



Alpha-synuclein aggregation, Ubiquitin proteasome system impairment, and L-Dopa response in zinc-induced Parkinsonism: resemblance to sporadic Parkinson's disease

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Abstract

Alpha-synuclein (α -synuclein) aggregation and impairment of the Ubiquitin proteasome system (UPS) are implicated in Parkinson's disease (PD) pathogenesis. While zinc (Zn) induces dopaminergic neurodegeneration resulting in PD phenotype, its effect on protein aggregation and UPS has not yet been deciphered. The current study investigated the role of α -synuclein aggregation and UPS in Zn-induced Parkinsonism. Additionally, levodopa (L-Dopa) response was assessed in Zn-induced Parkinsonian model to establish its closeness with idiopathic PD. Male Wistar rats were treated with zinc sulfate (Zn; 20 mg/kg; i.p.) twice weekly for 12 weeks along with respective controls. In few subsets, animals were subsequently treated with L-Dopa for 21 consecutive days following Zn exposure. A significant increase in total and free Zn content was observed in the substantia nigra of the brain of exposed groups. Zn treatment caused neurobehavioral anomalies, striatal dopamine decline, and dopaminergic neuronal cell loss accompanied with a marked increase in α -synuclein expression/aggregation and Ubiquitin-conjugated protein levels in the exposed groups. Zn exposure substantially reduced UPS-associated trypsin-like, chymotrypsin-like, and caspase-like activities along with the expression of SUG1 and β -5 subunits of UPS in the nigrostriatal tissues of exposed groups. L-Dopa treatment rescued from Zn-induced neurobehavioral deficits and restored dopamine levels towards normalcy; however, Zn-induced dopaminergic neuronal loss, reduction in tyrosine hydroxylase expression, and increase in oxidative stress were unaffected. The results suggest that Zn caused UPS impairment, resulting in α -synuclein aggregation subsequently leading to dopaminergic neurodegeneration, and that Zn-induced Parkinsonism exhibited positive L-Dopa response similar to sporadic PD.

Keywords Zinc · α -Synuclein aggregation · Ubiquitin proteasome system · Parkinson's disease · L-Dopa

Introduction

Parkinson's disease (PD) is a debilitating progressive movement disorder resulting from declined striatal dopamine levels due to selective dopaminergic neuronal loss in the substantia nigra pars compacta (SNpc) of ventral midbrain. Cardinal visual symptoms of PD include resting tremor, muscular rigidity, bradykinesia, and postural instability, while the presence of Lewy bodies and Lewy neurites in surviving dopaminergic neurons is considered as the anatomical hallmark of the sporadic PD [1]. Although the exact cause and mechanisms responsible for PD onset are not yet completely understood, clinical and experimental evidences suggest contribution of age and environmental and genetic factors in the manifestations of PD [2, 3]. Postmortem studies showing increased zinc (Zn) accumulation in dopaminergic

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neurons in the substantia nigra (SN) of PD patients implicated the contribution of heavy metals in the etiology of PD [4], which was further supported by experimental studies documenting Zn-induced dopaminergic neuronal degeneration in rodents [5–7].

Protein misfolding and aggregation play a central role in PD pathogenesis, which results in the formation of Lewy bodies in the surviving dopaminergic neurons in PD. Aggregates of presynaptic neuronal protein, α -synuclein, a 140-amino-acid protein, constitute the major component of Lewy bodies in PD [8, 9]. Association of α -synuclein with Lewy bodies and the link between point mutations or multiplications in α -synuclein encoding gene (SNCA) with autosomal-dominant familial PD established by genome-wide studies [10] collectively implicated α -synuclein in both familial and idiopathic PD. Genetic PD models developed by mutated α -synuclein genes (A53T, A30P, E46K) or overexpression of α -synuclein in experimental animals and involvement of α -synuclein aggregation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-, paraquat-, and rotenone-induced PD phenotypes have established its pathological role in PD [11–13]. Mutations in SNCA gene and environmental toxins increase the propensity of α -synuclein for misfolding and aggregation, thereby leading to the formation of protofibrils, Lewy neurites, and Lewy bodies [14].

Under normal physiological conditions, α -synuclein exists in monomeric/tetrameric form and is mostly degraded by Ubiquitin proteasome system (UPS). The UPS, a large multi-catalytic proteinase complex found in the nucleus and cytoplasm of eukaryotic cells, is primarily responsible for the degradation of normal, damaged, misfolded, mutated, or unfolded intracellular proteins [15, 16], and the malfunctioning of UPS leads to aberrant protein aggregation or accumulation subsequently resulting in cell death [17, 18]. The alleviated proteasome activity and the reduced expression of 20S and 19S UPS subunits reported in brains of PD patients suggested its involvement in the toxic manifestations of PD, which was substantiated by increased accumulation of non-ubiquitinated and ubiquitinated proteins observed in the substantia nigra and Lewy bodies of PD patients [8, 17, 19]. Single-nucleotide polymorphism-based studies have also linked mutations in two UPS enzyme coding genes, parkin and Ubiquitin C-terminal hydrolase L1 (UCH-L1) with familial PD [1, 15]. Although the exact mechanism for UPS impairment in sporadic PD pathogenesis is not yet established, conditions leading to increased oxidative stress and energy depletion such as age and environmental toxins are considered as plausible causes [16, 20, 21]. Experimental studies on toxin-based PD models like paraquat-, MPTP-, and rotenone-induced PD phenotypes have documented the contribution of UPS impairment in PD pathogenesis [22–24]. In vitro studies also revealed a marked decline in

proteasome activity in cultured cells following paraquat and rotenone exposures [22, 25]. Previous studies reported oxidative stress as one of the major perpetrators in Zn-induced dopaminergic neuronal degeneration; however, the contribution of protein aggregation and UPS in Zn-induced Parkinsonism is not yet explored.

Over the last two decades, extensive research carried out to explore the cause and mechanisms of PD onset and progression has led to the development of several genetic and neurotoxin-based animal models of PD [26]. None of the existing models completely mimic the human PD; however, response to dopamine precursor—levodopa (L-Dopa), the gold standard for PD treatment—is one of the criteria to ascertain its resemblance to idiopathic PD and whether Zn-induced Parkinsonian model responds to L-Dopa treatment is not yet known. The present study, therefore, aimed to explore the role of α -synuclein aggregation and UPS in Zn-induced Parkinsonism. Additionally, it assessed L-Dopa responsiveness in Zn-induced PD phenotype to establish its closeness with sporadic PD.

Materials and methods

Chemicals

Acetic acid, disodium hydrogen phosphate, Folin–Ciocalteu reagent, magnesium chloride (MgCl_2), nicotinamide adenine dinucleotide reduced form (NADH), potassium chloride, sodium chloride (NaCl), sodium dihydrogen phosphate, and sodium fluoride (NaF) were procured from Sisco Research Laboratories (SRL, Mumbai, India). Acrylamide, bisacrylamide, bovine serum albumin (BSA), 5-bromo-4-chloro-3'-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) system, bromophenol blue, β -mercaptoethanol, ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), hydrogen peroxide (H_2O_2), nitroblue tetrazolium (NBT), phenylmethane sulphonyl fluoride (PMSF), protease inhibitor (PI) cocktail, sodium deoxycholate, sodium dodecyl sulfate (SDS), sodium orthovanadate, sodium pyrophosphate, *N,N,N',N'*-tetramethyl ethylene diamine (TEMED), thiobarbituric acid (TBA), Tris-base, Triton X-100, Tween-20, proteasome inhibitor—MG-132, N-Suc-Leu-Leu-Val-Tyr-AMC substrate, Z-Leu-Leu-Glu-AMC substrate, TRITC-conjugated goat anti-mouse antibody and zinc sulfate (ZnSO_4) were procured from Sigma-Aldrich (St. Louis, MO, USA). Neg-50 was purchased from Richard Allen Scientific (Kalamazoo, MI). Formic acid was purchased from Ranbaxy Private Limited (New Delhi, India). Glycerol, potassium dihydrogen orthophosphate, methanol, and sodium carbonate were obtained from Merck Limited (Mumbai, India). Mouse monoclonal anti-tyrosine

hydroxylase (TH) antibody, anti- β -actin antibody, goat polyclonal anti- β -5 subunit antibody, goat polyclonal anti-SUG1 antibody, rabbit polyclonal anti-Ubiquitin conjugate antibody, rabbit polyclonal anti- α -synuclein antibody, alkaline phosphatase (AP)-conjugated bovine anti-mouse, anti-rabbit, and rabbit anti-goat secondary antibodies, and FITC-conjugated goat anti-rabbit antibody were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Z-Ala-Arg-Arg-AMC substrate and polyvinylidene fluoride (PVDF) membrane were purchased from Millipore Corporation, MA, USA. TSQ [N-(6-methoxy-8-quinoly)-p-toluene sulphonamide] and Alexa Fluor[®] 594 goat anti-rabbit and Alexa Fluor[®] 488 goat anti-mouse secondary antibodies along with Prolong gold anti-fade mounting medium were procured from Invitrogen. Levodopa (L-Dopa)/syndopa was of pharmaceutical grade and purchased from local firm. Other chemicals used were purchased locally.

Animal treatment

Experiments were performed using male Wistar rats (150–180 g) provided by animal facility of CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, with the approval of the Institutional Animal Ethics Committee. The animals were maintained under standard conditions of temperature and humidity and provided standard pellet diet and water. Animals were treated with zinc sulfate (Zn; 20 mg/kg b.w.) intraperitoneally twice weekly for 12 weeks along with vehicle controls. In few subsets, the animals were treated with dopamine precursor—levodopa/L-Dopa (combined with peripheral 3,4-dihydroxyphenylalanine decarboxylase inhibitor—carbidopa; 7.5 mg/kg b.w.; i.p.) daily for 21 consecutive days after 12 weeks of Zn exposure along with respective controls [27].

Decapitation and dissection of the brain

Animals were sacrificed by cervical dislocation and brains were dissected in ice-cold conditions. Striatum and SN were removed, pooled, and used as nigrostriatal tissues for experimental purposes except for dopamine estimation and immunofluorescence (IF) studies. Striatal tissue was used for dopamine estimation, while frozen brain sections were used for IF experiments. A minimum of at least four independent sets of experiments were performed.

Measurement of nigrostriatal Zn content

Zn content in the nigrostriatal tissues of brains of the control and Zn-exposed groups was estimated using flame atomic absorption spectrophotometer (Analytikjena ZEE nit 700, Germany) by a standard procedure described elsewhere [28]. The Zn contents in the nigrostriatal tissues of the control and

Zn-treated animals were calculated using standard Zn plot developed simultaneously.

Behavioral activity and striatal dopamine content

To assess the effect of Zn in the absence or presence of L-Dopa on motor activity and coordination, spontaneous locomotor activity (SLA) and rotarod performance were analyzed. SLA was measured using an Opto-Varimex animal activity meter as described elsewhere [6]. Rotarod performance test in control and treated animals was carried out as described previously [7]. In brief, the animals were first trained for 3 consecutive days to stay on a rotating rod at a fixed speed (5 rpm) for 5 min and then the time of stay on the rod was determined in 5 min test time. A minimum of five animals were taken in each group and at least three independent sets of experiments were performed for both SLA and rotarod performance test. Both SLA and rotarod performance are expressed in terms of percent of controls.

Striatal dopamine content of brains of the control and treated rats was estimated using liquid chromatography–mass spectrometry (LC–MS) as described previously [29]. Briefly, striatal tissue was homogenized employing 0.1% formic acid, sonicated, and centrifuged at 15,000 \times g for 30 min at 4 °C. The supernatant obtained was filtered through 0.22- μ m filters and the samples were analyzed through ultraperformance LC (UPLC) coupled to a mass spectrometer along with standard. Dopamine content was calculated in ng/mg tissue and the results are expressed as % of control.

Immunofluorescence (IF) studies

IF studies were performed to assess the effect of Zn exposure on free Zn content in the brain, α -synuclein aggregation, Ubiquitin-conjugated proteins, and TH-positive dopaminergic neurons. In brief, the animals were anesthetized with ether; the brain was perfused with chilled 0.9% saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The brains were cryoprotected in gradient sucrose solutions and serial sections (15 μ m) were cut using cryostat. Sections were blocked using PBS containing 5% normal goat serum, 1% BSA, and 0.1% Triton X-100 for 2 h, incubated for 48 h in either a single specific primary antibody or an equimolar mixture of antibodies in the case of co-immunofluorescence experiments, and washed with PBS. Further the sections were incubated with the respective fluorescently labeled secondary antibodies (1:300) for 2 h at room temperature, washed with PBS, and finally mounted using an anti-fade mounting medium. The images were captured at 20 \times with a fluorescent microscope. For TSQ staining, TH-labeled sections were incubated with TSQ solution for 2 min, washed with normal saline, mounted as mentioned

above, and viewed under a fluorescence microscope. The results for TH-positive neurons are expressed as % of controls and the results of free Zn content are expressed in terms of TSQ intensity in arbitrary fluorescence units (A.F.U.) measured via free ImageJ software.

Oxidative stress indexes

Lipid peroxidation (LPO) levels in the control and Zn-exposed groups with or without L-Dopa treatment were estimated using standard TBA method as described previously [6]. LPO levels are measured as nmoles malondialdehyde (MDA)/mg tissue. Superoxide dismutase (SOD) activity was measured in the control and treated groups using NBT method as described elsewhere [6]. Catalase activity was estimated according to the method described earlier [6]. The results of LPO, SOD, and catalase activities are expressed as % of controls.

Ubiquitin proteasome system (UPS) activity

UPS-related enzyme activities were estimated by a standard method as described elsewhere [30]. The brain was immediately washed with PBS and homogenized in the ratio of 1:5 using ice-cold proteasome lysis buffer [0.2% Triton X-100 in proteasome assay buffer (10 mM HEPES pH 8.0, 50 mM NaCl, 1 mM EDTA, and 50 mM sucrose)]. The homogenate was centrifuged for 5 min at $500\times g$ at $4\text{ }^{\circ}\text{C}$ and the supernatant was stored. The pellet was re-homogenized and centrifuged as described above. Both the supernatants were pooled and centrifuged for 10 min at $10,000\times g$ at $4\text{ }^{\circ}\text{C}$. The supernatant thus obtained was mixed with assay buffer and incubated at $37\text{ }^{\circ}\text{C}$ for 10 min followed by the addition of specific substrate for chymotrypsin-like activity (N-Suc-Leu-Leu-Val-Tyr-AMC/21 μM), trypsin-like activity (Z-Leu-Leu-Glu-AMC/34 μM), or caspase-like activity (Z-Ala-Arg-Arg-AMC/105 μM) and mixed properly. The fluorescence of AMC dye was measured using 380 nm excitation wavelength and 460 nm emission wavelength for 20 min. The catalytic activities were calculated using fluorescence of standard AMC dye and the results are expressed as % of control.

Preparation of Triton X-100 soluble and insoluble fractions for α -synuclein expression

Nigrostriatal tissues from the control and Zn-treated groups were homogenized in HEPES buffer [20 mM HEPES (pH 7.4), NaCl (150 mM), glycerol (10%), Triton X-100 (1%), EGTA (1 mM), MgCl_2 (1.5 mM), lactacystin (1 μM), NaF (50 mM), sodium pyruvate (10 mM), sodium orthovanadate (2 mM), PMSF (1 mM), and PI cocktail]. Homogenate was subjected to four consecutive freeze and thaw cycles and centrifuged at $100,000\times g$ for 30 min at $4\text{ }^{\circ}\text{C}$.

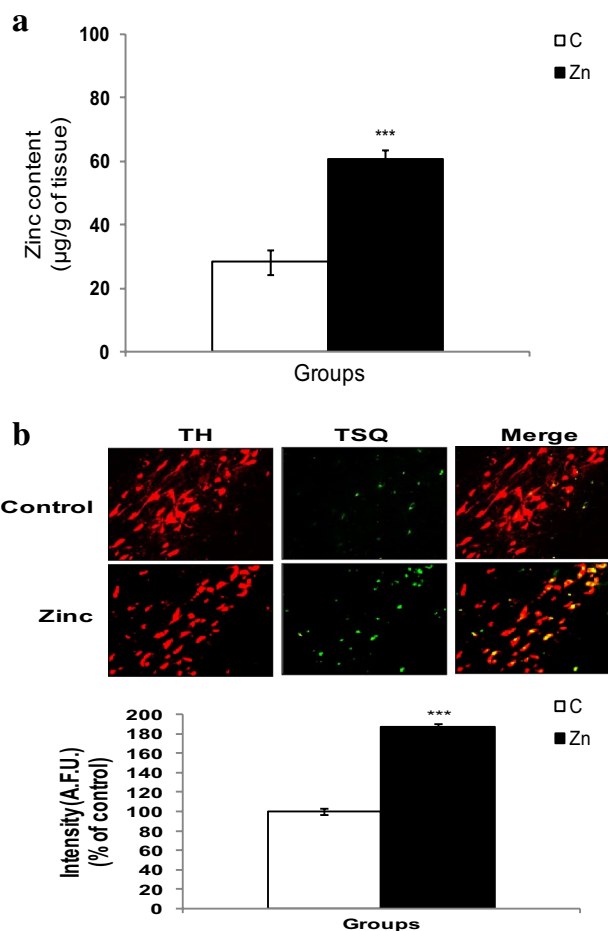


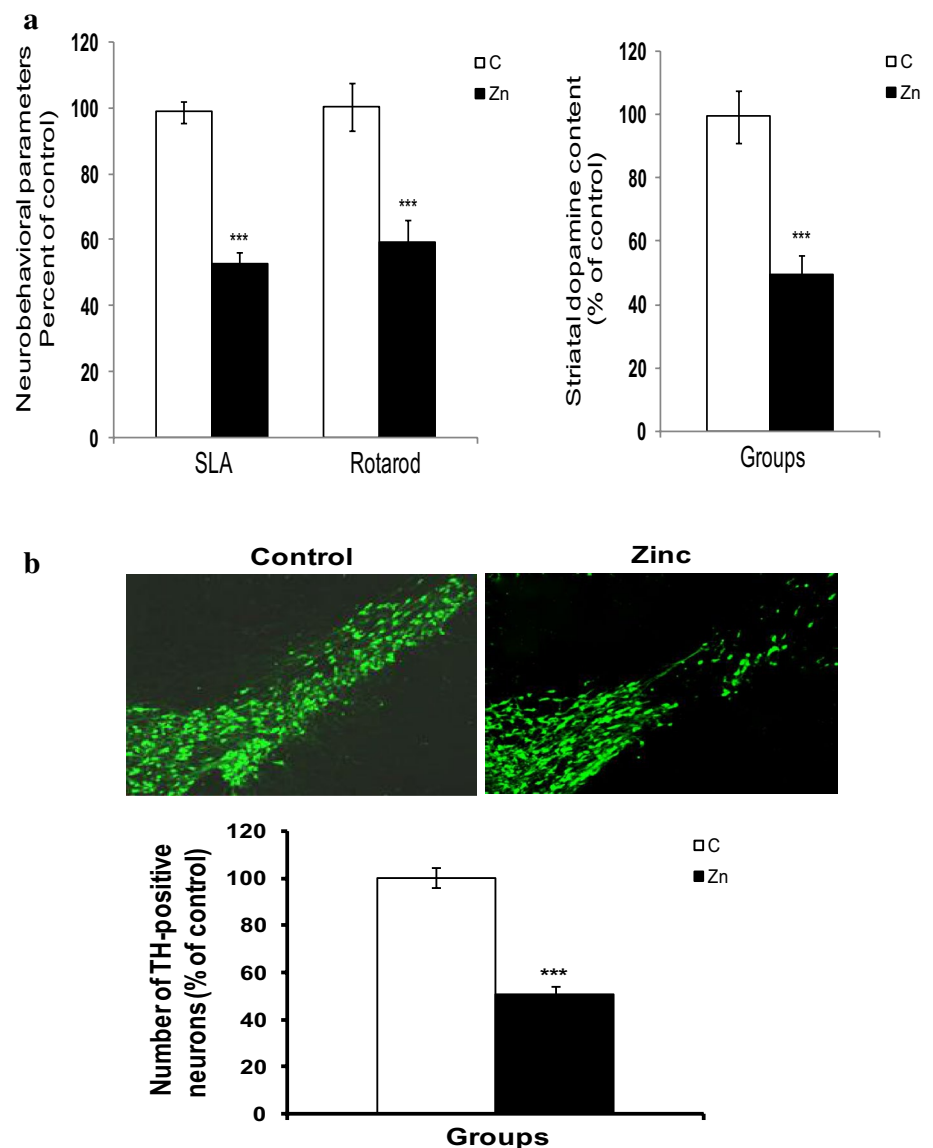
Fig. 1 **a** Measurement of total Zn content in the nigrostriatal tissue of the control and Zn-treated groups. **b** IF analysis to assess free Zn content in dopaminergic neurons in the control and treated groups. The upper panel shows the representative picture illustrating dopaminergic neurons (red color) and TSQ staining for free zinc (green color) in the SN region of the brain, while the lower panel shows the bar diagram of TSQ fluorescence intensity linked to dopaminergic neurons in the control and treated rats. The data are expressed as means \pm SEM ($n=4$) (***) $p < 0.001$ as compared with the control group). (Color figure online)

The supernatant obtained was taken as Triton X-100 soluble fraction. Pellet was resuspended in Tris buffer [50 mM; pH 7.4] containing 5% SDS, then sonicated, and boiled for 20 min followed by centrifugation for 10 min at $16,000\times g$ at $25\text{ }^{\circ}\text{C}$. The supernatant thus obtained was taken as Triton X-100 insoluble fraction. The soluble and insoluble fractions of α -synuclein protein were used for western blotting.

Western blotting

The protein levels of TH, Ubiquitin-conjugated proteins, Rpt6/SUG1, and beta-5 (β -5) subunits of UPS along with Triton X-100 soluble and insoluble fractions of α -synuclein were analyzed in the control and treated groups. The

Fig. 2 a Zn-induced alterations in neurobehavioral parameters and striatal dopamine content following 12 weeks of exposure. **b** Immunofluorescence analysis of TH-positive neurons in the substantia nigra of the rat brain following 12 weeks of treatment. The upper panel shows the representative IF picture for TH-positive dopaminergic neurons in brain sections of the control and treated groups. The lower panel shows the bar diagram of the number of TH-positive neurons in the control and treated groups. The data are expressed as means \pm SEM ($n=4$). ($***p < 0.001$ as compared with the control group)



nigrostriatal tissues from the control and treated groups were homogenized using RIPA buffer as described previously [6] and centrifuged, and the supernatant was used for western blotting of Ubiquitin-conjugated proteins and UPS subunits (SUG1/Rpt6 and β -5). Briefly, proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane with trans-blot semi-dry transfer cell. Blots were incubated in respective primary antibodies for 2 h, washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer, and further incubated in appropriate AP-conjugated secondary antibodies. Finally, the blots were developed with BCIP/NBT. The results are expressed as band density ratio of protein of interest and β -actin. Data are expressed as means \pm standard error of means (SEM) of band density ratio of at least four individual experiments.

Protein estimation

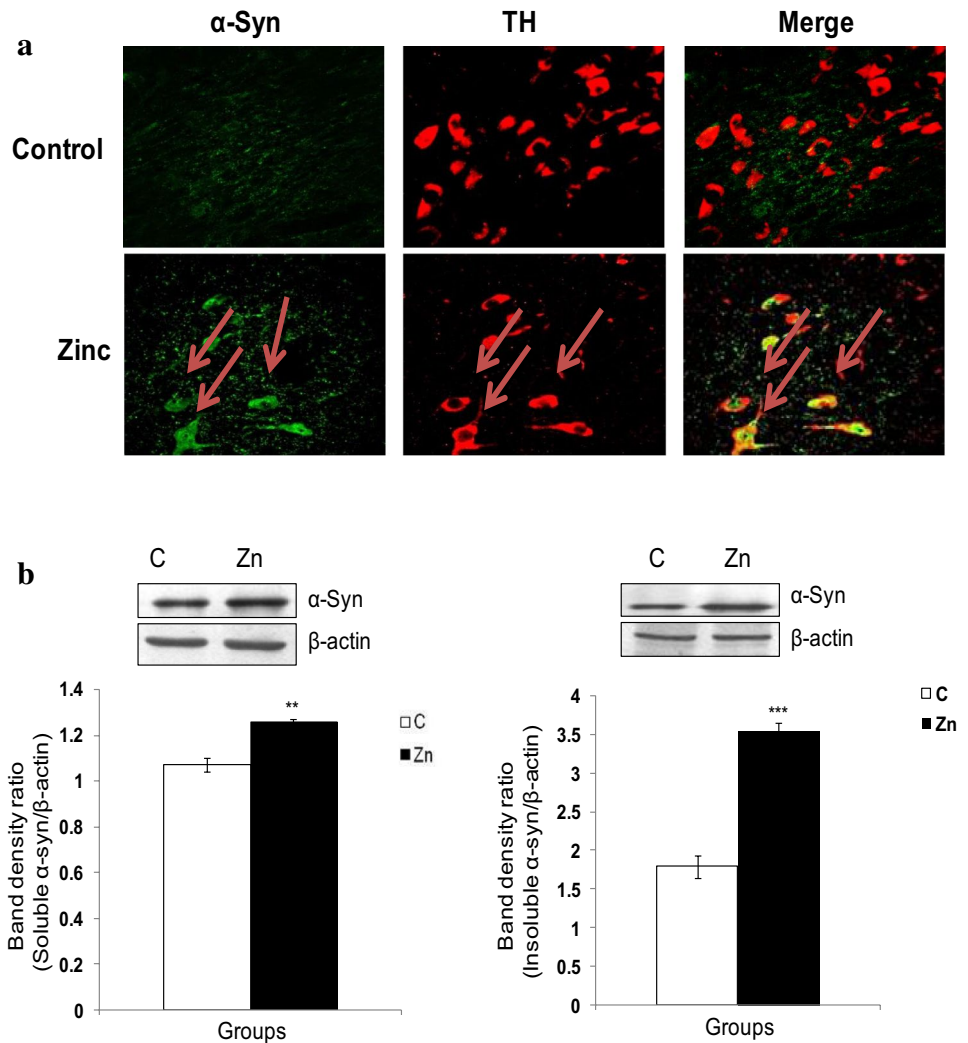
Protein content in the tissue homogenates was estimated using Lowry method [31] with BSA as the standard and protein content was calculated in mg/ml employing the graph plotted with BSA.

Statistical analysis

The results are expressed as means \pm standard error of means (SEM) of a minimum of four independent sets of experiments. Student's *t* test and one-way ANOVA were used for statistical analysis. Newman–Keuls post hoc test was used for multiple comparisons in few sets of experiments. The

Fig. 3 Expression and aggregation of α -synuclein/ α -Syn in dopaminergic neurons.

a Representative picture of immunofluorescence staining of α -synuclein (green color) and tyrosine hydroxylase (TH) in dopaminergic neurons (red color) of the SN region of the control and Zn-treated groups. Arrows indicate the accumulation of α -synuclein in dopaminergic neurons. **b** The upper panel shows representative western blot of Triton X-100 soluble and insoluble fractions of α -synuclein. The lower panel shows the densitometric analysis of the same with β -actin as the reference. Results are expressed as means \pm SEM ($n=4-6$). (** $p < 0.001$ and ** $p < 0.01$ as compared with the control group). (Color figure online)



results were considered statistically significant only if the ‘ p ’ value was less than 0.05.

Results

Effect of Zn exposure on nigrostriatal Zn content

Atomic absorption spectroscopic analysis of total Zn content of nigrostriatal tissues revealed a significant increase of the Zn content in the exposed groups as compared to the control groups (Fig. 1a). Further, co-immunofluorescence analysis of TH-positive neurons and free Zn-associated TSQ dye in the brain sections of the control and treated groups exhibited a marked elevation of free Zn content in the dopaminergic neurons of the SN region in brains of exposed animals (Fig. 1b).

Neurobehavioral analysis and striatal dopamine level

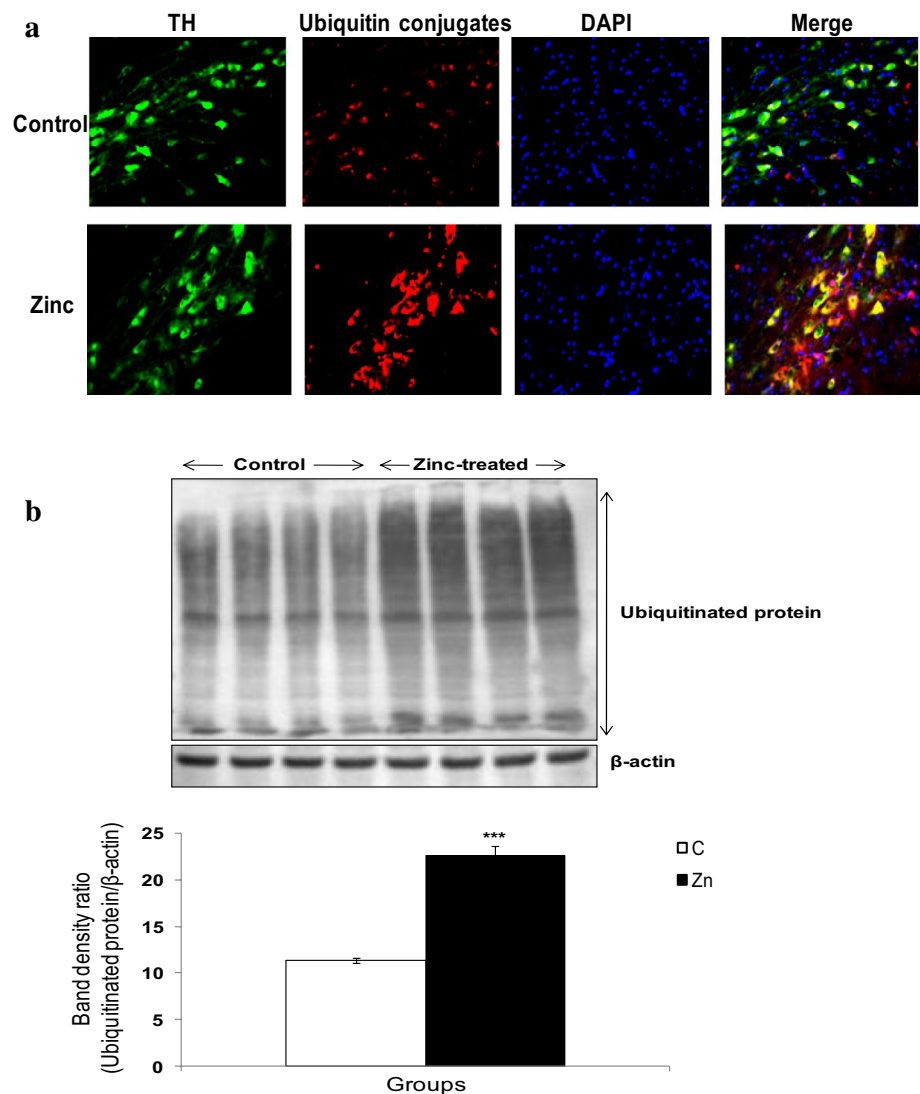
Neurobehavioral studies were performed in the control and Zn-treated groups to assess the effect of zinc on SLA and motor coordination. Zn exposure led to a significant reduction in both SLA and rotarod performance in exposed rats, showing that Zn induced PD-like symptoms in exposed rats (Fig. 2a).

Striatal dopamine content was also markedly attenuated in the Zn-exposed groups as compared with the control animals further supporting the neurobehavioral analysis (Fig. 2a).

IF analysis of TH-positive neurons

Effect of Zn on dopaminergic neurons was assessed by IF analysis of TH-positive cells present in the substantia

Fig. 4 Effect of Zn on Ubiquitin-conjugated/ubiquitinated proteins in nigrostriatal tissues of the rat brain. **a** Representative picture of immunofluorescence staining showing TH (green), Ubiquitin-conjugated proteins (red), and nuclei (blue) in the substantia nigra region of brain sections of the control and Zn-exposed groups. **b** The upper panel depicts the representative western blot of Ubiquitin-conjugated proteins in nigrostriatal tissues of the control and Zn-treated groups and the lower panel shows the densitometric analysis of the same using β -actin as the reference. Results are expressed as means \pm SEM ($n=4-6$). (***) $p < 0.001$ as compared with the control group). (Color figure online)



nigra region of the rat brain. Zn exposure caused a significant decrease in the number of TH-positive neurons in the substantia nigra region of the brain of exposed animals as compared with controls (Fig. 2b).

Effect of Zn on protein expression and aggregation of α -synuclein

Co-immunofluorescence analyses of TH and α -synuclein protein exhibited increased α -synuclein accumulation in the dopaminergic neurons in the SN region of the brain of Zn-exposed animals as compared with the control group (Fig. 3a).

Furthermore, western blot analysis of α -synuclein showed a marked elevation in the protein levels of both Triton X-100 soluble (monomeric form) as well as Triton X-100 insoluble (aggregated form) fractions of α -synuclein, suggesting

that Zn augments both protein expression and aggregation of α -synuclein in the brains of exposed animals (Fig. 3b).

Zinc enhanced the levels of Ubiquitin-conjugated proteins in dopaminergic neurons

Since the aggregates of α -synuclein act as an indicator of UPS malfunction, IF analysis of Ubiquitin-conjugated proteins was performed in dopaminergic neurons of the SN region of the control and Zn-treated groups by co-immunofluorescence studies. IF analysis revealed a significant augmentation in the levels of Ubiquitin-conjugated proteins in dopaminergic neurons in Zn-exposed animals as compared to controls (Fig. 4a). Moreover, western blot analysis also exhibited an elevated expression of Ubiquitin-conjugated proteins substantiating the IF analysis results (Fig. 4b).

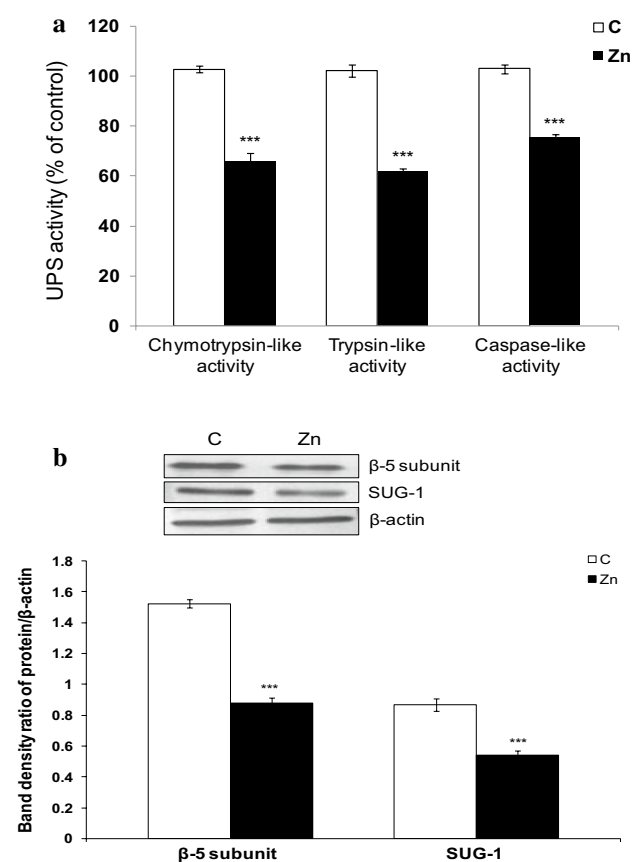


Fig. 5 **a** Bar diagram showing UPS-associated chymotrypsin-like, trypsin-like, and caspase-like activities in the nigrostriatal tissues of the control and Zn-exposed groups. **b** Protein expression of SUG-1 and β-5 subunits of UPS in the brains of control and Zn-exposed groups. The upper panel shows the representative western blot of protein expression of SUG-1 and β-5 subunits of UPS in the control and Zn-treated groups. The lower panel shows the densitometric analysis of the same with β-actin as the reference. Results are expressed as means ± SEM ($n=6$). (***) $p < 0.001$ as compared with the control group)

Zinc attenuated the UPS activity and the expression of UPS subunits

Elevated levels of Ubiquitin-conjugated proteins instigated us to estimate UPS-linked enzyme activities. Zn treatment yielded a noteworthy reduction in chymotrypsin-, trypsin-, and caspase-like activities associated with UPS as compared to the unexposed groups (Fig. 5a). Furthermore, Zn exposure resulted in a marked decline in the protein levels of SUG1/Rpt6 and β-5 UPS subunits as compared with controls (Fig. 5b).

L-Dopa rescued from Zn-induced behavioral impairments and dopamine depletion

Zn-treated animals displayed a significant decrease in SLA and rotarod performance of exposed animals; however, post-treatment of L-Dopa markedly alleviated Zn-induced neurobehavioral deficits in exposed rats (Fig. 6a).

Zn caused a significant attenuation in striatal dopamine content, which was noticeably restored towards normalcy with L-Dopa treatment in the exposed groups (Fig. 6b).

Effect of L-Dopa on Zn-induced dopaminergic neuronal cell loss and TH expression

Immunofluorescence analysis of TH-positive neurons showed a noteworthy reduction in TH-positive cells in the SNpc of Zn-treated animals. Post-treatment of L-Dopa did not alter Zn-induced dopaminergic cell loss in SNpc of exposed rats (Fig. 7a). L-Dopa alone did not affect dopaminergic neurons in exposed groups (Fig. 7a).

Western blot analysis exhibited a marked reduction in the TH protein expression in the Zn-exposed groups, which was unaltered by L-Dopa treatment (Fig. 7b). L-Dopa per se did not cause any significant change in TH protein levels in the control groups.

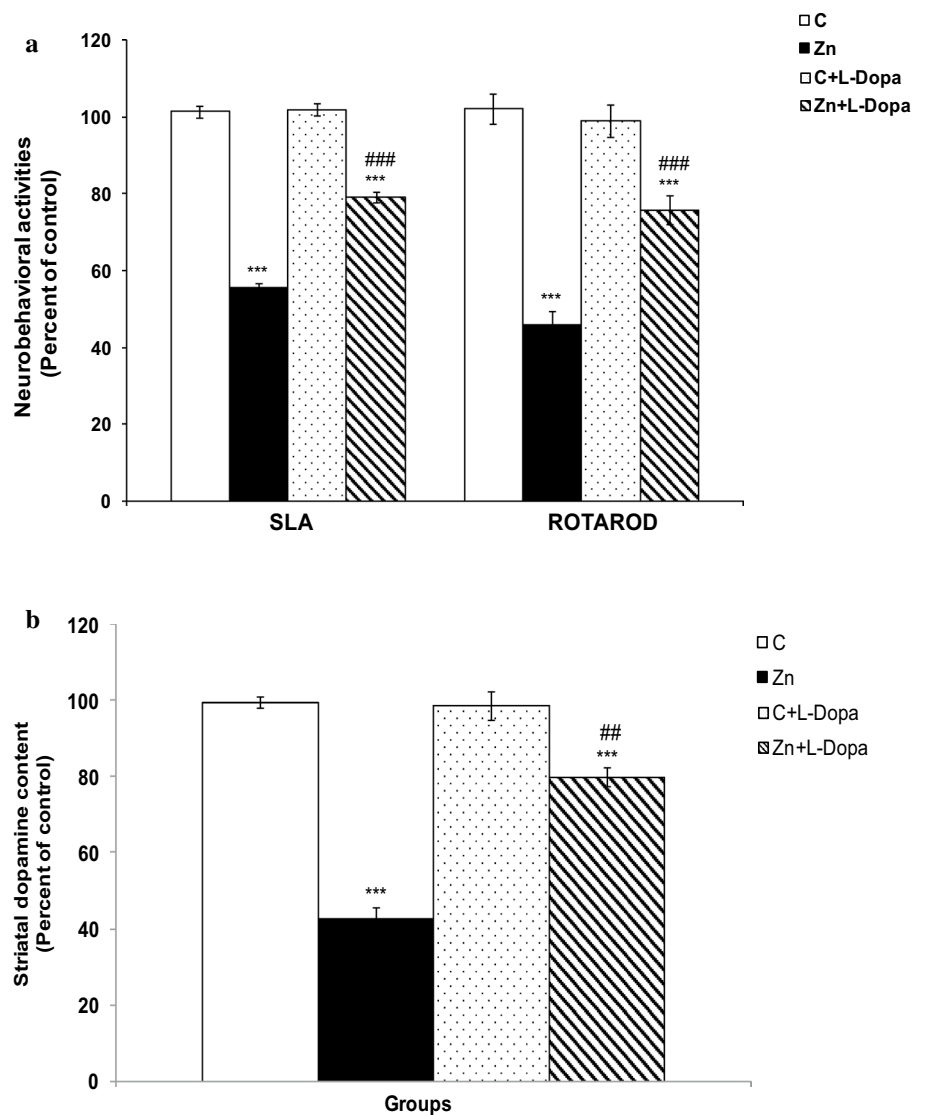
Effect of L-Dopa on Zn-mediated oxidative stress indices

Zn treatment noticeably increased LPO levels and SOD activity, while catalase activity was attenuated in the treated groups. L-Dopa post-treatment did not affect Zn-induced modulations in the oxidative stress indices in the exposed animals (Fig. 7c). Oxidative stress parameters were also not altered in the groups treated with L-Dopa alone.

Discussion

The present study explored the involvement of α-synuclein aggregation, UPS impairment, and L-Dopa response in Zn-induced Parkinsonian model to establish its closeness with idiopathic PD. Clinical and experimental evidences have implicated high Zn levels in PD pathogenesis [4–6]. Zn is largely present in bound form inside cells under normal physiological conditions and availability of free Zn is strictly regulated as it is linked to deleterious effects. Elevated total Zn content in the nigrostriatal tissue of brains of the Zn-exposed rats and increased TSQ fluorescence, specific for free Zn, observed in the dopaminergic neurons of brains of exposed groups suggested an accumulation of free Zn in the SN region of brains of the exposed groups as reported in PD patients and experimental animals [4, 28]. The

Fig. 6 Effect of Zn and/or L-Dopa on neurobehavioral parameters (a) and striatal dopamine content (b) in exposed rats. Results are expressed as means \pm SEM ($n=4$). (***) $p<0.001$ as compared with the control group and ### $p<0.001$; ## $p<0.001$ as compared with the zinc-treated group)



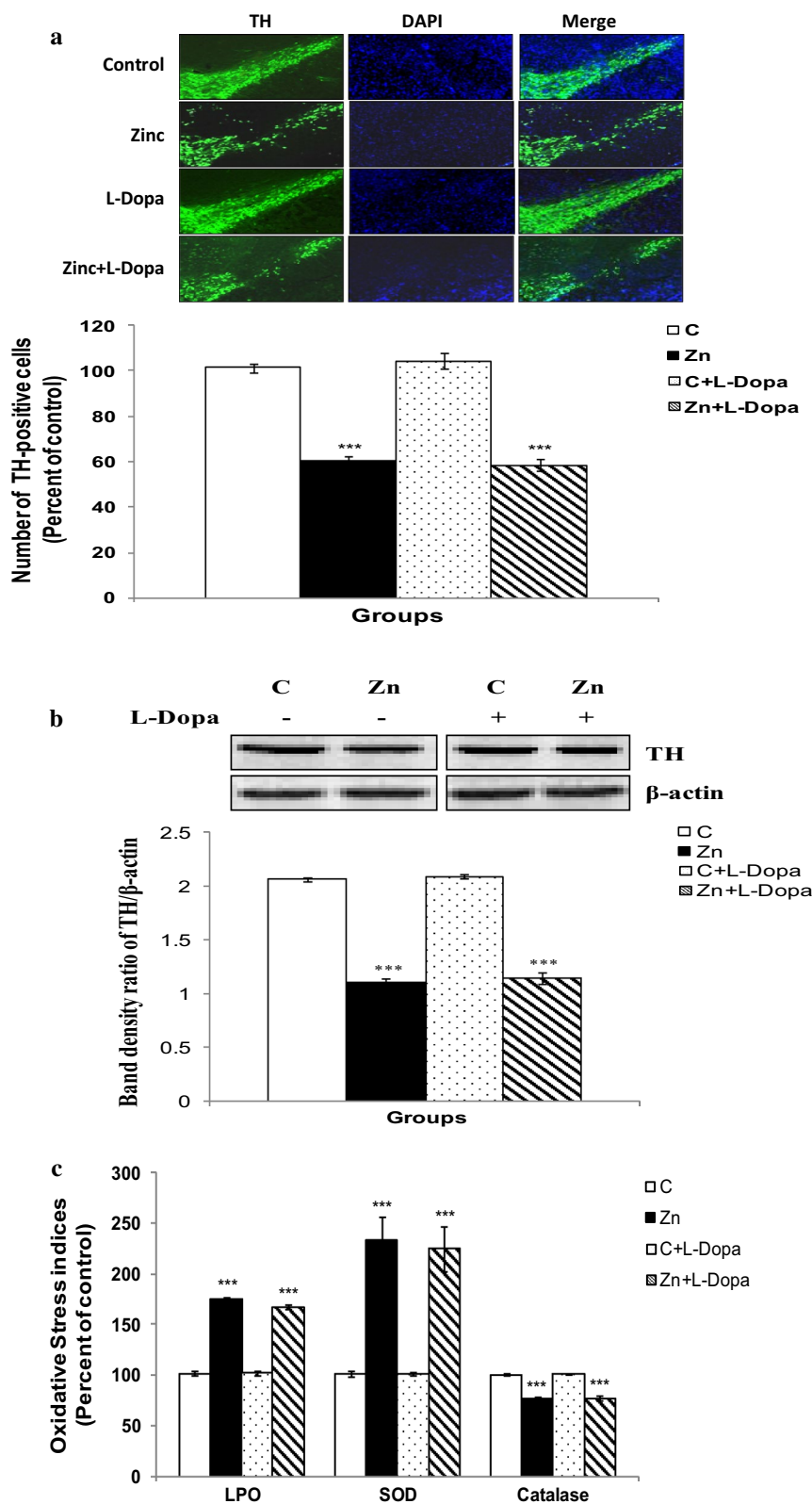
neurodegenerative indexes, viz. selective loss of TH-positive dopaminergic neurons, neurobehavioral impairments, and striatal dopamine decline, were assessed to reaffirm that Zn induces PD phenotype as reported previously by our group [5, 32].

Oxidative stress plays a critical role in aberrant protein aggregation in sporadic PD and toxin-induced PD phenotypes [15, 33]. Oxidative stress is established as the malfactor in Zn-induced neurodegeneration and Zn promotes protofibril formation in purified α -synuclein protein in vitro [34]; therefore, the study explored the effect of Zn exposure on α -synuclein expression and accumulation. Increased α -synuclein expression/accumulation observed in the dopaminergic neurons along with elevated levels of non-aggregated/aggregated forms of α -synuclein protein in nigrostriatal tissues suggested that Zn induced both the expression and aggregation of α -synuclein in dopaminergic neurons

of the SN region of the brain. The aggregated α -synuclein might further contribute to Zn-mediated dopaminergic neuronal death as reported in sporadic and chemical-induced PD [13, 22, 35].

Aggregated α -synuclein is known to inhibit proteasomal function [36] and conversely dysfunctional UPS leads to the accumulation of protein aggregates [22]. Polyubiquitination of protein prepares it for degradation via UPS and therefore the accumulation of Ubiquitin-conjugated proteins serves as a marker for impaired UPS function and is reported in PD patients and toxin-induced PD models. Augmented levels of Ubiquitin-conjugated proteins illustrated in dopaminergic neurons of the Zn-exposed groups by IF analysis and increased levels of Ubiquitin-conjugated proteins in the nigrostriatal tissues of the exposed groups observed by western blot analysis pointed towards UPS malfunctioning [30, 37]. Attenuated catalytic activities of UPS-associated

Fig. 7 **a** Effect of L-Dopa treatment on TH immunoreactivity in the substantia nigra of the brains of control and Zn-exposed rats. The upper panel shows the representative picture of TH-positive neurons (green) and nuclei (blue) in brain sections of the control and treated animals, while the lower panel depicts the bar diagram showing the number of TH-positive neurons in the substantia nigra region of the control and treated groups. **b** Effect of Zn and/or L-Dopa on TH protein expression. The upper panel shows the representative western blot and the lower panel shows the densitometric analysis of the same. **c** Effect of Zn and/or L-Dopa on oxidative stress indices (LPO, SOD, and catalase) in the nigrostriatal tissues. The data are expressed as means \pm SEM ($n=4-6$). (** $p < 0.001$ as compared with the control group). (Color figure online)



enzymes in this study further confirmed reduced UPS function in Zn-exposed animals, which was in accordance with the studies reporting UPS dysfunction in sporadic PD and

toxin-induced PD models [17, 22, 25, 30, 37] and Zn-mediated inhibition of UPS activity and accumulation of Ubiquitin-conjugated proteins in ischemic hippocampal neuronal

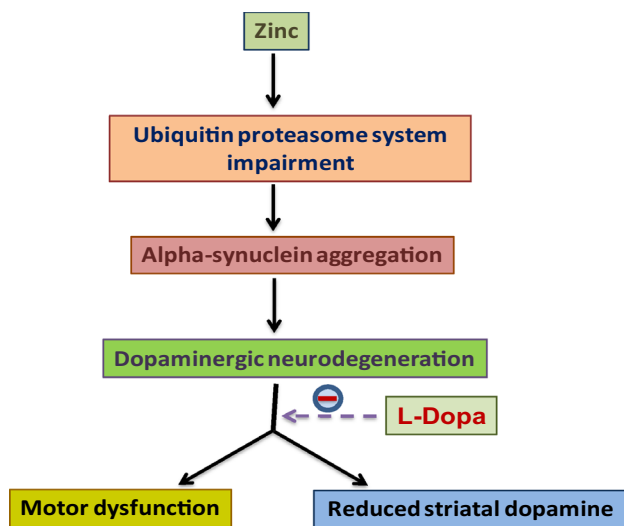


Fig. 8 A schematic representation illustrating the involvement of UPS impairment and α -synuclein aggregation in Zn-induced Parkinsonism and its responsiveness to L-Dopa therapy

culture [38]. Additionally, diminished protein levels of SUG1/Rpt6 and β -5 UPS subunits obtained in the Zn-treated groups corroborated with the activity results, suggesting that Zn causes UPS impairment at the translational level implying that dysfunctional UPS machinery could be responsible for increased α -synuclein accumulation and aggregation as documented in other PD models [21, 37].

Several animal PD models are available till date to study the mechanistic and therapeutic aspects of PD pathogenesis; however, not a single model mimics sporadic PD completely. In order to assess the resemblance of Zn-induced Parkinsonian model with sporadic PD, the present study explored the L-Dopa response in this model. L-Dopa rescued against Zn-induced neurobehavioral anomalies and markedly restored the striatal dopamine content in the Zn-exposed groups, indicating that Zn-induced PD phenotype resembles sporadic PD in terms of L-Dopa responsiveness [39, 40]. However, no marked change observed in the number of dopaminergic neurons and TH protein expression in the Zn-exposed groups following L-Dopa treatment suggested that the protective effect of L-Dopa could be due to the restoration of dopamine levels. Zn-induced modulations in oxidative stress indexes were also unaffected by L-Dopa, indicating that the treatment paradigm used in the current study did not aggravate oxidative stress in the Zn-exposed groups, which was in accordance with previous studies reporting that L-Dopa treatment promoted functional recovery without inducing any toxicity to the remaining dopaminergic neurons [41, 42]. The results suggested that Zn exposure causes UPS impairment by attenuating its proteolytic activity and expression resulting in the augmentation of α -synuclein aggregation finally leading to dopaminergic neurodegeneration. Furthermore,

post-treatment of L-Dopa restored the behavioral activity and striatal dopamine levels towards normalcy without affecting surviving dopaminergic neurons in the brain of Zn-induced PD phenotype (Fig. 8).

Conclusion

In conclusion, this study demonstrated that Zn-induced UPS impairment led to α -synuclein aggregation, subsequently resulting in dopaminergic neurodegeneration, and that Zn-induced Parkinsonian model responds positively to L-Dopa treatment exhibiting resemblance to idiopathic PD.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

Ethical approval This study was approved by the Institutional Animal Ethics Committee. The experiments were performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) throughout the study.

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