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Amlodipine Abridged Calcium Associated Complex-I Inhibition in 6-OHDA Lesioned Parkinson's Rat

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Authors' contributions

This work was carried out in collaboration between both authors. Author ASA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author PV managed the script correction and analyses of the study. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The present study was carried out to evaluate Amlodipine, a L-type calcium channel blocker for alleviating or reducing the neurodegeneration in 6-OHDA lesioned rat models.
Place and Duration of Study: Department of Pharmacology, JSS College of Pharmacy, Rocklands, Ooty, India between October 2011 and may 2012.
Methodology: Male adult Wistar rats were given with intra-cerebroventricular injection of 6-hydroxydopamine (6-OHDA) into the median forebrain bundle and treated post 48 hours with Amlodipine (10 mg/kg and 20 mg/kg) per oral for 30 days. Motor coordination, striatal dopamine, mid brain calcium and complex-I activity were estimated. Data were statistically analyzed and $p<0.05$ was considered significant.
Results: Amlodipine regained motor coordination, mid-brain dopamine content, and prevented calcium overload and complex I activity at both dose levels when compared with 6-OHDA control group. Alleviation of calcium overload and complex I inhibition with subsequent increase in dopamine level in Parkinson's rats were observed at the end of treatment period.
Conclusion: The experimental study gave light to a new therapeutic intervention of

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Amlodipine in preventing neuronal morbidity in Parkinson's disease (PD). So, further neuro-molecular study with Amlodipine in experimental PD is warranted in future.

Keywords: Parkinson's disease; dopamine; calcium; complex I; Amlodipine.

1. INTRODUCTION

PD is a progressive neurodegenerative disorder of movement, consisting of four cardinal features: Bradykinesia, muscular rigidity, resting tremor and postural imbalance. PD is the second most common neurodegenerative disease, primarily affecting approximately 1.5-2.0% people over 55 years although young adults and even children can also be affected. The global burden in 2005 is expected to double by 2030, mostly outside the western world due to increase in ageing population. The pathological hallmark of PD is loss of the dopaminergic neurons of SNpc in nigrostraital pathway, with the appearance of intracellular inclusions known as Lewy bodies. Current therapy is focused on restoring the neurotransmitter imbalance, not alleviating the underlying cause [1,2].

High mitochondrial calcium level and low complex I activity in the electron transport chain are implicated in neuronal apoptosis in PD [3]. Mitochondrial calcium overload results via various mechanisms, namely influx into the cell through L-type calcium channels, release from the sarco and endoplasmic reticulum stores via two major proteins, sarco and endoplasmic reticulum calcium ATPases and inositol triphosphate receptors, over expression of voltage dependent anionic channels, opening of permeability transition pore and increased outer mitochondrial membrane permeation. This leads to complex I (NADH: ubiquinone oxidoreductase) inhibition, blocking the electron flow along the mitochondrial respiratory chain and increased reactive oxygen species production with subsequent release of pro-apoptotic factors like cytochrome C from inter-membrane space to cytoplasm, caspase activation, eventually leading to apoptosis [4-6].

Calcium channel blockers (CCBs) have been hypothesized to be neuroprotective against PD with reduced risk of incidence and mortality among patients with PD [7,8]. Calcium channel blockers have been reported to prevent1-methyl-4 phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and 6-OHDA induced neurotoxicity in mouse models [9,10]. Thus, in this study, we hypothesize that Amlodipine, belonging to dihydropyridine class of CCBs, could alleviate midbrain calcium overload to protect complex-I and treat further neurodegeneration in 6-OHDA Parkinson's rats.

2. MATERIALS AND METHODS

2.1 Chemicals

6-OHDA and Amlodipine were procured from Sigma-Aldrich, USA. All chemicals used were of analytical grade.

2.2 Animals

Healthy, adult Wistar rats of both sexes (180-220 g) were obtained from the central animal house facility of JSS College of Pharmacy, Udhagamandalam, Tamilnadu. The animals were housed in a well-ventilated room, exposed to 12 h day and night cycle with a temperature of

22±2ºC and fed with water and rat feed *ad libitum*. All the experiments were performed with prior approval from Institutional Animal Ethics Committee, bearing the No: JSSCP/IAEC/PH.D/PH.COLOGY-2/2010-11. Animals were divided into four groups, each with 10 rats:

- Group I : Sham control
- Group II : 6-OHDA control
- Group III : 6-OHDA+Amlodipine (10 mg/kg orally)
- Group IV : 6-OHDA+ Amlodipine (20 mg/kg orally)

2.3 Induction of Parkinsonism by 6-OHDA and Drug Dosing

Surgery was conducted using stereotaxic apparatus (Steotling) as previously described with slight modification [11]. Briefly, desipramine hydrochloride (25 mg/kg, i.p.) was administered 30 min prior surgery to protect adrenergic neurons from 6-OHDA. Rats were anesthetized with ketamine (100mg/kg, i.p.), xylaxine (15mg/kg, i.m.) and received an injection of 8 µg 6-OHDA hydrochloride in 4 µl 0.9% saline containing 0.05% ascorbic acid into the medial forebrain bundle using the following coordinates, with respect to bregma: Antero-posterior, -2.5mm; lateral, +2.0; dorso-ventral, -8.5mm at the rate of 1 ml/min. The needle was left in place for 2 min and then withdrawn. Sham control received vehicle alone. Three weeks after surgery, 6-OHDA lesioned rats were challenged with apomorphine hydrochloride (0.2 mg/kg, s.c.) and those showing fewer than 20 contralateral rotations per 5 min were excluded from the studies. After the Apomorphine challenge test Amlodipine was suspended in 0.3% carboxymethyl cellulose and administered orally to Group III and IV respectively for 30 days, Group II received vehicle alone.

2.4 Experiments

2.4.1 Grip strength

Muscle coordination was assessed using rotarod apparatus (INCO-India Ltd) after 30 days treatment with minor modification from previously described method [12]. Briefly, rats were trained to remain on rotating rod at 5, 10 and 20 rpm on two consecutive days with a cut-off time of 180 seconds. The final test was carried out at 20 rpm and fall off time was noted.

2.4.2 Estimation of striatal dopamine

Rats were sacrificed by excess anesthesia and dissected striatum was sonicated and centrifuged in pre-cold 0.1 M perchloric acid (about 100 µl/mg tissue) and the supernatant was used to estimate dopamine using HPLC (Waters) as per previously described method with minor modification [13]. Briefly, 20 µl supernatant was isocratically eluted at a flow rate of 1 ml/min through an 150×4.6-mm C_{18} column (PRINCETON SPHERE) with a mobile phase containing 50 mM ammonium phosphate pH 4.6, 25mM hexane sulfonic acid pH 4.04 and 5% acetonitrile and detected by UV detector. Dopamine concentration was expressed as ng/mg protein. The protein content in tissue homogenates was measured by Lowry's method [14].

2.4.3 Estimation of midbrain calcium level

Rat midbrain calcium content was estimated by atomic absorption spectrophotometer (AAS) 6300 (Shimadzu) with modification of previously described method [15]. Briefly, rat midbrain was isolated and weighed on aluminum foil and then dried at 115ºC for 20 h and weighed again. Dry samples were then made ash in a quartz crucible by gradual heating up to 550ºC for 24 h. The ash was dissolved in a variable volume of 3M nitric acid to ensure optimum concentration of calcium for estimation. Standards of different calcium concentrations (i.e., 1, 1.5, 2 and 2.5 µg/ml) were prepared from stock standard. The blank, standards and samples were atomized using air-acetylene flame and absorbance peaks were recorded. The concentration of calcium in the brain was calculated by extrapolating from the standard curve.

2.4.4 Complex I assay

Complex I activity was estimated in mitochondria isolated from rat midbrain as previously described [16,17] Briefly, tissue was homogenized in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM TrisHCl, 1 mM EDTA; pH 7.4) and centrifuged at 800 *g*, 4ºC for 10 min. Supernatant was centrifuged at 13,000 *g*, 4ºC for 10 min and the pellet was washed with mitochondrial isolation buffer and centrifuged at 13,000 *g*, 4ºC for 10 min to obtain crude mitochondrial fraction.

The crude mitochondrial fraction was lysed by freeze-thawing in hypotonic buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$; pH 7.4). 50 µg mitochondria was added to the assay buffer [hypotonic buffer containing 65 µM ubiquinone, 130 µM NADH, 2 µg/mL antimycin A and 2.5 mg/mL defatted bovine serum albumin] and the oxidation of NADH by Complex I was monitored at 340 nm for 2 min at 30ºC in UV-visible spectrophotometer 1700 (Shimadzu). The activity was monitored for further 2 min after adding rotenone (2 µg/ml). The difference between the rate of oxidation before and after the addition of rotenone was used to calculate complex I activity using an extinction coefficient of 6.81 $mM^{-1}cm^{-1}$ at 340 nm.

2.4.5 Statistical analysis

Data were analyzed using one way ANOVA followed by Dunnett's multiple comparison test in Graph Pad Prism software V 5.01 (Graph Pad Software, Inc.). $p<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

Parkinsonism was successfully induced three weeks post-surgery in 6-OHDA lesioned rats. The rats showed more than 20 contralateral rotations within five minutes when challenged with apomorphine hydrochloride, owing to the proof of 6-OHDA mediated destruction of presynaptic dopaminergic neurons in lesioned brain hemisphere, followed by stimulation of intact but hypersensitive postsynaptic dopamine D_2 receptors by apomorphine hydrochloride. Followed by the treatment with Amlodipine, rats were tested for grip strength and striatal dopamine, midbrain calcium and complex I activity was measured. Amlodipine showed significant improvements of disease in 6-OHDA rats. The corresponding results were shown in Fig. 1. Our experimental results revealed the dopaminergic restorative effects of Amlodipine in 6-OHDA rat models. Amlodipine is known to have anti-inflammatory, anti-oxidant and anti-apoptotic properties [18,19]. The antiparkinsons activity in this experimental study might be offered by any of the therapeutic properties of Amlodipine.

In the rotarod test to access motor impairment seen in PD rats (Fig. 1A), 6-OHDA group showed significant loss of grip strength and muscular coordination when compared to sham control, which indicated by significant (*p<.001*) reduction in retention time in rotarod apparatus. The Amlodipine treatment improved muscle grip strength and coordination in dose dependent manner with respect to 6-OHDA control (Fig. 1A).

Dopamine content in striatal region was measured by HPLC. The concentration of dopamine was significantly (*p<.001*) reduced in 6-OHDA control when compared with sham control rats. This correlated well with the loss of grip strength, contributed by significant destruction of dopaminergic neurons in basal ganglia by 6-OHDA. Amlodipine treated group showed significant regaining of dopamine level at both dose of Amlodipine when compared to 6-OHDA control (Fig. 1B).

The calcium concentration in the midbrain region was measured by AAS. When compared with sham control, in 6-OHDA treated rats there was a significant (*p<.001*) increase in calcium overload, probably which caused neuronal excitotoxicity. When compared with 6-OHDA group, Amlodipine treatment did not elevate mid brain calcium concentration, probably this contributed through blockade of L-type calcium channels (Fig. 1C).

Further, complex I activity was significantly (*p<.001*) reduced in 6-OHDA lesioned rats when compared to sham control, which may mediate free radical induced caspase activation and neuronal apoptosis as reported earlier [6]. Amlodipine at a dose of 10 mg/kg and 20 mg/kg improved complex I activity when compared with 6-0HDA treated animals (Fig. 1D).

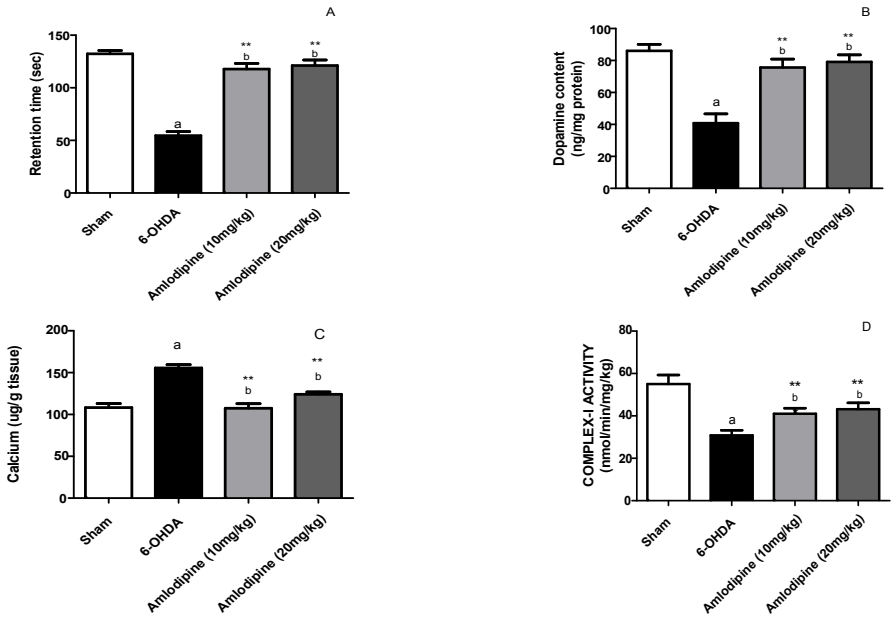

Fig. 1. Effect of Amlodipine on (A) retention time in rotorod, B) striatal dopamine content, (C) midbrain calcium concentration and (D) complex I activity in 6-OHDA

Bars represent mean ± SEM; *n=6* in each group, $^{a}p < 0.001$, $^{b}p<0.05$ compared with sham control; $^{**}p<0.01$ when compared with 6-OHDA control. One way ANOVA followed by Dunnett's multiple comparison test.

4. CONCLUSION

Treatment with Amlodipine, a calcium channel blocker showed reliable neuroprotection by regaining the levels of brain dopamine, mitochondrial calcium and motor functions in rat model of PD. The investigations revealed that, the test drug is having a reliable anti-Parkinson's activity. The clinical acceptability of this molecule for treating PD needs continuation of toxicity and pharmacological investigation. We conclude that, further investigations are needed to exemplify Amlodipine as a drug of choice for Parkinson's disease.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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