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Effect of zinc and paraquat co-exposure on neurodegeneration: Modulation of oxidative stress and expression of metallothioneins, toxicant responsive and transporter genes in rats

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Abstract

Oxidative stress is implicated in Parkinson's disease (PD). Metallothioneins (MT), cytochrome P450 IIE1 (CYP2E1) and glutathione S-transferases alpha4-4 (GSTA4-4) are involved in oxidative stress-mediated damage. Altered dopamine transporter (DAT) and vesicular monoamine transporter-2 (VMAT-2) are also documented in PD. The present study was undertaken to investigate the effect of Zn and PQ co-exposure on neurodegeneration in rats. A significant reduction was observed in spontaneous locomotor activity (SLA), striatal dopamine (DA) levels, tyrosine hydroxylase (TH) immunoreactivity, glutathione reductase (GR) and catalase activity along with increased lipid peroxidation (LPO) and glutathione peroxidase (GPx) activity after Zn and/or PQ exposure. Zn and/or PQ exposure increased gene expression of DAT, CYP2E1, GSTA4-4, MT-I and MT-II, but reduced the expression of VMAT-2. Protein expression analysis of TH, VMAT-2 and DAT showed results similar to those obtained with gene expression study. Zn and PQ co-exposure caused a more pronounced effect than that of individual exposure. The results obtained in this study suggest that, similar to PQ, Zn induced neurodegeneration via alterations in oxidative stress and expression of the above-mentioned genes. However, the effect of Zn+PQ was only slightly higher than that of alone, indicating that probably Zn and PQ follow some different molecular events leading to neurodegeneration.

Keywords: *Paraquat, zinc, oxidative stress, metallothioneins, dopamine transporter genes, toxicant responsive genes*

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Introduction

Parkinson's disease (PD) is a multi-factorial disease contributed to by age, genetic and environmental factors. Recent studies have shown that environmental factors in combination with age and genetic susceptibility play a critical role in PD. Among environmental factors implicated in PD, pesticides, heavy metals, drinking well water and rural habitat are of major importance [1,2]. Epidemiological and experimental studies have shown strong association of heavy metals and pesticides exposure with PD onset [3–7]. Paraquat (1,1'-dimethyl-4,4'-bipyridium; PQ), a widely used herbicide, induces neurodegeneration in both animal models and humans [8–13]. Systemic administration of PQ causes selective degeneration of dopaminergic (DAergic) neurons in mice and its exposure in humans has shown strong association with the increased risk of PD development [9,14]. PQ results in mitochondrial dysfunction and microglial activation leading to increased generation of reactive oxygen species (ROS), which in turn damages DAergic neurons. PQ also decreases the binding of dopamine (DA) to dopamine transporter (DAT) and inhibits DA uptake, thereby disturbing DA homeostasis [15].

Zinc (Zn) is an essential metal required for normal functioning of the brain, particularly during neuronal development. Its deficiency is linked with several physiological and behavioural abnormalities including impaired memory and learning functions, while increased zinc is associated with epilepsy, traumatic brain injury, Alzheimer's disease (AD) and PD [16,17]. Intra-nigral infusion of zinc results in DAergic neuronal

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damage in rat brain [18]. Clinical studies have also reported increased levels of iron (Fe) and Zn in the substantia nigra of brain of sporadic PD patients, implicating the role of heavy metals in PD [19,20]. Zn potentiates 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), DA and iron-induced neurotoxicities [21–23]. Although Zn does not participate directly in free radical generation like iron and copper, Zn-induced neurotoxicity is contributed by oxidative stress. Zn facilitates generation of ROS via activation of NADPH oxidase through protein kinase C in cortical neuron cultures and microglial cell cultures [23–26]. Zn also possesses antioxidant property, which is attributed to metallothioneins (MTs). MTs are cysteine-rich small molecular weight proteins having a free radical scavenging property and play a protective role against oxidative stress-induced toxicity. MTs are mainly induced by toxic or essential metals (zinc, copper, cadmium, mercury, etc.), oxidative stress, pesticides, neurotoxins, radiation, etc. [27–31].

Pesticides, MPTP and heavy metals are reported to decrease striatal DA levels and impair its homeostasis by interfering with either functioning and/or expression of transporters involved in maintaining normal DA levels, i.e. DAT and vesicular monoamine transporter (VMAT-2) [32–37]. DAT facilitates re-uptake of dopamine from the synaptic cleft and VMAT-2 is responsible for its storage inside vesicles in the cytoplasm, thereby regulating DA levels. PQ is known to decrease the dopamine binding to DAT in rats and decreased DAT levels are reported in MB+PQ-induced PD phenotype in mouse, but a direct effect of Zn on DAT levels has not been investigated yet [15,38].

Cytochrome P450s (CYPs) and glutathione S-transferases (GSTs) are involved in the metabolism of several pesticides viz. methoxychlor, anachlor, parathion, mancozeb, PQ, etc. [38–43]. Pesticide metabolism by CYPs results in ROS formation, which subsequently leads to cell death. Involvement of CYP2E1 is established in pesticides-induced free radical generation and in maneb (MB)+PQ-induced PD phenotype in mouse [38,44,45]. Increased level of 4-hydroxy nonenals (4-HNE) is reported in plasma and cerebrospinal fluid (CSF) of PD patients [46] along with increased 4-HNE protein adducts in substantia nigra of PD patients [47], implicating a deleterious role of 4-HNE in PD. Glutathione S-transferase alpha4-4 (GSTA4-4), an isoform of GST alpha, possesses highest affinity for 4-HNE, a major by-product of lipid peroxidation and other hydroxyl alkenals, and facilitates their conjugation with glutathione, therefore GSTA4-4 might play a protective role in these diseases. Increased levels of GSTA4-4 are reported in MB+PQ-induced PD phenotypes and MPTP-induced neurotoxicity in mice [38,46]. GSTA4-4 null mice are also reported to show increased sensitivity against PQ-induced toxicity [48]. The role of GSTA4-4 in Zn-induced neurotoxicity has not yet been investigated.

Epidemiological and experimental data have proved that combined exposure to heavy metals and/or pesticides is a more potent inducer of neurological damage than individual agent and co-exposure also mimics the real life situation as farmers use pesticides and fertilizers simultaneously, thereby being co-exposed to a mixture of metals and/or pesticides [4,10,11,23,38,49]. The present study was, therefore, undertaken to investigate the effect of Zn and PQ co-exposure on neurobehavioural changes, indices of oxidative stress, expression of metallothionein genes (MT-I, MT-II), toxicant responsive genes (CYP2E1, GSTA4-4) and transporter genes (VMAT-2, DAT) in nigrostriatal tissue of rats.

Experimental procedures

Materials

Acetic acid, disodium hydrogen phosphate, glutathione reduced (GSH), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), potassium chloride and sodium dihydrogen phosphate were procured from Sisco Research Laboratories (SRL) (Mumbai, India). Folin Ciocalteau reagent, hydrogen peroxide (H_2O_2), glutathione reductase (GR), and potassium-dichromate were purchased from Merck (Darmstadt, Germany). 1-chloro 2,4-dinitrobenzene (CDNB), dextran, histopaque 1119/1077, paraquat dihydrochloride (PQ), sodium dodecyl sulphate (SDS), thiobarbituric acid (TBA), tri-reagent, and zinc sulphate ($ZnSO_4$) were procured from Sigma-Aldrich (St. Louis, MO, USA). cDNA synthesis kits were procured from MBI Fermentas (York, Ireland, UK). dNTPs, $MgCl_2$, Taq buffer and Taq DNA polymerase were purchased from Bangalore Genei India Pvt. Ltd. (Bangalore, India). Gene specific primers were synthesized from Metabion GmbH (Martinsried, Germany). Monoclonal anti-TH antibody, biotinylated anti-mouse secondary antibody for and rabbit anti-VMAT2 antibody were procured from Sigma Aldrich. Polyclonal goat anti-DAT, monoclonal anti-β-actin antibody, bovine anti-mouse alkaline phosphatase (AP) conjugated, rabbit anti-goat AP conjugated and bovine anti-rabbit (AP) conjugated secondary antibodies were from Santacruz Biotechnology (Santa Cruz, CA, USA). Streptavidin-peroxidase, normal goat serum and DAB system were procured from Bangalore Genei Pvt. India Ltd. Polyvinylidene difloride (PVDF) membrane was procured from Millipore Corporation (MA, USA).

Animal treatment

Male Wistar rats (150–180 g) were obtained from the animal facility centre of Indian Institute of Toxicology Research (IITR), Lucknow. The study was approved by the Institutional Animal Ethics Committee. The

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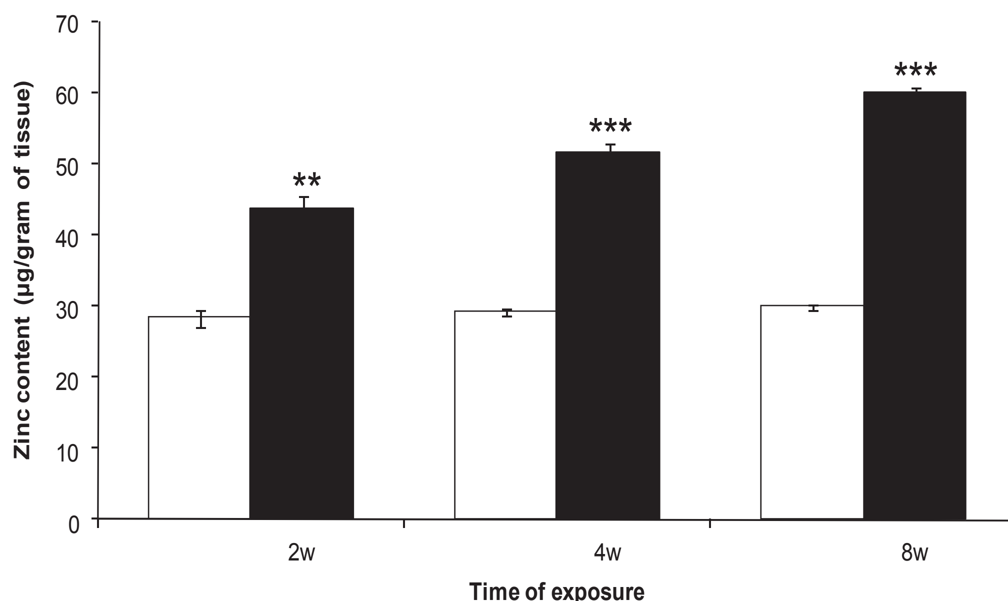

Figure 1. Effect of intraperitoneal Zn administration on the Zn content in the nigrostriatal tissue of brain in rats following 2, 4 and 8 weeks of exposure. First and second bar of each panel represents control and Zn-treated groups, respectively ($^{***}p < 0.001$ and $^{**}p < 0.01$ as compared with control).

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animals were maintained under standard conditions of temperature and humidity with 12 h light/dark cycle and fed *ad libitum*. Animals were divided into controls, zinc sulphate (Zn), paraquat (PQ) and Zn+PQ treated groups. Zn (20 mg/kg body wt.) and PQ (5 mg/kg body wt.) were administered intraperitoneally either alone or in combination twice a week for 2, 4 and 8 weeks. Control animals received normal saline.

Decapitation and dissection of brain

Animals were sacrificed by cervical dislocation followed by decapitation to ensure minimum pain. The brain was dissected out and frozen immediately. Dissection of brain was performed in ice cold conditions to isolate nigrostriatal and striatal tissue, which were stored at −80°C until further use.

Zinc estimation in brain tissue

In order to assess the effect of intraperitoneal zinc administration on the brain, zinc concentration was estimated in nigrostriatal tissue by a flame atomic absorption spectrophotometer (Analytikjena ZEEnit 700, Jena, Germany) in control and treated groups by a standard procedure described elsewhere [50]. Standard zinc graph was used to calculate the zinc concentration in experimental samples.

Spontaneous locomotory activity (SLA)

SLA in an open field was determined by an Opto-Varimex animal activity meter. In brief, rat was placed in the chamber for 1 min and locomotor activity was measured for a further 5 min. SLA was recorded in both control and treated animals. Results are expressed in terms of total distance travelled in cm/5 min.

Immunohistochemical analysis (IHC)

IHC staining of tyrosine hydroxylase (TH)-positive DAergic neurons was performed in rat brain sections of control and treated groups using standard procedure [51]. Rats were ether anaesthetized and intracardiac perfusion was performed using chilled normal saline followed by ice-cold paraformaldehyde (4%) in phosphate buffered saline (PBS). Briefly, the perfused rat brains were post-fixed with paraformaldehyde, cryoprotected in sucrose (10%, 20% and 30% in PBS) and sections (20 µm thick) were cut serially using a cryostat. Sections were incubated with 0.5% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity followed by incubation in blocking buffer (5% normal goat serum, 1% BSA and 0.1% Triton X-100 in PBS) for 2 h. The sections were incubated in monoclonal anti-TH antibody (1:2000) at 4°C for 48 h, washed with PBS three times for 15 min each and then labelled with biotinylated secondary antibody (1:300) for 2 h and streptavidin peroxidase complex for 30 min. The colour was developed with 3, 3 diaminobenzidine and the sections were permanently mounted with DPX after dehydrating them in graded ethanol [52]. The mounted sections were examined under bright field microscopy (Leica Mikroskopie-GMBH; Wetzlar, Germany) and images were captured at 10× magnification. Counting of TH-positive cells was also done using a standard procedure reported earlier [53]. In brief, an experimenter coded the slides

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and another experimenter performed cell counting of TH-positive neurons. The person who performed unbiased counting was unaware of the treatment schedule. The first section was taken at a fixed distance from the Bregma and TH-positive neurons were counted bilaterally in every fourth serial section. TH-positive neurons were counted in three-to-four fields per section and the cell number per section was calculated. Average number of cells per slice was calculated by dividing total cell number by the slice number. A minimum of three animals were assessed for TH-immunoreactive neurons in each group. The results are expressed as a percentage of control.

Dopamine content

Striatal dopamine content was estimated through HPLC method described elsewhere [54]. Striatum from brain of control and treated rats were suspended in 0.1 M perchloric acid, homogenized for 20 s, sonicated for 30 s and centrifuged at 21 000 ×g for 30 min at 4°C. The supernatant was filtered and dopamine levels were detected by HPLC in a mobile phase consisting of 1 mM heptane sulphonic acid, 0.1 M potassium phosphate and 10% methanol (pH 4.0) using reverse phase C-18 column at a constant flow rate of 1 ml/min. The dopamine content was determined by taking the ratio of known amounts of dopamine and a constant amount of internal standard dihydrobenzylamine (DHBA). The values were calculated as ng dopamine/mg tissue.

Oxidative stress indices

Oxidative stress was assessed in control, Zn and/or PQ exposed animals by measuring lipid peroxidation (LPO) levels along with catalase, glutathione reductase (GR) and glutathione peroxidase (GPx) activities in the nigrostriatal tissue. LPO level was assessed using the method described by Ohkawa et al. [55]. The final product formed in the assay was read at 532 nm and results are expressed as nmoles MDA/mg tissue. Catalase was estimated by spectrophotometric method at 570 nm as described by Sinha [56]. The enzymatic activity was calculated in μmoles/min/mg protein. GR and GPx activities were measured in nigrostriatal tissue by standard methods as described earlier [57,58]. The enzyme activities are expressed as nM/min/mg protein.

Protein estimation

Protein content was estimated in control and treated groups using Lowry method [59]. Protein concentration was calculated using the standard curve of bovine serum albumin (BSA).

Amplification of MT-I, MT-II, CYP2E1, GSTA4-4, VMAT-2 and DAT genes by RT-PCR

Total RNA was isolated from nigrostriatal tissue of control and treated rats using Trizol as per standard procedure. cDNA was synthesized using total RNA (5 μg) by RT-Mul M reverse transcriptase kit as per manufacturer's protocol. Amplification of MT-I, MT-II, CYP2E1, VMAT-2, DAT and GAPDH genes was carried out as reported elsewhere. The primers used are given below: For MT-I: forward primer: 5'-GAATTCCGTTGCTCCAGATTCACCA-GATC-3', reverse primer: 5'-GAATTCTCACATGC TCGGTAGAAAACGG-3' and MT-II: forward primer: 5'-TAGATCTCCACCTGCCGCCTCCA-3', reverse primer: 5'-TAGATCTACACCATTGTGAG-GACG CCC-3' [60]; for CYP2E1: forward primer: 5'-CTCCTCGTCATATCCATCTG-3', reverse primer: 5'-GCAGCCAATCAGAAATGTGG-3' [61]; for GAPDH: forward primer, 5'-CCATGGAGAAGGCT GGGG-3', reverse primer, 5'- CAAAGTTGTCATG GATGACC-3' [62]; for VMAT-2: forward primer: 5'-CCTCTAACGTCGCCAAATGT-3', reverse primer: 5'-TAACGCGCTGTCTTACATGG-3' and for DAT: forward primer: 5'-GGTCAACAATGTC-CCTGTCC-3', reverse primer: 5'-GGGTGTGTCT CCCAACCTTA-3' [63]. GSTA4-4 gene primers were designed using DNA star software: forward primer: 5'-TCGATCTGCTGGCTGCTGGTT-3', reverse primer: 5'-CTGCCCGACTGAGTTGGTTGC-3' and conditions used were: denaturation at 95°C/5 min followed by 30 cycles of denaturation at 95°C/1 min, annealing at 61°C/1 min and extension at 72°C/2 min and final extension at 72°C for 7 min. The PCR products were visualized through agarose gel electrophoresis and densitometry was performed using Alpha Imager software taking GAPDH as a reference.

Western blotting

The expression of TH, DAT and VMAT-2 proteins in nigrostriatal tissue of control and treated animals was analysed using Western blotting technique. In brief, denatured proteins were resolved on 10% SDS-polyacrylamide gel 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 to block non-specific binding and subsequently incubated with primary antibodies against TH (1:5000), DAT (1:1000) and VMAT-2 (1:500) for 2 h. Alkaline phosphatase (AP) conjugated anti-mouse, anti-goat and anti-rabbit secondary antibodies in TBS-T buffer were used to detect TH, DAT and VMAT-2, respectively. Blots were visualized using BCIP/NBT as substrates. Relative band density was calculated with β-actin as the reference and data are expressed

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as mean ± SE of band density ratio of at least four experiments.

Statistical analysis

Results are expressed as mean ± standard error of means (SEM) for separate groups. Two-way analysis of variance (ANOVA) was used for statistical analysis. Bonferroni post-test was used for multiple comparisons. The differences were considered statistically significant when p-value was less than 0.05.

Results

Zinc concentration in nigrostriatal tissue

Intraperitoneal administration of zinc sulphate exhibited a significant and time of exposure dependent increase in the nigrostriatal Zn concentration of treated animals following 2, 4 and 8 weeks of exposure (Figure 1).

Locomotor activity and striatal dopamine content

Zn alone also produced significant reduction in SLA in a time-dependent manner similar to PQ and combined treatment showed a more pronounced effect as compared to either of the individual treatments after 4 weeks of exposure. Maximum decrease was obtained in the Zn+PQ treated group after 8 weeks of exposure, which was significantly greater than Zn and PQ alone (Figure 2A). Zn and PQ either alone or in combination reduced striatal dopamine content significantly and in an exposure time-dependent manner. Co-exposure resulted in higher reduction than either Zn or PQ alone. Eight weeks of combined exposure resulted in maximum reduction of striatal dopamine content, which was significantly greater than either Zn or PQ (Figure 2B).

TH-immunoreactivity

The effect of Zn and/or PQ treatment groups on DAergic neurons in substantia nigra region of brain was estimated by immunohistochemical (IHC) analysis of TH-positive DAergic neurons in frozen brain sections. The number of TH-positive neurons in controls was 344.7 ± 19.5. This is in accordance with the previously reported observations [64]. The values of the treated groups are expressed in terms of percentage of control. A significant reduction in TH-positive DAergic neurons in the substantia nigra (SN) region of the brain was observed in treated groups as compared with controls. The decrease was dependent on time of exposure, i.e. 8 weeks of exposure resulted in maximum degeneration of DAergic neurons. Combined treatment with Zn+PQ resulted in greater loss of DAergic neurons than Zn or PQ alone (Figures 3A and B).

Oxidative stress parameters

Lipid peroxidation. Zn and/or PQ significantly augmented LPO level in the nigrostriatal tissue of brain of treated animals in a time of exposure-dependent manner. Zn and PQ in combination yielded greater augmentation in lipid peroxidation as compared with individual treatment and 8 weeks of co-exposure resulted in maximum increase in LPO (Figure 4A).

Catalase, GR and GPx activity. Zn and/or PQ significantly reduced catalase and GR activity and combined exposure yielded a more pronounced effect than Zn or PQ alone. Maximum reduction in catalase and GR activity was obtained in the co-exposed group after 8 weeks of treatment (Figures 4B and C).

Contrary to catalase and GR, Zn and PQ treatment augmented GPx activity in the nigrostriatal region of the brain of exposed animals either alone or in combination. Augmentation was more pronounced in the co-treated group. Maximum augmentation was observed in co-treated animals after 8 weeks of exposure (Figure 4D).

Expression of MT-I, MT-II, CYP2E1, GSTA4-4, VMAT-2 and DAT genes

Both Zn and PQ induced expression of MT-I gene and Zn was a more potent inducer of MT-I than PQ. Co-exposure with Zn+PQ resulted in increased induction of MT-I (Figure 5A). MT-II gene was also induced significantly by Zn and PQ but combined treatment resulted in significantly higher induction of MT-II (Figure 5B). Expression of toxicant responsive genes-CYP2E1 and GSTA4-4 was also increased significantly by Zn and/or PQ and combined treatment resulted in significantly greater augmentation than individual treatment (Figures 6A and B). DAT expression was increased in a time of exposure-dependent manner following Zn and/or PQ exposure and combined exposure showed significantly higher augmentation in DAT expression than either Zn or PQ alone. On the other hand, VMAT-2 expression was attenuated significantly by Zn and/or PQ exposure. Although a greater decrease was observed following Zn and PQ co-exposure, it was not significantly higher than Zn or PQ alone (Figures 7A and B).

Western blot analysis

Expression of TH, DAT and VMAT-2 proteins was also analysed in control, Zn and/or PQ exposed animals using Western blotting technique. Western blot analysis of TH showed a significant and time

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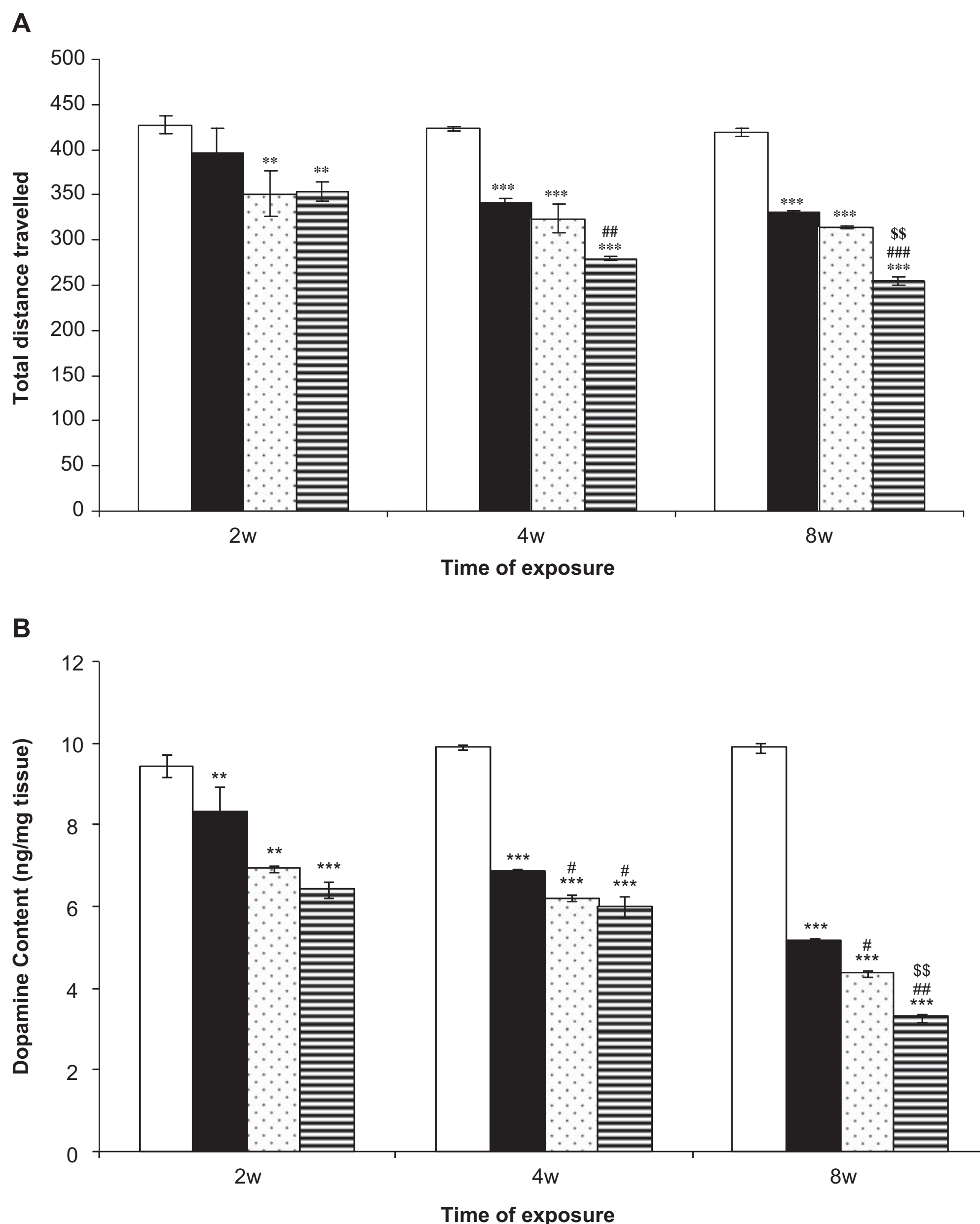

Figure 2. (A) Effect of zinc and/or PQ on SLA on exposed rats following 2, 4 and 8 weeks of exposure. (B) Effect of Zn and/or PQ on striatal dopamine content in control and exposed rats. Data are expressed as mean ± SE (n = 5–7). ($^{***}p < 0.001$ and $^{**}p < 0.01$ as compared with control; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ as compared with Zn treated group; $^{\$\$}p < 0.01$ as compared to PQ treated group). First, second, third and fourth bar in each panel represents control, Zn, PQ and Zn+PQ treated groups, respectively.

of exposure-dependent decrease in protein expression following exposure with Zn and/or PQ, which correlated with the results obtained by IHC analysis. Combined treatment yielded greater attenuation than the individual exposure (Figure 8A). A significant augmentation was observed in protein expression of DAT, which was in concurrence with the RT-PCR results mentioned above. Zn+PQ in combination were more effective in causing augmentation of DAT protein than Zn or PQ alone (Figure 8B). Western blotting of VMAT-2 showed significant attenuation of protein expression following exposure to Zn and/or PQ. The reduction in protein level was time of exposure-dependent, i.e. 8 weeks of exposure showed maximum effect and Zn+PQ yielded slightly greater reduction than Zn or PQ alone (Figure 8C).

Discussion

Epidemiological and experimental studies have established a strong association of environmental factors with PD [4,10,11,38,49]. Pesticides and heavy metals are implicated as major environmental risk factors for PD [3,4,7]. The synergistic effect of iron with PQ and zinc in inducing neurodegeneration has been reported in animal models [23,49]. The increased accumulation of iron and zinc is reported in the substantia

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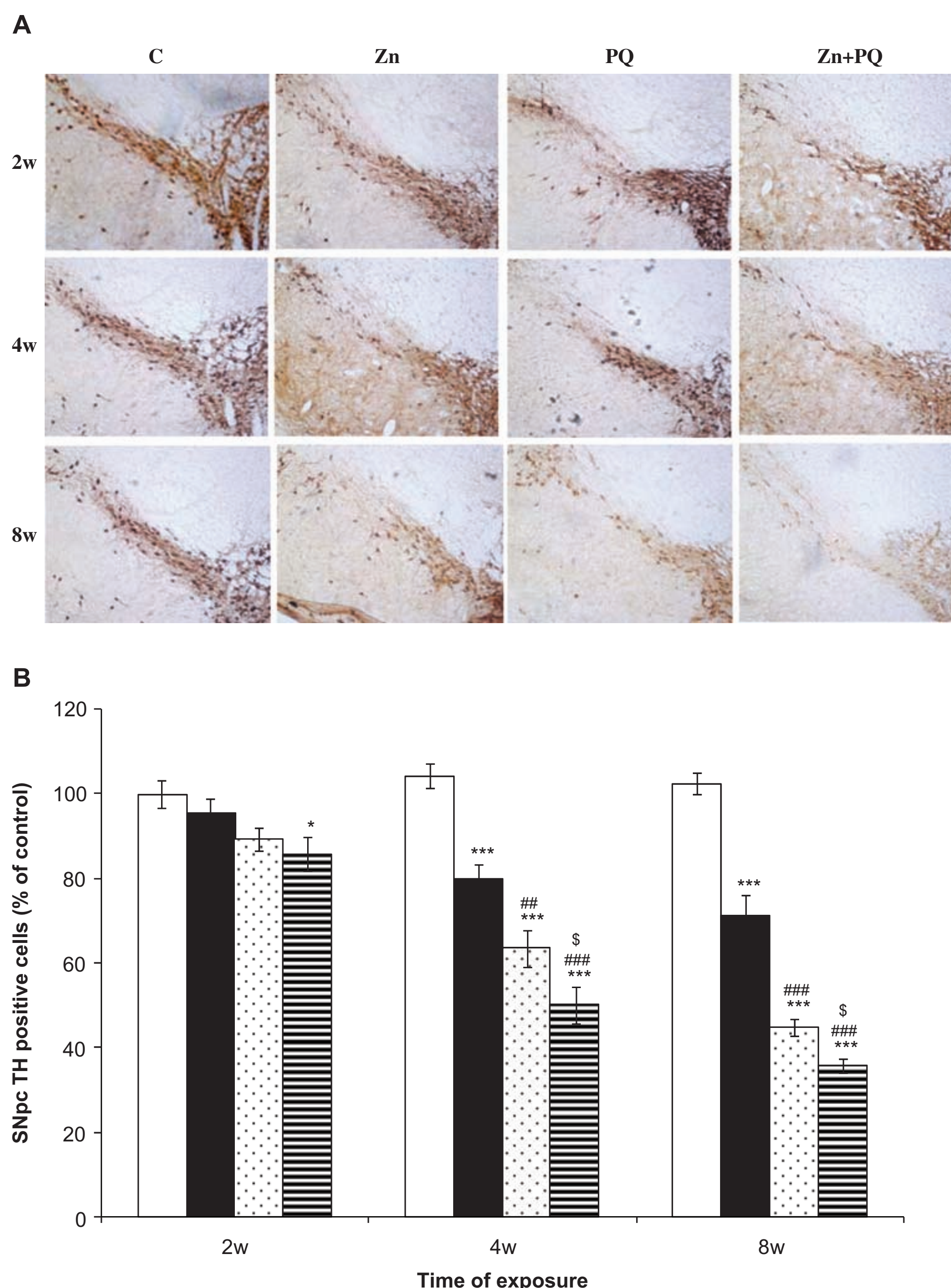

Figure 3. Effect of Zn and/or PQ exposure on TH immunoreactivity in the substantia nigra of rat brain following 2, 4 and 8 weeks of treatment. (A) Upper panel shows TH immunoreactivity in frozen brain sections of control and treated animals. (B) Lower panel shows number of TH positive neurons in substantia nigral region of control and treated groups. ($^{***}p < 0.001$ and $^{*}p < 0.05$ as compared with control; $^{\#\#\#}p < 0.001$ and $^{\#\#}p < 0.01$ as compared with Zn treated group; $^{\$}p < 0.05$ as compared to PQ treated group). First, second, third and fourth bar in each panel represents control, Zn, PQ and Zn+PQ treated groups, respectively.

nigra of PD patients, implicating their roles in PD pathogenesis [20]. Although zinc does not directly generate ROS, oxidative stress is suspected to be the key player in zinc-induced neurotoxicity [18].

Parkinson's disease is characterized by the progressive and selective degeneration of dopaminergic neurons of the nigrostriatal pathways that include the striatum and the substantia nigra pars compacta. The loss of dopaminergic neurons of the substantia nigra reduces dopamine level in the striatum, as these neurons project into the striatum. Dopamine, a neurotransmitter, stimulates motor neurons and controls movement. The depletion of dopamine in the striatum leads to the loss of control over movement and co-ordination, which results into major clinical symptoms of PD [65]. As the cell bodies of these neurons lie in the substantia nigra and axons project into the striatum, therefore, in the present study, most of the parameters were assessed in the nigrostriatal tissues and dopamine in the striatum.

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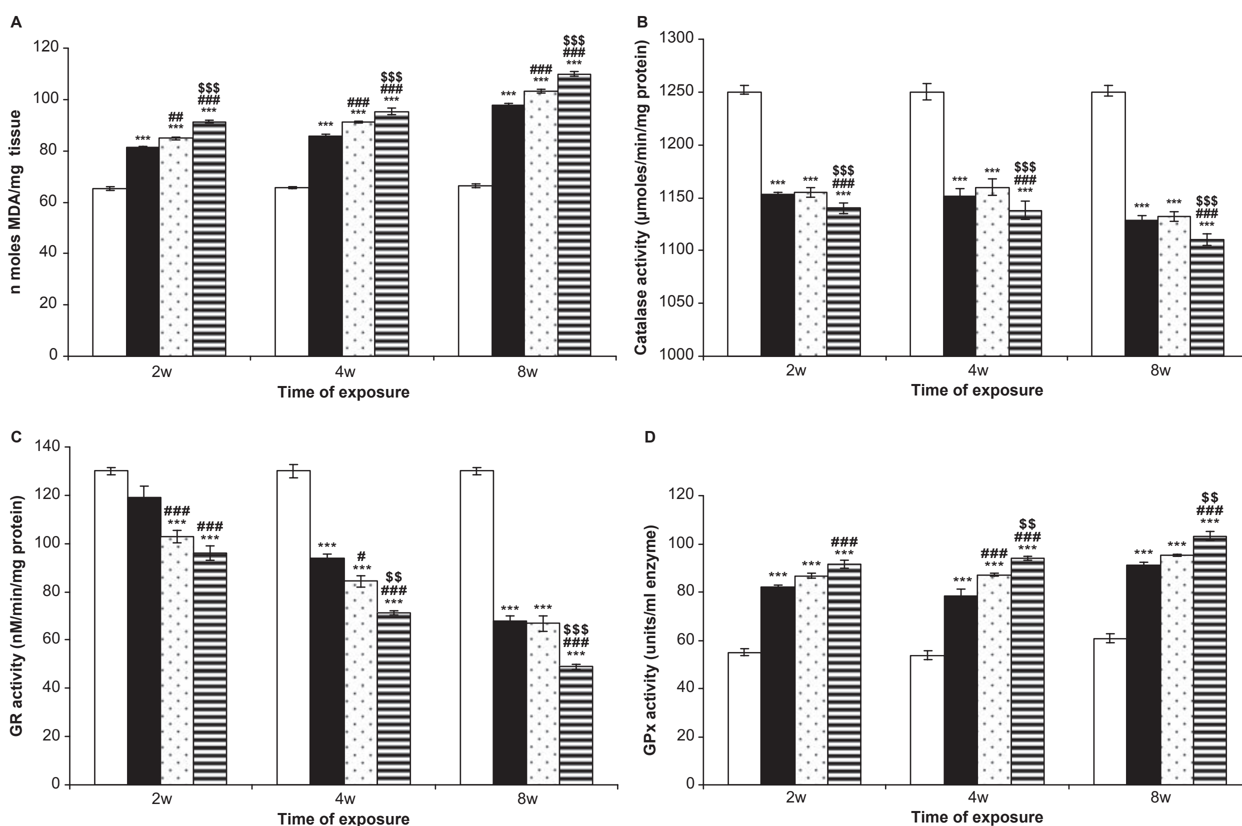

Figure 4. (A) Alterations induced by Zn and/or PQ on lipid peroxidation in the nigrostriatal tissue of control and exposed animals. (B) Catalase activity in nigrostriatal region of brain of control, Zn, PQ and Zn+PQ exposed animals. (C) Effect of Zn and/or PQ on GR activity of nigrostriatal tissue of control and exposed rats. (D) Alterations caused by Zn and/or PQ in GPx activity of nigrostriatal tissue of control and treated groups. Data are expressed as mean ± SE (n = 5–7). ($^{***}p < 0.001$ as compared with control; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ as compared with Zn treated group and $^{\$\$\$}p < 0.001$ and $^{\$\$}p < 0.01$ as compared with PQ treated group). First, second, third and fourth bar in each panel represents control, Zn, PQ and Zn+PQ treated groups, respectively.

Intraperitoneal zinc administration led to an increase in the zinc level in the nigrostriatal tissue of treated animals as also reported in a previous study conducted by Opoka et al. [66]. The basal value obtained in the present study was in accordance with previous observations reported elsewhere [67,68].

The reduced locomotor activity, muscular rigidity and dopamine depletion are major characteristic symptoms of PD. PQ-induced locomotor abnormality and reduced striatal DA content is in accordance with earlier studies showing induction of rotations and reduced dopamine content following intra-nigral injection of PQ in rats and systemic exposure of MB+PQ induced alterations in locomotor activity and decreased DA levels in mouse model for PD phenotype [69,70]. Zn alone decreased SLA and DA content at the tested dose, implying that Zn also induced neurobehavioural and biochemical changes characteristic of PD phenotype similar to PQ. Zn and PQ in combination showed further attenuation in these parameters. Dopamine metabolism plays a vital role in normal functioning of brain and alterations in DA metabolism, i.e. either decreased dopamine synthesis or increased dopamine catabolism is associated with PD progression [21,71]. Decrease in striatal dopamine content observed after PQ and Zn exposure was in concurrence with earlier reports showing a reduction in dopamine levels in rodents following PQ exposure [69] and intra-nigral infusion of Zn showing a decrease in dopamine content after 7 days [18]. Zinc is also reported to enhance MPTP-induced reduction of striatal dopamine levels in mice brain [21].

TH-immunoreactivity, characteristic of DAergic neurons, assessed in this study showed a significant reduction in the SN region of the brain of Zn and/or PQ exposed groups, confirming that reduced DA levels obtained by HPLC analysis were due to the loss of DAergic neurons and not due to increased DA metabolism. The loss of TH immunoreactivity was found to be time of exposure-dependent. Combined treatment resulted in more increased degeneration of DAergic neurons than Zn or PQ alone. The results of the present study are in accordance with earlier reports showing selective DAergic neuronal loss following exposure to several PD-inducing neurotoxins including pesticides like rotenone, PQ and MB+PQ in rodents [9,11,69,71,72].

Oxidative stress is established to precede PQ-induced neurodegeneration [38,70,73,74]. The

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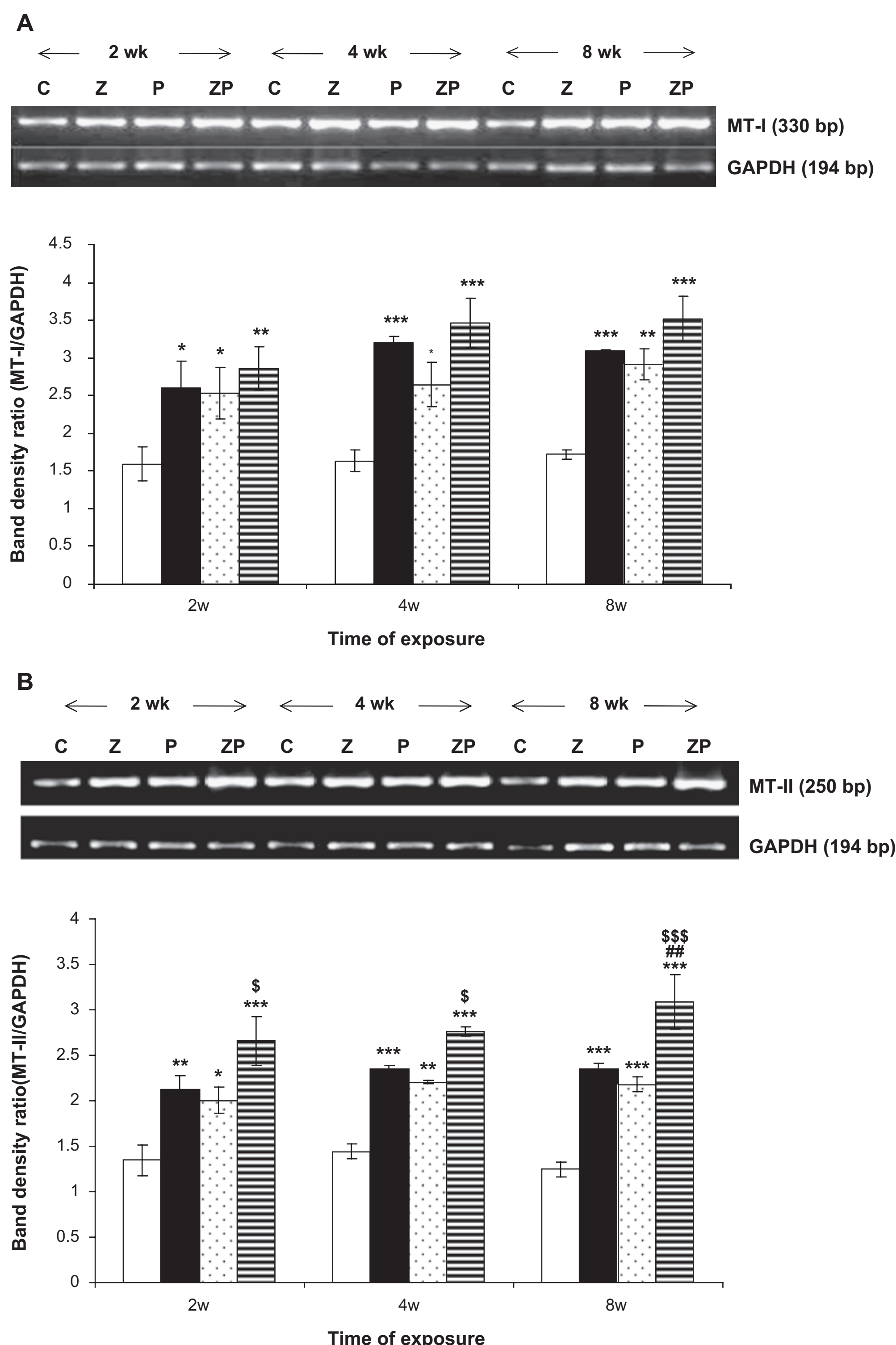

Figure 5. Effect of Zn and/or PQ on the gene expression of MT-I and MT-II in nigrostriatal tissue in brain of exposed rats. (A) Upper panel shows representative picture of effect of Zn and/or PQ on the gene expression of MT-I after 2, 4 and 8 weeks of exposure. Lower panel shows densitometric analysis of the same with GAPDH as a reference. (B) Upper panel shows representative picture of effect of Zn and/or PQ on the expression of MT-II gene after 2, 4 and 8 weeks of exposure. Lower panel shows densitometric analysis of expression of MT-II gene with GAPDH as a reference. ($^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ as compared with control; $^{\#\#}p < 0.01$ as compared with Zn treated group; $^{\$\$\$}p < 0.001$ and $^{\$}p < 0.05$ as compared to PQ treated group). First, second, third and fourth bar in each panel represents control, Zn, PQ and Zn+PQ treated groups, respectively.

reduction in GR and catalase activity and increased LPO levels observed by PQ exposure reaffirmed the role of oxidative stress in PQ-induced toxicity. Zn alone exhibited identical results as PQ, but combined treatment resulting in more increased oxidative stress than individual exposure suggested that Zn also contributed in oxidative stress generation. The increased GPx activity might be a defense

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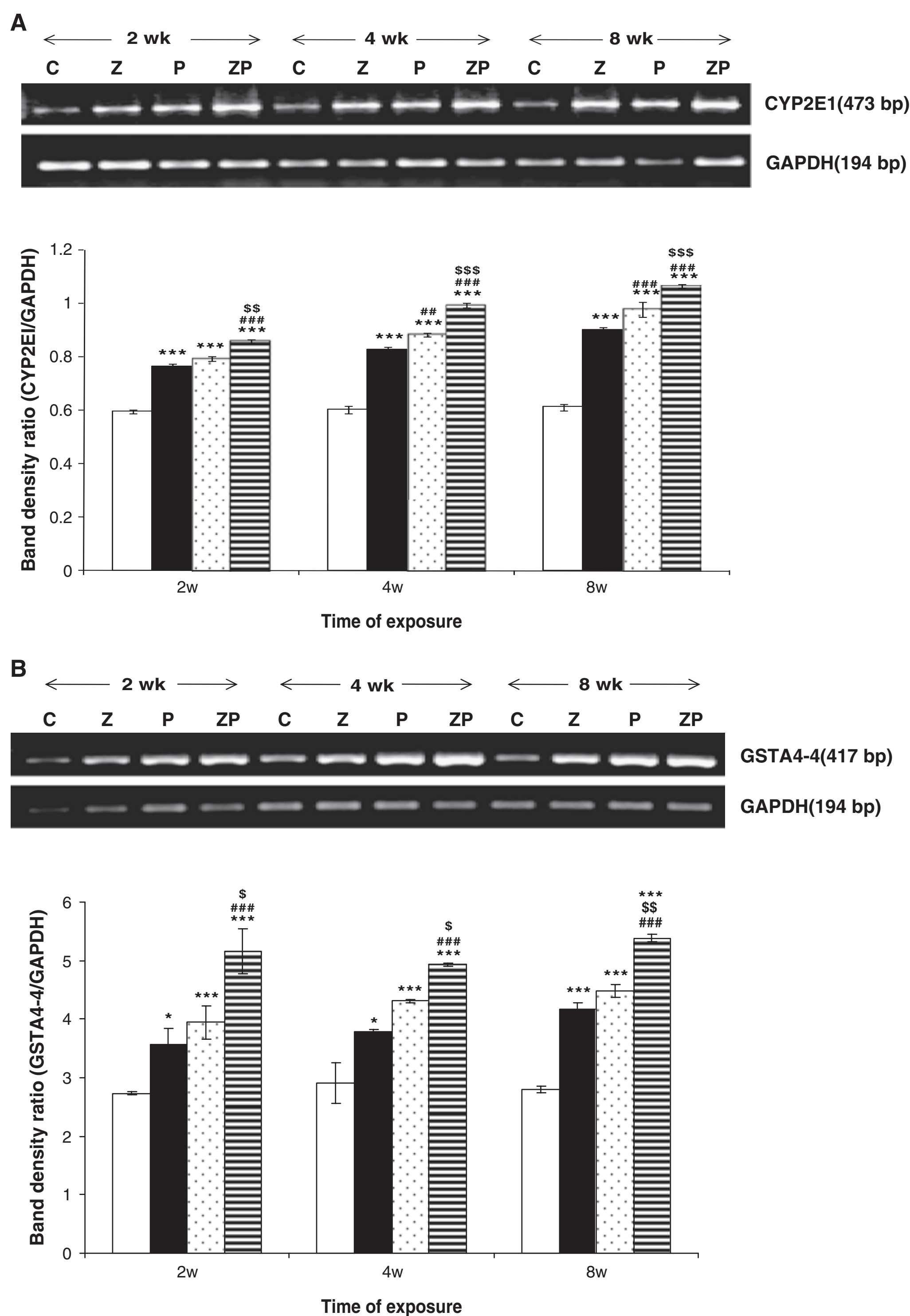

Figure 6. Analysis of gene expression of CYP2E1 and GSTA4-4 following Zn and/or PQ exposure in nigrostriatal tissue of rat brain. (A) Upper panel shows representative picture of effect of Zn and/or PQ on the expression of CYP2E1 gene after 2, 4 and 8 weeks of exposure. Lower panel shows densitometric analysis of the same with GAPDH taken as a reference. (B) Upper panel shows representative picture of effect of Zn and/or PQ on the expression of GSTA4-4 gene after 2, 4 and 8 weeks of exposure. Lower panel shows densitometric analysis of the same with GAPDH taken as a reference. ($^{***}p < 0.001$ and $^{*}p < 0.05$ as compared with control; $^{\#\#\#}p < 0.001$ and $^{\#\#}p < 0.01$ as compared with Zn treated group; $^{\$\$\$}p < 0.001$, $^{\$\$}p < 0.01$ and $^{\$}p < 0.05$ as compared to PQ treated group). First, second, third and fourth bar in each panel represents control, Zn, PQ and Zn+PQ treated groups, respectively.

mechanism to compensate for reduced catalase activity. The results from the present study show that exogenous Zn exposure induces oxidative stress *in vivo*. This is in accordance with previous findings that have shown an increase in oxidative stress after intra-nigral injection of Zn in rat brain and also in cortical and astrocytes cell cultures following Zn treatment [18,75].

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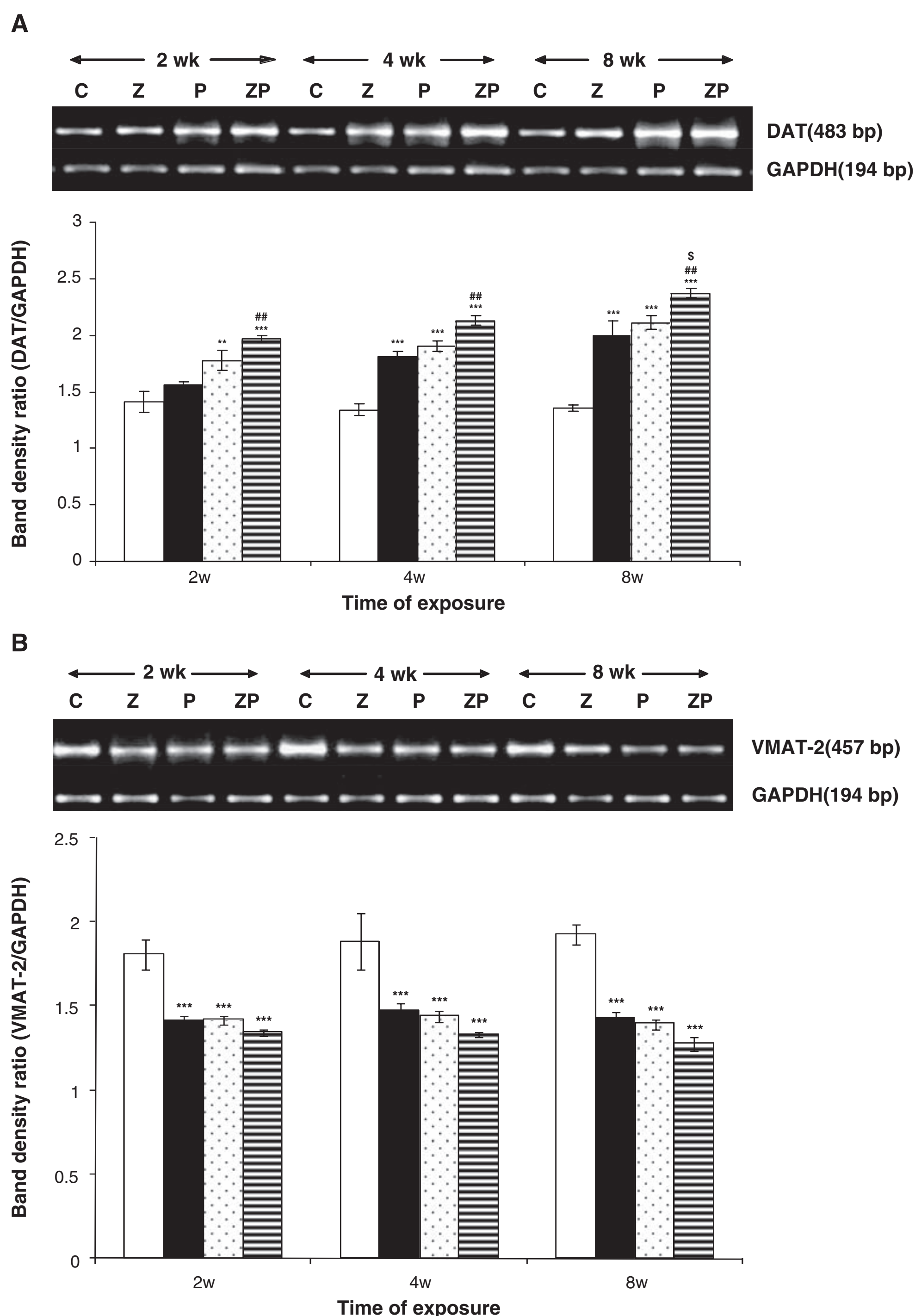

Figure 7. Effect of Zn and/or PQ exposure on gene expression of DAT and VMAT-2 in the nigrostriatal tissue of rat brain. (A) Upper panel shows representative picture of effect of Zn and/or PQ on the expression of DAT gene after 2, 4 and 8 weeks of exposure. Lower panel shows densitometric analysis of the same with GAPDH taken as a reference. (B) Upper panel shows representative picture of effect of Zn and/or PQ on the expression of VMAT-2 gene after 2, 4 and 8 weeks of exposure. Lower panel shows densitometric analysis of expression of VMAT-2 gene with GAPDH taken as a reference. ($^{***}p < 0.001$ and $^{**}p < 0.01$ as compared with control; $^{\#\#}p < 0.01$ as compared with Zn treated group and $^{\$}p < 0.05$ as compared to PQ treated group). First, second, third and fourth bar in each panel represents control, Zn, PQ and Zn+PQ treated groups, respectively.

MTs are cysteine rich, small molecular weight, metal binding proteins having free radical scavenging properties, and play a protective role against oxidative stress. MTs are mainly induced by heavy metals and oxidative stress-inducing agents and also involved in the detoxification of heavy metals [27,76–79]. Reports showing MT-I induction by PQ in liver, lung and kidney of rodents are also available [80–82]. Our study also exhibited induction of MT-I and MT-II by Zn and PQ in nigrostriatal tissue of exposed rats. Combined treatment resulting in more pronounced MT induction suggest that increased MT expression could

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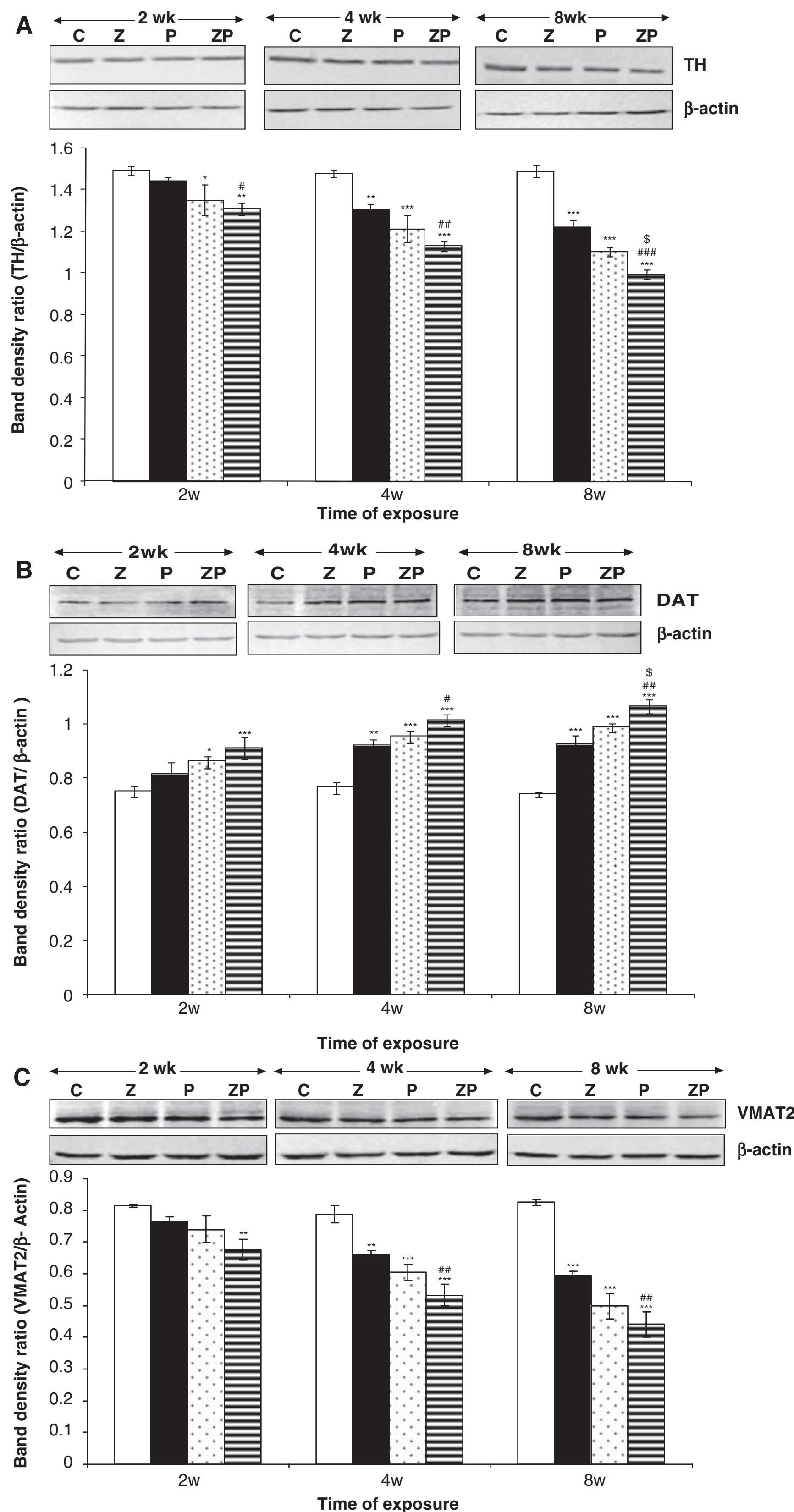

Figure 8. Western blot analysis of TH, DAT and VMAT-2 proteins in nigrostriatal tissue of rat brain following Zn and/or PQ treatment. (A) Upper panel depicts the representative blot of TH following 2, 4 and 8 weeks of exposure of Zn and/or PQ and lower panel shows densitometric analysis of the same with β-actin as the reference. (B) Upper panel depicts the representative blot showing expression of DAT protein in control, Zn and/or PQ treated groups and lower panel shows densitometric analysis of the same with β-actin as the reference. (C) Upper panel represents Western blot for VMAT-2 protein expression following exposure of Zn and/or PQ and lower panel shows densitometric analysis of the same with β-actin as the reference. Data are expressed as mean ± SE (n=4–5). ($^{***}p < 0.001$ and $^{**}p < 0.01$ as compared with control; $^{\#\#\#}p < 0.001$ and $^{\#\#}p < 0.01$ as compared with Zn treated group and $^{\$}p<0.05$ as compared with PQ treated group). First, second, third and fourth bar in each panel represents control, Zn, PQ and Zn+PQ treated groups, respectively.

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be a defense mechanism against oxidative stress generated by Zn and/or PQ due to the antioxidant property of MTs. Co-treatment resulted in increased oxidative stress, therefore yielded higher MT induction than that of individually exposed groups.

PQ-induced CYP2E1 expression observed in our study is in accordance with an earlier study showing CYP2E1 induction in MB+PQ-induced PD phenotype in mouse model [38]. In this study, Zn induced CYP2E1 expression both alone as well as in combination with PQ, suggesting that CYP2E1 might be involved in increasing free radical generation after Zn treatment, thereby enabling it to enhance the PQ-induced ROS production and finally aggravating PQ-induced neurotoxicity. GSTs, a family of phase II detoxification enzymes, participate in pesticides metabolism. GSTA4-4 is particularly involved in the neutralization of 4-HNE and other hydroxyl alkenals formed as by-products of lipid peroxidation, thus providing protection against oxidative stress-induced toxicity. Total GST activity and expression of GSTA4-4 is reported to increase in the striatum of MB+PQ-induced PD phenotype [38]. Increased sensitivity to PQ-induced toxicity in GSTA4-4 null mice confirms the protective role of GSTA4-4 against oxidative stress-mediated damage caused by PQ [48]. The increased level of 4-HNE in plasma and CSF and augmented levels of protein adducts of 4-HNE in SN of PD patients are also documented [46]. Since GSTA4-4 possesses high affinity for detoxifying 4-HNE, it might play a protective role against diseases involving oxidative stress-induced damage. The present study therefore, investigated the effect of Zn or PQ exposure on GSTA4-4 in exposed animals. A significant increase observed in GSTA4-4 expression after Zn and/or PQ exposure in this study further confirms the role of oxidative stress in Zn and/or PQ-induced neurotoxicity and implies that both these act via a common mechanism. The augmented GSTA4-4 expression may be the defense mechanism to protect against damage caused by 4-HNE and other by-products formed via increased lipid peroxidation induced by Zn and/or PQ treatment.

Several chemically-induced PD models developed for understanding the mechanism of PD have shown modulation of dopamine metabolism by alteration in the expression or functioning of VMAT-2 and DAT [33–35,71,83]. Both proteins play a critical role in dopamine homeostasis and alterations in these transporters might result in neuronal damage. DAT facilitates re-uptake of dopamine from synaptic cleft following neurotransmission, thus maintaining intercellular levels of dopamine. VMAT-2, on the other hand, helps in the storage of dopamine in the vesicles inside the cell and protects the neurons from oxidative stress-induced damage caused by ROS produced via auto-oxidation of dopamine. Modulation of DAT and VMAT-2 at gene and protein levels is reported in a MPTP-induced PD model [32,33,84]. Rotenone, another PD-inducing pesticide, up-regulates DAT expression and down-regulates VMAT-2 expression in PC-12 neuronal cell lines leading to oxidative stress-induced cell death [83]. PQ is reported to decrease binding of dopamine with DAT and Zn is also reported to modulate DAT functioning by causing conformational changes in DAT, which results in the inhibition of uptake/influx process of DAT [15,85], but the effect of Zn alone and in combination with PQ has not yet been reported. The present study exhibited increased gene and protein expression of DAT by PQ and/or Zn exposure, suggesting that this could either be a compensatory mechanism against decreased binding of dopamine to DAT or Zn and PQ might be affecting DAT at the level of function as well as protein expression, thereby disturbing the dopamine homeostasis. The increased DA accumulation inside the cytoplasm via increased DAT results in increased ROS generation through auto-oxidation of DA and may lead to oxidative stress-induced neuronal degeneration. As DAT and VMAT-2 are responsible for dopamine homeostasis, we also investigated the effect of PQ and Zn on VMAT-2 gene expression and protein levels. PQ and/or Zn both attenuated VMAT-2 gene expression as well as its protein levels and combined treatment showed greater attenuation than individual exposure. Some disparity was observed between RT-PCR and Western blotting results, which might be due to the semi-quantitative nature of the RT-PCR technique. An increase in DAT protein upon combined treatment with Zn and PQ along with decreased VMAT-2 protein expression might result in accumulation of dopamine in the cytoplasm, which would result in production of ROS via its auto oxidation and subsequently lead to dopaminergic neuronal death causing PD-like symptoms. Western blot of TH also supported the IHC and striatal dopamine data confirming that reduction in DA level was due to loss of TH positive neurons and not due to increased breakdown of dopamine. This further supports our hypothesis that Zn also causes degeneration of dopaminergic neurons like PQ.

The results obtained from the present study showed that zinc alone induced neurodegeneration by modulation of oxidative stress, toxicant responsive genes (CYP2E1 and GSTA4-4), metallothioneins (MT-I and MT-II) and dopamine transporter proteins (DAT and VMAT-2) similar to PQ. Zn and PQ in combination resulted in increased alterations in the levels of assessed parameters; however, the effect was neither additive nor synergistic and could possibly be the result of different molecular events involved in Zn and PQ-induced neurodegeneration. Thus, from the results of the present study we can conclude that Zn and PQ alter the assessed parameters in the same way, but possibly through different mechanisms, which need to be investigated. In the present study, altered

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gene/protein expression of CYP2E1, GSTA4-4, DAT and VMAT-2 by Zn were observed probably for the first time to the best of our knowledge.

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