1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one Attenuates Oxidative Trauma and Recuperate Beam Walk and Adhesive Removal Behavior in MPTP Parkinsonian Mice Model

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Recent researches have suggested 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor may attenuate motor impairments in Parkinson's disease (PD). The antiparkinsonian activity of ODQ were studied on motor abnormalities induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to provide a better understanding of this drug group. The objective of the present study is to evaluate the effect of ODQ on behavioral parameters such as Beam walk test, Adhesive removal test and to assess the biochemical changes due to ODQ against MPTP induced PD mice model. Eighteen C57BL/6J male mice were included in the study, divided into three groups of 6 each. Group A mice were treated with vehicle (Normal saline). Group B mice were subjected to MPTP sub acute protocol. Group C mice were treated with MPTP as according to sub acute protocol and administered with ODQ subcutaneous injection after final MPTP dose. Behavioral tests like Beam walk test, Adhesive removal test, along with Biochemical correlation were done using standard methods. Narrow beam walk and adhesive removal behavior were significantly reversed, and Superoxide dismutase (SOD) levels were enhanced in ODQ treated group compared to MPTP intoxicated mice group. Soluble guanylate cyclase inhibitor ODQ, could be a potential treatment for maintaining the balance of antioxidant and oxidant biochemical environment during oxidative stress which may be helpful for treating PD, targeting one or more factors of its multiple etiological factors.

Keywords: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, Parkinson's disease, Superoxide dismutase, Behaviour, MPTP.
(cGMP) from guanosine triphosphate (GTP)\(^1\). cGMP causes glutamate toxicity upon over regulation of sGC\(^2\). ODQ inhibits sGC by binding to heme site and affects sGC-cGMP signalling pathway\(^3\). Excitotoxicity was a known factor for Parkinson's disease (PD) due to high glutamergic inputs in Basal ganglia pathway. Also, it was considered for aggravating the disease condition and striatal neuronal cell death\(^4\). Over regulation of glutamate receptors causes intracellular calcium ion accumulation resulting in formation of reactive oxygen species. Activation of glutamate receptors causes opening of calcium channel that are coupled to glutamate receptors, results calcium influx and accumulation. This leads to neurocyte excitation that may initiate apoptosis and neurodegeneration\(^5\).

Glutamate toxicity is also related to superoxide dismutase levels (SOD). SOD catalyses the free radicals. Compromise in SOD levels increased glutamate toxicity\(^6\). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) increases SOD activity due to increased free radical generation which finally gets compromised\(^7\). The imbalance in glutamergic transmission results in akinesia\(^8\). This akinesia is a movement disorder causing difficulty in mobility. This is measured by behavioral tests. In this study we performed narrow beam walk test and Adhesive removal test. These tests were among the behavioral tests considered sensitive for identifying classical motor manifestations of PD in MPTP mice and also the functional recovery of the animal and treatment efficacy. The present study was performed to evaluate the effects of ODQ against MPTP induced oxidative stress and behavioral impairments in C57BL/6J mouse model.

MATERIALS AND METHODS

Animals
C57BL/6J male mice (25-30gm) were obtained for the study from Centre for Toxicology and Developmental Research (CEFT), Sri Ramachandra Institute of Higher Education and Research - Deemed University, Porur, Chennai, Tamil Nadu, India. Animals were housed in groups of 4 per cage. The temperature and humidity of the room was well controlled and maintained light-dark cycle on 12L:12D. They were provided with water and food \textit{ad libitum}. The study was done according to national guidelines of proper care and use of animals in laboratory research (Indian National Science Academy, New Delhi, 2000). Ethical clearance was obtained from Institutional Animal Ethics Committee (IAEC/49\(^{th}\)/SRU/502/2016), Sri Ramachandra Medical College and Research Institute- Deemed University, Porur, Chennai, India.

Chemicals
MPTP, ODQ were purchased from TCI Chemicals (India) Pvt. Ltd. Avery dotted Adhesives (Amazon Export Sales LLC, USA) were purchased. Mouse anti-Tyrosine hydroxylase (Sigma Aldrich, USA), goat anti-mouse IgG and ImmunoCruz™ ABC Staining kit (Santa Cruz, USA). All other chemicals used for SOD assay were of analytical grade.

Experimental Procedures
Eighteen C57BL/6J mice included in the study were randomized into three groups. Group A (n=6) included mice treated with normal saline (0.9%). Group B (n=6) included mice treated with intraperitoneal injection (IP) of MPTP-HCL, Group C (n=6) included mice treated with IP injection of MPTP-HCL and subcutaneous injection of ODQ. MPTP-HCL was administered for five days of 7 doses. 20mg/kg dose was given twice daily, 12\(^{th}\) hourly for two days followed by 20mg/kg dose once daily for subsequent three days\(^9\). 10mg/kg ODQ was administered subcutaneously on the last day one hour after the last dose of MPTP-HCL.

Behavioral tests
Narrow beam walk test
The beam walking test apparatus was featured with a wooden beam (L100 cm×W1 cm) which was mounted on a height of 1m above ground with help of two wooden towers. One end of the beam had a box with a hole so that the mice can enter from the beam into the box. In this test the mouse was trained to cross the beam as described elsewhere\(^10\). To measure the motor coordination and balance, foot slips and traversal time to cross the beam were recorded as described previously\(^11\).

Adhesive removal test
In this test, the mice in the home cage were shifted to another cage. The test mouse remained in the home cage. With the help of forceps, the adhesive dot was applied to snout of the mice. Time of contact, time to remove the adhesive dot was recorded. The latency in touching the adhesive dots was used for evaluating sensory impairment.
Maximum time for the test was 60s. If the mouse did not remove the adhesive dot within 60s, then it was removed manually. Three trials were performed for each mouse.

**SOD assay**

Estimation of SOD activity in brain homogenate was done by spectrophotometric method. Inhibition reaction of NADH- Phenantrene methosulphate - nitroblue tetrazolium formazan was measured at 560nm optical density. The enzyme activity was expressed as milliunits/mg protein. One single unit of enzyme activity implies the concentration of enzyme required to inhibit the chromogen production by 50% in one min at an optical density of 560nm.

**Tyrosine hydroxylase (TH) immunohistochemical staining**

Immunohistochemical evaluation of TH positive neurons in mice brain was investigated. Five micrometer - thick paraffin sections through matched coronal levels were stained with mouse anti-TH (1:100) using standard immunoperoxidase techniques. TH immunoreactivity was detected with a biotinylated goat anti-mouse for TH secondary antibody at 1:100 dilutions and ImmunoCruz™ ABC Staining kit. All slides were counterstained with Mayer’s hematoxylin and visualised in light microscopy. The expression of immunopositivity in sections was evaluated at a magnification of x10 under light microscopy. The number of immunopositive cells were counted manually as described previously by Guo et al.(2016) with minor modification.

**Statistical analysis**

The means of all groups in the study was analysed by one-way ANOVA and for multiple comparisons Post hoc test was done using SPSS software version 16.0.

**RESULTS**

The biochemical and behavioral tests mean values of all groups in study, their comparison using one-way ANOVA and Post hoc test are tabulated in Table 1.

**Effect of ODQ on SOD expression in Substantia nigra of MPTP treated mice**

In comparison to MPTP treated mice, ODQ treated MPTP mice showed enhancement in SOD levels (125.60±13.10). MPTP group

| Table 1. Statistical analysis of Biochemical and Behavioral tests in study. | SOD=superoxide dismutase, SD= standard deviation, *=P value between Group A and B, **=P value between Group B and C. P value < 0.05 is considered significant |
|---|---|---|---|
| S.No | Test | Group | Mean ± SD | P value |
| 1 | SOD | A | 132.61 ± 14.84 | 0.0005* |
| | | B | 74.8 ± 12.38 | 0.0005* |
| | | C | 125.59 ± 13.1 | 0.0005** |
| 2 | Adhesive removal test | Time Duration (sec) | A | 18.13 ± 5.64 | 0.0005* |
| | | B | 42.25 ± 10.8 | 0.0005* |
| | | C | 26.13 ± 2.70 | 0.0005** |
| | Number of Attempts | A | 2 ± 0.92 | 0.0005* |
| | | B | 5.75 ± 1.6 | 0.0005** |
| | | C | 2.88 ± 0.835 | 0.0005** |
| 3 | Beam walk test | Traversal time (sec) | A | 11.88 ± 2.8 | 0.0005* |
| | | B | 47.38 ± 9 | 0.0005* |
| | | C | 25.6 ± 7.5 | 0.0005** |
| | Number of slips | A | 0.75 ± 0.46 | 0.0005* |
| | | B | 4.88 ± 2.29 | 0.0005** |
| | | C | 2.88 ± 1.35 | 0.0005** |
mice exhibited decreased levels of SOD levels (74.80±12.38) in comparison to control mice group (132.62±14.84) [Figure 1].

**Effect of ODQ on narrow beam walk test against MPTP treated mice**

Beam walking test detects the dopaminergic neuronal loss in nigro-striatal pathway (central nervous system lesions). There was significant increase in beam walk traversal time of MPTP treated mice group (47.375±9.0) in comparison to control mouse group (11.875±2.79). ODQ reversed the beam walking ability (25.625±7.52) which was statistically significant. Number slips while crossing the beam was noted which was noticed high in MPTP treated mice (4.875±2.29) compared to control group (0.75±0.46). ODQ showed significant improvement compared to MPTP treated group (2.875±1.35) [Figure 2].

**Effect of ODQ on Adhesive removal test against MPTP treated mice**

The time taken for removing the adhesive material by mice from its snout signifies the sensory and motor coordination abilities of the mice. MPTP treated mice showed poor ability to remove the adhesive dot, took more time and attempts compared to normal control mice and ODQ treated MPTP mice [Figure 3].

**Effects of ODQ on TH- positive neurons in substantia nigra of MPTP treated mice**

The TH-positive neurons in substantia nigra were shown as mean ± SE. The MPTP treated mice of group B (17.2±2.08) showed decrease in TH positive neurons when compared to group A, normal mice (45±3.24). Group C, ODQ treated mice showed higher expression of TH positive neurons (38.25±2.25) when compared to Group B [Figure 4].

![Fig. 1. Effects of ODQ on SOD levels of MPTP treated mice brain. SOD level were represented in Y-axis as unit/mg protein and the study groups were mentioned in X-axis. # P value compared between Group A and Group B is 0.0005 (< 0.05), ** P value compared between Group B and Group C is 0.0005(<0.05). P value < 0.05 is significant](image1)

![Fig. 2. Effect of ODQ on Beam walking ability. A- shows traversal time, # P value compared between Group A and Group B is 0.0005(< 0.05), ** P value compared between Group B and Group C is 0.0005(<0.05). B- shows number of slips, # P value compared between Group A and Group B is 0.0005 (<0.05), ** P value compared between Group B and Group C is 0.007 (< 0.05). P value < 0.05 is significant](image2)
DISCUSSION

PD pathology includes mostly pronounced antioxidant role. The antioxidant enzyme role in dopaminergic neurons of basal ganglia is to compensate continuous generation of free radicals. MPTP toxicity alters status of antioxidant enzymes by damaging mitochondria16. So MPTP toxicity may be measured by estimating the antioxidant enzyme levels. In our study efficacy of ODQ was evaluated by estimating the SOD enzyme and motor behavioral changes against MPTP treated mouse model of PD. Sub-acute protocol of MPTP used for induction of PD in mice of this study showed reduction in TH positive neurons. SOD levels were found to be reduced in MPTP treated mice of study group C than control group A.}

Fig. 3. Effect of ODQ on Sensory motor ability-Adhesive removal test. A- shows removal time in seconds on Y-axis, # P value compared with Group A is 0.0005(< 0.05), ** P value compared between Group B and Group C is 0.0005(< 0.05). B- shows number of attempts, # P value compared between Group A and Group B is 0.0005 (< 0.05), ** P value compared between Group B and Group C is 0.005 (< 0.05). P value < 0.05 is significant.

Fig. 4. TH positive neurons revealed by immunohisto-chemical technique and evaluated at a magnification of x10 under light microscopy. a, b, c corresponds to the study groups A, B, C respectively. The cells counted manually in ten random fields were expressed as Mean ± Standard Error of Mean.
model\textsuperscript{17, 18}. In our study MPTP decreased SOD levels in mice brain. There are studies which reported decreased levels of SOD enzyme in Parkinson’s disease patients\textsuperscript{19}. Excitotoxicity due to free radicals may be inhibited by administering superoxide dismutase\textsuperscript{20}. Glutamate antagonist was shown to decrease post synaptic membrane excitation mediated by glutamate receptor coupled calcium channels of neurocytes. This was found to be beneficial in patients of comatose state\textsuperscript{21, 22}. Another study stated that intense brain irritation by glutamate like compound may be alleviated by glutamate antagonists ensuring neuroprotection\textsuperscript{2}. In our study, ODQ enhanced superoxide dismutase activity compared to MPTP treated mice. Hypoactivity in mice within half an hour of MPTP treatment and lasting up to 40 wks post treatment was reported in many studies\textsuperscript{23, 24}. Onset of hypoactivity was same in our study. Behavioral assessment was done by beam walk test and adhesive removal test to evaluate motor impairments. Beam walking test was initially used for evaluating sensory motor abnormalities in stroke, Huntington’s disease and PD patients\textsuperscript{25-27}. This test also provides evaluation for fine motor initiation, coordination and postural balance of individual animal. MPTP toxicity was reported to increase the traversal time and increase the number of slips (in coordination) in beam walk test. Our study results showing mobility time was consistent with other studies\textsuperscript{28}. ODQ showed beneficial effects by increasing the mobility time and decreased the number of slips. Adhesive removal test confirms sensorimotor abilities of the animal\textsuperscript{29}. Increased adhesive removal time was reported in PD models and this is also seen in our study\textsuperscript{25, 30}. ODQ reduced the adhesive removal time indicating the reversal of motor ability. Taking our study results in to consideration it may be stated that ODQ attenuate glutamate toxicity resulted by MPTP toxicity and improved SOD enzyme activity. This also may show the role of sGC - cGMP pathway in MPTP toxicity and in treatment of PD.

**CONCLUSION**

The results of our study show that MPTP up-regulated glutamate receptor and affected SOD activity in brain which might result in compromised antioxidant activity of mice. This may result in neurological insult that might cause neurodegenerative diseases. As MPTP toxicity is specific to corpus striatum and striatal dopaminergic neurons, it results in Parkinson’s disease. ODQ which is a specific soluble guanylate cyclase inhibitor attenuated MPTP toxicity and showed beneficial effects in this preclinical study.

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**REFERENCES**


