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Chronic exposure of homocysteine in mice contributes to dopamine loss by enhancing oxidative stress in nigrostriatum and produces behavioral phenotypes of Parkinson's disease

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ABSTRACT

Increased homocysteine (Hcy) level has been implicated as an independent risk factor for various neurological disorders, including Parkinson's disease (PD). Hcy has been reported to cause dopaminergic neuronal loss in rodents and causes the behavioral abnormalities. This study is an attempt to investigate molecular mechanisms underlying Hcy-induced dopaminergic neurotoxicity after its chronic systemic administration. Male Swiss albino mice were injected with different doses of Hcy (100 and 250 mg/kg; intraperitoneal) for 60 days. Animals subjected to higher doses of Hcy, but not the lower dose, produces motor behavioral abnormalities with significant dopamine depletion in the striatum. Significant inhibition of mitochondrial complex-I activity in nigra with enhanced activity of antioxidant enzymes in the nigrostriatum have highlighted the involvement of Hcy-induced oxidative stress. While, chronic exposure to Hcy neither significantly alters the nigrostriatal glutathione level nor it causes any visible change in tyrosine hydroxylase-immunoreactivity of dopaminergic neurons. The finding set us to hypothesize that the mild oxidative stress due to prolonged Hcy exposure to mice is conducive to striatal dopamine depletion leading to behavioral abnormalities similar to that observed in PD.

1. Introduction

Homocysteine (Hcy) - a non-proteogenic sulphur containing amino acid, is known to increase in plasma of Parkinson's disease (PD) patients under treatment with the gold standard drug, L-DOPA (L-3,4-dihydroxyphenylalanine) [1–4]. The methylation of L-DOPA by catechol-O-methyl transferase is the leading cause of elevated level of plasma Hcy in PD patients [5,6]. Several *in vitro* studies have highlighted the neurotoxic potency of Hcy to various neuronal types, including dopaminergic neurons [7–10]. The toxic potency of Hcy to dopaminergic neurons is also reported from animal models of PD [8,10–12]. Intraperitoneal injection of Hcy at a high dose (500 mg/kg) for a long time (36 days) caused a reduction of tyrosine hydroxylase (TH)-positive neurons, while dopamine and its metabolite remained unchanged [13]. Chandra et al. [11] have shown for the first time the direct effect of Hcy on dopaminergic neurons by intranigral administration of Hcy. Unilateral, intranigral injection of Hcy caused a significant decrease in striatal dopamine levels and loss of striatal dopaminergic neurons [11]. However, the underlying mechanism of Hcy-induced neurotoxicity to nigrostriatal dopaminergic neurons remains unexplained. However, Hcy-induces excitotoxicity, oxidative stress and mitochondrial alterations in cellular neuronal model leading to apoptosis and neuronal cell death [14–16]. Also, Hcy itself can serve as a pro-oxidant that contributes to oxidative stress in neuronal cells [17]. Hoffman [18] suggested that Hcy is a putative marker of cellular oxidant status.

Although the exact molecular mechanism of death of midbrain dopamine containing neurons in PD is not known clearly, however, oxidative stress has been postulated as the foremost event in PD pathogenesis [19]. Human postmortem studies indicated down regulation of antioxidant protective mechanisms in PD brain [20,21]. The present study examined the involvement of oxidative stress mechanisms in Hcy-induced dopaminergic neurotoxicity in mice. The effect of prolonged (60 days) systemic administration of Hcy on motor behavior, striatal dopamine levels and nigrostriatal enzymatic (SOD, superoxide dismutase and catalase) as well as non-enzymatic (reduced glutathione) oxidative stress parameters were investigated in mice. We also investigated the status of

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nigrostriatal mitochondrial complex-I after chronic Hcy treatment in mice. The findings may have important implications for understanding the pathogenesis of PD.

2. Materials and methods

2.1. Animals

Eight-weeks-old male Swiss albino mice (23–26 g) were used in the present study. The animals were maintained under standard conditions of 12 h light/dark cycles, 24 ± 2 °C temperatures and 60 ± 5% humidity. They were provided with food and water *ad libitum*. The experimental protocols met the National Guidelines and were approved by the Animal Ethics Committee of the University.

2.2. Materials

D,L-homocysteine (Sigma-Aldrich Cat# H4628), reduced glutathione (GSH), hydrogen peroxide (H_2O_2), ortho-phthalaldehyde, phosphoric acid (H_3PO_4), Triton X-100, bovine serum albumin (BSA), and ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Rabbit anti-tyrosine hydroxylase (TH) primary antibody and *anti*-rabbit goat secondary antibody were purchased from Millipore Co. (USA). Pyrogallol and other reagents were procured from Sisco Research Laboratories Pvt. Ltd. Maharashtra, India.

2.3. Experimental design

Two groups of mice containing 6 animals were treated with Hcy (100 or 250 mg/kg daily, i.p.) dissolved in vehicle (0.9% Sodium Chloride) daily for 60 days and another group received the vehicle. The volume of Hcy and vehicle injected as per body weight of the animals. In the lower dose group, Hcy was given once a day at a dose 100 mg/kg (74 mM). In the highest dose group, Hcy was given twice a day at a dose 125 mg/kg (92 mM), so cumulative dose was 250 mg/kg per day. Motor behavioral tests (akinesia and catalepsy) were conducted on 14th, 28th, 42nd and 56th day and swim test on the 56th day of daily Hcy or vehicle administration. After the last dose of Hcy or vehicle administration, animals were sacrificed for analysis of striatal dopamine, oxidative stress parameters and mitochondrial complex activity from substantia nigra (SN) and striatum (NCP). Animals that received Hcy alone or vehicle for 60 days were perfused for TH-immunohistochemistry of SN and NCP. Each assay was repeated at least twice on separate days.

2.4. Behavioral test

2.4.1. Akinesia

Akinesia was measured by noting the latency of animals in seconds (s) to move all the fore and hind limbs on an elevated wooden platform (40 cm × 40 cm × 30 cm). The test terminated if the animal remains latent upto 180 s [22].

2.4.2. Catalepsy

Catalepsy can be defined as the inability of an animal to correct an externally imposed posture. Animals were placed on a flat horizontal surface with both hind limbs placed on a 3 cm high square wooden block and the time period (in seconds) that the animal took to move from the block to horizontal surface was noted down [22].

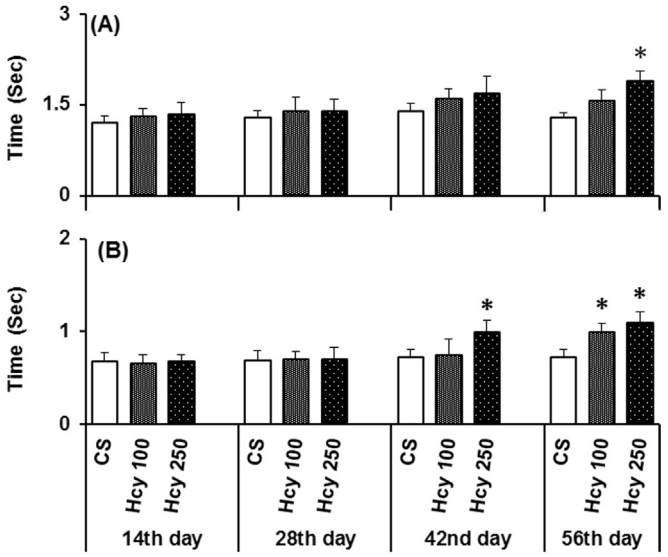

Fig. 1. Effect of chronic Hcy administration on (A) Akinesia and (B) Catalepsy. Akinesia and catalepsy were measured after daily administration of Hcy (100 mg/kg or 250 mg/kg) or vehicle on 14th, 28th, 42nd and 56th day. The effect of Hcy administration on the latency to move all four limbs (akinesia) as compared to the control group were examined (measured in sec). The effects of Hcy administration to correct an externally imposed posture (catalepsy) as compared to the control group were examined (measured in sec). The data represented are mean ± SEM. *P ≤ 0.05 as compared to control (n = 6).

2.4.3. Swim test

Swimming ability test [23] was carried out in tubs with 40 cm length, 25 cm width and 16 cm height. The level of water was maintained upto 12 cm at 27–28 °C. Animals were placed in water and the swimming ability was scored every min for a period of 10 min. The scoring scale is as follows: 3-continuous swimming, 2-swimming with occasional floating, 1-more floating with occasional swimming with hind limbs and 0-hind part sinks with only the head floating. Total swim score is the sum of scores obtained in each minute for the entire test period of 10 min.

2.5. Dopamine analysis

Striatum was dissected out from Hcy or vehicle treated animals after 60 daily injections and fresh 50 µl (1 µg/µl) striatal lysates (in STEN buffer) were detected with 50 µl primary antibody (1 h) and 100 µl anti-rabbit secondary antibody (30 min) at room temperature according to the manufacturer's protocols (Abnova, Taiwan) for dopamine [24].

2.6. Estimation of brain Hcy levels

For brain Hcy estimation, the animals were killed at 2 h after the last dose of Hcy or vehicle on 60th day of treatment. The right and left NCP were dissected out, whereas the right and left SN were micropunched from 1 mm frozen brain sections. The right and left hemisphere were processed together for Hcy estimation. The tissues were weighed and sonicated in PBS and stored overnight. After two freeze–thaw cycles, the homogenates were centrifuged at 5000 g for 5 min and the supernatant was analyzed for Hcy content. The total Hcy content was analyzed in brain homogenates using the Hcy ELISA kit (KA1242; Abnova, Taiwan) as per the manufacturer's protocol.

2.7. Mitochondrial complex-I activity

Complex-I activity was assayed as described earlier [25]. Mice were sacrificed after the last dose of Hcy or vehicle and complex-I