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Evaluation of Antiparkinson's activity of Ibuprofen and Monteleukast in 6 –Hydroxy Dopamine lesioned rat model

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A B S T R A C T

In our current study, we demonstrated the efficacy of Ibuprofen and Montelukast in order to alleviate or reduce neurodegeneration which cause neuronal death in Parkinson's disease. Many recent reports are showing that Ibuprofen and Montelukast can be used as a potential drug in Parkinson's disease, but these have not been properly worked out in preclinical testing. In our studies it was demonstrated that the complex I activity was significantly reduced for 6-OHDA control and in L-DOPA treated groups when compared to other treatment groups. It has been assumed that the ibuprofen and montelukast treatment may have some positive mechanism to normalize the complex I activity. Recent evidences suggested that the complex I abnormalities may induce apoptosis. Apoptosis refers to programmed cell death and was distinct from cell necrosis characteristically, morphologically and genetically. Morphological changes include chromatin condensation and the formation of chromatin bodies. Nuclear DNA undergoes extensive cleavage with the digestion of nucleosomes situated 180 base pairs of the part. The *MT-ND5* gene provides instructions for making a protein called NADH dehydrogenase 5. This protein is part of a large enzyme complex known as complex I, which is active in mitochondria; our mitochondrial ND5 gene sequencing studies have revealed that mutations might have taken place in 6-OHDA induced Parkinson's rat model and the results showed that the mutations might have been controlled with ibuprofen and montelukast treatment. The overall pharmacological evaluation explains a positive role of Ibuprofen and Montelukast in reducing neurodegeneration in 6-OHDA rat models.

Keywords: Parkinson's disease; neurodegeneration; complex I; Ibuprofen; Montelukast

A R T I C L E I N F O

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1. Introduction

The effective treatment for Parkinson's disease is still a mystery even though the advent and recent developments of levodopa therapy most of the PD patients experienced an intolerable drug related side effects, and continued disease progression, associated with development of clinical features that could not be controlled by levodopa therapy. Even though levodopa is considered to be a gold standard drug for the treatment of Parkinsonism but during its metabolism it converts to dopamine quinones (amidoquinones and paraquinones)[1] in the neurons and these are highly reactive species and produces neurodegeneration and neuronal death. Despite the advent of drugs like dopaminergic agonists, dopamine facilitator, virtually all patients developed an unacceptable level of disability and mortality rates.[2] The application of new techniques in neurobiotechnology, genetics, neurochemistry and molecular biology in animal models has not proved to be clinically satisfiable and efficient. More over the clinical trials with the above mentioned techniques are unacceptable and unapproachable.[3] with these evidences, we tried and tested on already existing, marketed and clinically acceptable drugs like ibuprofen[10] and montelukast for anti - parkinsonian activity. [4]

2. Materials and Methods

Animals:

Healthy, adult Wistar rats of both sexes (180-220 gm) were obtained from the Central animal house facility from J.S.S College of Pharmacy, Ootacamund, Tamilnadu. The animals were kept in a well ventilated room and the animals had exposed to 12 hrs day and night cycle with a temperature between 20 ± 3^0C. The animals were housed in large spacious, hygienic polypropylene cages during the course of the experimental period. The animals were fed with water and rat feed *ad libitum*. All the experiments were performed after obtaining prior approval from CPCSEA and IAEC.[5] *Approval No.*: JSSCP/ IAEC/ M. PHARM/ PH. COLOGY/ 07/ 2010-11.

Chemicals

The chemicals which were used for the present study were procured from Sd-Fine Chemicals Mumbai, Sigma Aldrich USA, Loba chemie Mumbai, Merk chemicals Mumbai. The pure drug Nimodipine and Amlodipine was gifted by Amanath Pharmaceuticals, Pondicherry-605502.

Grouping of animals[6]

Animals were divided into six groups of 3 male and 3 female rats in each group.

Group I: sham operated

Group II: 6-OHDA Control

Group III: 6-OHDA +L-DOPA (6mg/kg orally)

Group IV: 6-OHDA+ Ibuprofen (60mg/kg orally), Group V: 6-OHDA+ montelukast (30mg/kg orally).

Induction of Parkinsonism by 6-OHDA[7,8]

On the zero day desipramine (Sigma, St. Louis, MO) (25 mg/kg, IP) was administered 30 min before surgery, to protect noradrenaline containing terminals from the effects of 6-OHDA (Sigma). All animals were anaesthetised with ketamine (100mg/kg ip), xylaxine (15mg/Kg im) and then placed in a steotling stereotaxic apparatus (USA) (incisor bar: - 3.3 mm). The scalp was retracted and unilateral holes were drilled in the skull above the injection site. The needle of a 10µl Hamilton syringe (Bonaduz, Switzerland) was lowered to the appropriate coordinate [tooth bar: ±0.0mm; anterior/ posterior: -4.8 mm; medial/lateral: -2.2 mm; ventral/dorsal: -7.2 mm] were determined from bregma. Injection of 6-OHDA (20 µg of 6-OHDA hydrobromide in 4µl 0.9% saline with 0.02 µg/ml ascorbic acid) was then made over 5 min and the needle was left in place for a further 5 min. 6-OHDA lesions were made in an identical manner except that vehicle alone was injected. The scalp was then sutured; all efforts were made to minimize the animal pain and suffering. Three weeks after the lesion, the animals' ability to rotate in response to apomorphine (0.5 mg/kg, s.c.) was tested. Contralateral rotations induced by apomorphine were measured 2 times at weekly intervals. Only animals showing at least 7 turns/ min in both tests were included in this study. After the induction of Parkinsonism (6week) the animals were treated with L-DOPA, ibuprofen and montelukast. According to the groups of the animals at 09.00 hours up to 60 days.

Parameters Evaluated:

The following parameters were evaluated, after the 60th day of treatment.

Isolation of mitochondria:

Tissue was homogenized with a Dounce tissue grinder in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris HCl, 1 mM EDTA; pH 7.4) and suspensions were centrifuged at 800 g, 4°C, for 10 min. The supernatant fluids were centrifuged at 13000 g, 4°C, for 10 min, and the pellets were washed with mitochondrial isolation buffer and centrifuged at 13000 g, 4°C, for 10 min to obtain the crude mitochondrial fraction.[9,10]

Complex I activity assay: NADH:

ubiquinone oxidoreducase (Complex I) activity was measured in the SN as described in the literature. Brain mitochondria, isolated as above, were lysed by freeze–thawing in hypotonic buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.4). The reaction was initiated by the addition of 50 µg mitochondria 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 (BSA)]. The oxidation of NADH by Complex I was monitored spectrophotometrically at 340 nm for 2 min at 30°C. The activity was monitored for a further 2 min following the addition of rotenone (2µg/ml). The difference

between the rate of oxidation before and after the addition of rotenone was used to calculate Complex I activity.[11.12]

Localization of iron in substantia nigra:

(Perl's diaminobenzidene (Perl's-DAB) method:

Brain tissues were stained for ferric ion using the perl's-DAB method. The isolated and partially frozen brains were cut horizontally to get 30-40 µm sections on a vibratome and then mounted on a glass slide. The sections were immersed in 2% potassium ferrocyanide and 2% hydrochloric acid for thirty minutes at room temperature and then rinsed with deionized water for five minutes. The perl's reaction was intensified by placing the tissue in 0.5% diaminobenzidine (DAB) in cold phosphate buffer (pH 7.4) for fifteen minutes. Next, 2ml of 1% hydrogen peroxide was added for every 200 ml of DAB solution. The sections remained in the solution for twenty five minutes. Following DAB treatment the sections were rinsed in deionized water for fifteen minutes. [13,14]

$$\text{Iron asymmetry ratio} = \frac{\text{Ipsilateral densitometry value}}{\text{Contralateral densitometry value}}$$

Bleomycin Assay:

Wistar rats were sacrificed by excess anaesthesia and brain samples were obtained. Homogenates of brain samples were prepared. Mid brain section (0.5 g) was diced, added to ice-cold PBS solution (40ml) and homogenized with an Omni 5000 homogenizer over ice for 5 min. The homogenate was centrifuged (3000 rpm- 5 min) and the supernatant was separated and stored at -80 ^{0}C for iron determinations. The reaction mixture contained 0.5 ml of calf thymus DNA (1 mg/ml), 0.05 ml of bleomycin sulphate (1 mg/ml), 0.1 ml of MgCl2 (50 mM), 0.1 ml of sample or iron standard, 0.05 ml of HCl (10 mM), 0.1 ml of Chelex-treated pyrogen-free water and 0.1 ml of ascorbic acid solution. Sample blanks were identical except that bleomycin was omitted.[15,16] $FeCl_3$ (0, 3.125, 6.25, 12.5, 25, and 50 nmol per 0.1 ml) in Chelex-treated pyrogen-free water. Each standard also had a corresponding blank that was identical except that bleomycin was omitted. The amounts of bleomycin-detectable iron in test samples were calculated from the standard curve obtained in each experiment.

Isolation of Subcellular Midbrain Fractions:

Mitochondria were isolated from pooled midbrain of individual rats according to the method previously described (Liang and Patel, 2006). The midbrain was homogenized with a Dounce tissue grinder in mitochondrial isolation buffer (70 mm sucrose, 210 mm mannitol, 5 mm Tris HCl, 1 mm EDTA; pH 7.4. with the exception of isolation buffer for measurement of chelatable iron which did not contain EDTA.) The suspensions centrifuged at 800 × g 4°C for 10 min. The supernatants were centrifuged at 13,000 × g 4°C for 10 min, pellets washed with mitochondrial isolation buffer and centrifuged at 13,000 × g 4°C for 10 min to obtain mitochondrial fractions. The purity of mitochondrial fractions has been confined by complex-II assay.[17,18]

Sequencing of the MT-ND5 Gene in Rat Brain Mitochondrial DNA

Isolation of Mitochondrial DNA:

Total DNA was extracted from rat brain tissue using the **XIT™ Mitochondrial DNA** Kit (***G-Biosciences, St Louis, MO. USA)***. The presence of mtDNA was verified by electrophoresis on 0.8% agarose gels containing 0.5 mg/ml ethidium bromide. Bands corresponding to full-length mtDNA were excised and extracted.[19]

Statistical analysis

The collected data were subjected to appropriate statistical tests like one-way ANOVA (Analysis of Variance) followed by Dunnets multiple comparisions test. P values of less than 0.05 were considered significant. The analysis was carried using Graphpad Instat software of version 3

3. Results and Discussions

Ibuprofen and montelukast are biologically important medicinal compounds having diverse pharmacological activities. Owing to the importance of these compounds, attempts were made to evaluate them for anti-Parkinson's activity.[20]

Effect of Ibuprofen, Montelukast on complex I activity in rats: The complex I activity was estimated from mitochondrial fractions isolated from brain tissue homogenate. When compared with vehicle control animals the mitochondrial activity was significantly reduced for 6-OHDA, levodopa, Ibuprofen, Montelukas. When compared with 6-OHDA group, complex I activity was not significant with the value of ($P<0.01$), for levodopa treated group but significant with ibuprofen and montelukasst treated groups.[21]

Effect of Ibuprofen and montelukast on Cytosolic Iron Concentration in Brain Tissue Homogenates:

The cytosolic iron concentrations were estimated from brain tissue homogenates, When compared with vehicle control animals the cytosolic iron concentration was significantly increased for 6-OHDA, levodopa treated groups but not significant with Ibuprofen and Montelukast treated groups. When compared with 6-OHDA group, cytosolic iron concentration was not significant with the value of ($P<0.01$), for levodopa treated group but significant with ibuprofen and montelukast treated groups.[22]

Effect of Ibuprofen and Montelukast on Brain iron evaluation (Perl's DAB iron asymmetry) in rats

The mean Perl's DAB substantia nigra iron asymmetry ratio for the treatment and vehicle control groups were compared with population mean of 1.00 using one way ANOVA followed by Bonferroni. The vehicle control animals showed an iron asymmetry ratio of (1.083 ± 0.0078). The 6-OHDA animals showed an iron asymmetry ratio of (1.819 ± 0.02067). The levodopa treated animals showed an iron asymmetry ratio of (1.628 ± 0.080405). Ibuprofen treated animals showed an iron asymmetry ratio of (1.300 ± 0.08602). The Montelukast treated animals showed an iron asymmetry ratio of (1.438 ± 0.04082). [23]

Sequencing of the MT-Nd5 Gene (Codes for Complex-I) In Rat Brain Mitochondrial DNA.

The sequencing of mitochondrial ND5 Gene was performed using polymerase chain reaction. The fluorescent bands were observed which are taken from 1% Agarose gel. The ladder value is from 12000 BP TO 14000 BP. there was no band observed in the region for 6-OHDA treated group

which indicates that the MT-DNA is drastically mutated in that region. Significant bands were observed for sham control, Ibuprofen treated, and Montelukast treated groups which indicate the improvement of MT-DNA.

Table 1: Effect of Ibuprofen and Montelukast on mitochondrial Complex I activity in brain homogenate of treatment groups

S.No	Treatment Group	Complex I activity (nmol/min/mg) protein
1	Vehicle Control	90.888±4.123***
2	6-OHDA control	60.60±1.140
3	6-OHDA+L-DOPA	70.40±1.140^{NS}
4	6-OHDA+ibuprofen	81.00±1.581**
5	6-OHDA+montelukast	75.00±1.581**

Values are mean ± SEM; n=6 in each group. ***P<0.001, **P<0.01 when compared with 6-OHDA control; One-way ANOVA followed by Dunnets multiple comparision test.

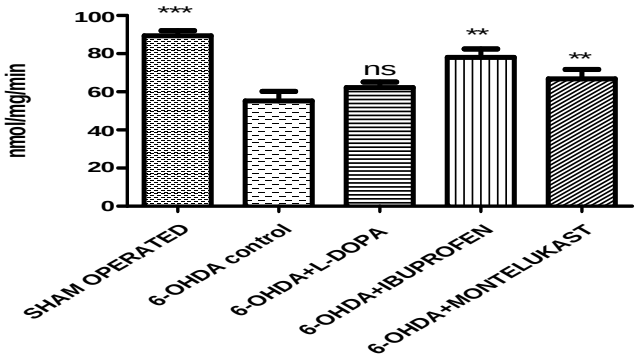

Figure 1: Effect of Ibuprofen and Montelukast on mitochondrial Complex I activity in brain homogenate of treatment groups

Table 2: Effect of Ibuprofen and montelukast On Cytosolic Iron Concentration in Brain Tissue Homogenates

S.No	Group	Concentration ng/mg tissue
1	Vehicle control	14.75±0.553**
2	6-OHDA Control	23.19±0.584
3	6-OHDA+L-DOPA	19.58±0.432^{NS}
4	6-OHDA+Ibuprofen	15.50±0.550**
5	6-OHDA+Montelukast	17.56±0.588*

Values are mean ± SEM; n=6 in each group. ***P<0.0001, **P<0.001, *P<0.01 when compared with 6-OHDA control; One-way ANOVA followed by Dunnets multiple comparision test.

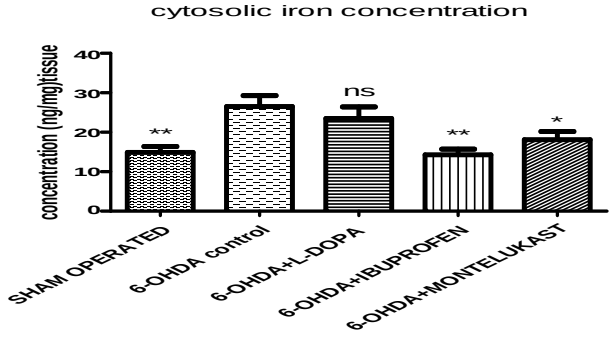

Figure 2: Effect of Ibuprofen and montelukast on Cytosolic Iron Concentration in Brain Tissue Homogenates

Table 3: Effect of L-DOPA, Ibuprofen, Montelukast on brain iron asymmetry ratio

S.No	Treatment group	Brain iron asymmetry ratio
1	Vehicle control	1.083±0.007829***
2	6-OHDA control	1.819±0.02067
3	6-OHDA+L-DOPA	1.628±0.08040^{ns}
4	6-OHDA+Ibuprofen	1.300±0.08602**
5	6-OHDA+montelukast	1.438±0.04082**

Values are mean ± SEM; n=6 in each group. ***P<0.0001, **P<0.001, *P<0.01 when compared with 6-OHDA control; One-way ANOVA followed by Dunnets multiple comparision test.

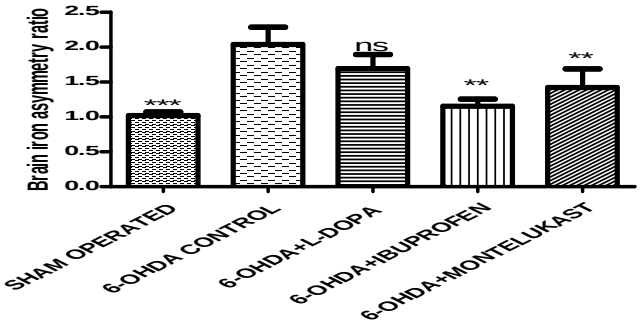

Figure 3: Effect of L-DOPA, Ibuprofen, Montelukast on brain iron asymmetry ratio

Table 4: Effect of Fenoprofen on Cytosolic Unbound Iron in Brain Tissue Homogenate

SNO	Group	Concentration ng/mg tissue
1	Control	14.15±0.2306***
2	6-OHDA Control	23.03±0.4817
3	6-OHDA+L-DOPA	22.25±0.2734 ns
4	6-OHDA+Fenoprofen	14.77±0.3676***

Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with 6-OHDA control; One-way ANOVA followed by Dunnets multiple comparision test.

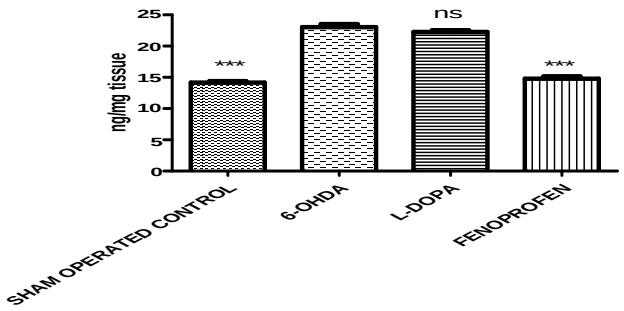

Figure 4: Effect of Ibuprofen on Cytosolic Unbound Iron in Brain Tissue Homogenate

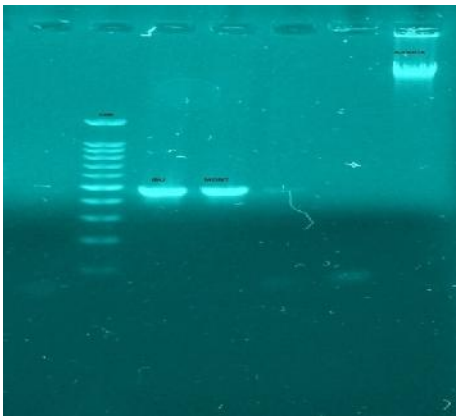

Figure 5: Sequencing of the MT-Nd5 Gene (Codes for Complex-I) In Rat Brain Mitochondrial DNA.

4. Conclusion

The Ibuprofen and Montelukast were tested for anti-Parkinson's activity in 6-OHDA rat model. Both showed reliable neuroprotection with respect to iron degeneration, complex-I activity and mtND5 gene mutations. The investigations revealed that test drugs could be future molecules for treating clinical PD. Reliability or clinical acceptability of these molecules for treating PD needs further toxicity and pharmacological investigation. Hence we suggest that, these molecules have to be further investigated to make it a clinically acceptable drug for Parkinson's disease in man.

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