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RESEARCH ARTICLE

Rajan et al: TUDCA and Syndopa in PD mouse model

TUDCA combined with Syndopa protects the midbrain and gut from MPTP toxicity in a Parkinson's disease mouse model: Immunohistochemical evidence

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ABSTRACT

Neuro-inflammation plays a significant role in the neurodegenerative processes associated with Parkinson's disease (PD). A hallmark of PD is the degeneration of dopaminergic neurons within the nigrostriatal pathway. The standard treatment for PD is Syndopa (a combination of levodopa and carbidopa). However, while Syndopa alleviates symptoms, it is also associated with numerous side effects in patients. Research has demonstrated the protective effects of Tauroursodeoxycholic acid (TUDCA) in mitigating the neuropathological consequences of PD in several preclinical studies. Nonetheless, further investigation is necessary to delineate the role of TUDCA in PD therapeutics. Although the efficacy of TUDCA monotherapy in PD has been explored, there is a lack of preclinical research examining the additive effects of TUDCA in conjunction with Syndopa therapy. In this study, we utilized an MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of PD to evaluate the potential therapeutic benefits of TUDCA monotherapy and the combined effects of TUDCA and Syndopa therapy, compared to standard Syndopa treatment. We conducted immunohistochemical (IHC) assessments of α-synuclein expression in the gut and substantia nigra pars compacta (SNpc), as well as tyrosine hydroxylase and NF-kB expression in the striatum and SNpc regions, to investigate the efficacy of the test drugs. The immunohistochemical findings indicate that both TUDCA monotherapy and the combination therapy of TUDCA and Syndopa significantly reduced MPTP-induced alterations in the expression levels of α-synuclein, tyrosine hydroxylase, and NFκB in the striatum and SNpc regions. Additionally, the MPTP-induced changes in α-synuclein expression in the gut were notably reversed by these treatments. Collectively, these results suggest that incorporating TUDCA with Syndopa may represent a promising therapeutic strategy to address the pathophysiological challenges associated with PD.

Keywords: Parkinson’s disease, PD, MPTP, TUDCA, Syndopa, α-synuclein, substantia nigra

INTRODUCTION

Parkinson's disease (PD) often referred to as paralysis agitants, is a brain degenerative condition that affects the central nervous system's extra pyramidal motor neurons. It is one of the detrimental neurodegenerative conditions in the elderly, next to Alzheimer's disease (AD) [1]. James Parkinson was the first to describe the cardinal manifestations of PD. Pathologically it is classified as synucleinopathy, along with other conditions that have Lewy bodies [2]. It is predicted following dopaminergic neuronal death in the SNpc pars compacta (SNpc), depleted striatal dopamine levels and proteinaceous clumps within neuronal cell bodies called Lewy bodies, which stain for α-synuclein [3]. Neuronal degeneration with Lewy bodies is not only restricted to the dopaminergic system, and it also affects the non-dopaminergic neuronal system and peripheral nervous system [4].

About 90 % cases of PD occur sporadically and are due to unknown etiology. The remaining 10 % cases are familial which occur due to mutations in SNCA, GBA, LRRK2, and PINK1/parkin genes [5,6]. Although the exact cellular mechanisms contributing to dopaminergic degeneration in PD are ambiguous, it is believed that oxidative stress, neuroinflammation, and mitochondrial dysfunction play a major role in the depletion of dopaminergic cells in sporadic & familial PD [7,8]. Glial cells which have significant role in antioxidant defense [3] are known to release several pro-inflammatory mediators and phagocytose cellular debris when activated by dying neurons [7,9,10]. This further contributes to the degeneration of neurons with provoked inflammation and cell death that aggravates to intensify the neurodegenerative process [11]. In fact, glial activation contributes significantly to the development of neuronal dysfunction in PD.

Clinically PD is manifested by motor symptoms viz., resting tremor, bradykinesia, muscle rigidity, and postural imbalance [12]. In addition to the above mentioned “cardinal” motor symptoms, PD also manifests in the form of other non-motor symptoms that include dysphagia, speech problems, constipation, anxiety, depression, orthostatic hypotension, micturition abnormalities and cognitive problems. Several of these are recognized as prodromal symptoms of PD or risk factors for the illness [13,14].

In PD patients, the caudate nucleus and putamen become excessively active and continuously produce excitatory signals to the corticospinal tract due to the destruction of dopaminergic neurons, which causes rigidity. Dopamine is the inhibitory transmitter released in these regions. Levodopa, or L-dopa (LD), a precursor of dopamine, is a commonly used treatment

drug for PD symptoms. However, there are certain harmful effects associated with L-dopa therapy, such as hypotension, nausea, gastrointestinal hemorrhage, sleeplessness, disorientation, auditory hallucinations [15].

1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) is used for developing PD in animal model, which reproduce the neurodegeneration. MPTP being a lipophilic component easily crosses the blood brain barrier and combines with astrocytes where it is metabolized by monoamine oxidase (MAO) into its active metabolite MPP+ [16,17]. MPP+ has tropism for dopaminergic neurons and actively taken up by the DA neurons, where it is believed to cause neurodegeneration in those neurons by inducing mitochondrial dysfunction, oxidative stress, inflammation, excitotoxicity and aggregation of inclusion bodies [18-21].

LD, a commonly used drug to relieve PD symptoms poses many adverse responses. The episodes of various motor symptoms due to dopa resistance and non-motor symptoms which encompass autonomic dysfunctions, mood and cognitive impairment and drug linked side effects (psychosis, motor fluctuations and dyskinesia) are the notable features [22,23]. Owing to longer half-life of dopamine agonists, they are being increasingly used in the treatment of PD as monotherapy or as a combination drug [24]. The present research scenario focuses on identifying an effective treatment which will be better than LD or by implementing pharmacological approaches to reduce the LD dose levels to prevent side effects. Although, α-synuclein accumulation seems to play a major role in PD and considered as one of the most supported hypotheses among several, the exact pathogenic mechanisms of PD is still unclear and warrants more detailed investigations. The course of PD involves several events which includes mitochondrial dysfunction, neuroinflammation and faulty protein clearance mechanisms [19]. But how these various events cooperate and integrate with each other remains incompletely understood [25]. While this movement disorder occurs due to damage of dopaminergic neurons in the SNpc, other brain regions are also critically affected [11,26] and extensive investigations are underway. Several seminal works have indicated the importance and significance of gut dysfunctions in PD pathogenesis. Although the LBs are identified as pathological hallmarks of PD containing mostly the aggregated α-synuclein protein, how they lead to neurodegeneration is still obscure.

Tauroursodeoxycholic acid (TUDCA), a natural bile acid is an endogenous taurine conjugate of ursodeoxycholic acid (UDCA), that is employed in the management of cholestatic liver diseases. It crosses the blood-brain barrier and found to be nontoxic. Research has been

conducted widely to know the beneficial role of TUDCA in many non-liver diseases such as neurodegenerative diseases. In fact, TUDCA has shown considerable neuroprotective role in mouse models of Alzheimer's and Huntington's diseases [27-30].

The proposed mechanisms of TUDCA's neuroprotective activity in an experimental model of PD includes activation of the pro-survival Ser/Thr kinase Akt and anti-oxidative mechanisms dependent on nuclear factor E2-related factor 2 (Nrf2) pathway, as well as parkin mediated mitochondrial turnover [31-34]**.** However, studies that support the gut protective mechanisms of TUDCA in PD are not found.

Brain histomorphological studies can be used to delineate TUDCA's neuroprotective role and efficacy in PD. So far, the therapeutic potential of TUDCA has been tested as monotherapy in PD preclinical research. In the present work, we tested TUDCA's pharmacological potential in MPTP intoxicated mice through immunohistochemical studies involving brain and gut samples. Notably, in the present work, TUDCA was tested either alone and as an additional drug along with syndopa to explore the neuroprotective benefits.

MATERIALS AND METHODS

Animals

The Mass Biotech animal facility in Chengalpattu, India, supplied C57BL/6 mice (male), which were 2-3 months old and weighed 30-40 g. The mice were allowed for 7-days acclimatization followed by 26 days of experimentation. Mice were kept in a clean environment with a relative humidity of 30 to 40% and a photoperiod of 12 hours of light and 12 hours of darkness. Throughout the investigation, the mice had unrestricted access to food and water.

Mice grouping and experimental protocol

The mice were randomly segregated into five groups, each having six mice as below: The randomization of animals was done by Random Number Table method: Each mouse was labelled with a specific number code (Non-sequential), and then numbers were drawn randomly from a table to assign individual mouse into various groups. The group allocation was masked until interventions started. Group 1 was considered as control which received normal saline (0.2 mL/mouse i.p.). Group 2 received MPTP (30 mg/kg/day i.p.) dissolved in saline for 5 days. Group 3 was given TUDCA (150 mg/kg/day i.p.) dissolved in phosphate buffer saline (PBS) for 21 days following MPTP administration for 5 days (30 mg/kg/day

i.p.). Group 4 received syndopa (12 mg/kg/day p.o.) dissolved in filtered water for 21 days following MPTP administration for 5 days (30 mg/kg/day i.p.). Group 5 received TUDCA (150 mg/kg/day i.p.) and syndopa (12 mg/kg day p.o.) for 21 days following MPTP administration (30 mg/kg/day i.p.) for 5 days. For additive/enhanced therapy: the time interval maintained each day between the TUDCA and Syndopa treatments for mice was 2 hrs.

Cardiac perfusion and preservation of gut and midbrain tissues

At the end of experimentation procedures, an isoflurane inhalation anesthesia protocol was followed to perform a cardiac perfusion with normal saline (0.9% NaCl). The mice skull was carefully opened to dissect the midbrain without mechanical damage. The midbrain was kept on ice and subsequently cleaned with ice-cold phosphate-buffered saline (PBS), pH 7.4. A portion of cut midbrain and gut tissues were fixed with 10% neutral buffered formalin, dehydrated and then subjected to paraffin embedding and sectioning (5 μm thickness) for the purpose of immunohistochemical analysis. While sampling the gut tissues, the colon (distal) region was processed for IHC staining. The technician who processed and stained the tissues for histological studies as well as the pathologist who did the interpretation of slides were blinded to the group identity at all stages of histological processing and quantification.

Immunostaining of tyrosine hydroxylase protein expression in striatum and SNpc

Slides containing midbrain sections were de-paraffinized with washes in xylene for 5-min, 1-min washes in a descending series of ethanol: 100%, 95%, 80%, 70%. The antigen retrieval was performed with sodium citrate buffer. Briefly, 2.94g of trisodium citrate dihydrate was dissolved in 95mL distilled water and then the pH was adjusted to 6.0 using 1N NaOH to obtain antigen retrieval buffer. Added 5mL of 0.1% Tween-20 and mixed well before adjusting the final volume to 100mL with distilled water. After antigen retrieval, slides were kept in 5% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Slides were washed in running tap water for 10 min, 5 min in 0.1 M Tris, blocked in 3% fetal bovine serum (FBS) (SD Fine Chem, India.). Slides were incubated in primary antibody overnight at 4°C. The Tyrosine hydroxylase (Sigma-Aldrich) primary antibody was used at 1:2000 dilution with 0.5-1% formic acid (SRL Pvt Ltd, India**)**. After washing the primary antibody with 0.1 M Tris for 5 min, incubation with goat anti-rabbit biotinylated IgG (Thermo Fisher Scientific, USA) at 1:1000 for 1 h was performed. 0.1 M Tris buffer was used to rinse off the biotinylated antibody for 5 min, then incubated with avidin-biotin

solution (Abcam, USA) for 1 h. Slides were then rinsed for 5 min with 0.1 M Tris, then the sections were stained with diaminobenzidine (DAB) (SRL Pvt Ltd, India) to visualize the immune positive cells. TH immune positive areas were observed under light binocular microscope (OLYMPUS CX23 Model).

Immunohistochemical studies of NF-kb protein expression in striatum and SNpc

The de-paraffinization of slides with xylene and washing protocol in descending series of ethanol were as detailed in previous IHC methods section. After antigen retrieval (as described in previous tyrosine hydroxylase IHC methods section) slides were incubated in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Slices were blocked in PBS containing 0.2% Triton X-100 and 10% normal goat serum at 37 °C for 45 min. Slides were incubated at 4°C temperature in primary antibodies overnight. The rabbit anti-NFκB (Sigma-Aldrich) primary antibody was used at 1:2000 dilution. After rinsing the primary antibody with 0.1 M Tris for 5 min, the sections were incubated with goat anti-rabbit biotinylated IgG in 1:500 at 37 °C for 45 min. The subsequent steps such as washing of biotinylated antibody, incubation with avidin-biotin solution, rinsing with 0.1 M Tris and staining process with DAB to visualize the immune positive cells were exactly followed as indicated above in TH immunohistochemical study protocol.

Immunohistochemical evaluation of α-synuclein protein expression in gut, and SNpc

For the evaluation of gut alpha-synuclein IHC expression, the transverse section of mucosa and submucosa of colon were analyzed. The gut and midbrain sections were de-paraffinized with 2 sequential 5-min washes in xylene, 1-min washes in a descending grade of ethanol: 100%, 100%, 95%, 80%, 70%. After antigen retrieval, slides were incubated in 5% H_2O_2 in methanol to quench endogenous peroxidase activity. Using running tap water the slides were washed off (10 min), in 0.1 M Tris (5 min), then blocked with 2% fetal bovine serum (FBS). Incubation with primary antibody was performed overnight at 4°C. The anti-α-synuclein primary antibody (Abacam, USA) was used at 1:1000 dilution. After rinsing off the primary antibody, the slides were treated with secondary antibody (goat anti-rabbit biotinylated IgG) at 1:1000 dilution for 1 hr. The remaining steps were followed exactly as detailed in previous IHC methodology to visualize the α-synuclein positive immunoreactivity.

Analysis and Quantification of IHC slides

The tyrosine hydroxylase, NFκB and α-synuclein immune positive areas detected in the target tissues were detected under a light binocular microscope (OLYMPUS CX23 Model) and the captured images were measured and quantified using Image-J software version 1.54 (Source: http://rsbweb.nih.gov/ij/download.html).

Ethical statement

Animals were housed and well maintained by adhering to the Committee for the Control and Supervision of Experiments on Animals (CCSEA), India, guidelines and protocols. The study procedures adhered to the IAEC guidelines and principles, and the research proposal was accepted by the Saveetha Medical College Institutional Animal Ethics Committee (SU/CLAR/RD/34/2023).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA) using one-way ANOVA. Multiple comparisons were done using the Tukey test. The results are calculated as mean ± SD, and data are shown as mean + SD. *P* values of <.05 were considered statistically significant. In all the main figures, the statistically significant data are shown as '*' according to the *P* values.

RESULTS

Evaluation of tyrosine hydroxylase protein expression in striatum and SNpc

The immunoreactivity of TH positive fibres in striatum (Figure 1 and Supplemental Figure A1 with lower magnification-200x) of control, MPTP-PD, TUDCA, syndopa and TUDCA + syndopa groups were 94.6, 18.5, 72.2, 56.1, and 79.5% and if differences were significant ($P < 0.001$). Compared to control, MPTP-PD and syndopa group showed significant differences in TH expression levels ($P < 0.0001$, and <0.05, respectively). By contrast, compared to control group, TUDCA, TUDCA + syndopa group did not show significant ($P > 0.05$) changes respectively. The syndopa group compared with MPTP-PD showed statistically significant changes ($P < 0.001$). When syndopa group was compared with TUDCA monotherapy group, there was no statistical significance ($P > 0.05$). A similar trend was noticed when TUDCA group was compared with TUDCA + syndopa ($P > 0.05$). However, statistical significance exists when TUDCA + syndopa group was compared with syndopa group ($P < 0.05$).

The immunoreactivity of TH positive fibres in the SNpc (Figure 2) of control, MPTP-PD, TUDCA, syndopa and TUDCA + syndopa groups were 90.4, 45.3, 78.6, 64.1, and 83.9% respectively. Compared to control group, MPTP-PD group showed significant decrease in the TH protein expression levels ($P < 0.01$). Compared to control group, syndopa group did not show significant differences ($P > 0.05$) and likewise compared to control group, TUDCA group and TUDCA + syndopa group also did not reveal significant differences in the TH expression levels ($P > 0.05$). A comparison of MPTP-PD group with either TUDCA group or TUDCA + syndopa group revealed statistically significant difference ($P < 0.01$). There was no significant difference when syndopa group was compared with MPTP group ($P > 0.05$). When syndopa group was compared with either TUDCA or TUDCA + syndopa, no statistical significance was noticed ($P > 0.05$). Likewise, comparison of TUDCA group with TUDCA + syndopa demonstrated no statistical level significance ($P > 0.05$). However, the efficacy of TUDCA + syndopa was marginally better than TUDCA monotherapy, whereas syndopa alone treatment didn't significantly improve the TH positive fibres in SNpc ($P > 0.05$).

Evaluation of NF-kb protein expression in SNpc and striatum

In striatum (Figure 3 & Supplemental Figure A2 with magnification-400x), the intensity of NF-kB positive cells of control, MPTP-PD, TUDCA, syndopa and TUDCA + syndopa groups were estimated as 7.2, 43.3, 22.8, 29.3 and 18.2% respectively. Compared to control group, MPTP-PD group showed significant differences in the NF-kb expression levels ($P < 0.0001$). Compared to MPTP-PD group, TUDCA group & TUDCA + syndopa additive group exhibited significant differences in the expression levels ($P < 0.01$). A comparison made between syndopa group and MPTP-PD group revealed no statistical significance ($P > 0.05$) although syndopa treatment reduced NF-kB levels reasonably. There was no statistical significance ($P > 0.05$) when group comparison was performed across all the drug intervened groups [syndopa Vs TUDCA; syndopa Vs TUDCA + syndopa & TUDCA Vs TUDCA + syndopa]. Overall, the NF-kB protein expression levels were significantly reduced in the TUDCA monotherapy group and TUDCA plus syndopa intervention group when compared with MPTP-PD group.

The intensity of NF-kB indicating cells in the SNpc (Figure 4 & Supplemental Figure A3 with magnification-400x) of control, MPTP-PD, TUDCA, syndopa and TUDCA + syndopa groups were shown as 5.70, 35.48, 17.82, 23.15 and 13.00%. Compared with control, MPTP-PD showed significant differences in the expression levels ($P<0.0001$). Whereas, in various

drug treated groups (TUDCA, syndopa and TUDCA + syndopa), the NF-kB expression levels were significantly reduced compared to PD-MPTP group ($P < 0.01$, 0.05 and 0.001 respectively). As mentioned above for striatum, statistical significance didn't exist ($P > 0.05$) when the various drug intervention groups were compared with each other.

Gut level changes in α-synuclein protein expression

The intensity of α-synuclein positive cells in gut samples (Figure 5 & Supplemental Figure A4 with lower magnification-200x) of control, MPTP-PD, TUDCA, syndopa and TUDCA + syndopa groups were manifested as 2.90, 62.63, 31.90, 32.22, 22.56 %. Compared with control, MPTP-PD group revealed significant upregulation of α-synuclein positive expression ($P < 0.001$). By contrast, syndopa, TUDCA and TUDCA + syndopa treatments led to notable reductions in the gut α-synuclein expression levels as compared to MPTP-PD group and the values were found to be statistically significant ($P < 0.05$). The group comparisons made across the various drug treatment groups didn't exhibit statistical level significance ($P > 0.05$)

Assessment of α-synuclein protein expression in SNpc

The intensity of α-synuclein positive cells (Figure 6 & Supplemental Figure A5 with lower magnification-200x) in control, MPTP-PD, TUDCA, SYNDOPA and TUDCA+SYNDOPA groups were found to be 6.06, 75.80, 49.28, 55.08, and 47.4%. Compared with control group, MPTP-PD, TUDCA, syndopa and TUDCA + syndopa showed strikingly elevated levels of α-synuclein ($P < 0.0001$, 0.001, 0.0001 and 0.001 respectively). TUDCA ($P < 0.01$), syndopa ($P < 0.05$) and TUDCA + syndopa ($P < 0.01$) groups also showed significant difference in the expression levels when compared with MPTP-PD group implicating the efficacies of drugs. There was no detectable level of statistical significance when the drug intervention groups were compared with each other ($P > 0.05$).

DISCUSSION

While PD is clinically diagnosed with cardinal motor symptoms which arises due to nigrostriatal degeneration, multiple pathologies coincide with PD pathogenesis. But it remains unclear which pathology is initiated first to trigger nigrostriatal degeneration [35]**.** The current IHC data shows prominent expression of α-synuclein in the MPTP intoxicated mice gut samples when compared to control group. Several notable works have emphasized that misfolded α-synuclein is first formed in the enteric nerves before it could be detected in the brain. But, it is still intriguing whether PD starts first in the gut or brain region. While many investigations support evidence that MPTP administration increases alpha-synuclein

expression in gut tissue, metabolic perturbation in gut & gut dysbiosis, [36,37] the first and foremost region to be affected by MPTP is still under investigations. However, a recent publication by Heng et al (2022) has mentioned that MPTP stimulate synucleinopathies first in the gut to take part in the etiology and progression of PD. Heng et al confirmed that synucleinopathies existed in the stomachs of both chronic and acute (single) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injected mice. They first showed a significant elevation of aggregated and nitrated α-synuclein in the enteric glial cells (EGCs) of the gastric myenteric plexus. Next, they tried to prove this mechanism by giving single MPTP-injection to mice. Stomach synucleinopathies were noticed well before they could be visualized in the nigrostriatal system i.e. 12 h after MPTP injection. High monoamine oxidase-B (MAO-B) activity and low superoxide dismutase (SOD) activity possessed by the stomach made it more susceptible to MPTP-induced oxidative stress and hence manifested with increased reactive oxygen species (ROS) in the stomach and 4-hydroxynonenal (4-HNE) in the EGCs 3h after MPTP exposure. Noticeably, a considerable hike in nitrated α-synuclein in the EGCs occurred after 3 h and 12 h of MPTP exposure. Taken together, the work of Heng et al (2022) demonstrated that the enteric glial cells could be new contributors to synucleinopathies in the stomach. Therefore, it was reinforced that the early-initiated gut synucleinopathies may be influencing the adjacent neurons in the myenteric plexus and the CNS.

Based on epidemiological and histological findings, the gastrointestinal problems (constipation) and α-synuclein inclusions were detected early in the ENS many years before the propagation of motor symptoms and inclusions in the CNS [38].Another study depicted by Luk et al. (2012) [39] showed the ability of α-synuclein to undergo a 'prion-like' misfolding and aggregation process implicating that the disease may start in the peripheral organs such as the ENS and then progress to the CNS via the dorsal motor nucleus of the vagus thereby affecting the brain stem, mid- and fore-brain and ultimately the cerebral cortex [38,40].

The process by which misfolded α-synuclein present in the enteroendocrine cells (EECs) spreads from the gut epithelium to the brain was depicted by Haggerty et al. [41]. The same group have also shown how the neuron-like features of enteroendocrine cells (EECs) connects to enteric nerves and take part in the dissemination of α-synuclein misfolding from gut and brain. The ability of TUDCA-syndopa enhanced therapy in reducing the gut α-

synuclein levels is evident from the present IHC findings which implicates amelioration of MPTP toxicity and early PD symptoms in mice.

While loss of dopaminergic neurons is the primary cause of MPTP toxicity in brain, considerable number of research works have depicted that MPTP intoxication in mice and non-human primates can trigger α-synuclein expression in SNpc [42-44]. In another report, a significant decrease (80–89%) in tyrosine hydroxylase (TH)-positive SNpc neurons in the side ipsilateral to MPTP administration in young and old MPTP treated Rhesus Monkeys were manifested along with increased α-syn expression in the SN region [45]. Moreover, MPTP is still a widely used PD model for the preclinical testing of various pharmacological agents [46-48]. Although prolonged L-dopa treatment could be responsible for down-regulation of TH which is involved in dopamine synthesis, it may potentially lead to L-dopa-induced dyskinesia. This could be the plausible reason that restoration of TH expression levels in striatum by syndopa is not convincing like other drug treatment groups. In SNpc, the TH expression was improved in syndopa group, although the value was not statistically significant in comparison with MPTP group.

Neuroinflammation is an injurious pathological event in PD and AD. NF-κB, a key marker of neuroinflammation is widely studied in PD models to explore both the pathological states of the disease and the efficacy of test drugs [49]**.** Neuroinflammation in SNpc was greatly attributed to the progression of PD in mice [50]**.** NF-κB increased significantly in the striatum and SNpc of MPTP induced mice [51,52]**.** Using a mouse model of PD, it was proven that drugs which inhibited NF-κB activation in the SNpc region circumvented the dopaminergic neuronal loss effectively. So far, the anti-inflammatory potentials of TUDCA in PD midbrains are less studied and therefore the present research involving an MPTP model mice fills the research gap through IHC findings.

Decreased tyrosine hydroxylase and increased α-synuclein expression in midbrain and striatum has been depicted in MPTP and other chemical induced PD models [53-55]. PD patients brain tissues had shown significantly reduced DA content based on several published works [56,57]. Dopamine synthesis is triggered by TH via tyrosine hydroxylation to L-dopa (Precursor of dopamine). Importantly, the activity of tyrosine hydroxylase in the brains of PD patients and PD mice has been shown to be significantly reduced [58-60]. TH+ antibodies can detect the dopaminergic neurons in the SNpc pars compacta region in MPTP induced mice [61]**.** The number of TH+ neurons in the SNpc were significantly reduced with

increased numbers of activated microglia in MPTP-treated mice [62]. Therefore, pharmacological approaches aimed at reducing TH levels in striatum and SN regions in MPTP models were useful to reduce PD complications [63,64]. It is evident that MPTP can activate glial cells in both the striatum and SNpc regions of the brain to alter the proinflammatory markers, which is responsible for the promotion of neuroinflammation and neurodegeneration [65].

TUDCA intervention either alone or administered along with syndopa reversed these changes considerably by reducing the damage caused by MPTP toxicity. The syndopa treatment also rendered better effect to counteract the MPTP toxicity as shown in the present work. While striatal TH expression was significantly improved in syndopa treatment group, there was no significant restoration of TH in SNpc region following syndopa treatment. The region wise variations depicted by syndopa seems interesting and needs extensive investigations. The anti-inflammatory effect of syndopa in striatum is not pronounced in this MPTP model. All the three drug intervention groups significantly inhibited α-synuclein accumulation in gut tissues of MPTP intoxicated mice and the data is shown perhaps for the first time in the present study.

TUDCA crosses the blood–brain barrier (BBB) [66] to elicit neuroprotective response. TUDCA's anti-apoptotic mechanisms elicited in hepatocytes were found to mimic in neuronal cells as well [27-29]. Based on extensive literature evidence it appears that bile acids can be used to defend against neuronal cellular programmed death pathways. Drugs that restored the loss of TH levels in the subsantia nigra (SNpc) and striatal regions were found to be beneficial in ameliorating PD problems in MPTP, α-Synuclein and 6-hydroxydopamine models [67-70]. Protein oxidation, autophagy and α-synuclein aggregation were attenuated by TUDCA when administered to mice before MPTP induction [71]. According to Rosa et al. [34], TUDCA pre-treatment to mice prevented the MPTP toxicity by increasing the numbers of TH-positive cells in the striatum. By contrast, the present study supports the efficacy of TUDCA post-treatment in an MPTP model of PD. In addition, our current findings suggest therapeutic potentials of TUDCA and syndopa additive therapy than TUDCA monotherapy for PD. Although the comparisons made across the various drug treatment groups were not significant in most of the study parameters, the mean values of TUDCA + syndopa group showed a better trend compared to other drug groups tested.

Preclinical studies postulate that TUDCA's therapeutic functions rely on anti-apoptotic and anti-neuroinflammatory mechanisms apart from suppression of oxidative stress and mitochondrial damage. From the present research findings, it is evident that TUDCA's anti-inflammatory effect in PD brain (both striatum and SNpc) is convincing when administered alone and found to be better when syndopa treatment was added. It also acts as a chaperone to sustain the stability and correct folding of proteins. Intriguingly, phase II clinical trials in AD have shown TUDCA as a safe and a potential drug. AD is one of the common neurodegenerative diseases which tested hydrophilic bile acids as a therapeutic agent. While much more clinical evidence is being gathered for the other diseases, TUDCA holds promise for the treatment of neurodegenerative diseases [72]**.** Combinatorial therapy is gaining more attention in recent years for many neurological disorders including PD [73-75]. Our earlier works which tested embelin and levodopa combination therapy is an additional proof of preclinical evidence to substantiate the importance and efficacy of combined therapy in the amelioration of PD complications [24,76].

Study Limitations: (i) The present study didn't test the behavioral parameters (functional activity) to assess the additive effects of TUDCA and syndopa treatments in PD model mice. Therefore, future works rely on these preclinical data to validate the drug efficacies and to ensure the potential for cure in translational research (ii) the validity of the MPTP model is not high and this model does not feature all the problems/symptoms of PD patients, and (iii) it seems likely that antioxidant monotherapy is insufficient for disease modification in humans.

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TABLES AND FIGURES WITH LEGENDS

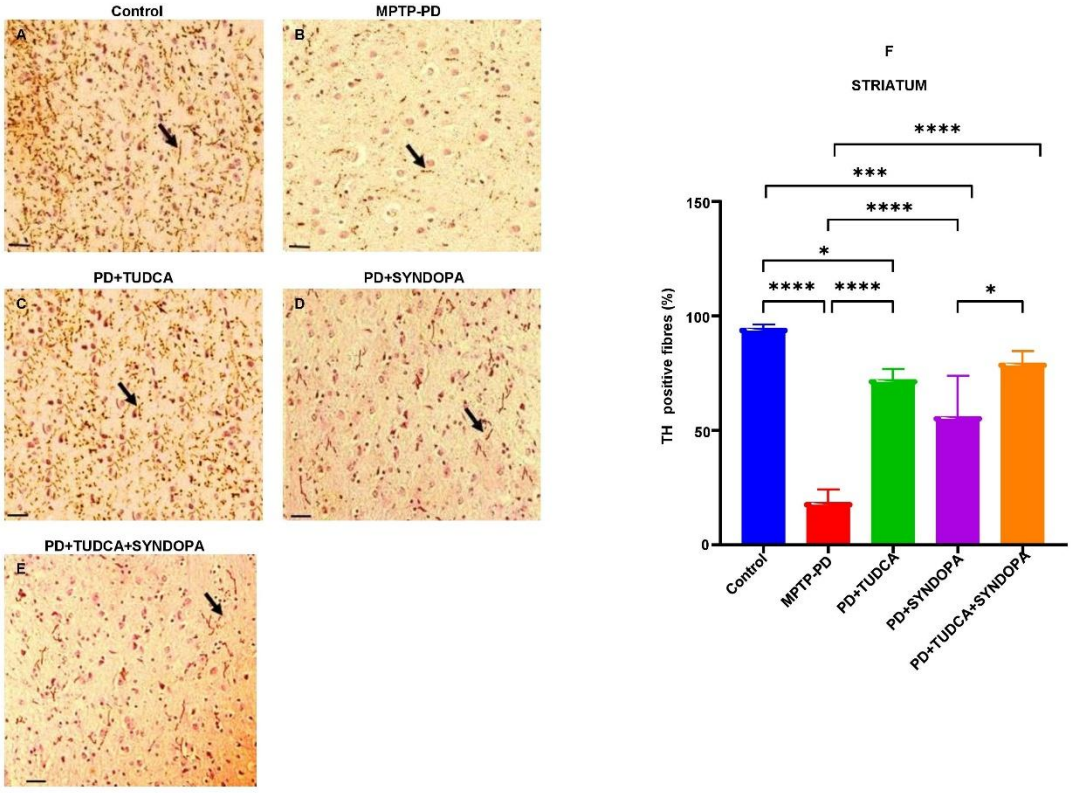

Figure 1. Tyrosine hydroxylase expression analysis in the brain striatum. The midbrain sections of mouse brains were immunostained with a specific anti-tyrosine hydroxylase (TH) antibody. The TH-positive fibers in the striatum were observed under the light microscope and counted. A-E) The representative microscopic images of the various study groups are shown here. Magnification – 400X; Scale bar – 20 µm. F) The bar diagram shows the % expression of TH fibers in the brain striatum. The data are represented as means + SD (n=5 or 6). *$P < .05$; ***$P < .001$; ****$P < .0001$.

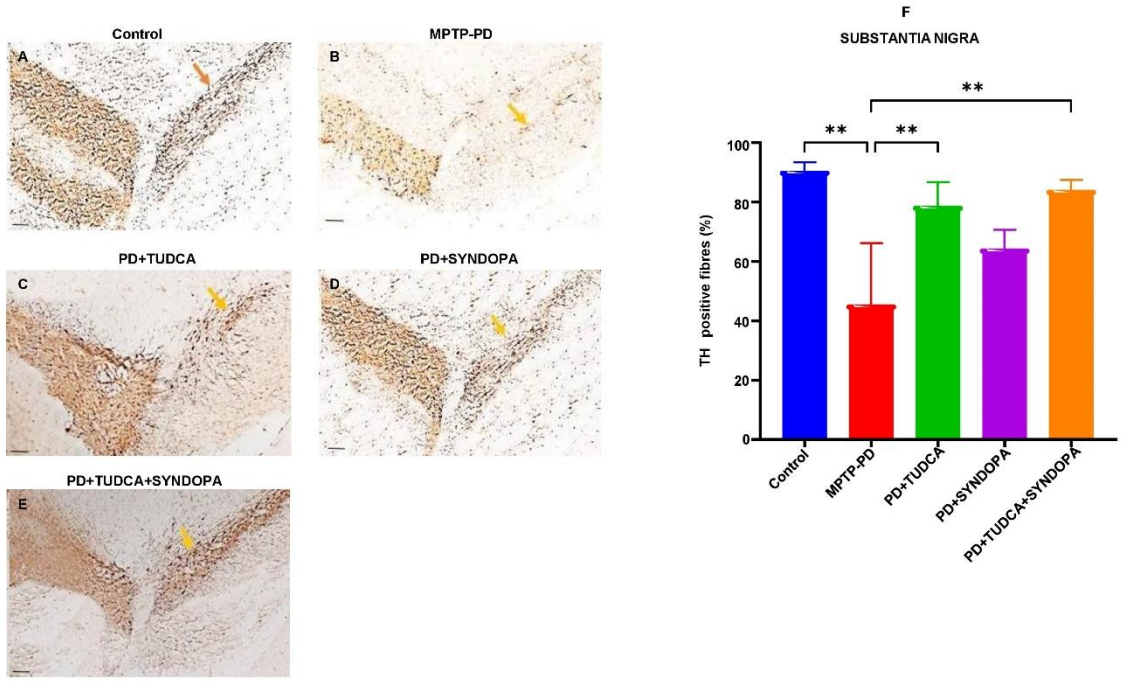

Figure 2. Tyrosine hydroxylase expression analysis in the brain SNpc. The midbrain sections of mouse brains were immunostained with a specific anti-tyrosine hydroxylase (TH) antibody. The TH-positive fibers in SNpc were observed under the light microscope and counted. A-E) The representative microscopic images of the various study groups are shown here. Magnification – 200X; Scale bar – 200 µm. F) The bar diagram shows the % expression of TH fibers in the brain SNpc. The data are represented as means + SD (n= 5 or 6). $^{**}P < .01$.

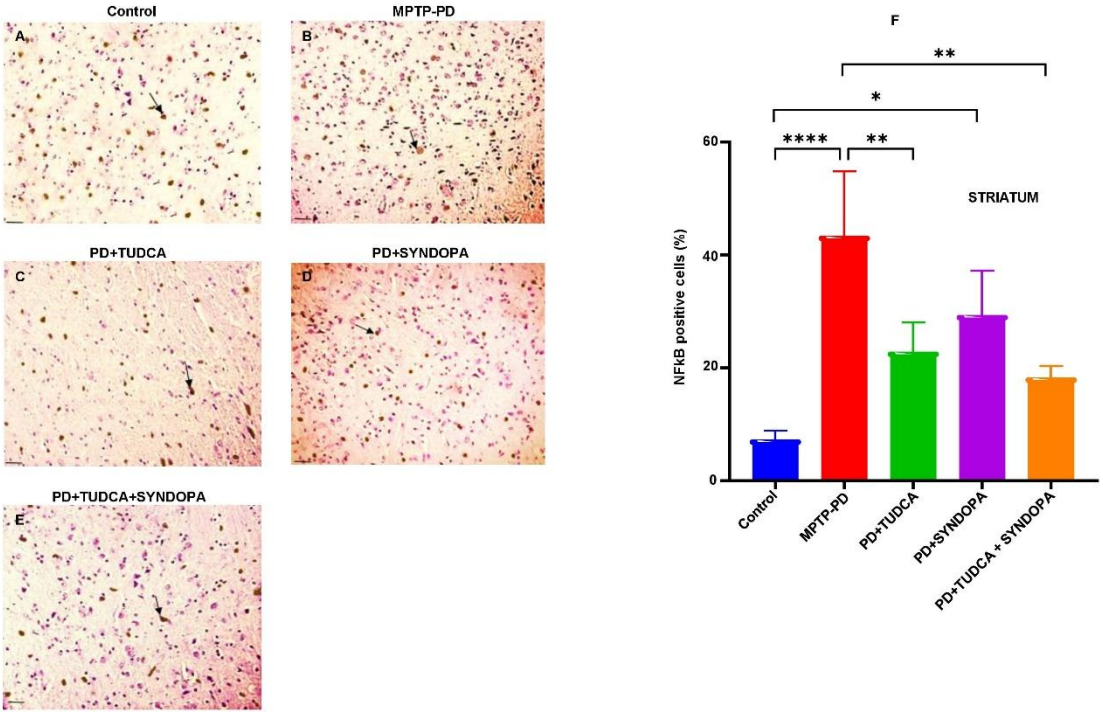

Figure 3. NF-kB expression analysis in the brain striatum. The midbrain sections of mouse brains were immunostained with a specific anti-NF-kB antibody. The NF-kB-positive cells in the striatum were observed under the light microscope and counted. A-E) The representative microscopic images of the various study groups are shown here. Magnification – 600X; Scale bar – 50 µm. F) The bar diagram shows the % expression of NF-kB in the brain striatum. The data are represented as means + SD (n=5 or 6). **P* < .05; ***P* < .01; *****P* < .0001.

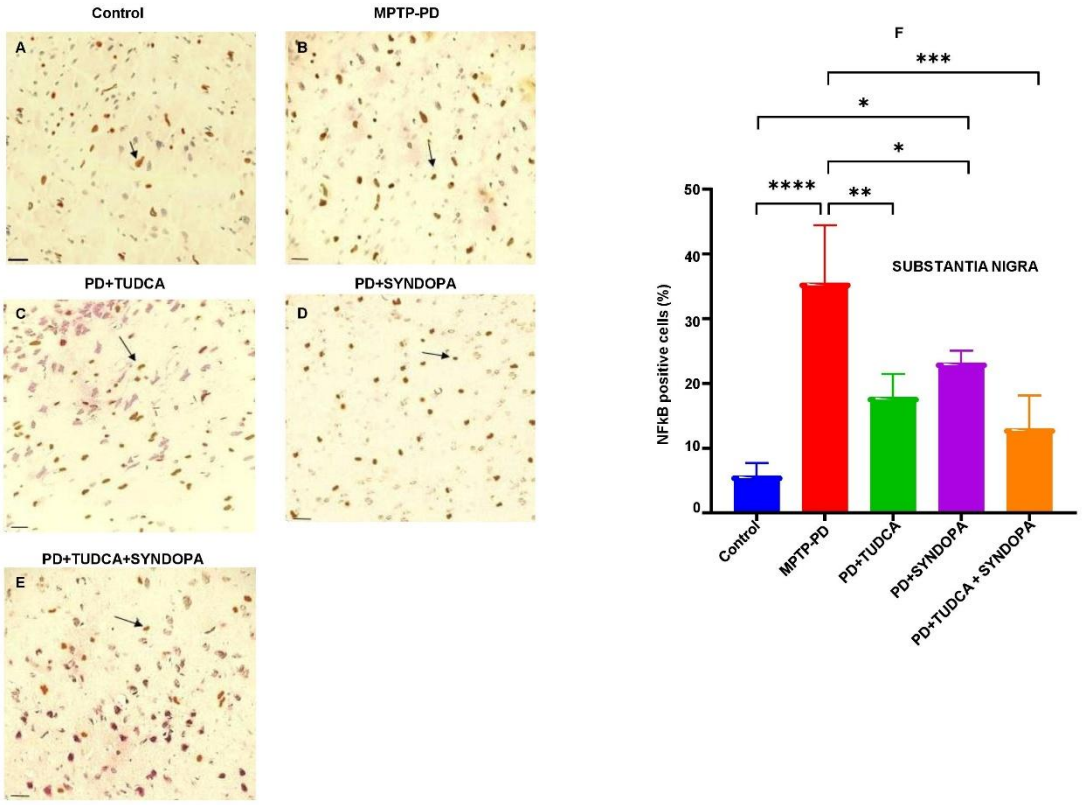

Figure 4. NF-kB expression analysis in the brain SNpc. The midbrain sections of mouse brains were immunostained with a specific anti-NF-kB antibody. The NF-kB-positive cells in the SNpc were observed under the light microscope and counted. A-E) The representative microscopic images of the various study groups are shown here. Magnification – 600X; Scale bar – 50 µm. F) The bar diagram shows the % expression of NF-kB in the brain SNpc. The data are represented as means + SD (n=5 or 6). **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

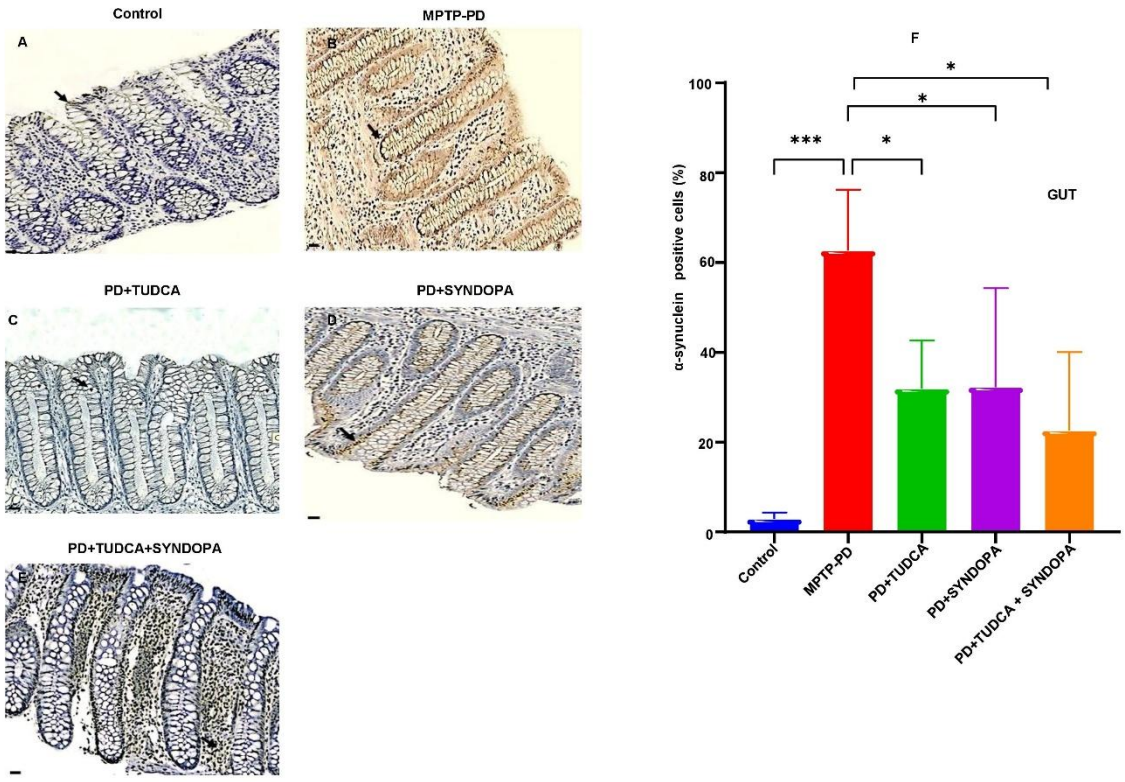

Figure 5. The expression analysis of α-synuclein in the gut. The transverse sections of mucosa and submucosa tissues were immunostained with a specific anti-α-synuclein antibody. The α-synuclein-positive cells in gut tissues were observed under the light microscope and counted. A-E) The representative microscopic images of the various study groups are shown here. Magnification – 400X; Scale bar – 200 µm. F) The bar diagram shows the % expression of α-synuclein in the mucosa and submucosa tissues of the gut. The data are represented as means + SD (n=5 or 6). **P* < .05; ****P* < .001.

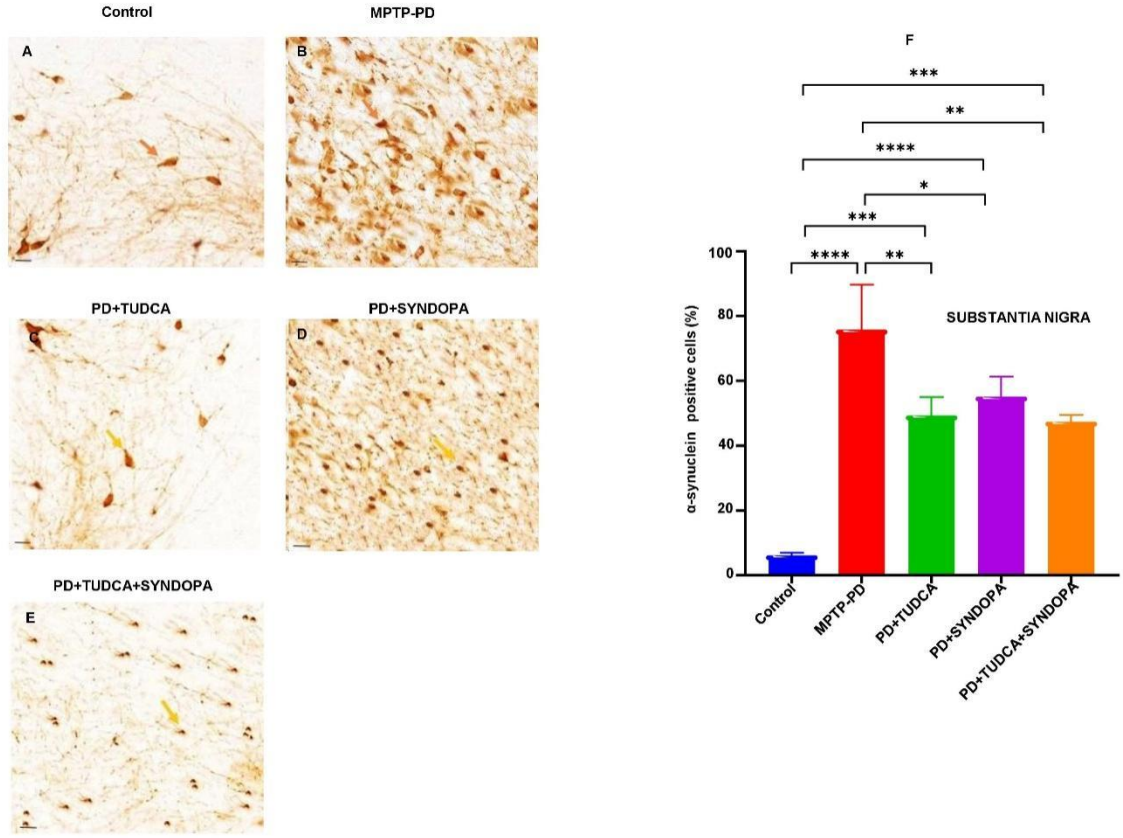

Figure 6. The expression analysis of α-synuclein in the brain SNpc. The midbrain sections of mouse brains were immunostained with a specific anti-α-synuclein antibody. The α-synuclein-positive cells in the SNpc were observed under the light microscope and counted. A-E) The representative microscopic images of the various study groups are shown here. Magnification – 400X; Scale bar – 50 µm. F) The bar diagram shows the % expression of α-synuclein in the brain SNpc. The data are represented as means + SD (n=5 or 6). *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

SUPPLEMENTAL DATA

Supplemental data are available at the following link:

https://www.bjbms.org/ojs/index.php/bjbms/article/view/12519/3979