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Oxidative stress in zinc-induced dopaminergic neurodegeneration: Implications of superoxide dismutase and heme oxygenase-1

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Abstract

The study was undertaken to investigate the effect of zinc (Zn) on glutathione S-transferase (GST) and superoxide dismutases (SOD) activities and on the expressions of cytosolic Cu, Zn-SOD (SOD1), mitochondrial Mn-SOD (SOD2), γ-glutamyl cysteine synthetase (γ-GCS) and heme oxygenase-1 (HO-1) in the nigrostriatal tissue of rats. Additionally, Zn-induced alterations in the neurobehavioral parameters, lipid peroxidation (LPO), striatal dopamine and its metabolites and tyrosine hydroxylase (TH) protein expression were measured to assess their correlations with the oxidative stress. Zn exposure reduced the locomotor activity, rotarod performance, striatal dopamine and its metabolites and TH protein expression. LPO, total SOD, SOD1 and SOD2 activities were increased while GST and catalase were reduced in a dose and time dependent manner. Expressions of SOD1 and HO-1 were increased while no change was observed in SOD2 and γ-GCS expressions. The results obtained suggest that Zn-induced augmentation of total SOD, SOD1, SOD2 and HO-1 was associated with increased oxidative stress and neurodegenerative indexes indicating the involvement of both cytosolic and mitochondrial machinery in Zn-induced oxidative stress leading to dopaminergic neurodegeneration.

Keywords: *zinc, oxidative stress, superoxide dismutase, heme oxygenase-1, neurodegeneration, Parkinson's disease*

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused by the selective loss of dopaminergic neurons in the substantia nigra (SN) region of brain and mainly caused by age and genetic and environmental factors. Epidemiological and experimental data have established pesticides and heavy metals exposure as the major environmental risk factors for PD [1,2]. Clinical studies have shown an increased accumulation of Zn in the SN of PD patients as compared with case controls [3,4] that has prompted investigators to look into the role of Zn in dopaminergic neurodegeneration [5]. It is not yet clear whether an increased accumulation of Zn is the cause of dopaminergic neurodegeneration or consequence of the disease progression. However, long term exposure to Zn is known to induce dopaminergic neurodegeneration in rats [5]. Zn is an essential transition element, which forms structural or functional component of several proteins including enzymes necessary for replication, transcription and translation, thereby facilitating normal physiological processes, cell division and differentiation [6]. Zn possesses both antioxidant and pro-oxidant properties and therefore, it may be neuroprotective or neurotoxic depending upon the concentration. Excess Zn levels are associated with neurodegenerative diseases including Alzheimer disease (AD) and PD [3,6–8].

Oxidative stress is implicated as the primary event in idiopathic and chemically-induced PD [9,10]. Increased free radical production, mitochondrial dysfunction and impaired antioxidant defense system are the key players contributing to oxidative stress in PD pathogenesis [10–12]. Although Zn is redox inactive,

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the role of oxidative stress in Zn-induced dopaminergic neurodegeneration is documented, which is supported by the protective effect of vitamin D3 against Zn-induced neurodegeneration in rats [5,8,13]. Zn is reported to potentiate the neurodegeneration induced by 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), dopamine and iron (Fe) via increased oxidative stress [13–15]. The role of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been implicated in sporadic and chemically induced PD, as it is responsible for the generation of superoxide free radicals, and therefore oxidative stress, which is one of the main culprits of PD pathogenesis [16–18]. Protective effect of superoxide dismutase (SOD)/catalase mimetics against PD has confirmed the deleterious role of free radical-induced oxidative stress in PD pathogenesis [19,20]. In vitro studies with cultured cortical cells have shown Zn-induced superoxide radical formation [21]. SOD is the enzyme responsible for the removal of superoxide radicals via conversion into hydrogen peroxide, which is then neutralized by peroxidases such as catalase, glutathione peroxidase (GPx), etc. SOD exists mainly in three isoforms- cytosolic/Cu, Zn-SOD/SOD1, mitochondrial/Mn-SOD/SOD2 and extracellular SOD/SOD3. Several studies have reported altered SOD activity in the SN region of brain of PD patients and chemically-induced PD phenotypes [4,22–25].

Heme oxygenase-1 (HO-1), an enzyme involved in heme metabolism, is a cellular stress protein, which protects against oxidative stress-induced damage [26,27]. Postmortem studies showing increased HO-1 expression in the SN of the brain and serum of PD patients implicated its role in PD pathology [28,29]. HO-1 is found to protect against 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxy dopamine (6-OHDA)-induced dopaminergic neurodegeneration [30,31]. Recent genetic studies have also reported 4-fold higher risk for PD in individuals possessing single nucleotide polymorphism (SNP) in HO-1 and glycogen synthase kinase-3β (GSK3β) genes [32]. HO-1 gene is regulated by Nrf-2 transcription factor via mitogen activated protein kinase (MAPK) and p38 pathways, which increase anti-inflammatory changes, thereby protecting against inflammation [33].

Glutathione (GSH) plays a protective role against oxidative stress-induced damage via neutralization of free radicals. GSH synthesis from amino acids is catalyzed by the enzymes- γ-glutamyl cysteine synthetase (γ-GCS) and glutathione synthase (GS). Reduced GSH levels are reported in both sporadic PD and animal models of PD [34–36]. Intra-nigral Zn infusion induced neurotoxicity is reported to be attenuated by GSH and zinc-induced GSH depletion is documented in cultured cortical neurons [13,37] but its effect on GCS is not yet known. Although systemic Zn exposure attenuates spontaneous locomotor activity (SLA), striatal dopamine content, tyrosine hydroxylase (TH) positive cells and increased lipid peroxidation in exposed rats [5], the effect of Zn on cytosolic and mitochondrial SOD, HO-1 and glutathione synthesizing enzyme γ-GCS is not yet investigated. The present study was therefore undertaken to investigate the effect of Zn on activities and/or expressions of total, cytosolic and mitochondrial SODs along with HO-1 and γ-GCS and to correlate the changes with the Zn-induced changes in the motor activities, striatal dopamine content and changes in TH protein expression in Zn-exposed animals to further explore the mechanism of oxidative stress in Zn-induced neurodegeneration in rats.

Materials and Methods

Materials

Acetic acid, dibutyl phthalate xylene (DPX), disodium hydrogen phosphate, nicotinamide adenine dinucleotide reduced form (NADH), methanol, potassium chloride and sodium dihydrogen phosphate were procured from Sisco Research Laboratories (SRL), India. Folin Ciocalteau reagent, hydrogen peroxide (H_2O_2), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), potassium dichromate and potassium cyanide were purchased from Merck, Germany. Bovine serum albumin (BSA), 1-chloro 2,4-dinitrobenzene (CDNB), sodium dodecyl sulphate (SDS), sodium pyrophosphate, thiobarbituric acid (TBA), tri-reagent and zinc sulphate ($ZnSO_4$) were procured from Sigma-Aldrich, USA. cDNA synthesis kits were procured from MBI Fermentas, USA. 5-Bromo-4-chloro-3'-indolyl phosphate p-toluidine/nitro-blue tetrazolium chloride (BCIP/NBT), dNTPs, $MgCl_2$, Taq buffer, Taq DNA polymerase, streptavidin-peroxidase complex, normal goat serum and 3,3' diaminobenzidine tetrahydrochloride (DAB) system were procured from Bangalore Genei Pvt. Ltd., India. Gene specific primers were obtained from Metabion GmbH, Germany. Monoclonal anti-TH antibody and biotinylated anti-mouse secondary antibody were procured from Sigma Aldrich, USA. Polyclonal anti-SOD1 and anti-SOD2 antibodies, monoclonal anti-β-actin antibody and bovine anti-mouse, rabbit anti-goat and bovine anti-rabbit alkaline phosphatase (AP)-conjugated secondary antibodies were purchased from Santacruz Biotechnology, USA. Polyvinylidene difluoride (PVDF) membrane was procured from Millipore Corporation, USA.

Animal treatment

Male Wistar rats weighing 150–200 g were obtained from the animal facility centre of Indian Institute of Toxicology Research (IITR), Lucknow, India. The study was approved by the Institutional Animal Ethics

Committee of IITR, which is a sub-committee of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). CPCSEA is governed by the Ministry of Environment and Forest. Animals were maintained on a 12 h light/dark cycle and fed proper pellet diet and water *ad libitum*. The animals were divided into control and zinc sulfate treated groups. Control animals received normal saline while treated animals were administered zinc as zinc sulfate at 10, 15 and 20 mg/kg body weight (equivalent to 2.3 mg/kg, 3.4 mg/kg and 4.5 mg/kg Zn^{2+} respectively) intraperitonially twice a week (Monday and Thursday of every week) for 2, 4, 8 and 12 weeks. Biochemical and protein/gene expression studies were performed with the nigrostriatal tissues obtained from control and exposed groups after 2, 4, 8 and 12 weeks of treatment and at least 4–5 animals were included per group.

Decapitation and collection of brain tissue

The animal was sacrificed three days after the administration of the last dose by cervical dislocation, decapitated and brain was dissected quickly. The brain was dissected under ice cold conditions to isolate the striatum and SN as described earlier [38,39]. In brief, the cerebral hemispheres were separated and a cut passing through corpus callosum was made. The cortex was peeled off carefully to expose the striatum, which was taken out. In order to isolate the SN, the mesencephalon was cut into two parts from the ventral side, perpendicular to its long axis at the caudal border exposing SN, which was carefully dissected out. The striatum and SN were pooled and used as nigrostriatal tissues in all experiments except in the measurement of dopamine and its metabolites and TH-immunoreactivity.

Motor behavioral studies

Spontaneous Locomotory Activity (SLA). SLA was assessed in control and zinc treated groups using infrared beam-activated movement monitoring chamber (OptoVarimax-Mini A; Columbus Instruments, Columbus, OH). The animals were placed in the chamber for 1 min before initiating the recording of the activity and locomotor activity was measured for the next 5 min [5]. For SLA, 5 animals were included in each group for one experiment and minimum of 3 independent sets of experiments were performed.

Rotarod performance test. The rotarod test was also performed in control and treated animals for assessing motor coordination using Omni rotor (Omnitech Electronics Inc., Columbus, OH, USA) consisting of a rod rotating at a constant speed (5 rpm) with 5 min cut-off time. The animals were trained for three consecutive days before the treatment and the final readings were taken by measuring the time spent on the rotating rod by the animals of control and treated groups [40]. Similar to SLA, minimum of 3 independent sets of experiments were performed and each set included 5 animals per group.

Dopamine and its metabolites. The striatal dopamine content and its metabolites i.e., 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) were measured through standard HPLC method described elsewhere [41]. In brief, the striatal tissue was homogenized in perchloric acid followed by sonication and centrifugation. The supernatant was filtered and dopamine, DOPAC and HVA levels were measured by HPLC as described previously [5]. The dopamine, DOPAC and HVA levels were calculated by taking the ratio of known amounts of respective standards and a constant amount of the internal standard dihydro benzyl amine (DHBA). The values are expressed as ng/mg tissue.

Immunohistochemistry. Dopamine synthesis is catalyzed by tyrosine hydroxylase (TH) located inside dopaminergic neurons in the SN of the brain and, therefore, immunohistochemical studies were performed to analyze the effect of Zn-exposure on TH-positive/dopaminergic neurons as described previously [5]. In brief, rats were anaesthetized and brain was perfused with chilled normal saline and 4% paraformaldehyde in phosphate buffered saline (PBS). The brain was removed, post fixed in paraformaldehyde solution overnight and cryoprotected in sucrose solution. The coronal brain sections (20 μm) were cut serially and incubated in H_2O_2 solution (0.5%) in methanol to block endogenous peroxidase activity. The sections were then kept in blocking buffer (PBS containing 5% normal goat serum, 1% BSA and 0.1% Triton X-100) followed by incubation in anti-TH antibody (1:2000) as described earlier [5]. The sections were rinsed with PBS, incubated in biotinylated anti-mouse secondary antibody (1:300) and then in Strepavidin peroxidase complex. The color was developed using DAB system and sections were mounted permanently using DPX. Bilateral counting was done using computerized analysis software (QWin Pro, Leica, Germany) to calculate the number of TH-positive neurons in minimum of four animals in each group by a researcher, unknown to experimental groups to ensure unbiased counting. The results are expressed as percentage of control.

Oxidative stress indices

LPO, catalase and GST activities. Malondialdehyde (MDA), an indicator of the level of LPO, was determined using the method of Ohkawa and coworkers

[42] with slight modifications as described previously [43]. The absorbance of the MDA formed was read at 532 nm and LPO levels are expressed as nmoles MDA/mg tissue.

Catalase was estimated according to the method described by Sinha [44]. In brief, 5.0 ml assay mixture containing 0.01 M phosphate buffer (pH 7.0), 0.2 M H_2O_2 and 0.1 ml sample was incubated at 37°C for 1 min. The reaction was stopped using potassium dichromate and glacial acetic acid and the reaction mixture was incubated in boiling water bath for 15 min. The samples were then cooled and absorbance was read at 570 nm against control devoid of H_2O_2. The enzymatic activity was calculated and is expressed as μmoles/min/mg protein.

GST activity was measured using spectrophotometric method [45]. In brief, tissue homogenate was mixed with 2.9 ml of 0.2 M phosphate buffer (pH 6.5). The reaction was initiated by the addition of CDNB and optical density was read at 340 nm for 3 min at the intervals of 30 sec. Enzyme activity is expressed in nM/min/mg protein.

SOD activity. SOD activity was estimated using standard method developed by Nishikimi et al., [46] with slight modifications as described in previous report [36]. To differentiate between the activity of SOD2/Mn-SOD and SOD1/Cu,Zn-SOD, the reaction was carried out in the presence of 4 mM potassium cyanide (KCN), which inhibits Cu,Zn-SOD. The optical

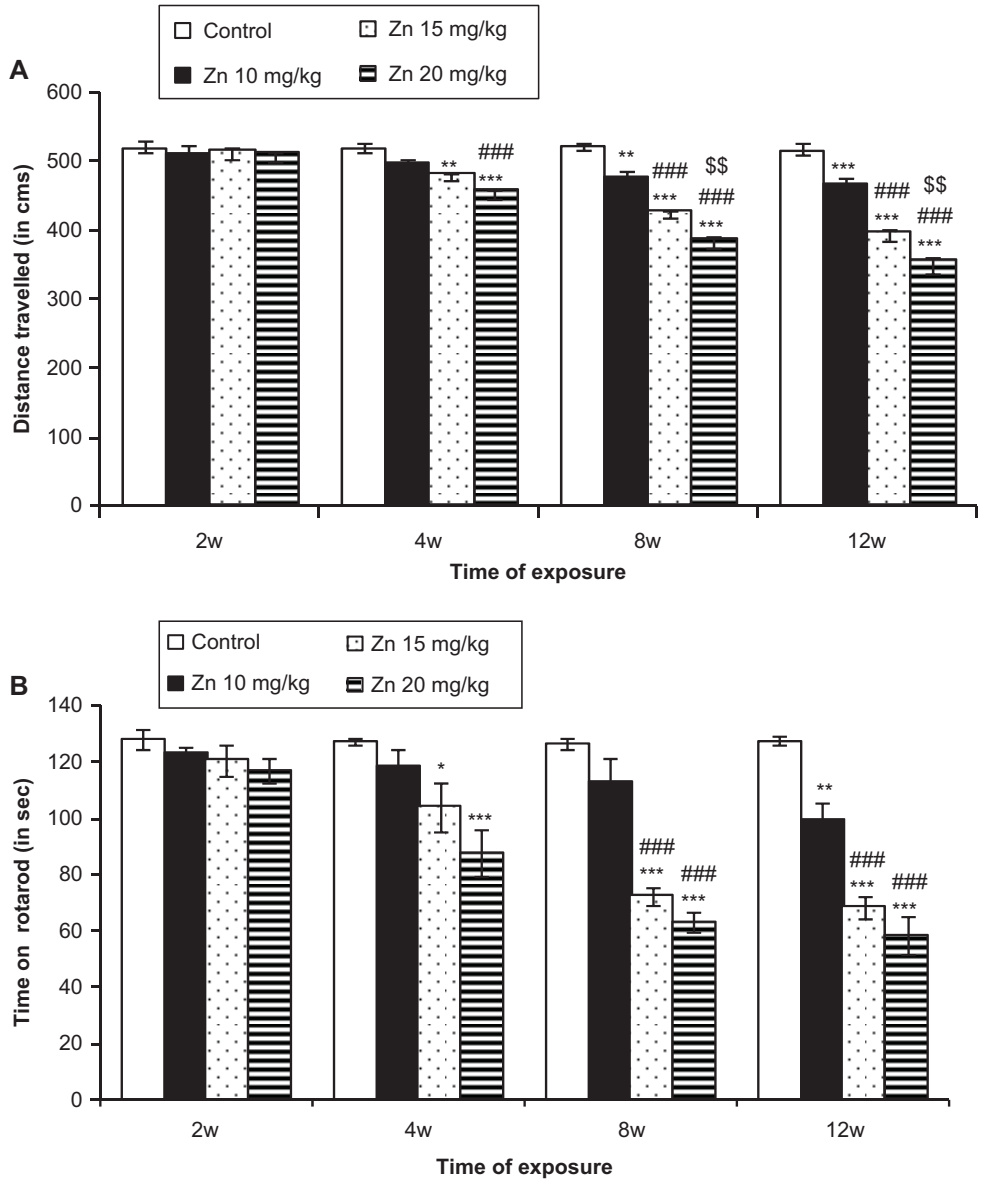

Figure 1. Effect of Zn exposure on the SLA (**A**) and rotarod performance of rats (**B**) following 2, 4, 8 and 12 weeks exposure at different doses. The data expressed are mean ± SEM (n = 3 independent set of experiments and each set included 5 animals per group). (*** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$ as compared with control; ### = $p < 0.001$ as compared with Zn 10 mg/kg treated group; $$ = $p < 0.01$ as compared with Zn 15 mg/kg treated group. F value for SLA = 18.3, 132.1, 94.79 and for rotarod = 6.406, 64.11, 34.09).

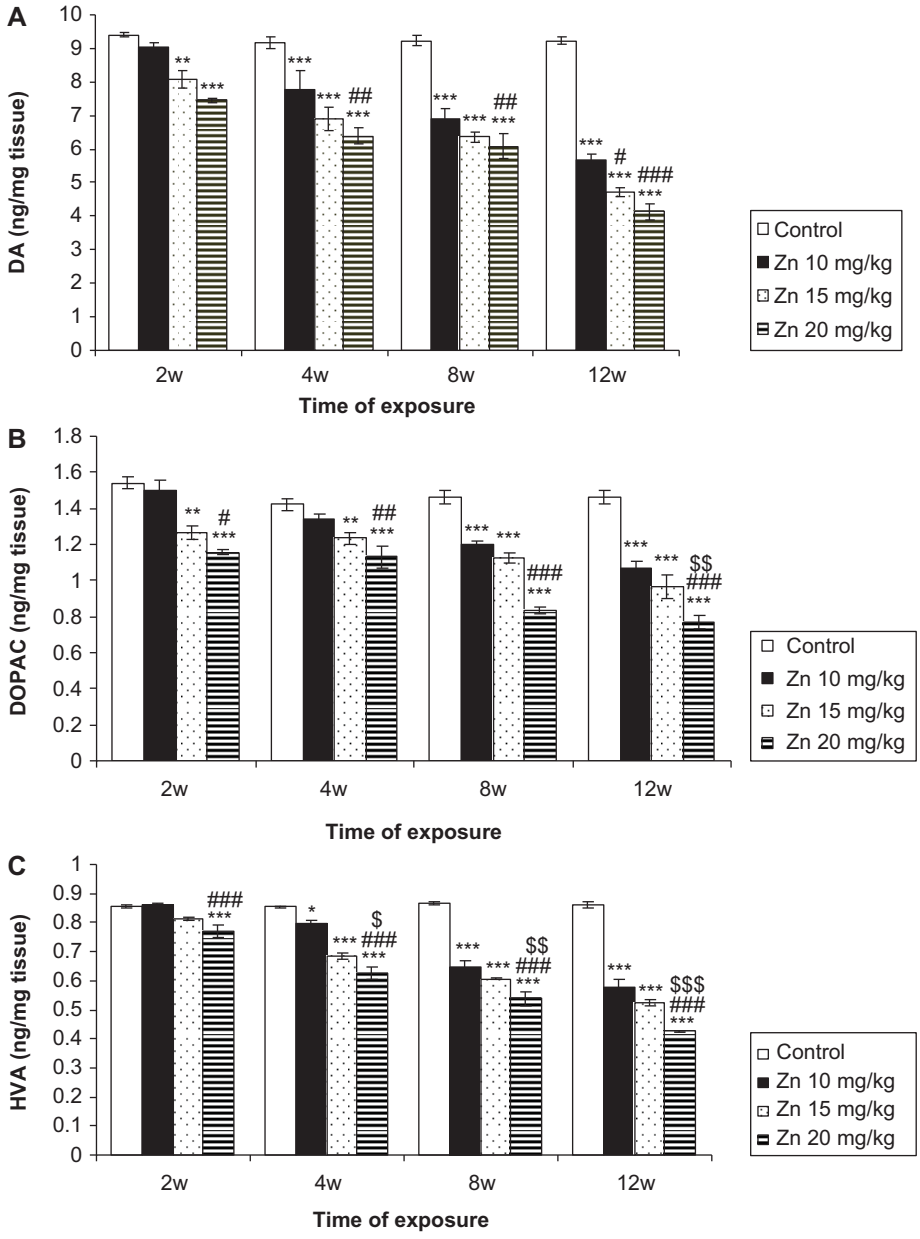

Figure 2. Effect of Zn on striatal dopamine level (**A**), DOPAC (**B**) and HVA levels (**C**) in rats following 2, 4, 8 and 12 weeks of exposure. The results are expressed as mean ± SEM (n = 4-5). (*** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$ as compared with control; ### = $p < 0.001$, ## = $p < 0.01$ and # = $p < 0.05$ as compared with Zn 10 mg/kg treated group; \$\$\$ = $p < 0.001$, \$\$ = $p < 0.01$ and \$ = $p < 0.05$ as compared with Zn 15 mg/kg treated group. F values for DA = 7.817, 136.3, 75.54; for DOPAC = 5.225, 92.35, 42.28 and HVA = 5.225, 92.35, 42.28).

density of the chromogen was measured at 560 nm. CuZn-SOD activity was calculated as the difference between total SOD and Mn-SOD activities i.e. the activity in the presence of KCN. The results are expressed as units/ml/min.

Protein estimation. Protein content was measured in control and Zn-treated groups using Lowry's method [47]. Protein concentration was calculated using the standard curve of BSA.

Reverse-transcription polymerase chain reaction (RT-PCR). mRNA expressions of SOD1, SOD2, HO-1 and γ-GCS were analyzed using RT-PCR. Total RNA was isolated from the nigrostriatal tissues of control and treated animals using Trizol method as per manufacturer's protocol. 5 μg of total RNA was used for the synthesis of cDNA using cDNA synthesis kit and gene specific primers were used to amplify SOD1, SOD2, HO-1, γ-GCS and β-actin genes in control and treated groups as described earlier [48–51]. PCR products were visualized using ethidium bromide under UV light. Densitometric analysis was performed using Alpha Imager software taking β-actin as the reference and data are expressed as mean ± standard error of means (SEM) of band density ratio.

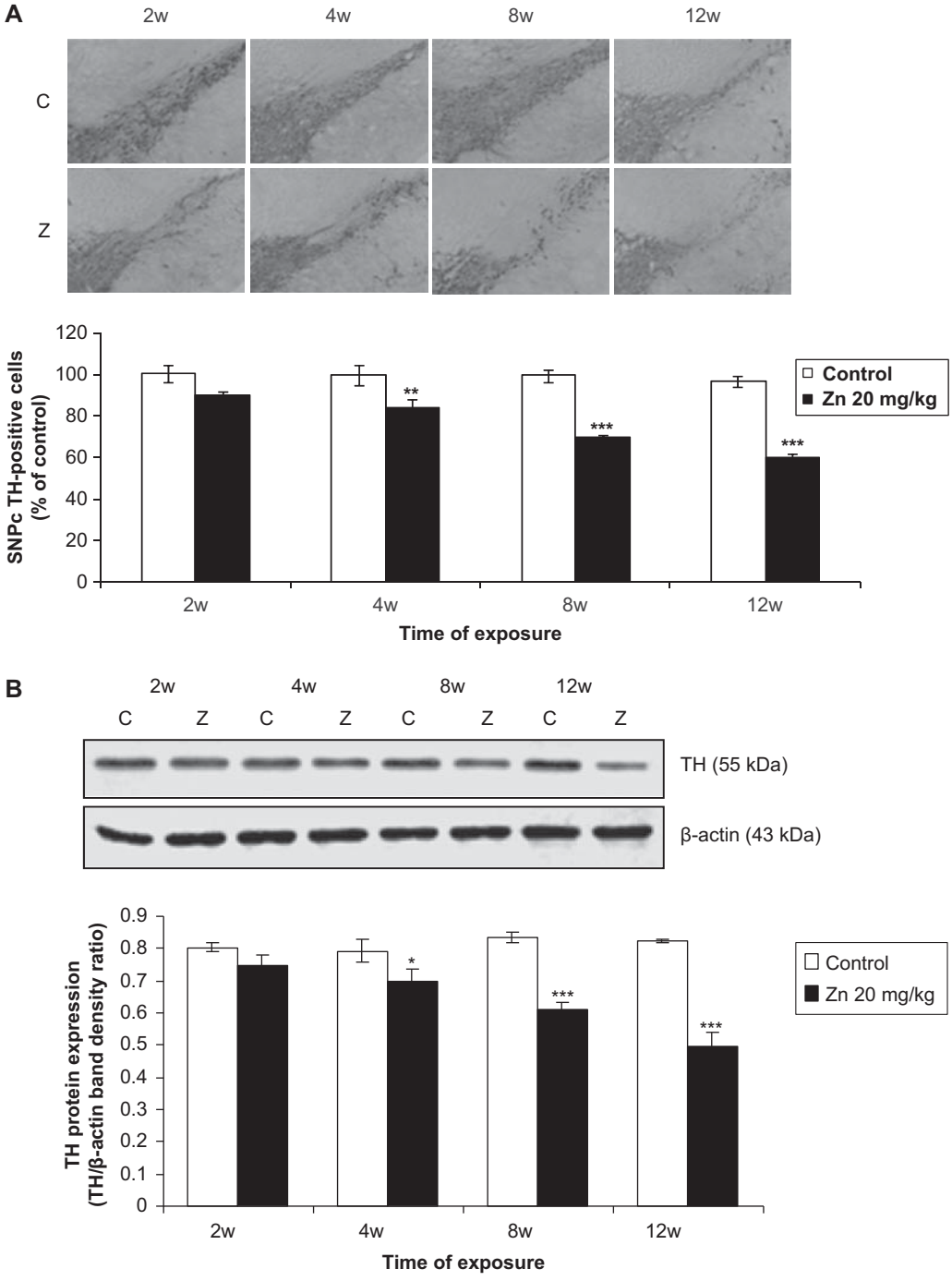

Figure 3. (**A**) Immunohistochemical analysis of TH-positive dopaminergic neurons in the SN of the control and Zn (20 mg/kg) treated animals. The upper panel shows the representative pictures of TH-immunoreactivity and lower panel depicts the results in bar diagram showing % change in a number of TH-positive neurons from control groups (n = 4). **(B)** Effect of Zn (20 mg/kg) on TH protein expression in control and treated rats after 2, 4, 8 and 12 weeks of exposure (n = 4). The results are expressed as mean ± SEM. (C = control; Z = Zn-treated; *** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$ as compared with control groups. F values for immunohistochemical analysis are 6.708, 97.5 and 10.37 and for TH protein expression are 9.08, 70.12, 5.65).

Western Blotting

The effect of zinc exposure on protein expression of TH, SOD1 and SOD2 proteins was analyzed in the nigrostriatal tissues of control and Zn-treated groups at 20 mg/kg dose. In brief, denatured proteins were resolved by SDS-polyacrylamide gel electrophoresis and electroblotted on PVDF membrane. Blots were blocked overnight with tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% non fat dry milk and subsequently incubated with specific primary antibodies against SOD1 (1:3000), SOD2 (1:4000), TH (1:5000) and β-actin (1:4000) for 2 h followed by 1 h incubation with anti-goat/anti-rabbit secondary antibodies and finally the blots were

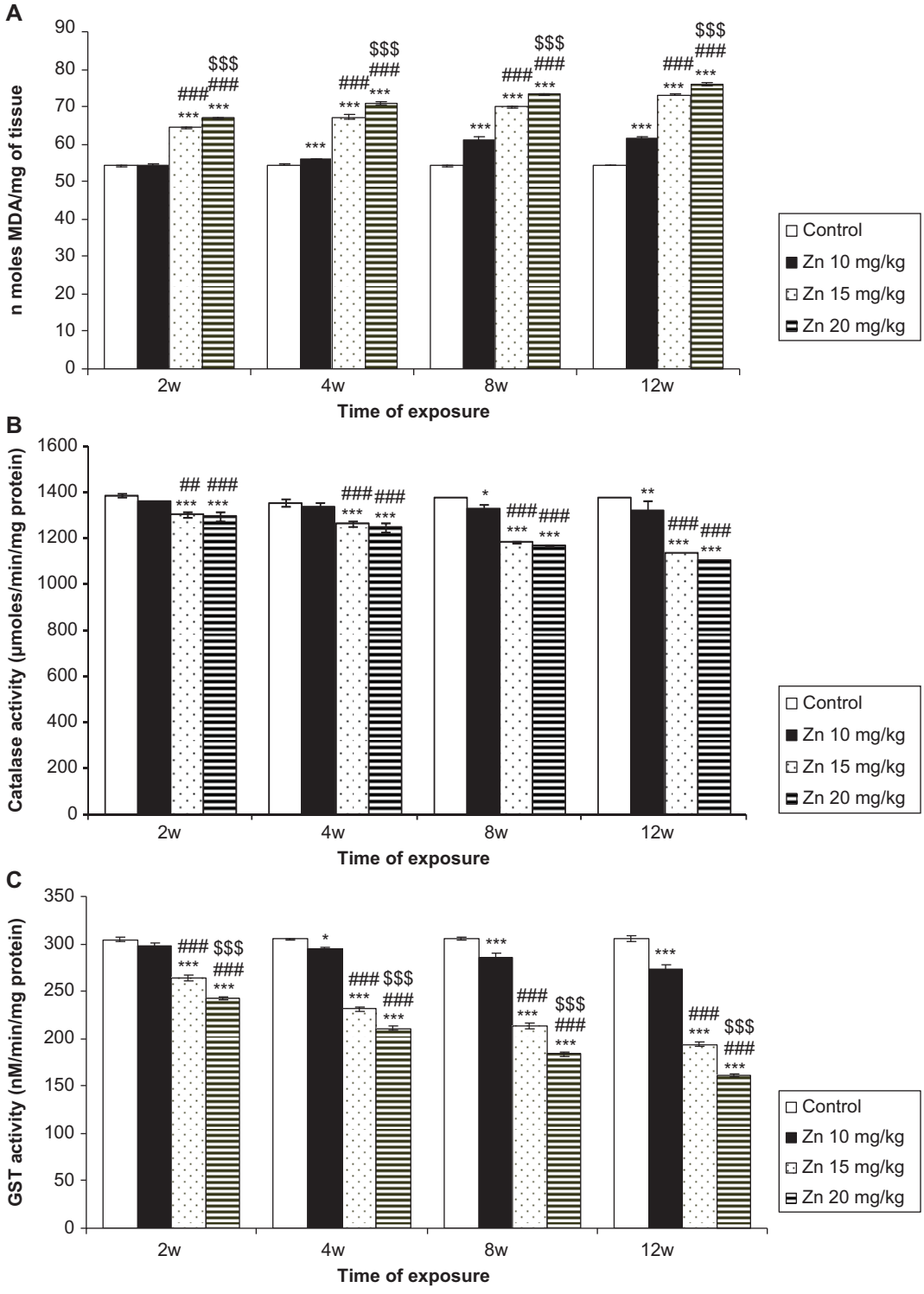

Figure 4. Effect of Zn on LPO (**A**), catalase (**B**) and total GST (**C**) activities in nigrostriatal tissue of rat brain at 10, 15 and 20 mg/kg doses following 2, 4, 8 and 12 weeks of exposure. The results are expressed as mean ± SEM (n = 4-5). (*** = $p < 0.001$, ** = $p < 0.01$ and * = $p < 0.05$ as compared with control; ### = $p < 0.001$ and ## = $p < 0.01$ as compared with Zn 10 mg/kg treated group; $$$ = $p < 0.001$ as compared with Zn 15 mg/kg treated group. F values for LPO = 41.09, 3061, 336.8; for catalase = 14.4, 227.2, 60.69 and for GST = 39.42, 1426, 197.3).

visualized using BCIP/NBT as substrates. Relative band density was calculated with β-actin as the reference and data are expressed as mean ± SEM of band density ratio.

Statistical analysis

Two-way analysis of variance (ANOVA) along with Bonferroni post-test was used for comparison between multiple groups. The results were expressed as

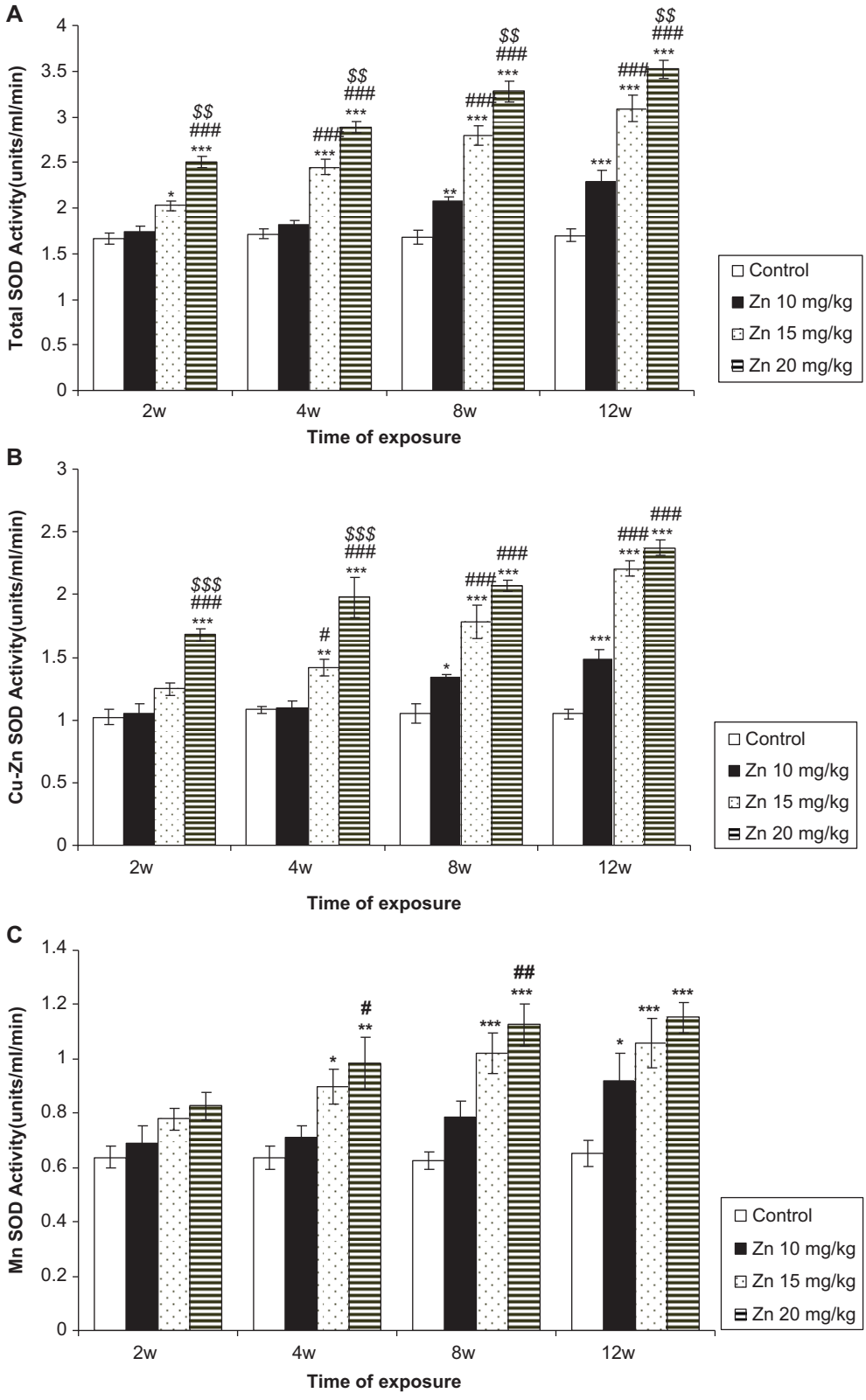

Figure 5. Effect of Zn treatment on the activity of total SOD along with its cytosolic (Cu,Zn-SOD/SOD1) and mitochondrial (Mn-SOD/SOD2) isoforms in the nigrostriatal tissue of rat brain following 2, 4, 8 and 12 weeks of exposure. Panel A shows total SOD activity at different doses of Zn. Panel B and C show effect of Zn exposure on the activity of SOD1 and SOD2 respectively. The results are expressed as mean ± SEM ($n = 4$-5). ($^{***} = p < 0.001$, $^{**} = p < 0.01$ and $^{*} = p < 0.05$ as compared with control; ### = $p < 0.001$, ## = $p < 0.01$ and # = $p < 0.05$ as compared with Zn 10 mg/kg treated group and \$\$\$ = $p < 0.001$ and \$\$ = $p < 0.01$ as compared with Zn 15 mg/kg treated group. F values are 7.255, 207.4, 49.15; 5.972, 134.6, 37.21 and 1.206, 28.16, 8.378 for total SOD, SOD1 and SOD2 respectively).

mean ± standard error of means (SEM) for separate groups. The differences were considered statistically significant when '*p*' value was less than 0.05.

Results

Neurobehavioral analyses

A significant dose and time dependent decrease was observed in SLA of treated groups following zinc exposure from 4 weeks onwards and the maximum attenuation was observed after 12 weeks of exposure at 20 mg/kg zinc sulfate dose (Figure1A).

Zinc treatment caused a significant decrease in the time of stay on rotarod in the Zn-exposed animals and the reduction was in a dose and time dependent manner. Animals treated with 15 and 20 mg/kg dose showed reduction after 4 weeks of treatment while the dose dependent effect was observed for all three doses after 8 and 12 weeks of exposures. Rats treated with

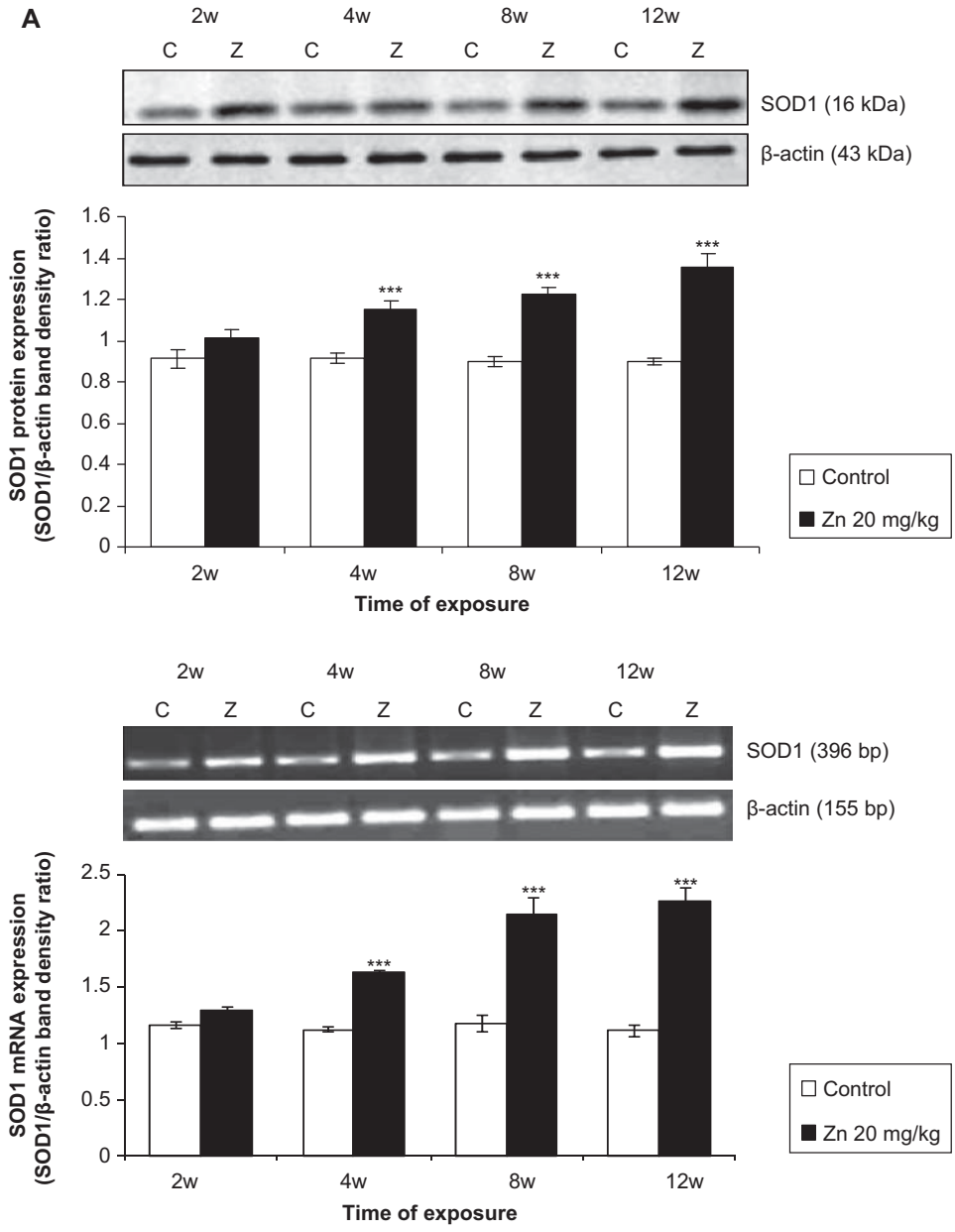

Figure 6. (**A**) Effect of Zn treatment on SOD1 protein and mRNA expression in nigrostriatal tissue of control and Zn (20 mg/kg) treated groups following 2, 4, 8 and 12 weeks of exposure. Upper panel shows representative picture of SOD1 protein expression along with its densitometric analysis. Lower panel shows mRNA expression of SOD1 in control and treated rats at different exposure time periods along with densitometric analysis of the same. **(B)** Effect of Zn treatment on SOD2 protein and mRNA expression in the nigrostriatal tissue of the control and Zn (20 mg/kg) treated groups following 2, 4, 8 and 12 weeks of exposure. Upper panel shows representative picture of SOD2 protein expression in the control and Zn-treated groups and densitometric analysis of the same. Lower panel shows representative gel picture of mRNA expression of SOD2 in the control and treated rats at different exposure time periods and its densitometric analysis. The results are expressed as mean ± SEM (n = 4). (C = control and Z = Zn-treated; *** = $p < 0.001$ as compared with control groups. F values for protein and mRNA expression of SOD1 are 5.589, 115, 4.443 and 19.1, 176.5, 18.9 respectively).

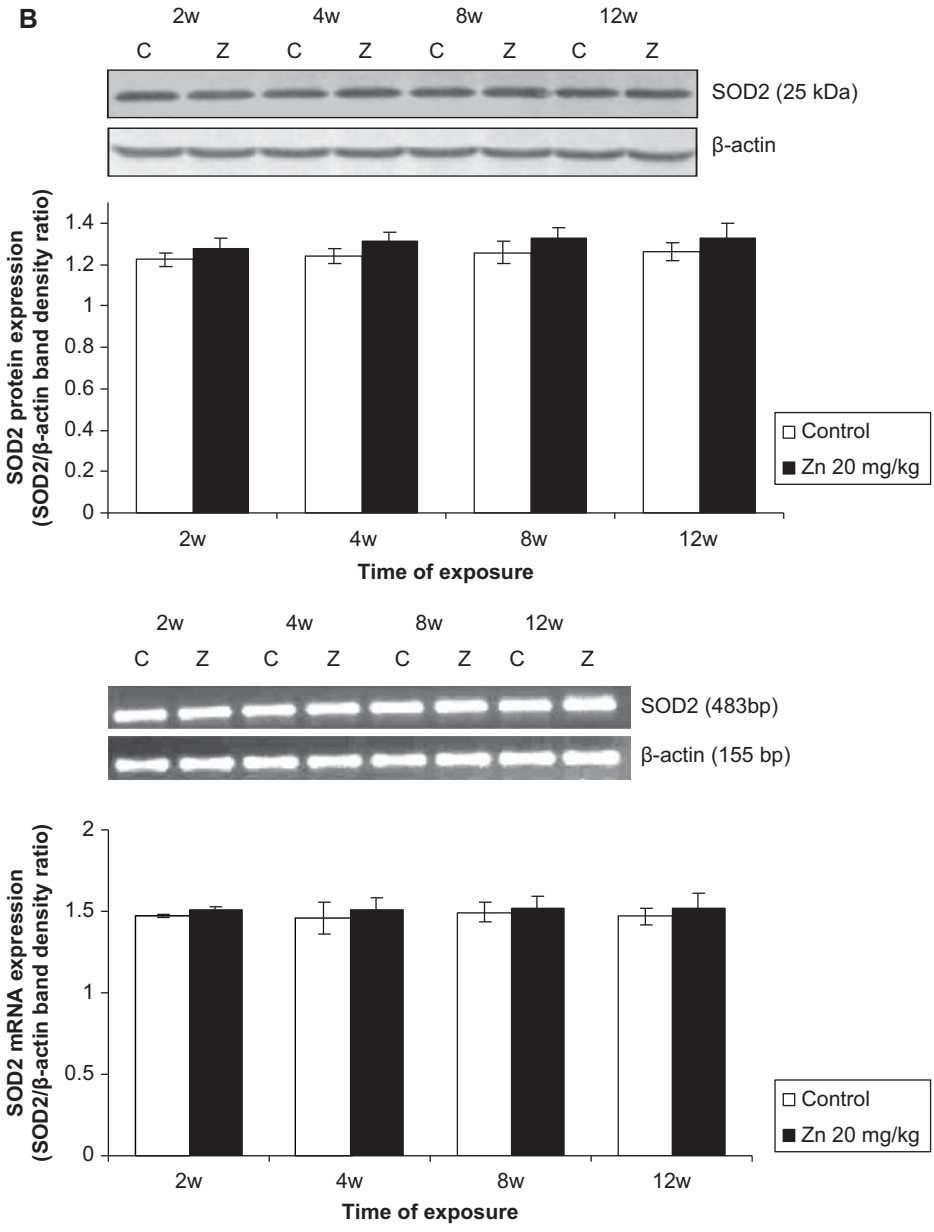

Figure 6. *(Continued)*

20 mg/kg dose showed significant reduction in the stay time at the rotating rod following 12 weeks of exposure (Figure 1B).

Striatal dopamine and its metabolites content

Zn exposure resulted in significant attenuation of striatal dopamine content along with its metabolites i.e., DOPAC and HVA in a dose and time dependent manner resulting in the maximum decrease following Zn exposure at 20 mg/kg dose after 12 weeks of treatment (Figures 2A, 2B & 2C).

TH-immunoreactivity

Immunohistochemical analysis of TH-positive neurons was performed in control and Zn-treated (20 mg/kg) animals at different exposure time periods to investigate the effect of Zn exposure on dopaminergic neurodegeneration. A significant and time dependent decrease was observed in the number of TH-positive neurons in the SN region of the brain of exposed groups as compared to control groups from 4 weeks onwards indicating that Zn induced dopaminergic neuronal loss characteristic of PD (Figure 3A).

TH protein expression

Western blot analysis of TH protein in control and treated groups yielded results similar to that obtained with immunohistochemical analysis. Zn treatment resulted in time dependent decrease in the TH protein levels in the nigrostriatal tissues of exposed groups from 4 weeks onwards (Figure 3B).

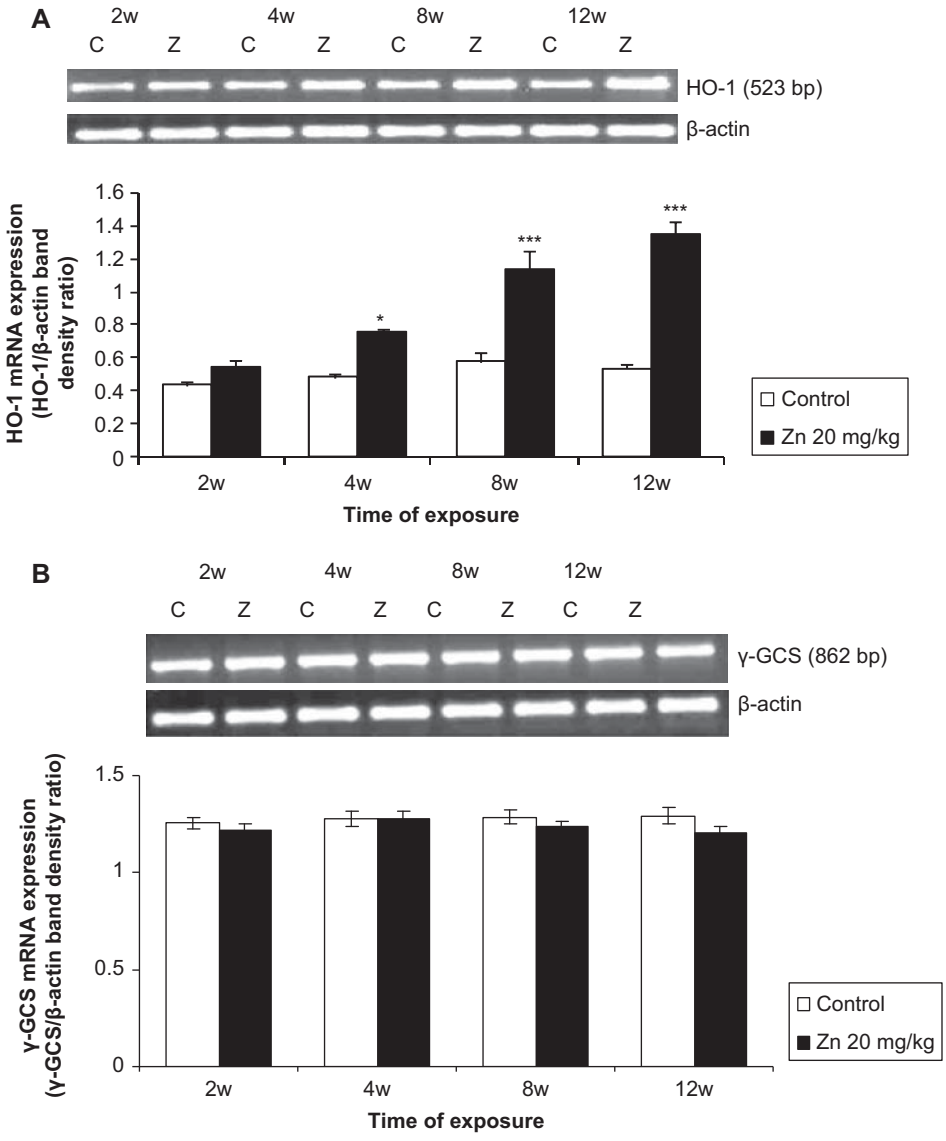

Figure 7. Effect of Zn exposure on gene expression of HO-1 and γ-GCS in nigrostriatal tissue of rat brain following 2, 4, 8 and 12 weeks of treatment at 20 mg/kg dose. **A:** Upper panel shows representative image of the gene expression of HO-1 in control and Zn exposed groups and lower panel shows the densitometric analysis of the same. **B:** Upper panel shows representative image of the gene expression of γ-GCS in control and Zn exposed groups and lower panel shows the densitometric analysis of the same. The results are expressed as mean ± SEM (n = 4). (C = control; Z = Zn-treated; *** = $p < 0.001$ and * = $p < 0.05$ as compared with control group. F values for HO-1 gene expression are 5.584, 43.67, 8.566).

Maximum reduction was obtained after 12 weeks of Zn exposure.

Oxidative stress indices

LPO, catalase and GST. A significant augmentation was observed in LPO levels in the nigrostriatal tissues of brain of Zn-treated animals in a dose and time dependent manner with maximum increase obtained after 12 weeks of exposure (Figure 4A). In contrast, Zn exposure resulted in the attenuation of both catalase and CDNB related total GST activities in the nigrostriatal tissues of treated groups in a dose and time dependent manner (Figures 4B & 4C).

SOD activity. The total SOD activity was increased significantly in treated groups after 2, 4, 8, and 12 weeks in a dose and time dependent manner (Figure 5A). Furthermore, in order to investigate whether the effect was due to cytosolic (SOD1/CuZn-SOD) or mitochondrial SOD (SOD2/ Mn-SOD), activities of SOD1 and SOD2 were also estimated. Zn exposure significantly augmented the activities of both SOD1 and SOD2 in a dose and time dependent manner (Figures 5B & 5C). Increase in SOD1 activity was observed from 2 weeks onwards while that in SOD2 activity was observed from 4 weeks exposure onwards (Figures 5B & 5C). Augmentation in SOD1 activity was greater than that obtained in SOD2 and preceded that observed in SOD2 activity.

Protein/gene expression of SOD1 and SOD2. Since SOD1 and SOD2 activity were induced following Zn exposure, effect of zinc exposure was also analyzed on protein/mRNA expression of SOD1 (Cu,Zn-SOD) and SOD2 (Mn-SOD) following 2, 4, 8 and 12 weeks of exposure at the highest dose i.e., 20 mg/kg body weight. A significant increase was observed in both the protein and mRNA expression of SOD1 following Zn treatment after 4 weeks in a time dependent manner while protein/mRNA expression of SOD2 was not altered following Zn treatment even after 12 weeks of exposure (Figures 6A & 6B).

Gene expression on HO-1 and γ-GCS. Effect of Zn on oxidative stress marker gene HO-1 and γ-GCS were also analyzed. mRNA expression analysis of HO-1 performed in control and Zn-treated groups at the highest dose and varying time periods of exposure revealed significant augmentation in HO-1 expression in Zn exposed groups in a time dependent manner indicating oxidative stress induction following Zn exposure while no significant change was observed in the γ-GCS expression after Zn treatment (Figures 7A & 7B).

Discussion

Clinical studies have shown variable zinc levels in the brain and biological fluids of PD patients. The increase of Zn concentration in the SN and decrease in its concentration in the cerebrospinal fluid (CSF) have been reported; however, no significant change in its concentration has been observed in the serum of PD patients [52–55]. These studies suggest that zinc dyshomeostasis could be critical in PD etiology. The role of agrochemicals that include metals and pesticides in PD pathogenesis is accepted globally. $ZnSO_4$ is globally preferred in the agricultural field by farmers to improve the quality and quantity of food grains production over other Zn formulations. The amount of $ZnSO_4$ used by farmers varies from 25–50 kg/ha (equivalent to 0.2–50 g per mm^3) depending upon soil type, cropping intensity and crop productivity. In addition, people involved in paint manufacturing, electrometallurgy and mine and smelting industries are also exposed to excessive level of Zn and might be prone to Zn-induced toxicity. $ZnSO_4$ is also used as anti-corrosive for lining water pipelines and may contaminate drinking water supply. We used $ZnSO_4$ in our previous study [5] and therefore the same is used in this study to further investigate the mechanism of Zn-induced dopaminergic neurodegeneration.

As PD is a progressive neurodegenerative disorder, the animal models to study the mechanistic or therapeutic aspects of PD have been developed using a treatment paradigm that produces slow and progressive neurodegeneration. The treatment schedule used in this study was standardized to achieve the same [5]. It is observed that when the animals are treated twice a week with $ZnSO_4$, they show progressive loss of dopaminergic neurons and reduced the level of dopamine, two major characteristics of PD in the experimental animals [5]. Therefore, the mentioned treatment schedule was used for this study. In the present study, Zn exposure resulted in dose and time dependent decrease in SLA and rotarod performance, which indicated that Zn induces neurobehavioral changes characteristic of PD in accordance to earlier reports [5,8]. The doses used in the present study were selected on the basis of the dose used earlier and animal response. Doses lower than 10 mg/kg i.e., 5 mg/kg body weight did not show any effect in the exposed groups and doses higher than 20 mg/kg induce anomalies in the skin that appears like skin ulcers/tumors after 2 weeks exposure. Therefore, the doses taken for the present study were 10–20 mg/kg. This dose (20 mg/kg) has also been used in our previous publications where the effect of zinc was monitored with paraquat [5]. Additionally, the LD_{50} of zinc sulphate in rat is ~200 mg/kg when administered intraperitoneally and the highest dose taken in this study was 20 mg/kg, which is $1/10^{th}$ of its LD_{50}.

The significant attenuation of striatal dopamine content along with the reduction of its metabolites (DOPAC and HVA) suggested that dopamine metabolism was affected by Zn exposure as previously depicted in sporadic and chemically-induced PD phenotypes, including Zn-induced dopaminergic neurodegeneration [5,8,13,56,57]. Reduced dopamine and its metabolites content might result from either impaired dopamine metabolism or due to loss of dopaminergic neurons in the SN of brain of exposed groups. The reduction observed in the number of TH-positive neurons by IHC studies and TH protein expression via blot analysis confirmed that the Zn-induced decrease in dopamine and its metabolites was due to the loss of dopaminergic neurons rather than impaired dopamine metabolism. The results are in accordance with earlier studies showing decreased TH-immunoreactivity and TH protein expression following intra-nigral Zn infusion and systemic Zn exposure in rats [5,13]. We have earlier reported that systemic Zn exposure causes reduced SLA, striatal dopamine content, TH protein expression and number of TH-positive dopaminergic neurons along with increased LPO levels [5] and these parameters were assessed in this study as well to correlate with Zn-induced alterations in SOD1, SOD2, HO-1 and GCS and Zn-induced dopaminergic neuronal degeneration.

Oxidative stress is established as a major player in the onset and progression of PD. Increased LPO levels are reported in the SN of PD patients and neurotoxicant-induced animal PD models [4,5,52,58,59]. The present study exhibited dose and time dependent augmentation in LPO levels of the nigrostriatal brain

tissues of Zn-treated animals similar to previous studies showing an increased LPO levels by intra-nigral Zn infusion and systemic Zn exposure in rats [5,8,13]. Involvement of free radical generating enzymes viz., NADPH oxidase, xanthine oxidase, nitric oxide synthase and mitochondrial dysfunction is reported in ROS production in sporadic and chemically-induced PD [16,60]. SOD facilitates dismutation of superoxide radicals to form H_2O_2, thereby protecting against free radical-mediated damage. Significant augmentation observed in the total SOD activity might result in the accumulation of end product H_2O_2, which could lead to inhibition of catalase activity due to feedback inhibition obtained in the present study and reported in our previous study also [5].

Differential modulation of cytosolic and mitochondrial SOD is reported in PD patients. A study has shown reduction of Cu,Zn-SOD in the SN of the brain of PD patients, while others have reported increased particulate SOD/Mn-SOD levels in the SN of the brain of sporadic PD patients [4,22,61]. Protective effect of SOD/catalase mimetics against chemically-induced experimental models of PD along with decreased dopaminergic neuronal toxicity in animals over-expressing Cu,Zn-SOD have established the protective role of SOD against oxidative stress-mediated PD pathogenesis [23,62-64]. Zn-induced augmentation of total SOD activity in exposed animals observed in this study is similar to previous reports showing increased SOD levels in PD patients' brain and toxin-induced animal PD phenotypes [22,65]. The increased SOD activity might be the defense mechanism against increased oxidative stress, which is supported by the previous study showing protective action of SOD/catalase mimetic EUK-139 against Zn-induced ROS generation in cortical neuronal cultures [19]. The present study exhibited differential modulation of cytosolic Cu,Zn-SOD/SOD1 and mitochondrial Mn-SOD/SOD2 following Zn exposure, which suggested the role of increased superoxide radical formation via both cytosolic and mitochondrial pathways in Zn-induced oxidative stress in rat nigrostriatal brain tissue. This is in accordance with earlier reports demonstrating the involvement of NADPH oxidase (comprised of cytosolic and plasma membrane bound subunits) and mitochondrial dysfunction in Zn-induced, increased free radical formation in vitro in cortical and microglial cell cultures [21,66]. SOD1 levels were elevated earlier and to a greater extent than the increase observed in SOD2 activity and protein/gene expression of only SOD1 was augmented suggesting that cytosolic pathway might be the major contributor in free radical production and the preceding event in the free radical generation cascade induced by Zn exposure in the rat brain; however, further investigation is needed to establish the actual sequence of the events.

HO-1 is documented to play a pivotal role in anti-oxidative and anti-inflammatory defense against oxidative stress-induced diseases including PD. HO-1 is reported to protect against oxidative stress-induced damage via activation of Nrf-2 transcription factor, which results in promoting cell survival mechanisms [27]. Increased HO-1 expression is reported in the brain of PD patients and HO-1 over-expression is reported to protect dopaminergic neurons against MPTP, rotenone and 6-OHDA-induced cell death [28,67,68]. Zn-induced time dependent increase in HO-1 expression further supported the role of oxidative stress in Zn-induced neurodegeneration and is in sync with the previous reports documenting increased HO-1 activity and expression by oxidative stress-inducing agents [69–71].

Protective role of GSTs, a family of phase II toxicant metabolizing enzymes is well documented in idiopathic PD and chemically-induced PD phenotypes [5,56,72,73]. Altered GST activity/expression is reported in the experimental PD models [56,58,72,73]. GSTs are known to facilitate the detoxification of reactive metabolites of toxicants formed during phase I reactions by conjugation with GSH and therefore the dose and time dependent attenuation of CDNB related GST activity observed in Zn-treated groups implies that either Zn might be directly inhibiting the GST activity or GSH depletion might be responsible for the reduction in GST activity. Since GSTs play a protective role against neurodegeneration, decreased GST activity might contribute to increased neuronal loss following Zn treatment. GSH is the main non-enzymatic antioxidant, which forms the major line of defense against oxidative stress mediated damage, including PD [74]. Reduction in GSH content is reported in both sporadic and chemically-induced PD [4,35]. GSH depletion and protective role of GSH in Zn-induced neurodegeneration is reported in literature but the direct effect of Zn on GCS, a rate limiting enzyme in GSH synthesis, is not yet investigated [13,37]. The present study revealed no significant alterations in the mRNA expression of GCS in Zn-exposed groups suggesting that Zn does not interfere with GSH synthesis at gene level and GSH depletion could be due to an increased consumption of neutralizing free radicals generated by Zn exposure or because of direct chelation of GSH by Zn as suggested earlier in the study with neonatal cortical neurons in vitro [37].

Although it is difficult to conclude that the dose used in this study is equivalent to human exposure, the study would be useful to understand the molecular basis of Zn-induced toxicity and may be extrapolated to humans, as farmers and workers working in Zn-based industries are exposed to this chemical in their day-to-day life. As the exact exposure level in humans is difficult to correlate with the animal exposure directly because the route of exposure in

experimental animals is fixed; however, in humans it may be different, the recurrent exposures to $ZnSO_4$ may result in the accumulation of zinc in the brain with increasing exposure time and may possibly reach the doses equivalent to that used in the present study.

The results obtained thus suggest that Zn-induced oxidative stress exhibited by increased LPO, SOD, HO-1 and decreased catalase and GST activities could be responsible for Zn-induced neurobehavioral, biochemical and molecular changes characteristic of PD phenotype. Differential modulation of SOD1 and SOD2 isozymes by Zn suggests that both cytosolic and mitochondrial pathways might be involved in Zn-induced oxidative stress leading to dopaminergic neurodegeneration.

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Declaration of interest

The authors report no conflicts of interest.

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