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Hypercholesterolemia causes psychomotor abnormalities in mice and alterations in cortico-striatal biogenic amine neurotransmitters: Relevance to Parkinson's disease

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ABSTRACT

The symptoms of Parkinson's disease (PD) include motor behavioral abnormalities, which appear as a result of the extensive loss of the striatal biogenic amine, dopamine. Various endogenous molecules, including cholesterol, have been put forward as putative contributors in the pathogenesis of PD. Earlier reports have provided a strong link between the elevated level of plasma cholesterol (hypercholesterolemia) and onset of PD. However, the role of hypercholesterolemia on brain functions in terms of neurotransmitter metabolism and associated behavioral manifestations remain elusive. We tested in Swiss albino mice whether hypercholesterolemia induced by high-cholesterol diet would affect dopamine and serotonin metabolism in discrete brain regions that would precipitate in psychomotor behavioral manifestations. High-cholesterol diet for 12 weeks caused a significant increase in blood total cholesterol level, which validated the model as hypercholesterolemic. Tests for akinesia, catalepsy, swimming ability and gait pattern (increased stride length) have revealed that hypercholesterolemic mice develop motor behavioral abnormalities, which are similar to the behavioral phenotypes of PD. Moreover, hypercholesterolemia caused depressive-like behavior in mice, as indicated by the increased immobility time in the forced swim test. We found a significant depletion of dopamine in striatum and serotonin in cortex of hypercholesterolemic mice. The significant decrease in tyrosine hydroxylase immunoreactivity in striatum supports the observed depleted level dopamine in striatum, which is relevant to the pathophysiology of PD. In conclusion, hypercholesterolemia-induced depleted levels of cortical and striatal biogenic amines reported hereby are similar to the PD pathology, which might be associated with the observed psychomotor behavioral abnormalities.

1. Introduction

The altered cholesterol metabolism with elevated level of plasma cholesterol (hypercholesterolemia) have been strongly associated with the pathophysiology of Alzheimer's disease as it not only causes cognitive impairment (Thirumangalakudi et al., 2008; Ullrich et al., 2010; Wood et al., 2014) but also influences the formation of the hallmark protein of the disease, the amyloid-β (Refolo et al., 2000; Thirumangalakudi et al., 2008; Xue-Shan et al., 2016). Studies of the last decade have provided a positive correlation between dietary factors, including cholesterol, and the occurrence of Parkinson's disease (PD) (Johnson et al., 1999; Hu et al., 2006, 2008; Miyake et al., 2010), however, some studies offered a contradictory inference (Huang et al., 2015; Tan et al., 2016). We have recently reviewed all the neuropathological implications

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caused by elevated cholesterol in the light of its putative contributions to the onset of PD pathology (Paul et al., 2015). *In vitro* studies have shown that excess cholesterol or its oxidation product (oxysterol) influences the aggregation of α-synuclein protein (Bar-On et al., 2008; Rantham Prabhakara et al., 2008; Marwarha et al., 2011), the hallmark pathology of PD (Di Maio et al., 2016). Rodents subjected to high-fat diet have been demonstrated to contribute towards the loss of striatal dopamine and tyrosine hydroxylase level in rodent models of PD (Choi et al., 2005; Bousquet et al., 2012). Moreover, treatment with cholesterol-lowering drugs (statins) has been reported to ameliorate the motor symptoms of PD as well as decrease the aggregation of α-synuclein protein (Bar-On et al., 2008; Roy and Pahan, 2011; Undela et al., 2013). In rodent models of hypercholesterolemia, the activity of mitochondrial complexes and antioxidant enzymes, as well as levels of antioxidant molecule have been reported to be reduced in non-dopaminergic regions of brain, such as cortex and hippocampus, which are indicative of oxidative stress (de Oliveira et al., 2011, 2013; Prasanthi et al., 2010; Otunola et al., 2014). However, the potential evidence that links cholesterol to brain functions, in terms of neurotransmitter metabolism and associated behavioral abnormalities, remains elusive. The present study investigated the effect of hypercholesterolemia on psychomotor behavior and neurochemical status in dopamine-rich region (striatum) and other regions of the brain of mice.

2. Materials and methods

2.1. Animals

Eight weeks old male Swiss albino mice (21–22 g) used in the present study were purchased from Pasteur Institute, Shillong, Meghalaya, India. The mice were housed under standard laboratory conditions of temperature (24 ± 2 °C) and humidity (60 ± 5%) and were provided with food and water *ad libitum*. An acclimatization time of 5 days was given prior to start of the experiment. The experimental protocols were in accordance with the National guidelines and the Institutional Animal Ethics Committee guidelines.

2.2. Chemicals and consumables

Cholesterol, acetonitrile, Cresyl violet, and paraformaldehyde were obtained from SISCO Research Laboratories (Mumbai, India). Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), norepinephrine hydrochloride, ethylenediaminetetraacetic acid disodium salt (EDTA), heptane sulfonic acid, triethylamine, orthophosphoric acid, chloral hydrate, hydrogen peroxide (H_2O_2), poly-L-lysine, Triton X-100 and 3,3-diaminobenzidine (DAB) liquid substrate system (D3939) kit were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Primary antibodies such as rabbit polyclonal anti-tyrosine hydroxylase (TH; ab112) and rabbit polyclonal anti-Glial fibrillary acidic protein (GFAP; ab7260) were purchased from Abcam (Cambridge, UK). Anti-rabbit goat secondary antibody tagged with horseradish peroxidase (HRP; ap307p) was purchased from Millipore Co. (USA). Plasma cholesterol estimation kit (CHOL, Autopak) was obtained from Siemens Ltd. (India).

2.3. Experimental design

Swiss albino mice used in the study were randomly divided into two experimental groups: Control group (CS) fed with standard diet (normal rodent chow), and the high-cholesterol diet group (HCD) fed with standard diet mixed with 5% cholesterol (Refolo et al., 2000) for 12 weeks (84 days). Body weight was monitored after every 2 weeks. Motor behavioral tests, such as akinesia and catalepsy were performed in these animals in an interval of two weeks from the start of treatment till 12th week (0^{th}, 14^{th}, 28^{th}, 42^{nd}, 56^{th}, 70^{th} and 84^{th} day). The behavioral parameters, such as elevated plus maze, forced swim test, gait, and swim test were performed on 80^{th}, 81^{st}, 82^{nd} and 83^{rd} day respectively. Scoring of the behavioral tests was performed by trained experimenters who were blind to the treatment paradigm. Serum total cholesterol level was analyzed after the 12^{th} week (84^{th} day). The animals were sacrificed and/or perfused (4% paraformaldehyde) after 12 weeks of treatment (84^{th} day) for analysis of neurotransmitters and immunoreactivity study from discrete brain regions.

2.4. Analysis of behavioral parameters

2.4.1. Akinesia

The latency in moving all the four limbs were tested for 180 s (Bhattacharjee et al., 2016a). The animals were placed on a wooden platform (40 cm × 40 cm × 30 cm) for 5 min, and then the latency was recorded.

2.4.2. Catalepsy

Catalepsy is the inability of an animal to correct an externally imposed posture (Bhattacharjee et al., 2016a). Mice were placed on a flat surface with both hind limbs placed on a wooden block of 3 cm height. The time taken by the animals in moving both hind limbs to the flat surface was counted.

2.4.3. Swim test

Swimming ability test was carried out in tubs with 12 cm high water, maintained at 27 ± 2° C. Animals were placed in water and the swimming ability for a period of 10 min was scored every min as: 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 (Haobam et al., 2005).

2.4.4. Walk test

To determine the gait abnormalities of mice, we performed footprint analyses as described earlier (Klapdor et al., 1997). Mice were acclimatized to walk on an inclining gangway lined with a white sheet leading to a dark chamber/platform. The fore- and hind-paws of the animals were coloured respectively with red and green non-toxic colour to get the foot impression. The footprints were analyzed for stride length, stride width, and footprint length, manually.

2.4.5. Forced swim test

Forced Swim test (FST) was carried out according to Porsolt et al. (1997) with slight modifications. The animals were individually forced to swim in a transparent glass vessel containing 12 cm high water, maintained at 27 ± 2 °C, for a period of 6 min. The duration of immobility or floating occurring during the last 4 min was counted. A mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only movements necessary to keep its head above water.

2.4.6. Elevated plus maze test

This test is the most popular test of anxiety, and was carried out according to Walf and Frye (2007) with slight modification. The apparatus is a plus-shaped maze, with two open and two closed arms opposite to each other interconnected by a central platform. Animal was placed on the central platform, facing the open arm, and allowed to explore the plus maze for 5 min. Time spent in the

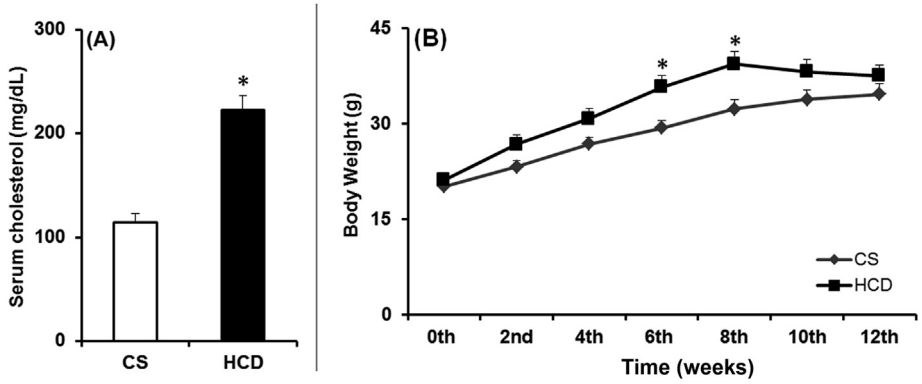

Fig. 1. Effect of high-cholesterol diet on (A) serum total cholesterol level and (B) body weight of mice. Mice were fed with high-cholesterol diet (HCD) or standard diet (control, CS) for 12 weeks. On the last day of the diet, blood was collected by the cardio-punctured method from both groups of mice and serum total cholesterol was estimated for validating the hypercholesterolemic model. Body weight (in gram) was measured two weeks apart from the start of treatment till 12 weeks. The results given are Mean ± S.E.M. *$p \leq 0.05$ as compared to CS (n = 8/experiment). Serum total cholesterol data was analyzed using an unpaired Student's t-test (two-tailed) and body weight data was analyzed using a two-way repeated measures ANOVA.

open arms and percentage of entries in the open arms with respect to the total number of arm entries is used as experimental indices of anxiety.

2.5. Estimation of total cholesterol

Mice were anesthetized with chloral hydrate (350 mg/kg; i.p.) on the last day of 12 weeks of the diet and blood was collected by cardiac puncture. The serum was separated by centrifugation at 3000×*g* for 4 min. The total serum cholesterol was estimated following the enzymatic method using a colorimetric kit (CHOL, Autopak, Siemens). Briefly, the serum was incubated with equal volume of buffer (Pipes buffer, pH 6.95 containing phenol and sodium cholate) and enzymes mixture (containing cholesterol esterase, cholesterol oxidase, peroxidase and 4-aminoantipyrine) for 5 min at 37 °C. The concentration of cholesterol in serum is directly proportional to the intensity of the red complex generated, which was measured at 500 nm using Microplate reader equipped with Spectrophotometer (Multiskan™, ThermoFisher Scientific, Finland) (Allain et al., 1974).

2.6. HPLC analysis of biogenic amines

To investigate the effect of hypercholesterolemia on brain biogenic amines (neurotransmitter) and their metabolites level, mice were sacrificed by decapitation on the last day of 12 weeks of feeding. The whole brain was removed from the calvarium and the cortex, nucleus caudatus putamen (NCP) and hippocampus regions were dissected out. The tissues were sonicated in ice-cold 0.1 M perchloric acid containing 0.01% EDTA, and were centrifuged for 5 min at 10,000×*g*. 10 μl supernatant was injected into the HPLC-ECD system at a flow rate 0.7 ml/min. The electrochemical detection was performed at +740 mV. The composition of the mobile phase was 8.65 mM heptane sulfonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.43% triethylamine and 0.22% orthophosphoric acid, as reported earlier (Borah and Mohanakumar, 2007).

2.7. Preparation of brain tissue for brain histology and immunohistochemical studies

The mice were deeply anesthetized with chloral hydrate (350 mg/kg b.w.; i.p.) on the last day of 12 weeks of diet and thoracotomy was performed immediately for perfusion. A 23-gauge needle attached to 50 ml syringe was cannulated in the left ventricle of heart and perfused with 50 ml of ice-cold phosphate-buffered saline (PBS; 0.1 M, pH 7.4) for a vascular rinse followed by 30 ml of 4% (w/v) paraformaldehyde (in PBS) for fixation. Brains were dissected out from calvarium, kept overnight in the same fixative and cryoprotected in 30% (w/v) sucrose solution till the brain sinks in the solution. Twenty micron thick coronal sections of brain passing through the cortex, striatum, hippocampus and substantia nigra were made using Cryostat (0620E, Thermo Shandon, UK). Sections were collected on poly-L-lysine coated slides for Nissl staining, and in well-plates for immunohistochemical studies of TH and GFAP (Mazumder et al., 2016; Bhattacharjee et al., 2016b).

2.8. Brain histology using Nissl stain

Histological analysis of different brain regions was performed by Nissl staining following Mazumder et al. (2016). Briefly, sections of brain regions viz. cortex, striatum, hippocampus and substantia nigra were hydrated in decreasing alcohol gradient (absolute, 90%, 70%, 50%, distilled water) for 1 min each followed by incubation in 0.5% cresyl violet for 3 min. The sections were washed in distilled water, dehydrated in increasing alcohol gradient (50%, 70%, 90%, absolute) for 1 min each, cleared in xylene, mounted in DPX and photographed using digital SLR camera attached with Trinocular Microscope (ECLIPSE Ci-L, Nikon, Japan).

2.9. Immunohistochemical analysis

Coronal sections of striatum and substantia nigra regions of brain were washed with 0.1 M Tris-buffered saline (TBS; 0.1 M, pH 7.4) three times for 5 min each. The endogenous peroxidase activity was blocked by incubating the sections with 3% (v/v) H_2O_2 (in TBS) for 5 min followed by washing in TBS to remove the bubbles generated due to H_2O_2 treatment. Blocking was done by incubating the sections with TBS containing 10% donkey serum and 0.3% Triton X-100 for 1 h at room temperature over the dancing shaker. Sections were incubated with polyclonal rabbit anti-TH or rabbit anti-GFAP primary antibody (1:500 dilution) in TBS containing 2% donkey serum and 0.3% Triton X-100 for overnight at 4 °C. The sections were washed with TBS and were incubated with HRP-conjugated anti-rabbit secondary antibody (1:700 for TH and 1:1000 for GFAP) in TBS containing 2% donkey serum and 0.3% Triton X-100 for 1 h at room temperature over the shaker. Colour development was performed by incubating the sections in DAB-liquid substrate solution for 3 min and then sections were

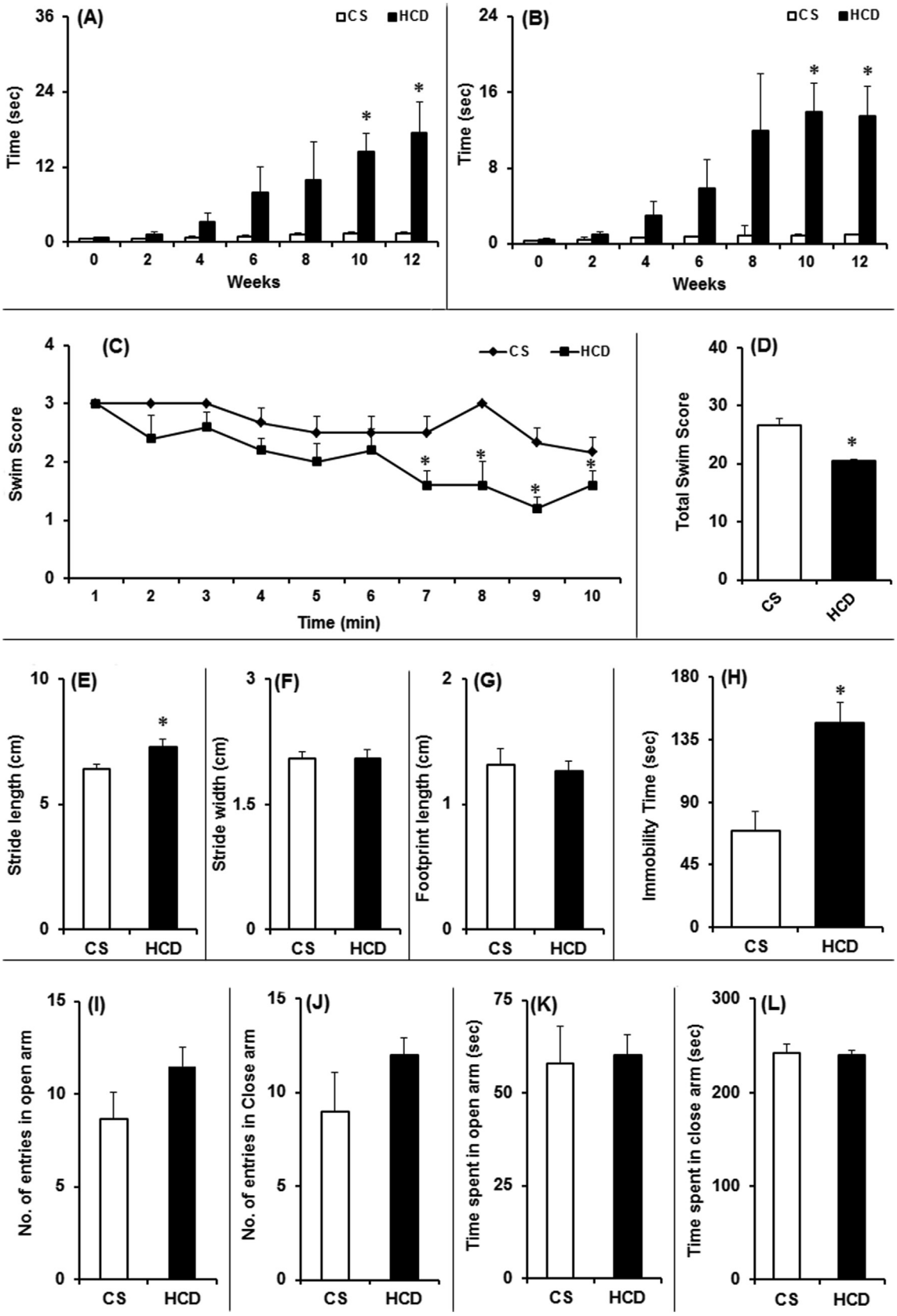

Fig. 2. Effect of hypercholesterolemia on (A–G) motor and (H–L) psychometric behavior. (A) Akinesia and (B) Catalepsy was measured at every two weeks till 12 weeks. High-cholesterol diet (HCD) fed animals showed akinetic and cataleptic behavior from 10th week onwards. (C) Swim-scores were recorded on a performance intensity scale of 0–3 for 10 min. (D) The total swim score in HCD mice was significantly reduced than the control (CS). (E–G) Gate pattern showed a significant increase in stride length in HCD mice. (H) The immobility time in seconds was recorded in forced swim test. Significant increase in the total immobility time in HCD mice demonstrates depressive-like behavior in the animal. (I–L) Exploratory activity in elevated plus maze demonstrated no significant anxiety-like behavior in HCD mice. The results are given as Mean ± S.E.M. *$p \leq 0.05$ as compared to CS (n = 6/group in each experiment). An unpaired student's t-test (two-tailed) was used to calculate P-values. Data of motor behavioral tests (2A-C) were analyzed using a two-way repeated measures ANOVA and remaining data (2D-L) were analyzed using unpaired Student's t-test (two-tailed).

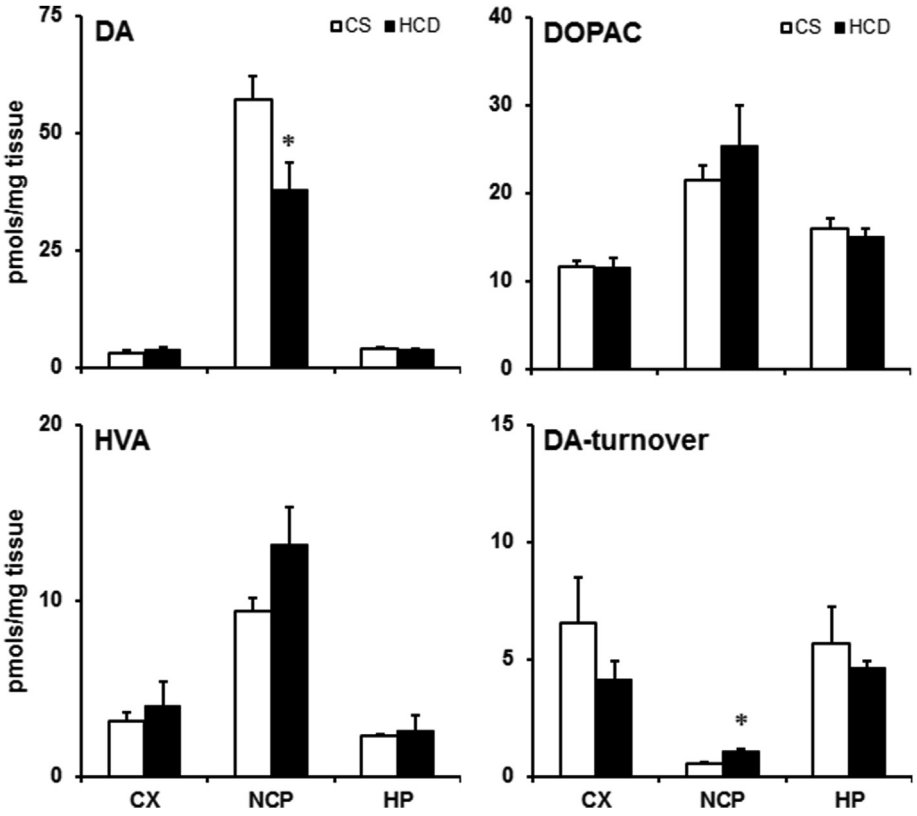

Fig. 3. Effect of hypercholesterolemia on brain dopamine (DA) and its metabolites level. Mice were fed with standard diet (control, CS) or high-cholesterol diet (HCD) was sacrificed after 12 weeks of diet. DA and its metabolites (3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were analyzed from cortex, striatum (NCP) and hippocampus (HP) regions of brain by using HPLC-ECD system. The results are given as Mean ± S.E.M. *p ≤ 0.05 as compared to CS (n = 6). P-values were calculated using a two-way repeated measures ANOVA.

washed, dehydrated in increasing grades of alcohol (30%, 50%, 70%, 90% and absolute alcohol; 30 s each), cleared in xylene, mounted in DPX and photographed using SLR camera attached to the Microscope. The striatal TH-immunoreactive sections were subjected to densiometric analysis to calculate optical density by using Fiji version of ImageJ software (Schindelin et al., 2012). Optical density was calculated from serial coronal sections of each group of mice (n = 5). Optical density is the logarithm of maximum intensity by mean intensity, where theoretical maximum intensity is 255. TH-positive nigral dopaminergic neurons were counted using ImageJ (Fiji version) software (Tripathy et al., 2014). The first section was chosen randomly and thereafter every sixth section was selected through the entire substantia nigra from control and hypercholesterolemic mice (n = 5; Tapias et al., 2010).

2.10. Statistical analysis

Statistical analysis was performed using the software GraphPad Prism version 7.0 for Windows (Graph Pad software, San Diego California USA, www.graphpad.com). The data, except, body weight, motor behaviors and neurochemical results, were analyzed employing an unpaired Student's *t*-test (two-tailed). The results of neurotransmitter levels were analyzed using a two-way, repeated measures ANOVA with factors 'intervention' (normal diet and HCD) and 'brain regions' (cortex, striatum, and hippocampus) was used to investigate the effect of 'intervention' on the level of neurotransmitter in different 'brain regions'. The results are presented as Mean ± S.E.M. P-value of 0.05 or less was considered to be significant.

3. Results

3.1. Total cholesterol assay

The hypercholesterolemic mouse model was validated by analyzing the serum level of total cholesterol. Mice that were subjected to high-cholesterol diet for 12 weeks had significantly elevated levels of serum total cholesterol by 1.94-fold ($P = 0.001$, $df = 14$, $F = 2.8$) compared to standard diet fed control mice (222.75 ± 13.86 mg/dL *vs.* 114.63 ± 8.27 mg/dL) (Fig. 1A). The elevated level of cholesterol in blood thus validates the animals as hypercholesterolemic (Refolo et al., 2000).

3.2. Effect of high-cholesterol diet on body weight

High-cholesterol diet caused a gradual increase in the body weight of mice, which was however not consistent throughout the treatment period. The body weight was increased significantly by 1.35-fold ($P = 0.001$, $df = 14$, $F = 2.09$) on 6th week and 1.31-fold ($P = 0.001$, $df = 14$, $F = 1.46$) on 8th weeks in high-cholesterol diet fed group compared to the control (normal diet). While from 8th week onwards till 12th week, there occurred no significant (at $p \leq 0.05$, n = 8) alterations in the body weight of mice receiving cholesterol-supplemented diet. On 10th and 12th week, body weight was increased respectively by 1.1-fold ($P = 0.0724$, $df = 14$, $F = 1.82$) and 1.0-fold ($P = 0.2512$, $df = 14$, $F = 1.46$) in high-cholesterol diet fed group compared to the control. Thus, high-cholesterol diet in mice did not produce the symptoms of obesity with respect to body weight. The body weight of the mice that were maintained on standard diet increased gradually after 4 weeks till

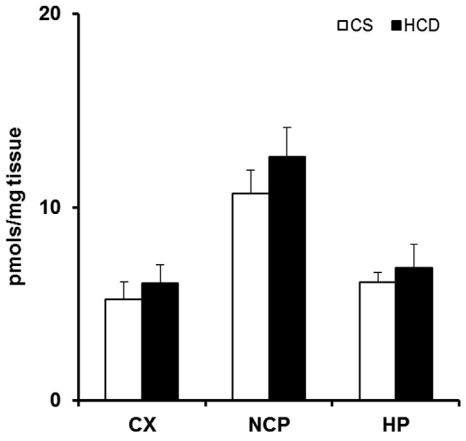

Fig. 4. Effect of hypercholesterolemia on norepinephrine level in brain. Mice, fed with standard diet (control, CS) or high-cholesterol diet (HCD), were sacrificed after 12 weeks. Norepinephrine level was analyzed from the cortex (CX), striatum (NCP) and hippocampus (HP) regions of brain by using HPLC-ECD system. The results given are Mean ± S.E.M. *p ≤ 0.05 as compared to CS (n = 5).

12 weeks period (Fig. 1B).

3.3. Hypercholesterolemia on akinesia and catalepsy

After successive two weeks interval, akinesia and catalepsy were tested up to 12 weeks. The mice fed with high-cholesterol diet were found to be akinetic and cataleptic from the 10th week of feeding. In hypercholesterolemic animals, the akinesia scores on 10th ($P = 0.0018$, $df = 10$, $F = 11.18$) and 12th ($P = 0.0105$, $df = 10$, $F = 58.42$) week were 14.43 ± 3.02 s and 17.4 ± 5.07 s, while in control animals the scores were found to be 1.36 ± 0.29 s and 1.45 ± 0.22 s respectively (Fig. 2A). In hypercholesterolemic animals compared to control, the catalepsy scores on 10th ($P = 0.0015$, $df = 10$, $F = 13.38$) and 12th ($P = 0.0022$, $df = 10$, $F = 55.7$) week were 14.06 ± 3.05 *vs.* 0.92 ± 0.14 s and 13.5 ± 3.2 *vs.* 0.96 ± 0.12 s respectively (Fig. 2B). However, at other time points the akinesia and catalepsy scores were not differed significantly ($p \leq 0.05$, $n = 6$) in hypercholesterolemic mice compared to the control.

3.4. Hypercholesterolemia on swimming performance

Swim test was performed to assess the effect of hypercholesterolemia on motor performance of animals (Haobam et al., 2005). Animals receiving high-cholesterol diet displayed a significantly poorer swimming ability as compared to the control animals. The swim score at the 1st min was 3.00 for both the groups, whereas from 2nd to 10th min there was a progressive decrease in swimming score, being increased significantly from 7th to 10th min, in high-cholesterol diet fed mice compared to the control group. The swimming scores were decreased significantly by 36% ($P = 0.0239$, $df = 10$, $F = 1.0$), 47% ($P = 0.0039$, $df = 10$, $F = 2.4$), 49% ($P = 0.0079$, $df = 10$, $F = 1.33$) and 35% ($P = 0.0259$, $df = 10$, $F = 1.8$) on 7th, 8th, 9th and 10th min respectively in hypercholesterolemic mice (Fig. 2C). The total swim score was decreased significantly by 23.5% ($P = 0.005$, $df = 10$, $F = 3.01$) in hypercholesterolemic mice as compared to the control (Fig. 2D).

3.5. Hypercholesterolemia on gait

Walk test was performed to estimate the gait abnormalities induced by hypercholesterolemia, which is a measure of motor coordination (Klapdor et al., 1997). From footprints of animals, it was observed that stride length was significantly affected in animals receiving high-cholesterol diet. Stride length - the distance between two successive hind limbs, was significantly increased by 1.13-fold ($P = 0.033$, $df = 10$, $F = 3$) in mice receiving high-cholesterol diet (Fig. 2E). However, no significant changes were observed in other measures of gait, such as stride width (Fig. 2F) and footprint length (Fig. 2G).

3.6. Hypercholesterolemia on psychometric assessment

The immobility time in the FST demonstrates desperation in animals (Porsolt et al., 1997). After an initial acclimatization for 2 min, the total immobility time (in sec) was noted for a period of 4 min. A significant increase in the duration of immobility was observed in mice receiving high-cholesterol diet. The duration of immobility or floating time was increased by 2.12-fold ($P = 0.02$, $df = 10$, $F = 1.06$) in mice on high-cholesterol diet as compared to the control (Fig. 2H).

High-cholesterol diet did not result in significant signs of anxiety-like behavior in mice when assayed using elevated plus maze test (Fig. 2I–L). There occurred no significant changes in scores of different parameters of the test, such as number of entries in close or open arm and time spent in close or open arm in high-cholesterol diet fed animals compared to the control ($p \leq 0.05$, $n = 6$).

3.7. Effect of hypercholesterolemia on dopamine and its metabolites level

Dopamine and its metabolites levels were analyzed from the three regions of brain, including cortex, striatum (NCP) and hippocampus (Fig. 3). In striatum of hypercholesterolemic mice, dopamine level was reduced by 34%, which differed significantly ($P = 0.003$, $df = 10$, $F = 1.4$) from the control group. While in cortex dopamine level was increased by 27% and decreased by 2% in hippocampus, which were statistically insignificant compared to control animals. In all the brain regions, the level of metabolites of dopamine (DOPAC and HVA) did not change significantly in hypercholesterolemic mice. DOPAC level in hypercholesterolemic mice decreased by 1% and 6% in cortex and hippocampus respectively, while in striatum the level of DOPAC was increased by 18%. However, HVA levels in hypercholesterolemic mice was increased by 25%, 40%, and 13% respectively in the cortex, striatum, and hippocampus. The turnover of dopamine, calculated as the ratio of the metabolites to the neurotransmitter [(DOPAC + HVA)/DA] was found to be significantly increased in striatum by 90% ($P = 0.0001$, $df = 10$, $F = 3.07$), whereas decreased by 36% in cortex, and increased by 18% in hippocampus of hypercholesterolemic mice, which were however statistically insignificant compared to control mice ($p \leq 0.05$, $n = 6$).

3.8. Effect of hypercholesterolemia on norepinephrine level

There occurred no significant changes in the level of norepinephrine in discrete brain regions of hypercholesterolemic mice (Fig. 4). In hypercholesterolemic animals, norepinephrine level was increased by 1.15-, 1.17- and 1.12-fold in the cortex, striatum and hippocampus regions of brain respectively, which were however not significant as compared to the control ($p \leq 0.05$, $n = 5$).

3.9. Effect of hypercholesterolemia on serotonin and its metabolite level

In the cortical region of brain of hypercholesterolemic mice, the level of serotonin was decreased significantly, while the level of its

metabolite (5-HIAA) and turnover of serotonin increased significantly compared to the control mice. In the hypercholesterolemic group, serotonin level was decreased by 48% in cortex ($P = 0.01$, $df = 8$, $F = 7.59$) and increased by 12% and 11% in striatum and hippocampus respectively. The level of 5-HIAA increased significantly by 55% in cortex ($P = 0.03$, $df = 8$, $F = 4.13$), while decreased in striatum and hippocampus by 21% and 3% respectively in hypercholesterolemic mice. The turnover of serotonin in cortex increased significantly by 151% ($P = 0.004$, $df = 8$, $F = 7.04$), while decreased respectively by 39% and 23% in striatum and hippocampus, which were not significantly different as compared to the control (Fig. 5).

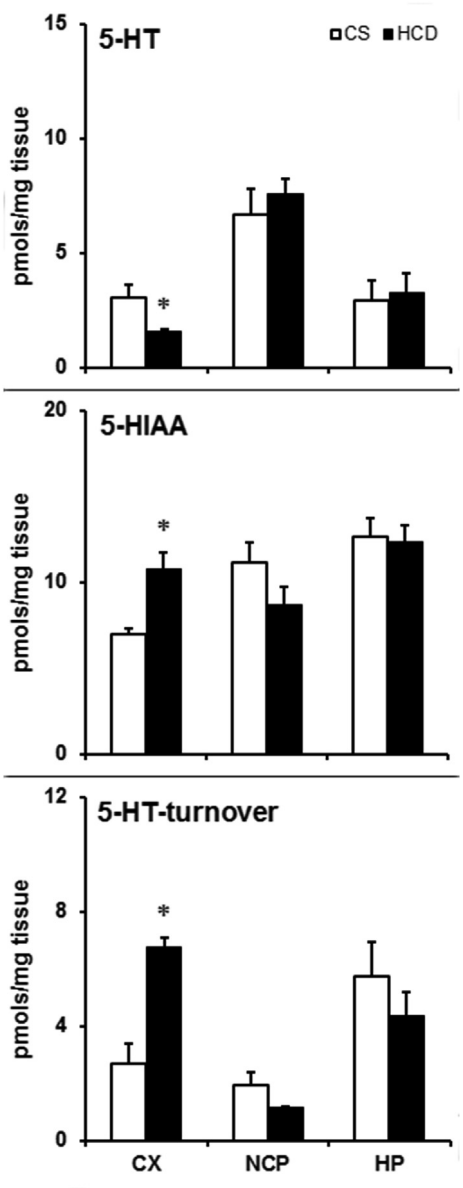

Fig. 5. Effect of hypercholesterolemia on brain serotonin (5-HT) and its metabolite level. Mice, fed with standard diet (control, CS) or high-cholesterol diet (HCD), were sacrificed after 12 weeks. 5-HT and its metabolite (5-hydroxyindoleacetic acid, 5-HIAA) levels were analyzed from the cortex, striatum (NCP) and hippocampus (HP) regions of brain by using HPLC-ECD system. The results given are Mean ± S.E.M. *p ≤ 0.05 as compared to CS (n = 5). A two-way repeated measures ANOVA was used to calculate P-values.

3.10. Effect of hypercholesterolemia on brain histology

Nissl staining was performed to see possible morphological aberrations in discrete brain regions: cortex, striatum, hippocampus and substantia nigra. The result revealed no visible morphological changes in the neurons of the studied brain regions in hypercholesterolemic animals as compared to control animals (Fig. 6).

3.11. Effect of hypercholesterolemia on dopaminergic neuronal integrity

There occurred a marked visible decrease in TH-immunoreactivity in the striatum region of brain of the animals subjected to high-cholesterol diet for 12 weeks (Fig. 7). The striatal TH-immunoreactivite staining intensity was found to be decreased significantly by 20% ($P = 0.001$, $df = 8$, $F = 1.23$) in hypercholesterolemic animals compared to the control (Fig. 7E). As the striatum region is rich in dopaminergic axon terminals (Wilson et al., 1996), the decrease in TH-immunoreactivity in striatum region signifies damages in the dopaminergic nerve terminals. However, high-cholesterol diet for 12 weeks did not cause a significant loss of TH-positive nigral dopaminergic neurons, which was found to be 8%, compared to the control animals (Fig. 7F).

3.12. Effect of hypercholesterolemia on GFAP-immunostaining

Glial fibrillary acidic protein (GFAP) is long being used as a marker for astrocyte activation and increased number of GFAP-positive cells signifies reactive astrogliosis (Pekny and Pekna, 2014), which is an indicative of inflammatory stress (Chakraborty et al., 2014). Marked increase in the number of GFAP-immunoreactive astrocytes was seen in striatum as well as in substantia nigra regions of brain of hypercholesterolemic animals compared to the corresponding brain regions of control mice (Fig. 8A–D). Examination of number of GFAP-positive astrocytes indicated that hypercholesterolemic mice had a significant increase by 5.1-fold in striatum ($P = 0.0182$, $df = 6$, $F = 2.51$; Fig. 8E) and 2.9-fold in susbstantia nigra ($P = 0.001$, $df = 6$, $F = 29.55$; Fig. 8F) regions of brain over the control animals.

4. Discussion

The most important outcome of the study is the significant decrease in dopamine content as well as TH-immunoreactivity in striatum and depletion of serotonin content in cortex region of brain in hypercholesterolemic animals. The results of the behavioral tests clearly showed that hypercholesterolemic mice developed motor as well as depressive-like behavior.

Mice on high-cholesterol diet for 12 weeks resulted in significant elevation of serum total cholesterol level which validates these animals to be hypercholesterolemic (Fig. 1A; Refolo et al., 2000; Ullrich et al., 2010). The observed elevation in blood cholesterol level may be due to increased rate of absorption of cholesterol from the intestine (Zulet et al., 1999; Hassan et al., 2011) as cholesterol level in plasma is contributed by *de novo* biosynthesis as well as from diet (Paul et al., 2016). Body weight of the animals subjected to high-cholesterol diet did not alter consistently during the 12 weeks of treatment period which indicates that animals were not obese (Woods et al., 2003). However, there was a trend of gradual increase in body weight till 8th week, being significantly increased in 6–8 weeks, and afterwards there occurred no significant alterations in body weight of high-cholesterol diet fed mice (Fig. 1B) which is a sign of hypercholesterolemia (Ullrich et al., 2010; Rao et al., 2016).

The present study provided evidence of motor behavioral

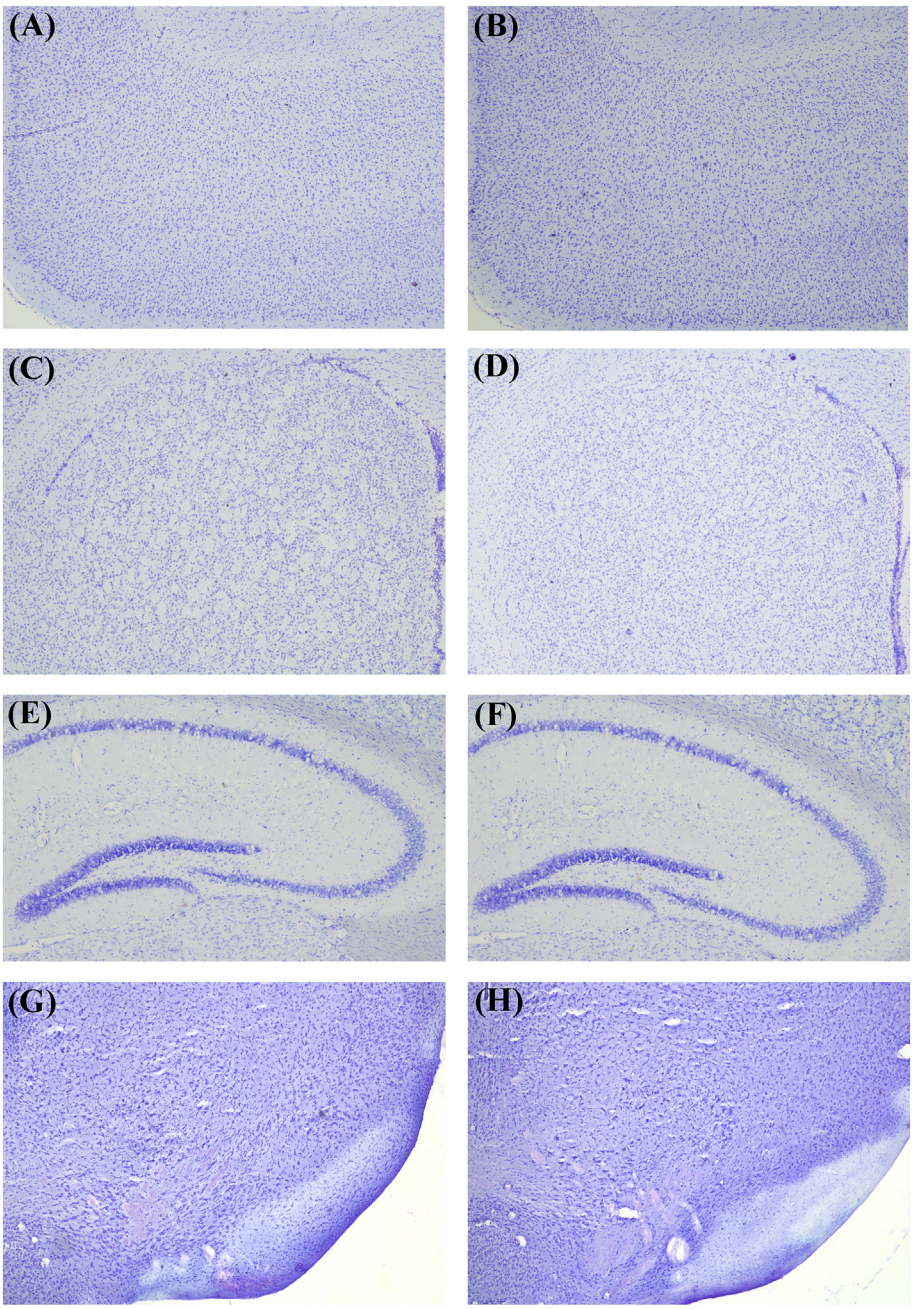

Fig. 6. Effect of hypercholesterolemia on brain histology using Nissl staining. Twenty micron thick coronal sections of brain from control (CS) and high-cholesterol diet (HCD) group passing through the cortex (A, B), striatum (C, D), hippocampus (E, F) and substantia nigra (G, H) were stained with cresyl violet. No morphological irregularity could be revealed in the cortex, striatum, and hippocampus of HCD mice, compared to the corresponding brain regions of CS mice. Photographs were taken at 4× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

abnormalities in hypercholesterolemic mice. Increase in akinesia and catalepsy score as well as poor swimming ability were indicative of motor deficits in the hypercholesterolemic mice, while the same was not observed from the gait (stride length). The indicative motor deficit parameters as observed in hypercholesterolemic mice are used to assess Parkinsonism in animals (Taylor et al., 2010; Naskar et al., 2015; Bhattacharjee et al., 2016b; Singh et al., 2016). In PD, the motor deficits are assessed by the decrease in stride length, however, giving extra weight to the PD patients has been reported to increase the stride length (Yoon et al., 2016). The increased stride length (Fig. 2E) as observed in the present hypercholesterolemic mice may be due to differences in body weight with control animals. In addition to motor behavioral abnormalities, we observed depressive-like behavior in hypercholesterolemic mice (Fig. 2H), while anxiety-like behavior was not evident (Fig. 2I–L). A significant increase in the duration of immobility time was observed in hypercholesterolemic mice (Fig. 2H). The increase in immobility time is an indication of depressive-like psychometric behavioral anomalies (Porsolt et al., 1997), thereby suggesting that hypercholesterolemia can cause despair in mice. Depressive-like behavior was reported earlier in animal models (De Bem et al., 2014; Engel et al., 2016) as well as human subjects of the disease (Bajwa et al., 1992; Kuczmierczyk et al., 1996). In patients with panic disorder such as anxiety, an elevated level of plasma

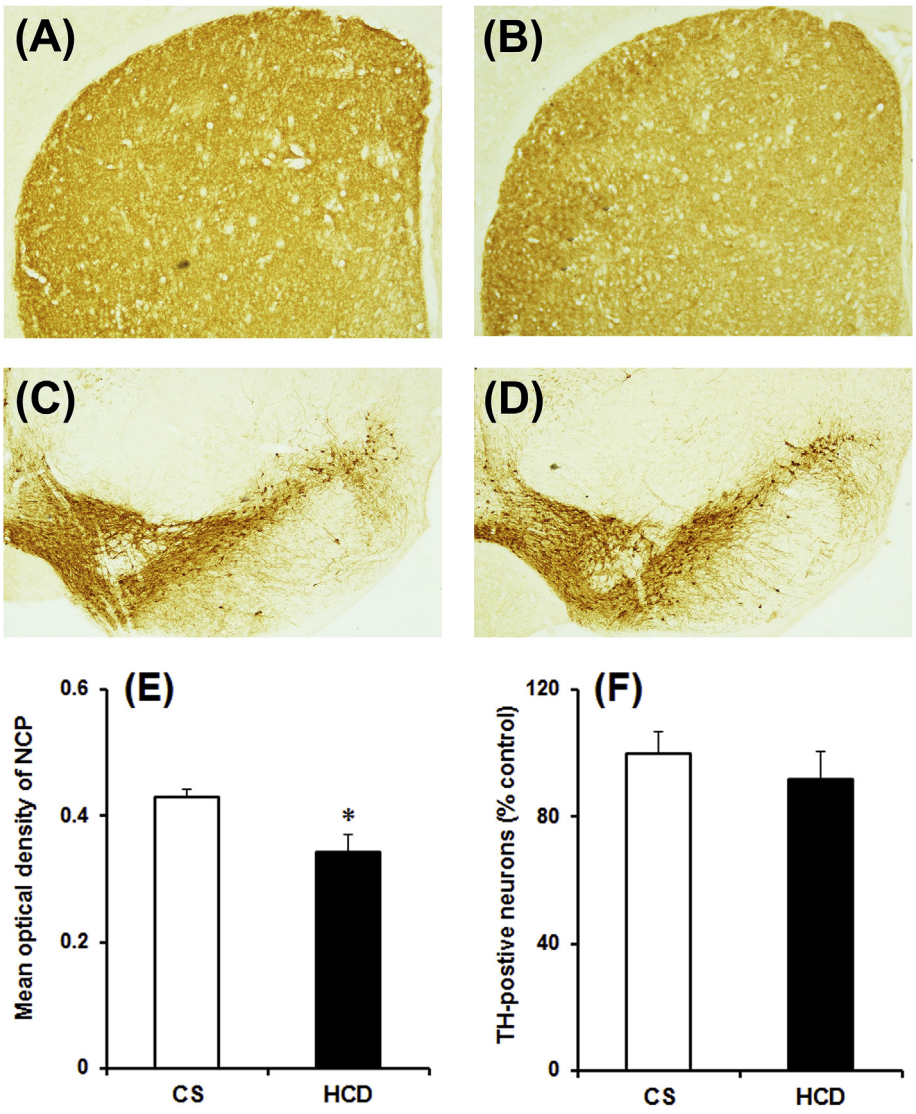

Fig. 7. Effect of hypercholesterolemia on tyrosine hydroxylase (TH)-immunoreactivity in striatum (NCP) and TH-positive substantia nigral (SN) neurons. Representative photographs of NCP (A–B) and SN (C–D) from animals subjected to standard diet (control, CS) or high-cholesterol diet (HCD) for 12 weeks. Photographs were taken at 4× magnification. (E) Quantification of optical density of TH-immunostaining in NCP and (F) neuronal count from SN region. Serial sections of NCP and SN from CS and HCD groups were analyzed using ImageJ software. There occurred a significant decrease in TH-immunoreactivity in NCP of HCD animals. Results are expressed as Mean ± SEM. $^{*}p \leq 0.05$ as compared to CS. P-values were calculated using an unpaired *t*-test (two-tailed). Sections from five different brain samples were considered for each group.

cholesterol has been reported (Kuczmierczyk et al., 1996; Peter et al., 2002). However, our result (Fig. 2I–L) is not in congruence with the previous report that hypercholesterolemia can cause age-dependent anxiety-like behavior in rats (Hu et al., 2014). The behavioral abnormalities in the hypercholesterolemic animals were observed from 10^{th} week onwards and interestingly since then the body weight of the animals was not altered significantly compared to control (Fig. 1B). Thus, the body weight has no influence on the observed behavioral abnormalities in hypercholesterolemic mice.

Till date, no studies reported the effect of hypercholesterolemia on the levels of biogenic amine neurotransmitters, such as dopamine, norepinephrine, serotonin and their metabolites, in discrete regions of brain of rodents. Nagaoka et al. (1986) have shown an elevated level of dopamine in the urine of hypercholesterolemic rat induced by polychlorinated biphenyls and excess tyrosine with diet. Hypercholesterolemia caused a significant depletion of dopamine content in the striatum (Fig. 3) and serotonin content in the cortex (Fig. 5) regions of brain which is the first report of such kind. However, we did not find any significant alteration in the level of norepinephrine in discrete brain regions of hypercholesterolemic mice. Also, the status of the dopaminergic neurons in the nigrostriatal pathway (SN and NCP) of brain was investigated using TH-immunoreactivity, which revealed a significant decrease in the immunoreactivity in the striatum of hypercholesterolemic mice (Fig. 7E). Since striatum is rich in dopaminergic axon terminals (Wilson et al., 1996), a decrease in TH-immunoreactivity in striatum signifies damages to the dopaminergic nerve terminals, which is prevalent in early stage of PD (Betarbet et al., 2000; Darvas et al., 2014; Kalia and Lang, 2016). This explains the underlying cause of depletion of striatal dopamine, revealed from the HPLC study, and resulting motor behavioral abnormalities in hypercholesterolemic condition. Loss of striatal dopamine and damage to dopaminergic nerve terminals found in the present study supports our (Paul et al., 2015) and those of Doria et al. (2016) contention that cholesterol is a putative contributor towards Parkinsonism.

Loss of striatal dopamine is an important factor for the

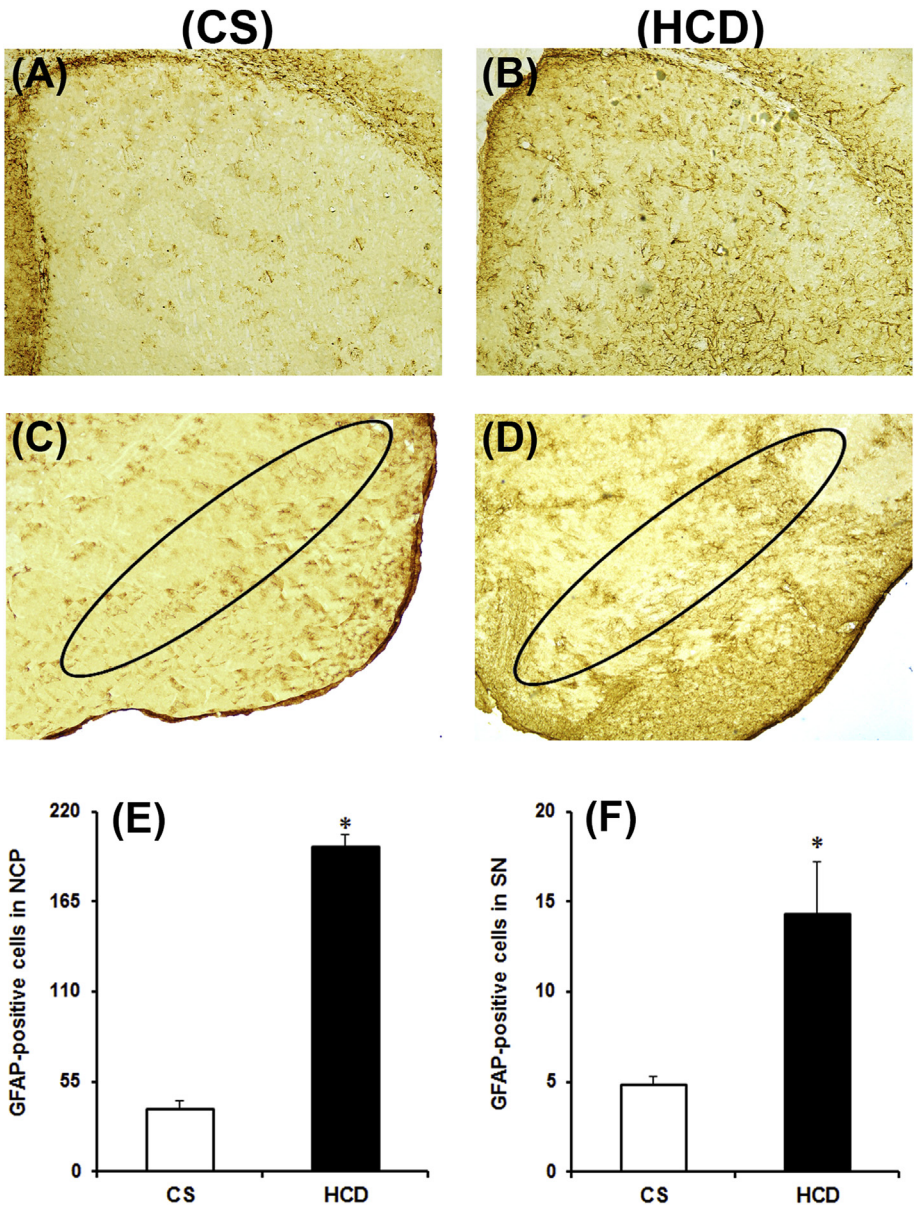

Fig. 8. Effect of hypercholesterolemia on astrogliosis. The coronal sections passing through (A–B) striatum (NCP) and (C–D) substantia nigra (SN) were processed for Glial fibrillary acidic protein (GFAP)-immunoreactivity. GFAP-reactivity was increased in NCP and SN regions of brain of high-cholesterol diet (HCD) fed mice, compared to the corresponding brain regions of control mice (CS), which signifies astrogliosis. The photographs were taken at 4× magnification. Four serial sections of NCP and SN were taken from each group (n = 4) to count the GFAP-positive cells using ImageJ software. The marked region in the photographs (C, D) represents SN region. Results are expressed as Mean ± SEM. *$p \leq 0.05$ as compared to CS (n = 4). P-values were calculated using an unpaired *t*-test (two-tailed).

appearance of the parkinsonian symptoms (Lotharius and Brundin, 2002; Kalia and Lang, 2016). To assess the degree of Parkinsonism in experimental models of PD, the motor behavioral parameters, such as akinesia, catalepsy, swim test and walk test are performed (Haobam et al., 2005; Taylor et al., 2010; Bhattacharjee et al., 2016b). The Parkinsonian motor behavioral deficits are reported to be associated with dopamine levels in the striatum (Haobam et al., 2005; Naskar et al., 2015; Singh et al., 2016). In hemiparkinsonian rat model, the behavioral phenotypes assessed were consistent with the degree of dopamine loss or nigral cell loss (Sindhu et al., 2006). A direct relationship exists between the striatal dopamine content and performances in motor behavioral tests (Sengupta et al., 2011). Moreover, age-related decrease in brain dopamine level has been reported to be associated with declined motor functions (Volkow et al., 1998). Interestingly, early stage of PD is characterized by loss of dopaminergic axon terminals in the striatum that projects from the substantia nigra region of brain (Darvas et al., 2014). Thus, not only mild reduction in striatal dopamine (by 34%) but also loss of dopaminergic terminals in this region might contributed to motor abnormalities as observed in the hypercholesterolemic mice, which has relevance to the early stage of pathology of PD (Darvas et al., 2014).

Depressive-like behavior in animal models, as well as patients, is known to be associated with altered levels of serotonin and norepinephrine in the cortical region of the brain (Seo et al., 2008; Boileau et al., 2008). Moreover, mood disturbances, particularly depression, in PD is generally being treated with drugs that elevate or increase the bioavailability of serotonin and norepinephrine in cortical regions (Boileau et al., 2008; Menza et al., 2009; Nayyar et al., 2009). In this regard, association of altered level of cortical

serotonin with depressive-like behavior in PD receives much attention (Scatton et al., 1983; Azmitia and Nixon, 2008; Nayyar et al., 2009). Although hypercholesterolemia did not cause any significant alterations in the level of norepinephrine (Fig. 4), but the serotonin level in cortex was decreased significantly (Fig. 5). Thus, the significant loss of cortical serotonin level in brain of hypercholesterolemic mice may be linked with the observed depressive-like behavior. Dopamine and serotonin systems are neurophysiologically interlinked with each other and have also been shown that impairment of one system can lead to functional alterations to the other (De Simoni et al., 1987; Daw et al., 2002).

Although the result of Nissl staining revealed no marked visible changes in neuronal architecture in discrete brain regions of hypercholesterolemia mice (Fig. 6) but the numbers of GFAP-positive astrocytes in dopaminergic regions of the brain, nigrostriatal pathway, were found to significantly more in hypercholesterolemia mice, which indicates astrogliosis. Astrogliosis is an outcome of inflammation that could result into neurodegeneration (Pekny and Pekna, 2014; Yates, 2015). Previous reports provided evidence of hypercholesterolemia-induced severe neuroinflammatory processes, including astrogliosis, in discrete brain regions mainly in the cortex and hippocampus of rodents (Thirumangalakudi et al., 2008; Ullrich et al., 2010); however our report of astrogliosis in nigrostriatal pathway of hypercholesterolemic mice is the first report of such kind (Fig. 8).

In conclusion, the present study reports psychomotor behavioral deficits and altered neurotransmitters metabolism in discrete brain regions, particularly depletion of serotonin in cortex and dopamine in striatum with decrease in TH-immunoreactivity in striatum of hypercholesterolemic mice. By and larger, the present experimental evidence strengthen the contention of the influence of cholesterol in the pathogenesis PD.

Conflict of interest

None declared.

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