

## Original Research

# Effects of Telmisartan, an AT1 receptor antagonist, on mitochondria-specific genes expression in a mouse MPTP model of Parkinsonism

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## 1. Abstract

**Background:** Mitochondrial dysfunction plays a crucial role in Parkinson's disease (PD) pathogenesis. The present study was undertaken to investigate the effects of Telmisartan (TEL), an angiotensin II type 1 receptor (AT1R) blocker, on the mitochondria-specific genes expression in a mouse model of Parkinsonism. **Materials and methods:** Mice were divided into 5 groups with 6

in each; Group I received 0.5% CMC (control) + saline, Group II received 0.5% CMC + 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (positive control), Group III & IV received MPTP + TEL 3 and 10 mg/kg, p.o. respectively, Group V received TEL 10 mg/kg, p.o. (drug control). MPTP was given 80 mg/kg intraperitoneal in two divided doses (40 mg/kg × 2 at 16 h time interval). Vehicle or TEL was administered 1 h before the MPTP injection. Motor function was assessed 48 h after the first

dose of MPTP and animals were euthanized to collect brain. **Results:** Mice intoxicated with MPTP showed locomotor deficits and significant upregulation of  $\alpha$ -synuclein ( $\alpha$ -syn), downregulation of metastasis-associated protein 1 (MTA1), and Ubiquitin C-terminal hydrolase L1 (UCHL1) in the substantia nigra pars compacta (SNpc) and Striatum (STr) regions of brains. In addition, MPTP intoxication down-regulated mitochondria-specific genes such as DJ-1, PTEN-induced putative kinase 1 (PINK1), Parkin, enriched with leucine repeats kinase 2 (LRRK2) gene expression. Pre-treatment with TEL restored locomotor functions and upregulated PINK1, Parkin, LRRK2, DJ-1, MTA1 and UCHL1. **Conclusion:** The present study evidences that TEL has the ability to improve mitochondrial functions in PD.

## 2. Introduction

Mitochondrial dysfunction plays a crucial role in the Parkinson's disease (PD) pathogenesis [1]. Neurons are particularly vulnerable to mitochondrial dysfunction as they are highly dependent on Electron transport chain (ETC) for energy requirements [2]. The first event in mitochondrial impairment in PD is the deficiency of Complex I in the ETC [3, 4]. Dopaminergic SNpc neurons are susceptible to oxidative stress as they have poorly myelinated long and thin axons [5].  $\alpha$ -synuclein ( $\alpha$ -syn), a presynaptic protein related to PD pathogenesis, was reported to aggregate within the mitochondria which causes cell death [6–8]. Deposition of Lewy bodies and Lewy neurites (composed of several proteins including  $\alpha$ -syn) were reported as early changes that occur before the degeneration of the SNpc and expression of clinical symptoms of Parkinsonism [9–11].

Renin angiotensin system (RAS) in brain play a crucial role in PD [12]. Activation of angiotensin II type 1 receptor (AT1R) triggers inflammatory pathways which increase ROS leading to dopaminergic cell death [12, 13]. Telmisartan (TEL), a lipophilic antihypertensive drug exerts its neuroprotective activity by blocking AT1R in the brain. In our earlier investigations, we have reported its neuroprotective activity acute and chronic model of Parkinsonism in mouse models [14, 15]. Blockade of AT1R using losartan was shown to exert anti-inflammatory activity in MPTP intoxicated mice model of PD [16, 17] and antioxidant properties against 6-OHDA model [18]. Recently, AT1R and AT2R have been discovered in brain mitochondrial outer and inner membrane, respectively [19]. Alleviation of mitochondrial function was shown to decrease oxidative stress and reduced the incidences of apoptosis in various neurological disorders [20–23].

Recent understandings of the existence of RAS components in mitochondrial structure, we proposed investigate whether AT1R antagonism modulates mitochondrial functions, particularly in PD. As a preliminary approach, the present experiment is designed to unravel the

role of AT1R antagonism using TEL on the mitochondria-specific genes expression in a MPTP model of Parkinsonism in mice.

## 3. Materials and method

### 3.1 Animals

Male C57BL/6J mouse (18–22 g b. wt.) were obtained from CPCSEA approved breeder and acclimatized for 7 days before the treatment, with an ambient temperature of  $22 \pm 3$  °C with 12 h light/12 h dark cycle. They were provided with the standard diet with purified water *ad libitum*.

### 3.2 Chemicals & reagents

Telmisartan API was received as a kind gift from Bal Pharma Limited, Bangalore, India. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and TRIzol reagent were purchased from Sigma Aldrich, USA. PCR master cyclor gradient was procured from Genet Bio, Korea. All the chemicals used in the experiment were analytical grade.

### 3.3 Experimental design

Animals were grouped into 6 per group, where Group I (Control) received 0.5% CMC + Saline as a vehicle, Group II (Positive Control) received MPTP + Saline, and Group III (TEL LD) & Group IV (TEL HD) received TEL 3 and 10 mg/kg p.o. respectively + MPTP and Group V received TEL 10 mg/kg p.o. as drug control. Neurotoxin, MPTP was given 80 mg/kg intraperitoneal in two divided doses ( $40 \text{ mg/kg} \times 2$  at 16 h time interval) from the first dose. All the treatment of TEL and vehicle were administered 1 h before the MPTP (Fig. 1). The motor function test was performed after 48th h of the first dose of MPTP administration [24]. After completion of the motor functions tests, animals were perfused with Phosphate Buffered Saline (PBS) intracardially. Further substantia nigra pars compacta (SNpc; ~ Bregma—3.16 mm, interaural 0.64 mm) and Striatum region (ST; Bregma—0.98 mm, interaural 4.78 mm) [25] was extracted from the mice brain.

### 3.4 Motor function test

#### 3.4.1 Beam walk experiment

Beam walk test was done after the MPTP intoxication, to cross a narrow beam of 100 cm length connecting to an escape box [26]. An aversive stimulus was created with an optimistic light (60 lux) above the beam to trigger the mice to a dark goal box on the other side of the beam. The mice were kept at the beginning of the beam after 48th h of the first exposure of MPTP injection. The time taken to reach the escape box on the other hand (sec), number of foot slip (s), and immobility period were noted. The observer was blinded to the treatment protocols.

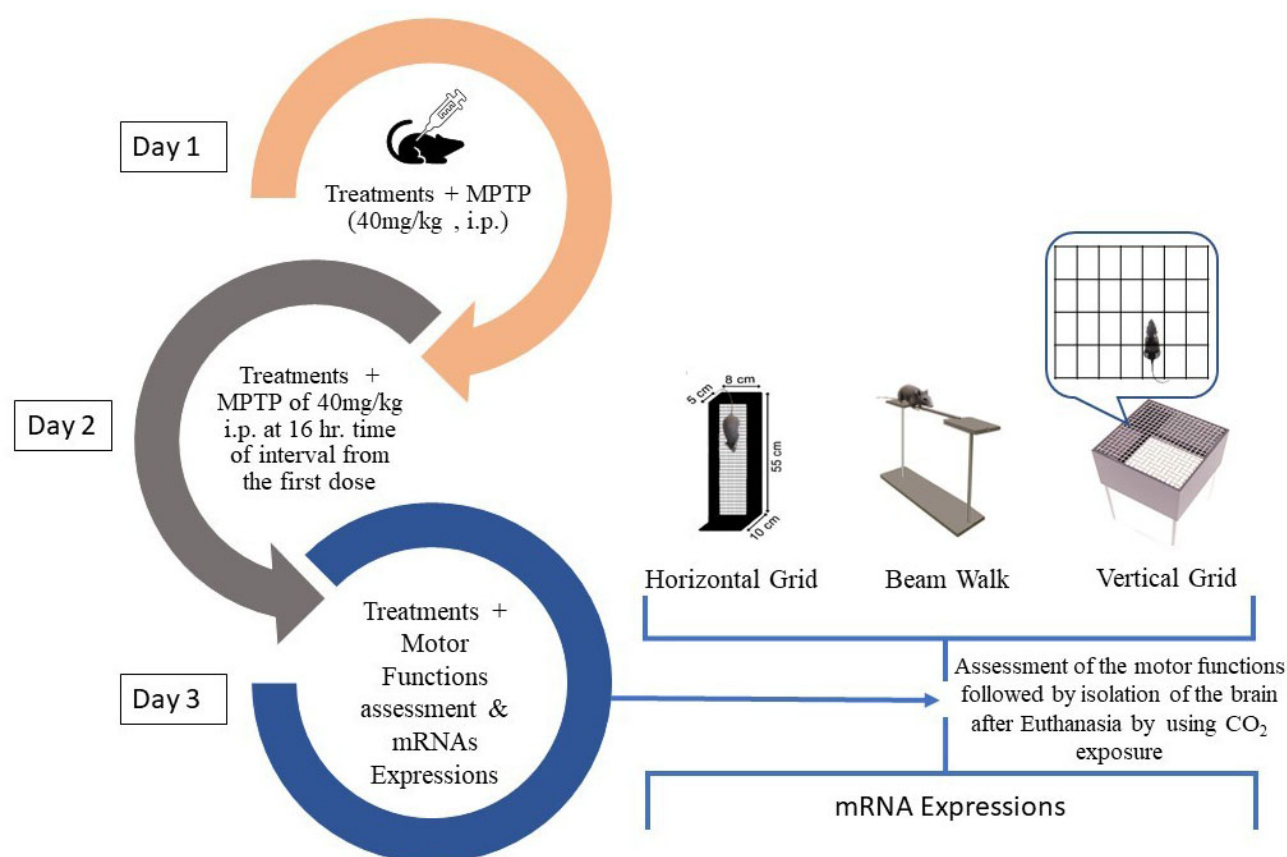


Fig. 1. Experimental design for the acute MPTP model of PD.

### 3.4.2 Horizontal grid

Horizontal grid test was done as per Kim *et al.* [27] method with minor changes. The apparatus was made up of horizontal grid mesh (size 12 cm<sup>2</sup>: with an opening of 0.5 cm<sup>2</sup>) mounted approximately 20 cm above a surface, supporting against the falling. Animals were kept independently in the center of the horizontal grid apparatus and given support until it grabs the grid. Then the grid was upturned to monitor the hanging time. The observer was blinded to the treatment protocols.

### 3.4.3 Vertical grid

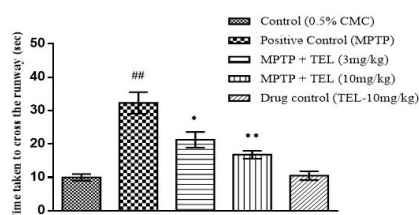
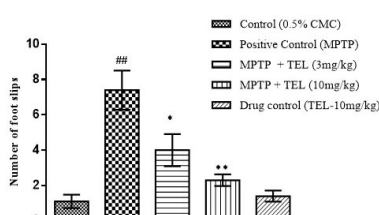
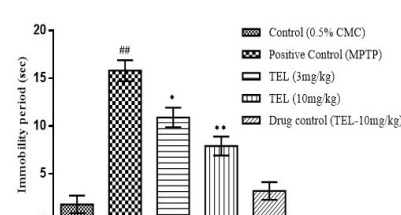
Vertical grid test was done to assess the motor function as per the method of Kim *et al.* [27] with minor modifications. The apparatus is made of an open box of size 8 × 55 × 5 cm and the vertically standing back side of the box was prepared of wire mesh, with an open front side covering the other four sides with black Plexiglas. Animals were kept individually in the apparatus at a height of 3 cm from the bottom, keeping the face upwards and allowed to climb down on the grid. Time to climb down the grid and immobility period were noted. The observer was blinded to the treatment protocols.

### 3.5 Reverse transcriptase PCR (RT-PCR) analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) was done to determine the level of mRNA expression of  $\alpha$ -syn, Parkin, MTA1, DJ-1, UCHL1, PINK1, and LRRK2. In brief, total RNA was extracted from SNpc and ST<sub>r</sub> regions of mice brains using TRIzol Reagent (Sigma, USA). Followed by the process of homogenization, the tubes were kept for incubation for 10 min then centrifuged at 1,000 rpm for 5 min. An amount of 200  $\mu$ L chloroform was put into the supernatant, further incubated for 5 min at room temperature followed by centrifugation at 12,000 rpm for 20 min. Total RNA was precipitated by adding 500  $\mu$ L of isopropyl alcohol to the supernatant and centrifuged at 12,000 rpm for 15 min and incubated for period of 10 min. The pellet obtained after decanting the supernatant was further washed 3 times with 75% ethanol then centrifuged at 12,000 rpm for 15 min and allowed to air-dry the pellet. The pellet was resuspended in 20  $\mu$ L of RNase-free water and stored at a temperature of  $-80^{\circ}\text{C}$ . In the next step, the isolated RNA was subjected to undergo reverse transcription and polymerization reaction to obtain cDNA using PCR master cyclor gradient. Then, the cDNA was processed for electrophoresis at 80 V for 30 min. Finally, the gene expression was analyzed using the bands formed in agarose gel. An amount of 200 nanograms of RNA were

**Table 1. Primer sequences.**

Genes	Forward	Reverse
<i>α-syn</i>	5'-GTTCTTCAGAAGCCTAGGGAGC-3'	5'-CCACACCCTGCTTGGTTTTC-3'
<i>Parkin</i>	5'-AAATGCATCTGGAGGGGACG-3'	5'-ACCTCTGGCTGCTTCTGAAT-3'
<i>MTA1</i>	5'-GTCAGCCCCTGTCATCAACA-3'	5'-GACCCCTACTGGGCATCAAG-3'
<i>DJ-1</i>	5'-CATGCGTGCTGGGTACACG-3'	5'-CCATCTCCTCTGCTCCTTTGG-3'
<i>UCHL1</i>	5'-GCAATGCCTGTGGAACGATT-3'	5'-TGACCTTCATGTGCACTGGT-3'
<i>PINK1</i>	5'-GTTGCAATGCCGCTGTGTAT-3'	5'-GCAAAGGGAAGGTGGGAGT-3'
<i>LRRK2</i>	5'-TGCCATGCACAGATATTCAGC-3'	5'-TGCAAGCAGCAAAGTACGTG-3'
<i>GAPDH</i>	5'-TTCACCACCATGGAGAAGGC-3'	5'-TCATGACCACAGTCCATGCC-3'

**a. Time is taken to cross the runway (sec)****b. Number of foot slips****c. Immobility period (sec)**

**Fig. 2. Effect of TEL on time taken to cross the runway (sec), number of foot slip (s), and immobility period (sec) in beam walk test. \* & \*\* indicates  $P < 0.05$  and  $0.01$ , respectively vs MPTP group. ## indicates  $P < 0.01$  vs control group.**

used for RT-PCR according to the manufacturer's instructions (Genet Bio, Korea). The primers used in the experiment are given in Table 1, the images were quantified by ImageJ software.

### 3.6 Data analysis

Data obtained in the experiment were expressed as mean  $\pm$  standard error of the mean (SEM). One-way ANOVA followed by Tukey's multiple comparisons as post hoc test was applied to analyse the mean differences between the experimental groups in GraphPad Prism 5.0 (San Diego, USA) software considering  $P \leq 0.05$  significant.

## 4. Results

### 4.1 Effect of TEL in motor function in MPTP intoxicated mice

#### 4.1.1 Beam walk test

MPTP intoxicated mice showed an increased immobility period and took a long time in crossing the narrow runway with more numbers of foot slips ( $P < 0.01$ ) when compared to the normal control group. No difference in foot slip was noted in the drug control and normal control mice. Pre-treatment with TEL at 3 mg/kg ( $P < 0.05$ ) and 10 mg/kg ( $P < 0.01$ ) decreased in the time taken in crossing, foot slips, and immobility periods, in a dose-dependent manner, when compared to MPTP intoxicated group (Fig. 2).

#### 4.1.2 Horizontal grid test

MPTP intoxicated mice showed a longer wall hanging time ( $P < 0.01$ ) when compared to normal control. Pre-treatment with TEL has significantly reduced the hanging time at 3 mg/kg ( $P < 0.05$ ) and 10 mg/kg ( $P < 0.01$ ) in a dose-dependent manner when compared to MPTP intoxicated mice. Drug control and normal control groups showed no significant difference in hanging time (Fig. 3).

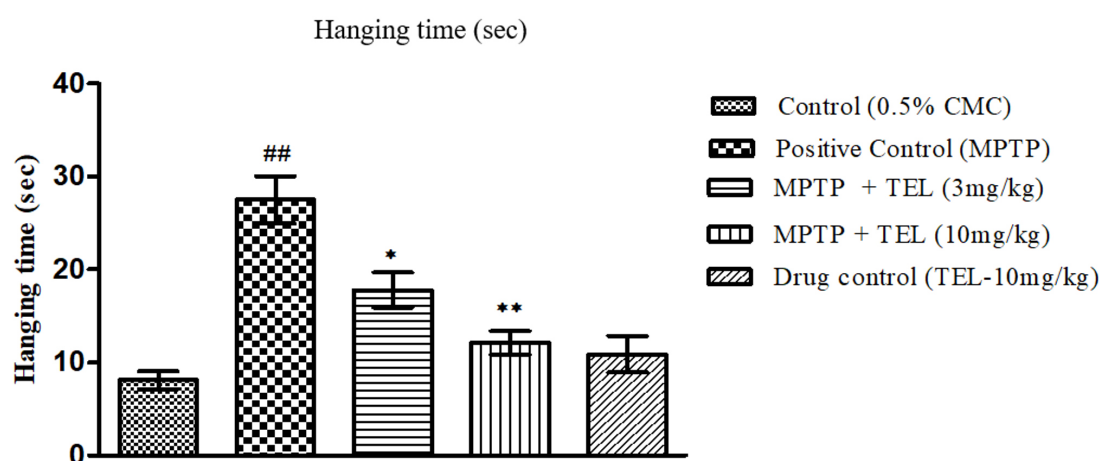
#### 4.1.3 Vertical grid test

MPTP intoxicated mice took a longer time ( $P < 0.01$ ) to climb down and increased immobility period ( $P < 0.01$ ) in comparison to normal control animals. TEL pre-treatment at 10 mg/kg reduced the climb down time ( $P < 0.05$ ) as well as reduced the immobility ( $P < 0.01$ ) period significantly when compared to MPTP intoxicated mice. No significant difference was observed at the lower dose of TEL (3 mg/kg) in the immobility period while compared to Parkinsonism mice (Fig. 4).

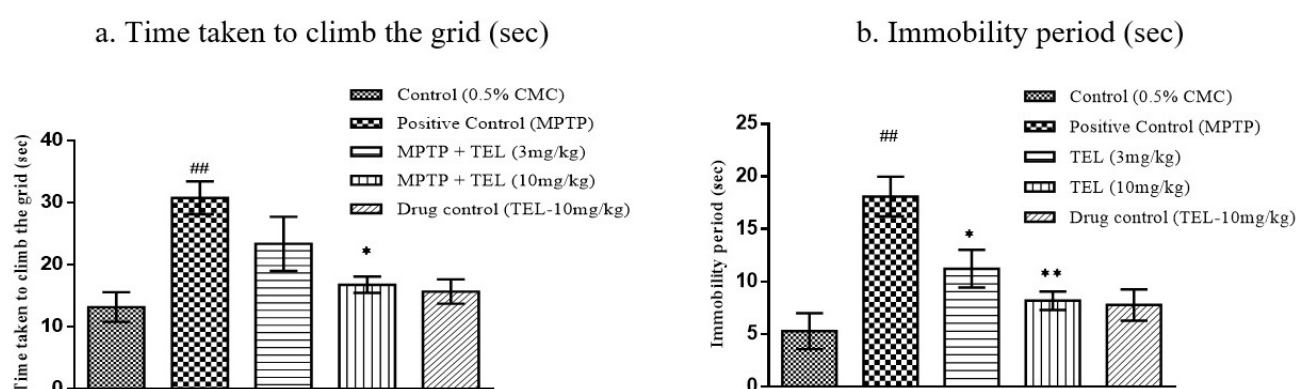
### 4.2 Reverse transcriptase PCR (RT-PCR) analysis

#### 4.2.1 SNpc region

Significant upregulations of  $\alpha$ -syn ( $P < 0.01$ ), downregulations of DJ-1 ( $P < 0.01$ ), LRRK2 ( $P < 0.01$ ), MTA1 ( $P < 0.01$ ), Parkin ( $P < 0.01$ ), PINK1 ( $P < 0.01$ ), UCHL1 ( $P < 0.01$ ) mRNA expressions were observed in vehicle treated MPTP mice when compared to control mice. Pre-treatment with TEL downregulated the  $\alpha$ -syn ( $P < 0.01$ ).



**Fig. 3.** Effect of TEL on the hanging times (sec) assessed on Vertical grid test. \* & \*\* indicates  $P < 0.05$  and  $0.01$ , respectively vs MPTP group. ## indicates  $P < 0.01$  vs control group.



**Fig. 4.** Effect of TEL on time taken to climb the grid (sec), immobility period (sec) on horizontal grid test. \* & \*\* indicates  $P < 0.05$  and  $0.01$ , respectively vs MPTP group. ## indicates  $P < 0.01$  vs control group.

0.01) and upregulated DJ-1 ( $P < 0.01$ ), LRRK2 ( $P < 0.01$ ), MTA1 ( $P < 0.01$ ), Parkin ( $P < 0.01$ ), PINK1 ( $P < 0.01$ ), UCHL1 ( $P < 0.01$ ) in MPTP intoxicated in SNpc region of mice.

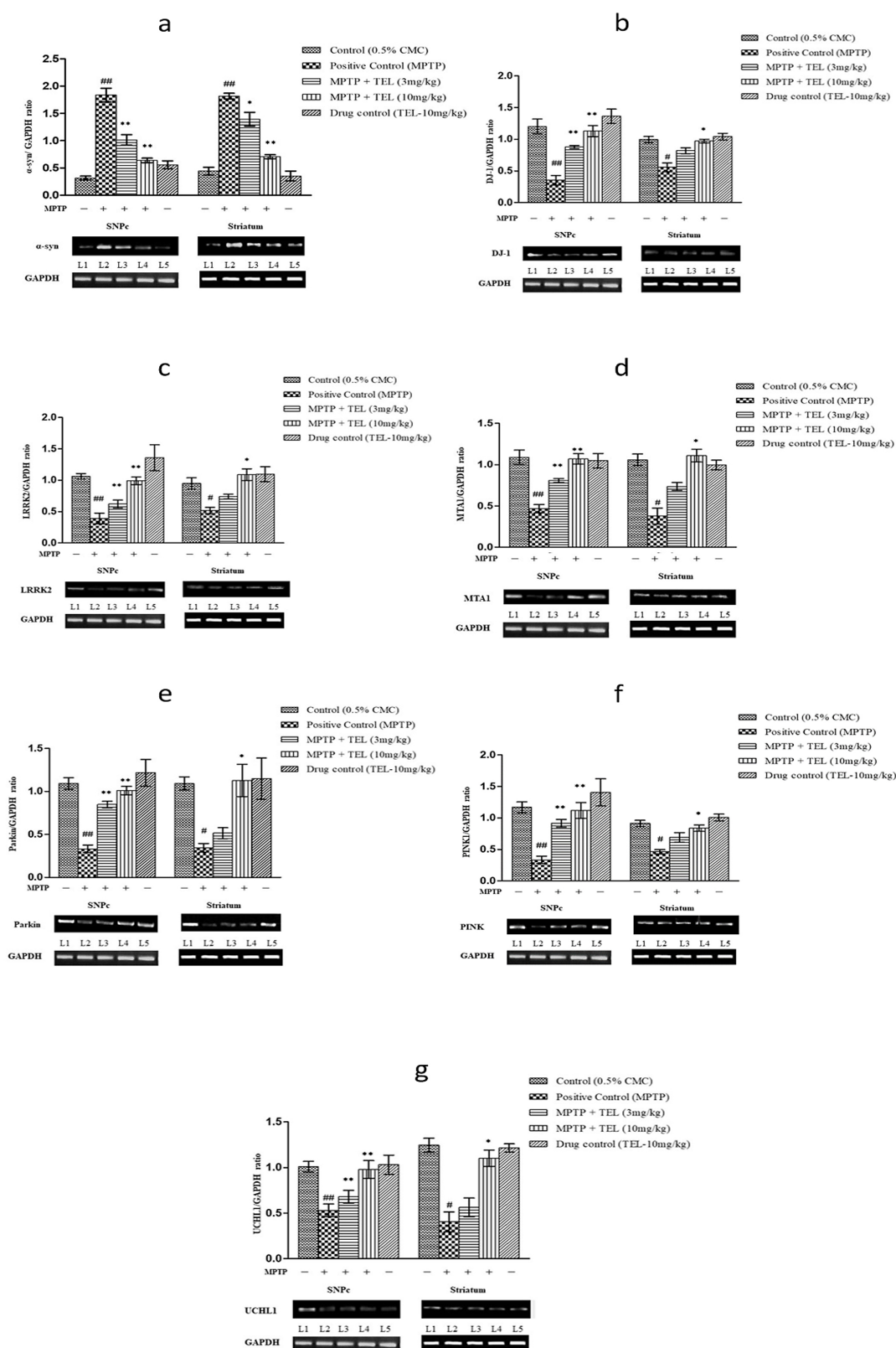
#### 4.2.2 STTr region

Significant upregulations of  $\alpha$ -syn ( $P < 0.01$ ), downregulations of DJ-1 ( $P < 0.05$ ), LRRK2 ( $P < 0.05$ ), MTA1 ( $P < 0.05$ ), Parkin ( $P < 0.05$ ), PINK1 ( $P < 0.05$ ), UCHL1 ( $P < 0.05$ ) mRNA expressions were observed in vehicle-treated MPTP mice when compared to control mice. Pre-treatment with TEL downregulated the  $\alpha$ -syn ( $P < 0.01$ ) and upregulated DJ-1 ( $P < 0.05$ ), LRRK2 ( $P < 0.05$ ), MTA1 ( $P < 0.05$ ), Parkin ( $P < 0.05$ ), PINK1 ( $P < 0.05$ ), UCHL1 ( $P < 0.05$ ) in MPTP intoxicated in STTr region of mice. However, there was no significant difference in mRNA expressions between the normal control and drug control groups (Fig. 5).

## 5. Discussion

The current study adds further evidence on the neuroprotective role of AT1R antagonism and also new information on its ameliorative effects on mitochondria-specific genes expression in PD. Inhibition of Complex I of ETC was reported in PD patient's brains [28, 29]. Neurotoxin MPTP produces PD-like conditions by inhibiting the Complex I. Recently, AT1R and AT2R (RAS) were identified on the mitochondrial surface and inner membrane, respectively [19, 30]. Activation of AT1R was shown to impair the mitochondrial functions via NAD(P)H mediated oxidative stress in the PD [31]. In our early study, we have shown that TEL suppresses nitrosative stress and elicits its neuroprotection in a mouse model of PD [32]. However, to date, there is no evidence on the effects of AT1R antagonism on mitochondria-specific genes expression which plays a crucial role in oxidative stress, inflammatory responses, and apoptosis. Hence, in the present study we investigated the effects of TEL on the mitochondria-specific genes like PINK1, Parkin, LRRK2, DJ-1 which may serve as newer therapeutic target in the treatment of PD.





**Fig. 5.** Effect of TEL on gene expressions such (a)  $\alpha$ -syn, (b) DJ-1, (c) LRRK2, (d) MTA1, (e) Parkin, (f) PINK1, (g) UCHL1 analysed by RT-PCR. The values were expressed in mean  $\pm$  SEM,  $n = 3$  animals/group. One-way ANOVA followed by Tukey's multiple comparison test was applied to compare mean differences between the experimental groups. ## indicates  $P < 0.05$  and  $0.01$ , respectively vs control group; \*, \*\* indicates  $P < 0.05$  and  $0.01$ , respectively vs MPTP intoxicated mice.

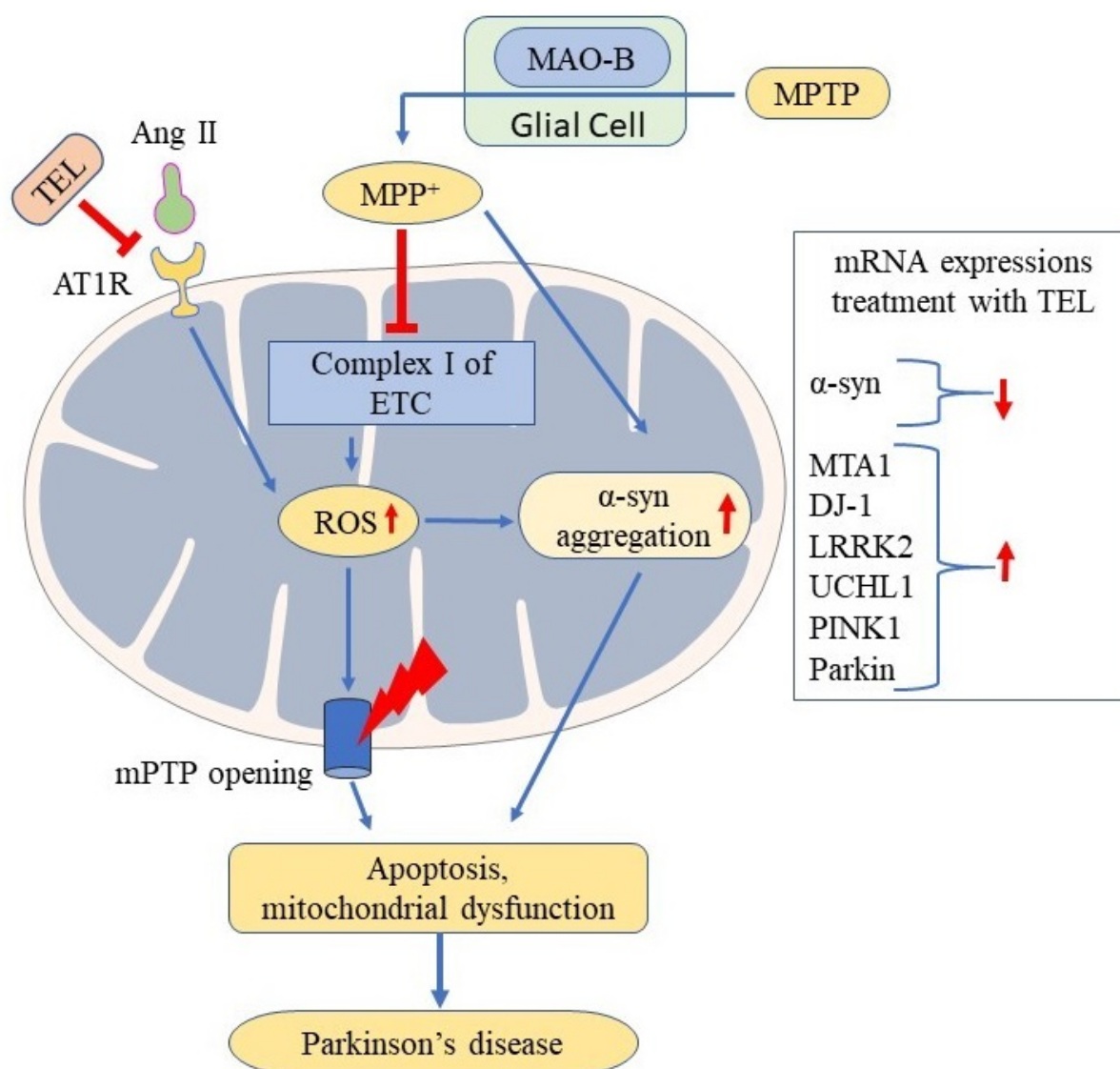


Fig. 6. Possible neuroprotective effect of TEL improving mitochondrial functions via inhibiting mitochondrial AT1R in PD.

Knockout/mutation in PINK1 and Parkin proteins are linked with PD pathogenesis [33–35]. Parkin is selectively recruited by PINK1 to the outer membrane of the damaged mitochondria to promote mitophagy in PD [36]. In the present study, pre-treatment with TEL upregulated the PINK1 and Parkin in MPTP intoxicated mice brains, which indicates that AT1R antagonism may support the PINK1-Parkin mediated mitophagy and in turn improving mitochondrial dynamics [37, 38].

Knockout/mutation (G2019S) in LRRK2 impair autophagy and aggregation of  $\alpha$ -syn [39–42]. Pre-treatment with TEL upregulated LRRK2 which might be the possible reason for the observed down-regulation in  $\alpha$ -syn expression and this can be speculated to improve neuronal clearance of  $\alpha$ -syn by autophagy process. This indicates that up-regulation of mitochondria-specific gene LRRK2 via central RAS modulation imparts neuroprotection in PD.

In physiological condition,  $\alpha$ -syn play a crucial role in the regulating the post-synaptic dopamine uptake [43, 44]. Another mitochondria-specific protein DJ-1 [45], which acts as an oxidative stress sensor and antioxidant [46] and its down-regulation is correlated to the increased aggregation of  $\alpha$ -syn via inhibiting the chaperone-mediated autophagy [47, 48]. UCHL1, an important component of the ubiquitin-proteasome system (UPS), and its down-regulation is linked to increased expression of  $\alpha$ -syn, confirming its direct link in PD [49–51]. TEL increased DJ-1 and UCHL1, which further indicates that restoration of the mitochondrial genomic profile imparted neuroprotection via improving  $\alpha$ -syn clearance, at least partly.

Finally, the upregulation of MTA1 expression is reported to increase dopamine synthesis [52] and its packaging and subsequent release of dopamine from neuronal vesicles through the upregulation of vesicular monoamine transporter-2 (VMAT2) [53, 54]. The improved motor func-

tion recorded in beam walk, horizontal grid, and vertical grid tests with TEL might be due to the increased dopamine levels which may be corroborated by increased expression of MTA1 and DJ-1 in PD mice. The current findings support that modulation of brain RAS function improves mitochondrial function and can impart neuroprotection in PD (Fig. 6).

## 6. Conclusions

In conclusion, modulation of central RAS, particularly AT1R antagonism improves mitochondrial functions and exerts neuroprotection in PD.

## 7. Author contributions

BR performed the study. GR, SV and SR analyzed the data. ST and AMM assisted the study. MME and SBC designed the study corrected and finalized the manuscript.

## 8. Ethics approval and consent to participate

Institutional animal ethical committee, Central animal house, JSS Academy of higher education and Research, Mysuru, Karnataka, India approved the study (JSSAHER/CPT/IAEC/016/2020).

## 9. Acknowledgment

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## 10. Funding

This research received no external funding.

## 11. Conflict of interest

The authors declare no conflict of interest.

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**Abbreviations:**  $\alpha$ -syn,  $\alpha$ -synuclein; AT1R, Angiotensin II type 1 receptor blocker; CMC, Carboxy methyl cellulose; IAEC, Institutional Animal Ethics Committee; LRRK2, Enriched with leucine repeats kinase 2; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTA1, Metastasis-associated protein 1; PD, Parkinson's disease; PINK1, PTEN-induced putative kinase 1; RAS, Renin angiotensin system; ROS, Reactive oxygen species; RT PCR, Reverse Transcriptase-Polymerase chain reaction; SEM, Standard error of the mean; SNpc, substantia nigra; STr, Striatum; TELM, Telmisartan; UCHL1, Ubiquitin C-terminal hydrolase L1.

**Keywords:** Parkinson's disease; Telmisartan; Mitochondria;  $\alpha$ -synuclein; PINK1; Parkin; DJ-1; LRRK2; MTA1; UCHL1

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