps to maintain methionine levels. Methionine is converted to S-adenosyl methionine (SAM) through activation by ATP. SAM acts as a major methyl donor in the cell which after transferring the methyl group, is converted to S-adenosyl homocysteine (SAH), and then to Hcy. The term hyperhomocysteinemia was initiated with the research of McCully in 1969 who suggested that metabolic effects of increased level of Hcy or a derivative of Hcy could be a cause of arterial damage in homocystinuria [2]. Abnormal levels of Hcy have been related to neurodegenerative and neuropsychiatric diseases, cardiovascular diseases, pregnancy related complications, congenital malformations etc. [3]. Variability in the clinical manifestations of hyperhomocysteinemia (human plasma Hcy levels above 15 µM) results from variability in the factors contributing to it. The factors responsible for metabolic disorders of Hcy are nutritional deficiencies of the vitamins and co-factors (vitamin B₆, vitamin B₁₂ and mainly folates), diseases like renal impairment, diabetes, anemia etc, physiological factors (age, sex etc.), medications (methotrexate etc.), mutations in the enzymes involved in Hcy metabolism (methylenetetrahydrofolate reductase, cystathionine synthase etc) and mode of lifestyle in some cases [4]. The effects of Hcy can be broadly categorized into neurotoxic and vascular. Its toxic potential includes endothelial cytotoxicity, lipid peroxidation, smooth muscle proliferation and neuronal cell apoptosis [5]. Hcy can undergo auto-oxidation, resulting in the formation of biologically reactive products that participate in signaling pathways associated with cyto-toxicity. It has been identified as a contributor to four fundamental disease mechanisms those are thrombosis [6] oxidative stress [7], apoptosis [8], and cellular proliferation [9]. The metabolism of Hcy occurs via three pathways: A. remethylation of Hcy to form methionine by methionine synthase in a vitamin B₁₂ and folate-dependent reaction, B. transsulfuration pathway, by which Hcy is converted to cystathionine by cystathionine β-synthase (CBS) by addition of a serine group, and C. remethylation of Hcy to methionine via betaine:homocysteine methyltransferase (BHMT) in certain tissues such as liver and kidney. Hyperhomocysteinemia is increasingly recognized as a risk factor for many neurological disorders. Hcy trespasses blood-brain barrier by activation of N-methyl-D-aspartate (NMDA) receptor and therefore Hcy concentration in cerebrospinal fluid (CSF) is correlated to that of plasma levels (approximately 5-10%) [10]. Thus the main possible causes of brain’s vulnerability to high Hcy levels are: A. Absence of the three above mentioned major metabolic pathways for Hcy breakdown in the brain [11] and
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B. Hcy is directly responsible for several mechanisms inducing neurotoxicity. Elevated Hcy levels have also been identified as risk factors for cognitive decline and Alzheimer disease (AD) [12-15] and linked to the pathogenesis of some psychiatric disorders such as depression and schizophrenia [16,17].

2. Plasma Homocysteine Level and Parkinson Disease

Parkinson Disease (PD) is the second most common neurodegenerative disorder after AD [18]. It is a progressive neurological disorder characterized by loss of dopaminergic neurons in the substantia nigra pars compacta, with consequent dopamine depletion from the striatum leading to motor control problems [19]. Pathophysiologically the disease is characterized by the presence of eosinophilic intracytoplasmic Lewy bodies, dystrophic neurites, mitochondrial dysfunction and neuroinflammation [20]. The non-motor complications of PD include cognitive decline, dementia, depression, and psychosis. Thus PD pathogenesis is multifactorial. Elevated levels of Hcy in PD patients compared to age-matched controls were first reported in a literature by Allain P et al., 1995 [21]. While the data suggesting that increased level of Hcy is a risk factor for PD development is lacking. Hcy levels are shown to directly correlate with the rate of PD progression which indicates its potential important role once the disease has been established. Later on it has been found that Plasma Hcy levels are elevated in patients with PD treated with L-DOPA (3, 4-dihydroxyphenylalanine), the precursor of dopamine, the most effective drug for treatment of PD. It is due to the metabolism of L-DOPA, where L-DOPA is O-methylated leading to the formation of 3-O-methyldopa, in a reaction catalysed by catechol-O-methyl-transferase (COMT) with SAM acting as a methyl donor [22]. The donation of a methyl group from SAM leads to the formation of SAH, which in turn is hydrolysed to produce Hcy. This causes elevated levels of Hcy in the blood plasma of L-DOPA treated patients. Thus hyperhomocysteinemia in PD is caused by L-DOPA therapy rather than pre-existing hyperhomocysteinemia. The condition of hyperhomocysteinemia has deleterious effect on further disease progression. In the present study we have shown the effect of L-DOPA treatment in north-Indian PD patient cohort in respect to the level of plasma homocysteine.

3. Materials and Methods

The study protocol was approved by Human Ethics Committee (Ref. no. F. Sc. /Ethics Committee/2015-16/1), Institute of Science, Banaras Hindu University, Varanasi, 221005, India.

3.1. Patients and controls

A total of 82 clinically diagnosed sporadic PD cases with of mean age of disease onset at 58.8±11 year ranging from 30 to 75 including 13 females were enrolled for this study with their written informed consent from Department of Neurosurgery and Department of Neurol-
ogy, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. All of them are supplemented with L-DOPA. The control group consists of 30 unrelated ethnically matched healthy volunteers with no positive family history for PD or any other neurological disorders. The mean age for control group is 49.8±6.9 year. Approximately, 6 ml of peripheral blood was collected in heparinized syringe.

3.2. Isolation of plasma

Within 20-30 min after blood sample collection 2 ml of blood was taken for preparation of plasma by centrifugation for 10 minutes at 1,000–2,000 x g. The resulting supernatant i.e. plasma was transferred in a fresh tube and stored at -80ºC. The Hcy levels were measured within three months using an automated high-performance liquid chromatography method with reverse phase separation and fluorescent detection, using monobromobimane.

3.3. High Performance Liquid Chromatography (HPLC)

The principle of separation in normal phase and reverse phase mode of HPLC is adsorption. When a mixture of components is introduced into a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower. The component which has less affinity towards the stationary phase travels faster. Thus the components are separated based on their retention time in stationary phase [Figure 2]. In the present study HPLC was used for estimation of plasma homocysteine, cysteine and glutathione. Briefly, estimation was done by standard curve method. Seven standard concentration points were taken for homocysteine (0, 3.125, 6.25, 12.5, 25, 50 and 100 µM), cysteine (0, 6.25, 12.5, 25, 50, 100 and 200 µM) and glutathione (0, 0.781, 1.562, 3.125, 6.25, 12.5 and 25 µM). Stocks of homocysteine, cysteine and glutathione were taken in 1.5 ml eppendorf tube and volume was maintained to 500 µl with MQ. A set of eight eppendorf tubes were taken (labeled T, T/2, T/4, T/8, T/16, T/32) for serial dilution of standard mix prepared above. Serial dilution was done by 100 µl of standard mix with 100 µl MQ water and further subsequent dilution using 100 µl of MQ in serial tubes. A set of eight tubes for standards (S1,S2,S3,S4,S5,S6,S7,S8) were added with 20 µl of MQ in S1 and S8 (for blank) and 20 µl from each serial dilution T/32,T/16,T/8,T/4,T/2,T in tubes S2,S3,S4,S5,S6,S7 respectively. 100 µl of plasma sample followed by 10 µl of isoamyl-alcohol was added to each standard tube. Fresh working solution of 1.43 M sodium borohydride was prepared in 0.1 M NaOH. Each tube was vortexed, added with 35 µl of 1M HCL and vortexed again. Working solution of 7 mM Monobromobimane (MBB) was prepared. 50 µl of 7mM MBB was added to each tube. All tubes were vortexed and minimal exposure to light was maintained after addition of MBB. Incubation was done at 42ºC for 12 minutes. Reaction was allowed to cool at RT for 24 minutes. 50 µl of ice cold 1.5M HCLO4 was added to precipitate the plasma proteins. Tubes were vortexed and incubated at RT for minimum 10 minutes. 100 µl of supernatant was
transferred to the insert already added with 6 µl of 2 M Trizma base to neutralize the acidic pH.

Fluorescent detector was used with excitation wavelength of 390 nm and emission wavelength 475 nm. Two mobile phase were used: Buffer A (for 1 litre buffer 50 ml HPLC grade methanol and 8.6 ml glacial acetic acid and rest volume of MQ were mixed and degassed) and Buffer B (absolute methanol). A gradient programme for binary solvent (buffer A and buffer B) was run at flow rate of 1ml/minute. Peaks were identified for homocysteine, cysteine and glutathione. Concentrations of unknown samples were calculated from standard curve.

3.4. Data Analysis

After performing HPLC peak area was calculated using LcSolution software and standard curve was prepared. Data was analyzed by the formula derived by standard curve.

4. Results

Standard curve was prepared using the following data tabulated in Table 1. Formula derived from this standard curve is $y = 33282x + 3000000$ with $R^2$ value of 0.998 [Figure 3]. Plasma Hcy concentration (x) of cases and controls were determined using this formula.

The average plasma Hcy concentration is 9.909 µmol/L ranging from 1.527 to 28.665 in cases as compared to 8.244 µmol/L ranging from 6.09 to 11.07 in controls [Figure 4]. The average concentration in both cases and controls lies within range (i.e., <15 µmol/L). Thus, there is no association found between elevated plasma Hcy level and PD (p-value=0.007) in our studied patient cohort treated with L-DOPA.

5. Discussion

Neurodegeneration is characterized by the death of specific group of neurons of the nervous system affecting its function. In an in vivo study using PD model developed by specific dopaminergic neurotoxin 1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine (MPTP), revealed that the proportion of murine midbrain dopaminergic neuro-degeneration was higher when plasma Hcy levels were elevated [23]. This demonstrates that Hcy is not a dopaminergic neurotoxin itself but enhances the neurotoxic effects of neurotoxins like MPTP. Thus Hcy potentiates the neurotoxic effects of other factors leading to the development of parkinsonian symptoms. Evidence for a central role for the NMDA receptor for causing neuronal death triggered by Hcy also arises from the finding that undifferentiated HT22 cells (a murine hippocampal neuronal cell line) are resistant to Hcy, where as differentiated HT22 cells are not. The susceptibility to Hcy arises with the onset of NMDA receptor expression [24]. Hcy has been shown to induce DNA hypomethylation by lowering the SAM: SAH ratio. When Hcy levels rise, SAM levels decrease and SAH levels increase. This induces DNA damage, cell death, and altera-
tions in gene expression [25]. Hcy also causes mitochondrial dysfunction. Homocysteic acid, a metabolite of Hcy, induces deficiencies in mitochondrial complex I which is strongly linked to PD, gives evidence that Hcy can modulate pathways that lead to Parkinson’s pathology.

The elevated level of Hcy is associated with the use of L-DOPA [Figure 5]. So L-DOPA therapy and the duration of its administration are important. Some studies (e.g. [26]) suggest the toxic effect of L-dopa on neuronal cell in vitro and that of in vivo studies in animal models are contradictory. This is because less than 5% of an oral dose of L-dopa is delivered to the brain, the remaining plasma levels of the drug undergo peripheral oxidative metabolism and may generate ROS. From the previous studies it has been found that elevated concentrations of plasma Hcy are common among PD patients and are associated with the use of L-DOPA [27]. However, in our studied patient cohort we did not find such association even though they are chronically treated with L-DOPA for variable time duration. This divergence may have been related to the influence of dietary Vit-B on Hcy concentrations. Some studies regarding this have revealed that higher dietary intake of vitamin B<sub>6</sub>, but not folate or B<sub>12</sub>, was associated with a decreased risk of developing PD [28,29]. Vitamin B<sub>6</sub> is known to act as a co-factor in the Hcy metabolism pathway. But there is no correlation between dietary folate or B<sub>12</sub> intake and PD, which suggests vitamin B<sub>6</sub>-mediated protection against PD is not directly related to Hcy levels. There are reports showing the association of polymorphisms in key genes that mediate folate metabolism, including MHTFR, with the risk of developing PD [30]. On the other hand there are reports suggesting that the MTHFR genotype and vitamin status have minor contributory effects to Hcy levels [31,32,33].

6. Conclusion

From the previous studies it is evident that elevated level of Hcy has a number of deleterious neurotoxic effects including neuro inflammation, DNA hypomethylation, oxidative stress, protein aggregation etc. those have significance for PD. However, it still remains a matter for debate, whether Hcy is a risk factor for PD development. It is known that in PD patients, treated with L-DOPA causes hyperhomocysteinemia though in our study we are unable to find such correlation. The cause behind it may be the effect of vitamin B<sub>12</sub> from dietary source or due to its oral supplement given by physicians while patients are chronically treated with L-DOPA. However, the correlation between elevated Hcy levels with L-DOPA naïve PD patients remains to be explored in this north-Indian patient cohort to better understand the disease mechanism.
7. Figures

Figure 1: Chemical structure of Homocysteine, Cysteine and Methionine

Figure 2: Separation of compounds in HPLC based on their retention time in stationary phase

Figure 3: Standard curve derived from the values of Table 1.

Figure 4: Box plot showing the distribution of data (plasma Hcy conc.) in PD cases and controls. Median value is 6.871 µmol/L in control and 8.693 µmol/L in Patients

Figure 5: Schematic representation of metabolism of Homocysteine in relation to L-DOPA intake. 1. COMT (catechol-O-methyl-transferase), 2. MTHFR (Methylene THF Reductase), 3. Cystathionine β-Synthase, 4. Y-Cystathionase
8. References


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Table 1: Peak area observed at seven standard concentration points of homocysteine


