Alpha Mangostin Confers Neuroprotection Against Rotenone-induced Parkinson's Disease via Modulating Autophagy and AMPK Signaling.

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Abstract

Intending to uncover bioactive phytochemicals that may halt or slow the progression of Parkinson's disease (PD), we previously studied the neuroprotective property of alpha mangostin (AM), obtained from the *Garcinia mangostana* Linn. in the rat model of PD. Albeit, the intracellular regulatory mechanism behind the observed effects remained to be elucidated. Thus, this study aimed to investigate AM's autophagic potential behind its neuroprotective effect in the rotenone-induced mouse model of PD. Adult C57BL/6 mice received the chronic treatment of rotenone for 9-consecutive weeks. While AM was administered one hour before the rotenone administration. Behavioral assessment was carried out every 2nd week till 9 weeks. The treatment with the AM significantly restored locomotor and cognitive impairments induced by rotenone. Western blot analysis revealed that rotenone reduced the expression of autophagic markers like LC3II/I, TFEB, Beclin-1 in the cortex, and striatum. It might be due to rotenone-induced reduction in activation of AMPK, the primary regulator of the autophagy process. Interestingly, AM treatment restored the levels of autophagic markers in the cortex and striatum. AM significantly reduced the expression of α -Syn in the striatum but not in the cortex, while it significantly reduced the levels of p- α -Syn in the cortex and observed a strong trend in the striatum, suggests its anti-aggregatory potential against α -Syn. AM also protected rotenone-induced loss of dopaminergic fibres in the striatum and TH⁺-cells in the SNc. These results demonstrate that AM decreased α -Syn accumulation via autophagy activation by up-regulating the AMPK pathway, elicits neuroprotection against rotenone-induced dopaminergic neurodegeneration.

Introduction

The second most common neurodegenerative disease, Parkinson's disease, is one of the chronic medical condition mainly affects people over 65 years of age, leads to progressive motor and non-motor disability(Marras et al., 2018). It is typically characterized by irreversible deterioration of dopaminergic neurons in substantia nigra pars compacta (SNc)(Ozansoy and Başak, 2013). From the last two decades, the pathophysiology of PD is linked with the presence of inclusions like Lewy bodies (LBs) in SNc, mainly consists of misfolded alpha-Synuclein (α -Syn)(Benskey et al., 2018). Intriguingly, α -Syn tends to form aggregates. In pathological conditions like PD, α -Syn forms toxic oligomeric species, which serves as a nexus for the formation of protein aggregates, responsible for the loss of nigral dopaminergic neurons(Wood et al., 1999). Moreover, upon the clearance of α -Syn dopaminergic dysfunction can be recovered(Ramirez-Moreno et al., 2019). Thus, promoting the removal of α -Syn aggregates by activating autophagy might be the key to rescue the neuronal loss in PD.

Autophagy, a conservative intracellular degradation process that involves the degradation of proteins, lipids, and cytoplasmic contents, including misfolded proteins and damaged organelles, gets impaired in PD and is central to the development of PD pathology(Parekh et al., 2019; Shacka et al., 2008). Moreover, the autophagy defects demonstrated to promote aggregation of α -Syn and subsequent neurodegeneration in animal models as well as in the cellular models of PD(Arotcarena et al., 2019). Conversely, autophagy augmentation, by using autophagy enhancers, effectively mitigated aggregation

of α -Syn in pre-clinical models of PD (Arotcarena et al., 2019). Examples of autophagy enhancers found to be neuroprotective in models of PD, were Metformin, Rapamycin, and Trehalose (Parekh et al., 2019). Therefore, enhancing the elimination of α -Syn aggregates by correcting the defects in the autophagic pathway may represent a viable therapeutic strategy for PD treatment.

Similarly, Beclin-1 overexpression (a vital regulator of the autophagic pathway) effectively ameliorated neurodegenerative PD pathology. It also reduced α -Syn aggregation in the *in-vivo* α -Syn model of PD, suggesting the critical involvement of the mammalian target of rapamycin (mTOR) signaling pathway (Spencer et al., 2009). The mTOR is a highly distinguished pathway required for autophagy regulation and is critically involved in PD pathology development. mTOR regulates autophagic activity by sensing the change in amino acid levels, insulin, and energy (Zhu et al., 2019). Compelling evidence suggests an increased activity of mTOR in PD patients' temporal cortex, the region displaying α -Syn aggregation, which further strengthens mTOR involvement in pathology development (Crews et al., 2010). Likewise, the accumulation of α -Syn is known to inhibit autophagy by modulating mTOR activity (Parkhe et al., 2020; Winslow et al., 2010). As reported previously, excessive activation of mTOR also leads to levodopa-induced dyskinesia (Santini et al., 2009). Simultaneously, mTOR inhibition promotes the clearance of toxic α -Syn through autophagy activation in mouse models of PD (Crews et al., 2010). 5 α -AMP-activated protein kinase (AMPK), a key regulator of autophagy, negatively regulates the mTORC1 and thereby facilitates autophagy (Suvorova and Pospelov, 2019).

Interestingly, Wu et. al demonstrated that resveratrol, an AMPK activator, protects nigral dopaminergic neurons in a cellular model of PD, which further supports the AMPK signaling pathway as a possible target for the management of PD (Wu et al., 2011). mTORC1 controls lysosomes' biogenesis by regulating transcription factor-EB (TFEB) (Napolitano et al., 2018; Settembre et al., 2012). TFEB transcriptionally regulates the biogenesis of lysosomes and autophagy-related genes (Beclin-1, Atg5) (Phillipson, 2017). AMPK was found to modulate TFEB activity and is altered in *in vivo* models of PD.

Alpha mangostin (AM), a polyphenolic xanthone, is extracted from the pericarp of the mangosteen (*Garcinia mangostana* Linn.), reported having an anti-oxidant, anti-inflammatory, anti-cancer, and neuroprotective activity (Ibrahim et al., 2016). Multitudinous studies investigated AM's autophagic potential in the different types of cancers (Chao et al., 2011; Wang et al., 2017). However, very few studies explored AM's autophagic activity in PD's cellular model, suggesting that AM mitigates rotenone-induced α -Syn accumulation and reduction of nigral TH⁺ (tyrosine hydroxylase)-neurons (Hao et al., 2017). Interestingly, AM has been shown to promote autophagy by activating the AMPK signaling pathway in glioblastoma cells (Chao et al., 2011). However, no study has been reported hitherto, demonstrating AM's autophagic potential in a chronic mouse model of PD. In our previous studies, we found AM's neuroprotective effect in the rotenone's chronic rat model. However, the exact mechanism behind neuroprotection was not reported (Parkhe et al., 2020).

Therefore, in the present study, we investigated AM's autophagic potential in a chronic mouse model of PD. We evaluated whether autophagy induction by AM cleared toxic α -Syn through the modulation of

AMPK activity and provided neuroprotection against TH⁺-dopaminergic neuronal loss in the striatum and SNc.

Materials And Methods

2.1 Chemicals

Alpha mangostin was isolated from the pericarp of *Garcinia mangostana* L. and characterized in our Chemical Biology laboratory at NIPER Ahmedabad, as reported previously (Parkhe et al., 2020). Rotenone, Acrylamide, Sodium dodecyl sulfate (SDS), Tween-20, Formaldehyde were procured from Sigma Aldrich. 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), Ammonium persulfate (APS), Tetramethyl-ethylenediamine (TEMED), Sodium chloride, Sodium lauryl sulfate (SLS), Bis-acrylamide, Sodium deoxycholate, Triton-X 100, Phenylmethyl sulfonyl fluoride (PMSF), Glycerol, Carboxymethylcellulose (CMC) were procured from Hi-Media Laboratories Pvt. Ltd. B-mercaptoethanol was procured from Alfa Aesar. Glacial acetic acid, Potassium chloride, Hydrochloric acid, Sodium hydroxide, chloroform were procured from Fischer scientific. Polyethylene glycol-400 was purchased from Merck. Tris-HCl was purchased from Thermo Fischer. Ladder and polyvinylidene difluoride (PVDF) membrane were procured from Bio-rad. HRP conjugated Enhanced Chemiluminescent Substrate Reagent kit was procured from Invitrogen. BCA (Bicinchoninic acid) reagent was purchased from Thermo Scientific. Primary antibody (TFEB) was procured from Sigma-Aldrich, while primary antibodies (LC3B, α -Syn, phospho- α -Syn, phospho-AMPK, total-AMPK, Beclin-1, β -actin) and secondary antibodies were purchased from Abcam. For immunohistochemistry, the Vectastain ABC kit was purchased from Vector Laboratories.

2.2 Animals

The Institutional Animal Ethics Committee approved all experimental procedures (IAEC: IAEC No.- IAEC/2018/033), and all the experiments were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India for the Care and Use of Laboratory Animals. Male C57BL/6 mice (7–8 weeks old, 20-28 g) were procured from the Zydus Research Center, Ahmedabad, and housed in a climate-controlled room at temperature: 20 ± 10 °C and humidity: $50 \pm 10\%$ (12 hours light/dark cycle), with free access to food and drinking water *ad libitum*. Behavioral experiments were performed in the light phase.

2.3 Study plan

Animals were habituated to the laboratory conditions for a week before starting the experiment. Behavioral parameters were recorded till 9 weeks at every two weeks. Animals were distributed blindly into 4 groups, namely: Group I: Vehicle control -Animals were administered with 0.5% CMC and a combination of Polyethylene glycol (PEG)-400 and H₂O (6:4), Group II: Rotenone group-

Animals received rotenone daily (10mg/kg, suspended in 0.5% CMC, Oral) for nine consecutive weeks. Group III: Rotenone plus alpha mangostin (R+AM) – Animals received co-treatment of rotenone (10mg/kg, suspended in 0.5% CMC, Oral) and alpha-mangostin (40mg/kg, suspended in PEG: H₂O; 6:4, Oral) for nine consecutive weeks. Group IV: Alpha-mangostin (AM) alone - Animals in these groups were administered with alpha mangostin (40mg/kg, suspended in PEG: H₂O, Oral) for nine consecutive weeks.

Rotenone is freely soluble in chloroform. A stock solution of 50 mg of rotenone in 1 ml of chloroform was prepared and stored at -20°C for not more than two weeks. For the model induction, rotenone emulsion was prepared in 0.5% CMC(Parkhe et al., 2020). AM was solubilized in the solution of PEG-400, and then water was added. (PEG:H₂O; 6:4). In all experiments, AM was co-administered daily for nine weeks before 1 hr of rotenone exposure. The dose and route of administration of AM used in the present experiment were chosen based on the previous results(Herrera-Aco et al., 2019). All the animals were sacrificed by decapitation using isoflurane at the end of the 9th week, and brains were quickly removed. Subsequently, striatum and cortex were isolated in ice-plate and processed for western blotting.

2.4 Behavioral assessments

2.4.1 Motor activity

Motor activity was measured using the round beam walk (RBW) test. The beam test was set up as described in a previous study(Suidan et al., 2013). Mice received the two training trials on two beams with different widths. In the first training trial, a wider beam (25mm in diameter) was placed 50cm above the tabletop with one end as the start platform, and another end is placed on the home cage. Mice were placed on the start platform and directed to move towards the home cage. For the second training trial, the wider beam was replaced by medium (15mm in diameter), and mice were placed on the start platform and then moved towards the home cage. After every training trial apparatus was clean with 1% acetic acid. In the test trial case, a medium beam was replaced by a narrow beam (10mm in diameter). To visualize the animal's feet on the beam, a video camera was placed at the end of the beam opposite the home cage. There was no interval between training and testing trials. Time is taken by the animal to cross the beam and the number of slips was analyzed from the recording by a blind evaluator to the groups.

2.4.2 Muscle strength

Muscle strength was measured using a grip strength meter. The grip strength meter is comprised of a grasping grid linked to a force transducer and digital display unit (IITC Life Science). To assess the grip strength, each mouse was placed on the grid, and the mouse was allowed to grasp the grid with all four paws. Once the mouse grasped the grid and then the mouse was slowly pulled back by the tail base until the grasp was overcome. The maximum force (in grams) required to hold the grid was recorded. Three

readings were taken with the time interval of 30 mins, and the mean of the obtained readings was used as representative grip force of the animal at that specific time(Meyer et al., 1979).

2.4.3 Memory impairment

Y-maze assists in evaluating short-term spatial memory based on rodents' inherent nature to explore a novel environment. Y-maze used was made up of wooden material with acrylic accessory and having three arms of equal length were interconnected at 120° angle with each other. Arms were defined as A, B, and C. Every time mice were placed in the center of the Y-maze facing A-arm, they freely explored the maze for 7 mins while being monitored by an experimenter. Mice were considered to visit the particular arm if they entered the arm with all the four-limbs inside. The sequence of entries in each arm by animal and spontaneous alternation was calculated. In the case of spontaneous alternation, we have allowed mice to explore the maze for 7 mins and considered made alternation if mice visited three arms sequentially without repeating any and counted the linear entries. We have also counted repeated entries, and further percent alternation was calculated by using the following equation(Garcia and Esquivel, 2018).

$$\% \text{ Alternations} = \text{Linear entries} / \text{Total entries} * 100.$$

The maze was wiped with 1% acetic acid before testing the next animal for memory impairment.

2.5 Western blotting

Frozen striatum and cortex samples were thawed, homogenized in RIPA buffer (25mM Tris, pH 7.8, 150mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% Triton X-100, PMSF, and protease inhibitor cocktail), and centrifuged at 16,000g and 4°C for 20 min. Further, the supernatants were collected, and the protein estimation was performed using the bicinchoninic acid (BCA) method. For Western blotting, the samples were loaded and resolved in 12% and 15% polyacrylamide gels based on the desired molecular weights of proteins. After that, electrophoresis was carried out at constant voltage. The resolved proteins were transferred onto the PVDF membrane followed by blocking with 3% BSA for 2 hours. Further, the incubation of membrane was carried out by using primary antibodies of LC3B (rabbit mAb, Abcam, AB192890 1:2000), TFEB (rabbit pAb, Merck, SAB4503154, 2:1000), Beclin-1 (rabbit mAb, Abcam, ab207612, 1:2000), p-AMPK (rabbit pAb, Merck, SAB4503754, 1:1000), T-AMPK (rabbit pAb, Abcam, AB131512, 1:1000), alpha-Synuclein (rabbit mAb, Abcam, AB212184, 1:1000), Phospho-alpha-Synuclein (rabbit mAb, Abcam, AB51253, 1:1000) and β-actin (mouse mAb, Abcam, AB6276, 0.5:5000) overnight at 4°C with gentle shaking. The next day, the antigen- primary complexed membranes were washed with TBST and further incubated for 1 hour with their respective horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit pAb, AB6721, 0.5:5000; mouse pAb, AB6789, 0.5:5000). β-actin was used as an internal control and used to normalize the protein levels. The expression level was quantified by densitometric analysis with the help of ImageJ software (NIH, USA)(Parkhe et al., 2020).

2.6. Immunohistochemistry for TH⁺-fibres in striatum and TH⁺-cells in SNc:

Mice were anesthetized using a ketamine-xylazine cocktail, accompanied by transcardial perfusion initially with PBS (phosphate buffer saline), followed by 4% paraformaldehyde in 0.05 M PBS, pH 7.4. Before processing, brains were rinsed with PBS for 1 hr. The brains were then cryoprotected with 10%, 20%, and 30% sucrose solution (dissolved in 0.1 M Phosphate buffer). Coronal sections (30 µm-thickness) were cut on a cryostat (Thermo Fisher Scientific) and immersed in 0.05M PBS. Mouse brain sections were taken at three different levels from bregma to A= +1.34, M= +1.18, P= +1.1 (for striatum) and A= -2.92, M= -3.16, P= -3.64 (for SNc) according to Paxinos and Watson atlas mouse brain. Immunohistochemistry was carried out as reported previously (Khairnar et al., 2010), with minor modifications.

Briefly, free-floating sections were pre-treated with 1% hydrogen peroxide and 0.1% Triton-X-100 for 20min to remove endogenous peroxidase activity followed by incubation in blocking buffer (Diluted blocking solution, Vectastain kit, Vector Laboratories) for 1 hr. Further, sections were incubated at 4°C overnight with anti-tyrosine hydroxylase (1:500 dilution, AB152, Merck) in a Blocking solution. The sections were then incubated with a biotinylated secondary antibody (Vectastain kit, Vector Laboratories) at 4 °C for 90 mins and avidin-biotin-peroxidase (Diluted ABC solution, Vector Laboratories) at 4°C for 30 min. The visualization was done by incubation with SIGMAFAST 3,3'-diaminobenzidine tablets (D4293, Sigma-Aldrich) for 8-15 mins. Finally, the sections were mounted on slides, air-dried and dehydrated in increasing grades of ethanol, coverslipped, and imaged using a bright-field microscope at 10X (Leica ICC50 E).

2.7. Quantitative Analysis

The striatal TH⁺-fibers density was quantified by using image densitometry analysis tool provided in the Image J software (<https://imagej.nih.gov> NIH, USA) and represented in terms of optical density (OD), as described previously (Altarche-Xifro et al., 2016; Jewett et al., 2017; Tian et al., 2016; Wang et al., 2018). To normalize the gray-scale range (0–255) into OD values, the Rodbard calibration function was employed given in the software. Each image was then converted into an 8-bit image. The OD values were normalized by eliminating OD values of background. At each level of the striatum, the obtained value was first normalized to the vehicle, and values from different levels were averaged after that. Here, OD values represent the strength of the TH⁺-fibers.

The number of dopaminergic neurons was determined as previously described (Kühn et al., 2003). Briefly, we manually counted TH-positive cells under bright-field illumination in the SNc using an x10 objective. Cell counts were determined blindly and it should be noted that the analyses of the TH-immunoreactive profiles were restricted to the SNpc and thus excluded the ventral tegmental area. In addition, neurons were only counted if they contained a nucleus that was surrounded by cytoplasm.

2.8. Statistical analysis:

Data were represented as mean $\pm$ SEM. Means of multiple groups were compared using Two-way and One-way ANOVA (analysis of variance), followed by Tukey's multiple comparison test. Differences were considered statistically significant if $p < 0.05$. Statistical software used for analyzing the data was GraphPad Prism, version 5.01, GraphPad Software, Inc.

Results

3.1 AM ameliorated rotenone-induced behavioral and cognitive deficits in mice

3.1.1 Effect of AM on motor activity in the round beam walk test:

Rotenone administration at 10mg/kg exhibited a prominent loss of motor coordination in the round beam walk test. Fig1. At the 9th week of rotenone administration, there was a significant increase in the time taken by the animal to cross the beam ($p < 0.01$) and the number of slips ($p < 0.05$) as compared to control mice. The time taken to cross the beam and number of slips was significantly reduced ($p < 0.01$ and $p < 0.05$, respectively) upon oral administration of AM at 40mg/kg compared to the rotenone administered group. (Fig.1A,1B)

3.1.2 Effect of AM on memory impairment in Y-Maze test:

Rotenone induced significant spatial memory impairment relative to the control group ($p < 0.001$) on the 9th week, which was significantly ($p < 0.001$) rescued by the treatment with AM. (Fig.1C)

3.1.3 Effect of AM on neuromuscular function in the grip strength test:

Rotenone induced a significant decrease in force (measured in grams) on the grip strength meter compared to the control group at the 9th week of rotenone exposure ($p < 0.01$). In contrast, AM treatment significantly reversed the rotenone-induced deficit of grip strength on the 9th week of treatment. (Fig.1D)

3.1.4 AM alleviate rotenone-induced α -Syn accumulation through AMPK dependent autophagy activation:

AMPK activation requires phosphorylation of threonine 172 (Thr172) in the kinase domain activation loop for maximal activity and is necessary for mTOR's negative regulation. Therefore, we analyzed the level of p-AMPK and t-AMPK in the striatum and cortex. The results showed that rotenone diminishes the activation of AMPK, as observed from a significant reduction in the expression of p-AMPK/t-AMPK in the striatum ($p < 0.05$) and cortex ($p < 0.05$) compared to the control group (Fig.2E, 3E). However, treatment with AM potentially mitigated the rotenone-induced down-regulation of AMPK in the cortex and striatum (Fig.2E, 3E). Since the AMPK is a key regulator of autophagy and we found activation of AMPK upon AM treatment, further we determined the levels of key autophagy markers (LC3B, Beclin-1) and marker of lysosomal biogenesis (TFEB). Chronic rotenone treatment significantly reduced the conversion of LC3-I to LC3-II as observed from decreased expression of LC3-II/I in the cortex and striatum compared to the control group ($p < 0.05$). Interestingly, AM treatment showed a significant increase in the LC3-II/I

expression in the cortex ($p<0.001$) and striatum ($p<0.05$) compared to rotenone (Fig.2B, 3B). Furthermore, rotenone significantly reduced the activation of Beclin-1 (an essential mediator of the autophagy process), the expression of which has been significantly restored by AM treatment in both the cortex and striatum ($p<0.05$) (Fig. 2D, 3D). Overall, western blotting analysis of p-AMPK, LC3B, and Beclin-1 in the striatum and cortex revealed that AM protected neuronal damage induced by rotenone by upregulation of autophagy. On the other hand, AM significantly blocked the rotenone-induced depletion of TFEB in the cortex ($p<0.05$) as well as striatum ($p<0.05$) (Fig.2C, 3C), which justifies the role of AM on the biogenesis of lysosomes, has been altered by rotenone treatment. Similar results were obtained with AM alone group. Following a previous report, induction of autophagy led to the substantial clearance of α -Syn. Thus, we determined the levels of α -Syn and p- α -Syn in the cortex and striatum. As expected, results showed that AM significantly alleviated rotenone-induced accumulation of α -Syn ($p<0.05$) and p- α -Syn (n.s.) in the striatum (Fig.3F, 3G). On the other hand, the treatment with AM did not affect the expression of α -Syn, but significantly reduced rotenone-induced accumulation of p- α -Syn in the cortex (Fig.2F, 2G). Together, these results strengthen the hypothesis that AM rescues the rotenone-induced damage to the neuronal cells through AMPK-dependent autophagy activation.

3.3. AM attenuates rotenone-induced neurodegeneration in the striatum and SNc:

To test the neuroprotective effect of AM in the rotenone-induced model of PD, we studied the immunoreactivity of TH in the SNc and striatum. All the groups were analyzed for the TH-immunoreactivity after the study. Treatment with the rotenone for 9 weeks induced a significant (45%) reduction in the density of striatal TH⁺-fibres, compared to the control ($p<0.05$). While co-administration of AM rescued the loss of TH⁺-fibres compared to the rotenone group in the striatum ($p<0.05$) (Fig.4B). Moreover, chronic rotenone treatment-induced ~30% degeneration of dopaminergic neurons in the SNc ($p<0.05$), and interestingly AM significantly restored the rotenone-induced loss of TH⁺-cells in the SNc ($p<0.05$).

Discussion

In the present study, we evaluated AM's autophagic potential, a natural polyphenolic xanthone obtained from *Garcinia Mangostana* Linn., in the clearance of toxic α -Syn using chronic rotenone treated mouse model of PD. It has been reported in our previous study that AM is neuroprotective in PD (Parkhe et al., 2020). However, the mechanisms behind neuroprotection remain unknown. Therefore, for the first time, in the current study, we found that AM (40 mg/kg) was able to clear the α -Syn load by inducing autophagy through modulating AMPK activity. Interestingly, we found that rotenone treatment significantly reduced the conversion of LC3-I to LC3-II (depicts autophagosome formation), reduced the expression of TFEB (involved in lysosomal biogenesis), and Beclin-1 (an upstream regulator of autophagy). The reduction in the expression of LC3-II/I, TFEB, and Beclin-1 was restored after treatment with AM, which might be due to the activation of AMPK. As studies in the past suggested the role of activation of autophagy in the clearance of α -Syn aggregates, herein we also found that treatment with the AM alleviated the rotenone-induced accumulation of normal α -Syn as well toxic p- α -Syn, which may have resulted in the

neuroprotection against rotenone-induced TH⁺-dopaminergic nerve terminal loss in the striatum and TH⁺-cells in the SNc. Moreover, AM also improved the motor and cognitive deficits induced by the chronic rotenone treatment.

Accumulation of oligomeric and fibrillar forms of α -Syn is central to PD's pathogenesis (Iwatsubo 2003). Studies reported that α -Syn accumulation induces degeneration of dopaminergic neurons of the SNc (Mori et al., 2006). With regard to this, autophagy, an intracellular nutrient recycling process, in physiological conditions responsible for clearance of aggregated α -Syn, was found to be impaired in the PD pathology (Curry et al., 2018). The process of autophagy is in control by several regulators; AMPK is one among them. Interestingly, Jang M and his colleagues reported that AMPK is responsible for the initiation of autophagosome maturation and lysosomal fusion by modulating ULK1, autophagy initiating kinase, and negatively regulating mTORC1 signaling (Jang et al., 2018).

On the other hand, increased activity of mTOR has been reported in clinical and pre-clinical models of PD (Crews et al., 2010), which further confirms the pathogenic role of mTOR in PD pathology. Simultaneously, an *in-vitro* study of α -Syn accumulation reported downregulation of AMPK activity (Dulovic et al., 2014). Extensive studies have indicated that upregulation of AMPK activity might be a better therapeutic approach in treating PD. About that, several AMPK activators like Trehalose, Resveratrol, and Metformin were found to be protective in PD (Curry et al., 2018; Dulovic et al., 2014; Gao et al., 2018; Lu et al., 2016; Wu et al., 2011; Yan et al., 2017). Therefore, in the present study, we found significant activation of AMPK upon treatment with AM in rotenone-treated mice in the cortex and striatum. As reported previously, AMPK activation induces autophagosome formation by increasing the conversion of LC3-I to LC3-II, which gets impaired in PD (Wu et al., 2011). Thus, we also checked the conversion of LC3-I to LC3-II in the striatum and cortex and found that rotenone administration significantly downregulated the formation of autophagosomes (reduced levels of LC3-II/I), which corresponds to impaired autophagy, which was found to reverse upon the treatment with AM. Similar to our study results, Zhou Q and his colleagues also demonstrated a reduction in LC3B-II levels in both the cerebral cortex and striatum after rotenone administration and aberrant protein deposition (Zhou et al., 2016). We also found an increase in the levels of α -Syn and p- α -Syn in the cortex and striatum after rotenone treatment. While AM was able to ameliorate the α -Syn aggregation in the striatum of rotenone-treated mice, might be due to the induction of autophagy, as observed from increased expression of LC3-II. Importantly, in the cortex AM significantly mitigated rotenone-induced aggregation of p- α -Syn, while observed modest trend in the expression of α -Syn.

AMPK is involved in autophagosome formation and also modulates lysosome biogenesis through nuclear translocation of TFEB (Phillipson, 2017). TFEB is a significant regulator for the transcription of genes involved in lysosome biogenesis (Decressac et al., 2013). In this context, we found a reduced level of TFEB in the cortex and striatum after rotenone treatment, which was restored after the treatment with AM by modulating AMPK activity. As a whole, an increase in autophagosome formation and lysosomal biogenesis by AM ultimately leads to induction of autophagy and may have reduced the α -Syn load in the

striatum. AM might have protected against rotenone-induced loss of dopaminergic fibres and neurons in the striatum and SNc by this mechanism, respectively.

Conclusion

In conclusion, we can say that AM possesses AMPK modulatory activity and can induce autophagy, which may have cleared the toxic α -Syn load and provided protection against rotenone-induced motor deficits and rotenone-induced loss of TH⁺-cells and dopaminergic fibres in the SNc and striatum, respectively.

Declarations

Ethics approval: All procedures and experiments were conducted under the guidelines approved by Institutional Animal Ethics Committee (IAEC: IAEC No.- IAEC/2018/033) and all the experiments were conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India for the Care and Use of Laboratory Animals.

Consent to participate: Not applicable

Consent for publication: All author gives consent for the publication.

Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Writing- Original Draft: [Pathik Parekh]; Methodology, Software, Writing- Original Draft, Validation: [Nishant Sharma]; Methodology, Software: [Anagha Gadepalli]; Methodology, Writing- Original Draft: [Monika Sharma]; Methodology, Writing- Review & Editing: [Sayan Chatterjee]; Supervision: [Abhijeet Kate]; Conceptualization, Writing- Original Draft and Review & Editing, Supervision: [Amit Khaimar]

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Figures

Figure 1

Effect of alpha-mangostin (AM) at 9th week on behavioral parameters in rotenone-induced Parkinson's mouse model. a) Time is taken to cross the beam, b) Number of foot slips are measured by round beam walk test c) Memory impairment by Y-maze test and d) Muscle strength by grip strength test. Data presented as Mean $\pm$ SEM (n=5). *p < 0.05, **p < 0.01, ***p < 0.001 vs control group, #p < 0.05, ##p < 0.01, ###p < 0.001 vs rotenone group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test.

Figure 2

Representative western blot image (A) and quantification graph showing the effect of alpha-mangostin (AM) on protein expression of B) LC3II/I, C) TFEB, D) Beclin-1, E) p-AMPK/t-AMPK, F) α-syn and G) p-α-syn in the cortex of rotenone-induced mouse model of PD. β-actin was probed as a housekeeping gene. Values are expressed to 100% for levels of the control group. The results were expressed as Mean ± SEM (n = 3). *p < 0.05, vs control group, #p < 0.05, ##p < 0.01, ###p < 0.001 vs rotenone group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test

Figure 3

Representative western blot image (A) and quantification graph showing the effect of alpha-mangostin (AM) on protein expression of B) LC3II/I, C) TFEB, D) Beclin-1, E) p-AMPK/t-AMPK, F) α -syn and G) p- α -syn in the striatum of a rotenone-induced mouse model of PD. β -actin was probed as a housekeeping gene. Values are expressed to 100% for levels of the control group. The results were expressed as Mean $\pm$ SEM (n = 3). *p< 0.05, vs control group, #p< 0.05, ##p< 0.01 vs rotenone group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test

A

B

Figure 4

Alpha mangostin attenuates rotenone-induced loss of striatal TH+fibres. A) Representative images of immunoreactivity of TH+fibres in the striatum AT 4x and 20x, B) Quantification of immunoreactivity of TH+fibres in terms of mean optical density (OD). The results are expressed as Mean $\pm$ SEM (n =3). *p< 0.05, vs control group, #p< 0.05 vs rotenone group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s test.

A

B

Figure 5

Alpha mangostin attenuates rotenone-induced loss of nigral TH⁺-neurons. A) Representative images of immunoreactivity of TH⁺-neurons in the SNc at 4x and 10x, B) Quantification of immunoreactivity of TH⁺-cells. The results are expressed as Mean $\pm$ SEM (n =3). *p< 0.05 vs control group, #p< 0.05 vs rotenone group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test.

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