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Silymarin Protects Against Impaired Autophagy Associated with 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Induced Parkinsonism

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Abstract

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exacerbates mitochondrial impairment and α-synuclein expression leading to Parkinsonism. Impaired mitochondria and over-expressed α-synuclein are degraded and eliminated via macroautophagy and chaperone-mediated autophagy. Owing to multiple properties, silymarin protects from oxidative stress–mediated cellular injury. However, its effect on MPTP-induced changes in autophagy is not yet known. The study aimed to decipher the effect of silymarin on MPTP-induced changes in autophagy. Male mice (20–25 g) were treated with silymarin (intraperitoneally, daily, 40 mg/kg) for 2 weeks. On day 7, a few animals were also administered with MPTP (intraperitoneally, 20 mg/kg, 4 injections at 2-h interval) along with vehicles. Striatal dopamine content was determined. Western blot analysis was done to assess α-synuclein, beclin-1, sequestosome, phosphorylated 5′ adenosine monophosphate–activated protein kinase (p-AMPK), lysosome-associated membrane protein-2 (LAMP-2), heat shock cognate-70 (Hsc-70), LAMP-2A, phosphorylated unc-51-like autophagy activating kinase (p-Ulk1), and phosphorylated mechanistic target of rapamycin (p-mTOR) levels in the nigrostriatal tissue. Silymarin rescued from MPTP-induced increase in beclin-1, sequestosome, p-AMPK, and p-Ulk1 and decrease in LAMP-2, p-mTOR, and LAMP-2A levels. Silymarin defended against MPTP-induced increase in α-synuclein and reduction in dopamine content. The results demonstrate that silymarin protects against MPTP-induced changes in autophagy leading to Parkinsonism.

Keywords MPTP · Silymarin · Macroautophagy · CMA · Neuroprotection

Introduction

Hypokinetic rigid syndrome, universally referred to as Parkinson's disease (PD), is a well-known age-related neurodegenerative disorder primarily characterized with motor disability (Olanow 2007; Singh et al. 2006; Schapira 2009; Yadav et al. 2012). Although the tangible contributors of disease have been mysterious, increased age and hereditary and environmental factors are documented as the key perpetrators that accelerate the degeneration of dopamine-producing neurons in the nigrostriatal pathway (Olanow 2007; Schapira 2009; Dauer and Przedborski 2003; Srivastava et al. 2010; Gupta et al. 2014; Rasheed et al. 2017). The discerning loss of tyrosine hydroxylase (TH)–positive cells in the substantia nigra ends up in the striatal dopamine deficiency (Yadav et al. 2012). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), initially found to be present in the synthetic heroin, induces the selective degeneration of dopamine-synthesizing cells in the nigrostriatal area of the brain (Yadav et al. 2012; Dauer and Przedborski 2003; Srivastava et al. 2010; Gupta et al. 2014; Rasheed et al. 2017; Langston 2017). Although MPTP is not a very powerful neurodegenerative agent, it does cross the blood-brain barrier owing to its lipophilic nature and subsequently enters the astrocytes where it gets converted to 1-methyl-4-phenylpyridinium cation (MPP^+), a highly potent neurotoxicant, by an enzyme monoamine oxidase (Yadav et al. 2012; Gupta et al. 2014; Langston 2017; Herraiz and Guillén 2011). The adjoining

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dopaminergic neurons take up MPP^+ from the nigrostriatal astrocytes through monoamine transporters (Langston 2017; Cui et al. 2009). Accumulation of MPP^+ after a threshold level inhibits the mitochondrial complex I leading to free radical generation, mitochondrial dysfunction, energy depletion, and neuronal cell death (Yadav et al. 2012; Srivastava et al. 2010; Rasheed et al. 2017; Przedborski et al. 2001).

Autophagy, an auto-sacrificing course of action of eukaryotic cells, is indispensable for the removal of impaired organelles and superfluous proteins. It is inevitable for cellular survival and homeostasis (Iwata et al. 2005; Pan et al. 2008; Mishra et al. 2015; Levine and Kroemer 2008). Macroautophagy, also referred to as autophagy in a less universal sense in multicellular animals, sequesters the abnormal proteins and defective intracellular organelles through the formation of a double-membrane vesicle, called autophagosome. It delivers the cargo to the lysosome for the degradation and elimination after fusion and formation of a specialized structure called autophagolysosome (Iwata et al. 2005; Mishra et al. 2015; Mishra et al. 2018). Reduction in the number of lysosomes, level of lysosome-associated membrane proteins, and accumulation of redundant vesicles are shown to be associated with impaired autophagy (Bové et al. 2014). Autophagy impairment in PD is often contributed by an excessive accrual of redundant α-synuclein and damaged organelles or malfunctioning of clearance machineries (Mishra et al. 2015; Levine and Kroemer 2008; Mishra et al. 2018; Bové et al. 2014; Tripathi et al. 2019). Macroautophagy eliminates insoluble (oligomeric) proteins along with the majority of soluble proteins. Nonetheless, approximately one-third of soluble proteins and monomeric α-synuclein are degraded and cleared off from the cell through chaperone-mediated autophagy (CMA) (Tripathi et al. 2019; Kaushik and Cuervo 2008). In neuronal survival, both macroautophagy and CMA have been implicated, so defect in any of these events leads to PD or Parkinsonism.

PD pathology is associated with soluble α-synuclein accumulation. Substantial fraction of this protein is eliminated by CMA (Schapira 2009; Gibb and Lees 1988; Mak et al. 2010). Under extreme stress or genetic changes, it undergoes the posttranslational modification leading to an altered protein product that does not correctly interact with chaperones or prevents other proteins to interact or does not properly deliver itself to the lysosome due to reduced interaction with lysosome-associated membrane protein-2A (LAMP-2A), leading to less degradation and elimination (Mishra et al. 2015; Tripathi et al. 2019; Cuervo et al. 2004; Xilouri et al. 2009). Defective CMA can also be associated with an altered level of CMA indicator proteins, such as heat shock cognate (Hsc-70) and LAMP-2A since such variables are directly implicated in recognition, transport, and degradation of soluble substrate proteins (Mishra et al. 2018; Tripathi et al. 2019; Cuervo et al. 2004; Majeski and Dice 2004). Similarly, macroautophagy is characterized by beclin-1 activation, sequestosome (p62) degradation, LAMP-2 expression, and phosphorylation/dephosphorylation of 5′ adenosine monophosphate–activated protein kinase (AMPK), unc-51-like autophagy activating kinase (p-Ulk1), and mechanistic target of rapamycin (p-mTOR) (Mishra et al. 2018).

MPTP is a widely recognized Parkinsonian toxicant that induces Parkinsonism in experimental rodents and primates. On the contrary, silymarin is a neuroprotective agent and is an incredibly safe naturally occurring antioxidant. It is known to possess anti-inflammatory, anti-apoptotic, and anti-Parkinsonian efficacies (Gazak et al. 2007; Singhal et al. 2011; Singhal et al. 2013; Haddadi et al. 2018; Haddadi et al. 2014; Lee et al. 2015; Pérez-H et al. 2014). In this study, the preference was given to silymarin over other agents owing to its natural origin and very less toxicity that it produces even at high doses along with unproblematic doorway in the brain through the blood-brain barrier (Singhal et al. 2011; Singhal et al. 2013). Silymarin is also shown to protect against apoptotic dopaminergic neurodegeneration in five consecutive days MPTP-intoxicated mouse model (Pérez-H et al. 2014). However, it is not yet known if silymarin possesses efficacy against MPTP-induced macroautophagy and CMA impairments. Thus, the present investigation aimed to investigate the effects of silymarin against MPTP-induced PD phenotype and impaired autophagy in mice.

Materials and Methods

Materials

Chemicals, consumables, and reagents required for this study were obtained through local purchase unless or otherwise stated in subsequent phrases. Protease inhibitor cocktail was procured from Thermo Fisher Scientific (Waltham, MA). Acrylamide, sodium dodecyl sulfate (SDS), Tween-20, *N*,*N*′-methylenebisacrylamide, ammonium persulfate, bovine serum albumin (BSA), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, dimethylsulfoxide (DMSO), polyvinylidene fluoride (PVDF), ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetra-sodium salt (EGTA or egtazic acid), glycine, Tris (hydroxymethyl) aminomethane (Tris), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitroblue tetrazolium chloride (NBT), phenyl methyl sulfonyl fluoride (PMSF), silymarin, MPTP, ethylene diaminetetraacetic acid disodium salt dehydrate (EDTA), dopamine hydrochloride, anti-β-actin, anti-beclin-1, anti-LAMP-2, anti-pAMPK-α, and Folin-Ciocalteu's reagent were obtained from Sigma-Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany). Anti-p-Ulk1 Ser 317 primary antibody was obtained from Cell Signalling Technology Inc., (Danvers, MA). Anti-p62, anti-Hsc-70, anti-α-synuclein, and anti-p-mTOR primary antibodies along

with secondary antibodies, such as goat anti-mouse IgG-AP and goat anti-rabbit IgG-AP, were supplied by Santa Cruz Biotechnology Inc., (Dallas, TX). Anti-LAMP-2A antibody was acquired from Abcam (Cambridge, UK).

Animal Treatment

Male Swiss albino mice (20–25 g) employed in the study were kept under benchmark conditions in the animal house and fed with pellet diet and water ad libitum (Srivastava et al. 2012). The present investigation was endorsed by the animal ethics committee of the institute. The mice were administered with silymarin (intraperitoneally; 40 mg/kg), daily, for 15 days with respective vehicle (0.1% DMSO in 0.9% sodium chloride/saline). Besides, subsets of experimental animals were also treated intraperitoneally with MPTP (20 mg/kg), four times a day (Jackson-Lewis and Przedborski 2007; Kuroiwa et al. 2010; Guo et al. 2016) on day 7 at the interval of 2 h in conjunction with respective control. Animals were killed through cervical dislocation 24 h after final silymarin/vehicle treatment. The brain was taken out, and corpus striatum and substantia nigra were separated. Dopamine was measured in the corpus striatum immediately. Remaining experiments were done in the nigrostriatal (corpus striatum and substantia nigra combined) tissue.

Estimation of Dopamine

The dopamine was estimated in the supernatant of corpus striatal tissue homogenate employing the method described elsewhere (Srivastava et al. 2012; Singh et al. 2010). In summary, 10% corpus striatal homogenate was prepared in perchloric acid (0.45 N) and then centrifuged, and supernatant was collected. It was further cleaned by passing through a syringe filter and injected into a C-18 (250 mm × 15 mm, 5 μm) column attached to a high-performance liquid chromatography system and detection was done using an electrochemical detector (Waters, Milford, MA, USA). *N*-Heptane sulfonic acid and potassium dihydrogen phosphate in 10% methanol were used for mobile phases.

Western Blotting

Nigrostriatal tissue was homogenized (50 mM Tris-HCl, 0.1% SDS, PMSF, and protease inhibitor cocktail, 2 mM EDTA, 200 mM sodium chloride, and 2 mM EGTA) and centrifuged. The protein content was estimated in the supernatant and the measured amount (approximately 40–100 μg) of protein was subjected to polyacrylamide gel electrophoresis followed by electrotransfer onto the PVDF membrane (Mishra et al. 2018). Non-specific interaction was minimized. Specific protein interaction was detected after incubating the membrane in the primary antibody, washing buffer, secondary antibody (goat anti-mouse IgG-AP/goat anti-rabbit IgG-AP; 1:10,000), and substrate solution (BCIP/NBT), respectively. Following dilution was used for primary antibody: anti-beclin-1 (1:2000), anti-p62 (1:2000), anti-LAMP-2 (1:5000), anti-α-synuclein (1:2000), anti-Hsc-70 (1:2000), anti-LAMP-2A (1:2000) anti-p-mTOR (1:2000), anti-p-Ulk1 (1:5000), anti-p-AMPK (1:2000). Image was captured, relative band density was calculated, and value is expressed in relation to a housekeeping protein, β-actin.

Statistical Analysis

One-way analysis of variance along with a Newman-Keuls post hoc test was employed for statistical comparison. Value was calculated as mean ± standard error (SE). Variation was declared considerable when the probability (p) value was < 0.05.

Results

Dopamine

MPTP reduced the dopamine content in the striatum and silymarin treatment ameliorated MPTP-induced alteration in dopamine. Silymarin per se could not make any change in dopamine content (Fig. 1).

Macroautophagy Proteins

Silymarin per se did not alter any indexes of autophagy while MPTP increased the level of beclin-1 and p62

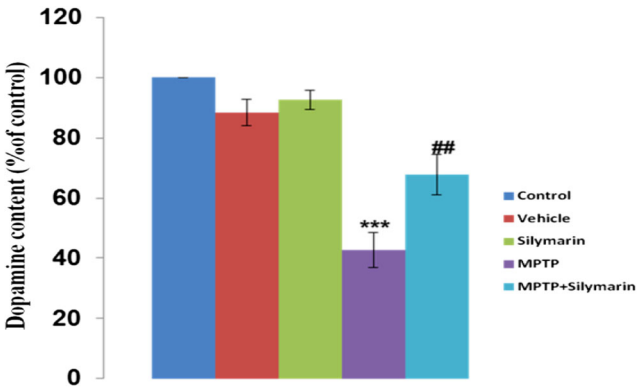

Fig. 1 Dopamine content in the corpus striatum. The dopamine content showed improvement in its level in the silymarin- and MPTP-exposed group in comparison with the MPTP-alone-treated group. Values (originally calculated in ng per mg tissue; controls are presented as 100% in all sets) are presented in mean ± SE (n = 3) and considerable changes are shown as $^{***}p < 0.001$ in relation to control and $^{\#\#}p < 0.01$ in relation to the MPTP-treated group

accumulation, which were significantly reduced when MPTP-treated animals were also administered silymarin (Fig. 2). On the contrary, MPTP attenuated LAMP-2 protein, which was significantly normalized by silymarin in MPTP-intoxicated animals.

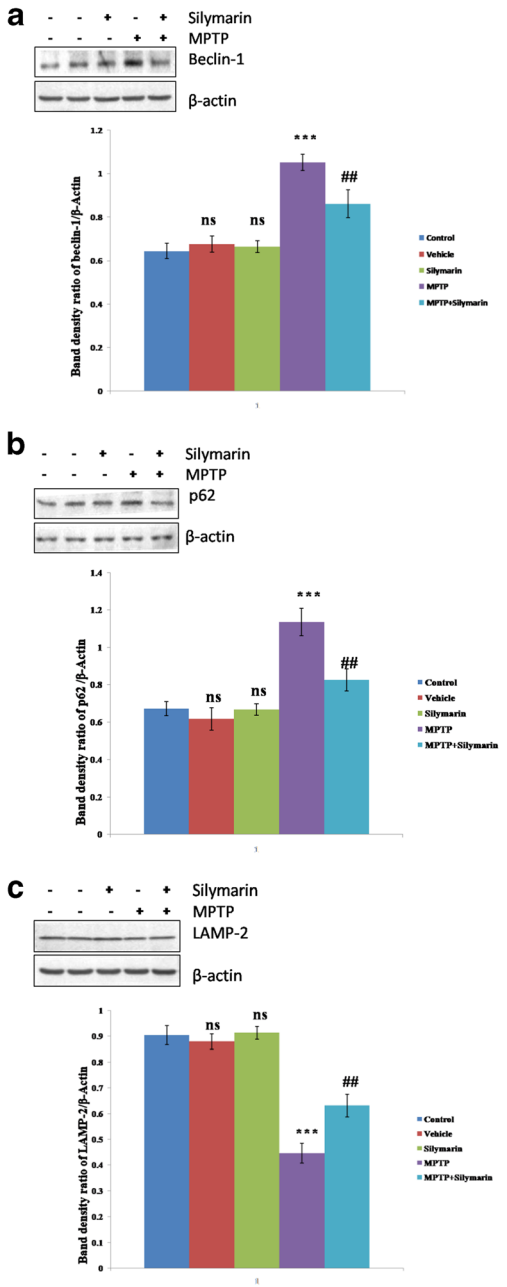

Fig. 2 Level of autophagy proteins in the nigrostriatal tissue. Silymarin per se did not alter the beclin-1 expression, p62 accumulation, and LAMP-2 content. MPTP increased the level of the first two and reduced the last one. MPTP-induced changes were altered towards normalcy if animals were also administered with silymarin. Western blots (upper panel) and band density ratio (lower panel) of beclin-1 (**a**), p62 accumulation (**b**), and LAMP-2 (**c**) with reference to β-actin are shown. Values are shown in mean ± SE ($n = 3$) and significant changes are stated as $^{***}p < 0.001$ in comparison with control and $^{\#\#}p < 0.01$ in comparison with the MPTP-treated group

Autophagy Regulatory Proteins

Expression of p-mTOR, p-AMPK, and p-Ulk1 proteins was not altered by silymarin per se. However, MPTP reduced p-mTOR level and augmented the expression of p-AMPK and p-Ulk1 proteins, which were significantly modulated towards normalcy in silymarin-co-treated animals (Fig. 3).

CMA Proteins

Silymarin per se could not change the expression of α-synuclein, LAMP-2A, and Hsc-70 proteins. However, MPTP augmented the expression of α-synuclein and reduced the level of LAMP-2A without producing any change in the expression of Hsc-70 protein. Silymarin treatment in the MPTP-intoxicated group significantly reduced the level of α-synuclein. Silymarin also augmented LAMP-2A protein expression in the MPTP-treated group in comparison with MPTP alone (Fig. 4).

Discussion

In MPTP-induced Parkinsonism, oxidative stress and mitochondrial dysfunction are found to be critical in addition to several other biological events (Ali et al. 1994). Acute MPTP mouse model is used in this study since it is widely employed to comprehend the molecular pathways involved in nigrostriatal dopaminergic neuronal loss and also to decipher the neuroprotective effectiveness of synthetic or natural antioxidants (Herraiz and Guillén 2011). MPTP was found to accelerate the depletion of striatal dopamine in the current experimental paradigm as observed in many previous investigations. Such variable was measured in order to ascertain if animals treated with MPTP possess Parkinsonian feature in the present scenario as reported elsewhere (Wong et al. 2011; Zhu et al. 2007; Lee et al. 2018). Silymarin ameliorated the striatal dopamine towards normalcy showing that silymarin rescued from MPTP-induced changes. This is in agreement with the previous study where silymarin is shown to act as a neuroprotective agent in a few toxicant models of Parkinsonism (Singhal et al. 2011; Haddadi et al. 2018; Haddadi et al. 2014; Lee et al. 2015; Pérez-H et al. 2014; Zhu et al. 2007).

MPTP- and MPP^+-based cellular and animal models of neurodegeneration have implicated the role of general and mitochondrion-specific macroautophagy (Wong et al. 2011; Zhu et al. 2007; Lee et al. 2018). MPP^+ increases the autophagic flux formation and mitochondrial impairment leading to cell death in neuroblastoma cells, which are significantly protected by an autophagy inhibitor, bafilomycin A1, indicating that MPTP regulated autophagy (Zhu et al. 2007). The present study measured the level of a few selected

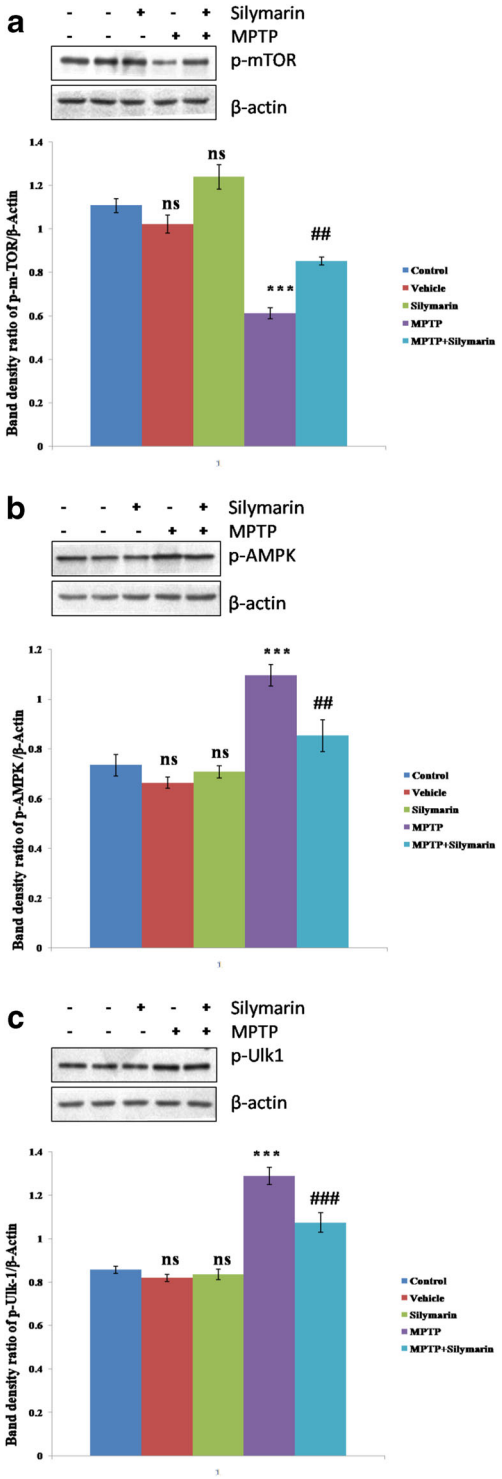

Fig. 3 Level of p-mTOR, p-AMPK, and p-Ulk1 proteins. Western blots (upper panel) and band density ratio (lower panel) of p-mTOR (**a**), p-AMPK (**b**), and p-Ulk1 (**c**) with reference to β-actin are shown. Silymarin per se did not change the expression of any proteins. MPTP decreased the levels of the first and increased the last two. MPTP-induced changes were altered towards normalcy when rodents were also administered with silymarin. Values are calculated in mean ± SE ($n = 3$) and considerable changes are shown in $^{***}p < 0.001$ in relation to control and $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ in relation to the MPTP-treated group

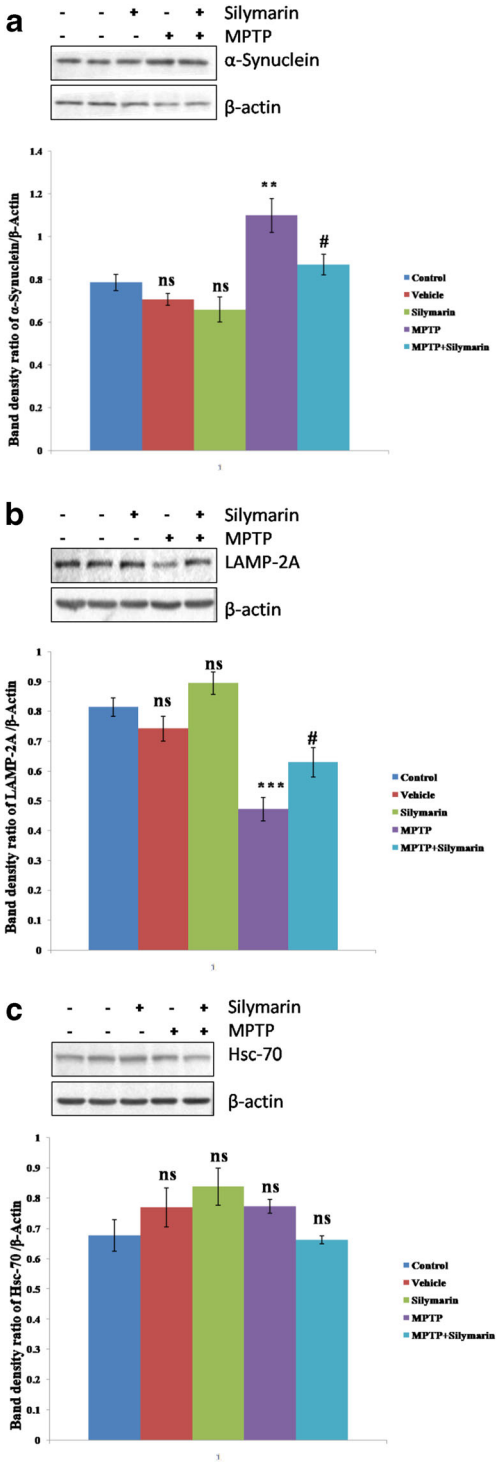

Fig. 4 Level of α-synuclein, Hsc-70, and LAMP-2A proteins. Silymarin rescued from MPTP-induced changes in the expression of α-synuclein and LAMP-2A proteins. The level of Hsc-70 protein was not altered by any of these two agents. Western blots (upper panel) and band density ratio (lower panel) of α-synuclein (**a**), LAMP-2A (**b**), and Hsc-70 (**c**) are shown in relation to β-actin. Values are shown in mean ± SE ($n = 3$) and noteworthy changes are presented in $^{**}p < 0.01$ and $^{***}p < 0.001$ in relation to control and $^{\#}p < 0.05$ in relation to the MPTP-treated group

macroautophagy and CMA indicators to investigate if MPTP regulates such events. An increased level of autophagosome formation markers, such as increased beclin-1 and p62 in MPTP-intoxicated mice, showed that MPTP modulated autophagy by regulating autophagosome formation. Silymarin modulated beclin-1 and p62 showing its ability to resist against MPTP-induced changes in macroautophagy. Reduced level of LAMP-2 in MPTP-intoxicated animals showed the involvement of aberrant autophagolysosome formation leading to an accumulation of autophagosome. This is not an unusual phenomenon since a similar observation is reported in a few other models (Mishra et al. 2018; Bové et al. 2014). Silymarin treatment altered the level of LAMP-2 protein towards normalcy showing that silymarin encountered MPTP-induced changes in macroautophagy and thereby offered neuroprotection. This is supported by previous studies, which have shown that silymarin induces protection in toxicant models of Parkinsonism (Singhal et al. 2011; Haddadi et al. 2018; Haddadi et al. 2014; Lee et al. 2015; Pérez-H et al. 2014; Zhu et al. 2007). Increased level of p-Ulk1 and p-AMPK proteins and reduced p-mTOR content following MPTP administration showed that MPTP induced p-AMPK-mediated Ulk1-dependent macroautophagy. Silymarin reduced the MPTP-induced increase in p-AMPK and p-Ulk1 levels, and decrease in p-mTOR content towards normalcy showed that it corrected macroautophagy probably at the initial steps of signalling cascade, which was reflected further in downstream effectors. Since silymarin is a potent antioxidant and extremely safe chemical entity, it could restore the antioxidant capacity of dopaminergic neurons leading to autophagy correction. That is supported by the fact that initial steps of autophagy get impaired due to increased oxidative stress since MPTP induces oxidative stress. It is in accordance with previous investigations where silymarin is found to possess antioxidant and anti-inflammatory properties and protects from oxidative stress and mitochondrial dysfunction (Singhal et al. 2011; Haddadi et al. 2018; Haddadi et al. 2014; Lee et al. 2015; Pérez-H et al. 2014; Zhu et al. 2007) leading to CMA impairment.

Occasionally, macroautophagy fails to degrade the proteins owing to excessive oxidative stress. In that condition, CMA takes over the process as a compensatory cellular event. CMA is regulated by substrate recognition units, such as Hsc-70 and LAMP-2A, along with normal functioning of the lysosome. In addition to various mutated pathogenic proteins, CMA degrades soluble α-synuclein, hence its level was measured in the study. Attenuated LAMP-2A expression and increased α-synuclein level in MPTP-intoxicated animals showed that CMA is impaired in MPTP-induced Parkinsonism. Silymarin co-treatment, on the other hand, reduced α-synuclein content and increased LAMP-2A level in MPTP-intoxicated animals showing that silymarin-induced neuroprotection could also be contributed owing to its ability to correct CMA, which increased the clearance of aggregated proteins. Correction of impaired lysosome quality could be an outcome of silymarin-mediated effects at the initial steps of macroautophagy and CMA owing to its antioxidant property (Singhal et al. 2011; Haddadi et al. 2018; Haddadi et al. 2014; Lee et al. 2015; Pérez-H et al. 2014; Zhu et al. 2007).

Conclusively, silymarin restored the antioxidant defense system of dopamine-producing neurons of the nigrostriatal area leading to correction of MPTP-induced impairments in macroautophagy, CMA, and lysosome quality.

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Conflict of Interest The authors declare that they have no conflicts of interest.

Author Contributions Manish Kumar Tripathi was involved in the data generation, acquisition, and analysis. Mohd Sami Ur Rasheed and Abhishek Kumar Mishra were involved in performing some initial experiments and treating the experimental animals if and when Manish Kumar Tripathi was on leave or busy in some other activities. Devendra Kumar Patel performed dopamine estimation. Mahendra Pratap Singh conceived the study plan and interpreted the analyzed data, which were provided by the first author. Manish Kumar Tripathi wrote the first version of the manuscript and Mahendra Pratap Singh revised the language. All authors have gone through the submitted version of the manuscript and endorsed the same.

Compliance with Ethical Standards

The present investigation was endorsed by the animal ethics committee of the institute.

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