

Evaluation of neuroprotective effect of flupirtine in brain of albino rats

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ABSTRACT

Background: Flupirtine (FP) is found to antagonize both glutamate and N methyl, D aspartate (NMDA) and the current study was undertaken to elucidate a possible neuroprotective role of flupirtine against NMDA induced neurotoxicity in experimental rat model.

Methods: Excitotoxicity was produced in rat and it is counteracted by flupirtine. The animals were grouped as Group 1 (vehicle treated), Group 2 (received NMDA+vehicle), Group 3 (received FP+NMDA only), and Group 4 (received FP+vehicle) and were observed of animal behavior and oxidative stress biomarkers and also mRNA expression using reverse transcriptase-polymerase chain reaction (PCR PCR) was performed to determine the level of mRNA expression of acetyl cholinesterase (AChE) and muscarinic cholinergic receptor (MChR) in brain samples (hippocampus) of experimental animals.

Results: Depression effect induced by NMDA was reversed by flupirtine. Decrease in oxidative stress bio-markers associated with increase in the antioxidant enzyme activities in group 3 and 4 compared to group 1 and 2. Gene expression were up-regulated in group 2 compared to 1, 3 and 4. Flupirtine treatment reversed these alterations.

Conclusions: This study represents the neuroprotective characteristics of flupirtine against the excitotoxicity induced by NMDA in an in vivo rat models.

Keywords: Flupirtine, NMDA, Malondialdehyde, Glutathione, mRNA, AChE, MChR

INTRODUCTION

The major excitatory neurotransmitter in brain is glutamate. It acts on different classes of receptors, G-protein-coupled metabotropic receptors and ionotropic glutaminergic receptors. The ionotropic receptors are further classified into N-methyl-D-aspartate (NMDA), kainate, and α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors.¹ Glutamate binds to NMDA receptors, which results in Ca^{2+} entry from extracellular space into the cell. This leads to activation of Ca^{2+} -dependent enzymes like calpain,² calcineurin,³ inducible nitric oxide synthase (iNOS) expression⁴ and cytosolic phospholipase A₂ (cPLA₂).^{5,6} In frontal cortex and hippocampal CA₁ region NMDA receptors (NMDAR) are present predominantly.⁷ Activation of NMDAR also induces signalling cascades involved in behaviour and memory, synaptic excitability and plasticity, and neuronal degeneration.⁸ Excessive activation of glutamate receptors can result in the death

of neurons through and excitotoxicity. Excitotoxicity has been implicated in several neurodegenerative diseases, like Alzheimer disease,⁹⁻¹¹ Huntington disease,¹² schizophrenia,¹³ and bipolar disorder.¹⁴⁻¹⁶ In in vivo animal models NMDA antagonists can reduce sustained seizures¹⁷ and neuronal injury resulting from experimental hypoxia-ischemia.⁸⁻²¹ Development of additional potential neuroprotective compounds like flupirtine would be help full in NMDA induced excitotoxicity and neuro degenerative disorders.

Flupirtine is a centrally acting non-opioid analgesic; it has been in clinical practice since 1984. In pain states have been reviewed extensively.²² Flupirtine exhibits potent cyto and neuro protective potential effects, in a clinically relevant dosage range. In our present study, the effect of flupirtine on chronic administration of NMDA to rats, as a model of excitotoxicity in brain. To the extent that this model represents clinical effect of flupirtine

which might be used in NMDA induced behavioural and neurodegenerative disorders.

METHODS

Flupirtine, NMDA, normal saline, experimental animals and the facility for harbouring the animals and the conduct of the experiments. Sprague dawley rats (170-200 g) were used in Center For Toxicology (CEFT), Sri Ramachandra university, Chennai, India and they were housed individually at $22 \pm 1^\circ\text{C}$ in cages with a 12 hours light dark cycle. All rats were allowed free access to the diet and water for 1 week for adaptation to the new environment. Rats were fed with standard laboratory pellets. The experimental protocol was approved by the Institutional Ethics Committee (IAEC) and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals. The animals were randomly assigned to 4 groups (n = 6 each) as group 1 (vehicle treated), group 2 (received NMDA + vehicle), group 3 (received FP + NMDA only), and group 4 (received FP + vehicle). Excitotoxicity was produced in rat by intra peritoneal administration of NMDA (25 mg/kg body weight for 21 days) and it is counteracted by intra peritoneal administration of flupirtine (5 mg/body weight for 21 days).

Behavioural analysis

Anxiety and depression - elevated plus maze:

Elevated plus maze is representative of those tests that are based upon the study of spontaneous behaviour pattern. Behavioural alteration was tested in control and treatment groups using elevated plus maze²⁵ with slight modification. Parameters such as time spent in open arm and closed arm, number of entries to open arm and closed arm and number of head dips were recorded and analysed.

Anxiety, depression and exploratory behavior - open field exploratory test:

The test provides a unique opportunity to systematically assess novel environment exploration, general locomotor activity, and provide an initial screen for anxiety-related behaviour in rodents. Animals were allowed to freely explore the chamber for the duration of the test session. Each square crossed by the animal was scored. In addition to horizontal units of activity, rearing behaviour, defecation, and grooming activity were scored and analysed.

Sacrifice of the animals and subsequent analysis

At the end of the experiment, the rats were fasted overnight, subjected to anaesthesia with diethyl ether and sacrificed. The whole brain of each rat was rapidly dissected, washed with isotonic saline and dried on filter paper. Each brain was divided sagittally into two

portions. The first portion was weighed and homogenized in ice-cold medium containing 50 mM Tris/HCl and 300 mM sucrose at pH 7.4 to give a 10% (w/v) homogenate.²⁶ This homogenate was centrifuged at $1400 \times g$ for 10 min at 4°C . The supernatant was stored at -80°C and used for biochemical analyses that included oxidative stress biomarkers (SOD and MDA), antioxidant status (GSH), and gene expression in rat brain, the second portion of the brain was fixed in 10% formalin for histological investigation.

Biochemical analysis

Determination of superoxide dismutase activity:

Superoxide dismutase (SOD) activity in 10% brain homogenate was assayed spectrophotometrically.²⁷ One unit of SOD activity was calculated as the amount of protein required to give 50% inhibition of phenazonium methosulphate-nitro blue tetrazolium (PMS-NBT) auto oxidation. The activity of SOD was expressed in unit/min/mg of protein.

Determination of lipid peroxidation (TBARS):

Lipid peroxidation was assessed as thiobarbituric acid-reactive substance concentrations in 10% tissue homogenate. Values are expressed in mg/dl homogenate.²⁸

Estimation of reduced glutathione:

Reduced glutathione (GSH) was estimated based on the reaction of GSH with dithiobistritro benzoic acid (DTNB). The absorbance was read at 412 nm. The values were expressed in μg of glutathione/ml homogenate.²⁹

mRNA expression of AChE and MChR

Reverse transcriptase (RT) - polymerase chain reaction (PCR) was performed to determine the level of mRNA expression of AChE and MChR in brain sample of experimental animals. Briefly, 50 mg of tissue was homogenized with 500 μl TRIzol and the tubes were incubated for 10 min and centrifuged at 1000 rpm for 5 min. 200 μl of chloroform was added to the supernatant, allowed to incubate for 5min at room temperature and centrifuged at 12000 rcf for 20 min. Then 500 μl of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12000 rcf for 15min following the incubation period of 10 min. The supernatant was decanted carefully; the pellet was washed three times with 75% ethanol, centrifuged at 12000 rcf for 15 min and the pellet was allowed to air dry. The pellet was resuspended in 20 μl of RNase free water and stored in -80°C until use. The isolated RNA was allowed to undergo reverse transcription and polymerization reaction to get cDNA using PCR master cyclor gradient. PCR products were electrophoresed at 80V for 30 min using 1.0% agarose gel with ethidium

bromide stain. 200 nanograms of RNA was used for reverse transcription polymerase chain reaction (RT-PCR) according to the manufacturer's instructions (Genet Bio, Korea). The following sequence was performed for each PCR reaction: 42°C for 30s, 94°C for 5 min (1 cycle); 94°C for 1 min, β -actin (55.4°C), AChE (60.6°C) and MChR (59.4°C) for 1 min, and 72°C for 1 min (with 35 cycles); and a final extension phase at 74°C for 10 min.³⁰

Histopathology analysis

All the surviving rats at the end of the experimental period were euthanized using overdose of anaesthetic ether. Brains were collected from rats of all experimental groups and fixed in 10% neutral buffered formalin. Representative tissue section of brain at the level of hippocampus region were trimmed and dehydrated in series of graded alcohol and embedded in paraffin wax. Tissue sections of 3-4 micron thickness were obtained, mounted on a clean glass slides and stained with haematoxylin and eosin (H&E) for microscopic evaluation.³¹

Statistical analysis

Data were expressed as mean (X) \pm standard error of mean (SEM). The results were analysed for statistical significance by one-way analysis of variance (ANOVA) test using prism 5.0.

RESULTS

Behavioural Analysis

In elevated plus maze method reduced entries into open arm, time spent on the open arms, was seen in the group 2 compared to the group 1 while entries into close arm, time spent on the close arms, head dip and number of fecal pellets were increased ($p < 0.05$) (Table 2). In comparison to the group 2, in the group 3 and 4, there were significant reduced entries into close arm, time spent on the close arm and entries into open arm, time spent on the open arms increased ($p < 0.05$). But head dip and number of fecal pellets were no reduced group 3, 4 compare to group 2 there was no significant.

In open field method a reduced in no. of crosses on squares was seen in the group 2 (NMDA induced) compared to the group 1 while immobility period (sec), rearing, grooming, fecal pellets were increased, ($p < 0.05$) (Table 1). In comparison to the group 2, in the group 3 and 4, there were significant reduced in immobility period (sec), rearing, grooming, fecal pellets and No. of squares crosses increased ($p < 0.05$).

Bio chemical analysis

Among the parameters evaluated in the brain tissue extracted from rats, a decrease in SOD, glutathione level

was seen in the group 2 compared to the group 1 while MDA levels were increased ($p < 0.05$) (Figure 1, 2, 3). In comparison to the group 2, in the group 3, 4 there were significant decreases in MDA while SOD and glutathione had increased ($p < 0.05$).

Gene (mRNA) expression of AChE and MChR

AChE and MChR gene (mRNA) expression were found to be up-regulated in group 2 when compared to group 1, 3 and 4. Flupritine treatment reversed these alterations (Figure 4 & Table 3).

Histopathology analysis

Histological profile in group 1 revealed normal architecture of neurons in the CA regions of hippocampus. In group 2 no treatment related changes were observed in the CA regions of hippocampus. Group 3 and group 4 remains to be similar and no treatment related changes have been noticed in the tissue sections of the CA region of hippocampus (Figure 5).

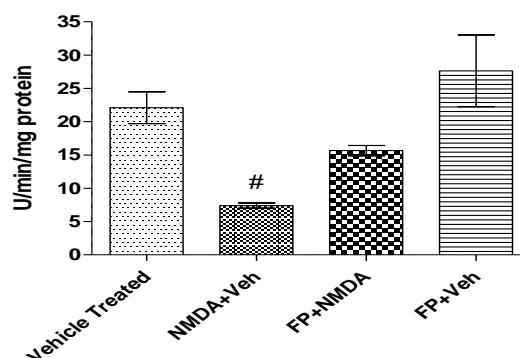


Figure 1: Effect of flupritine on SOD activity in NMDA induced rats.

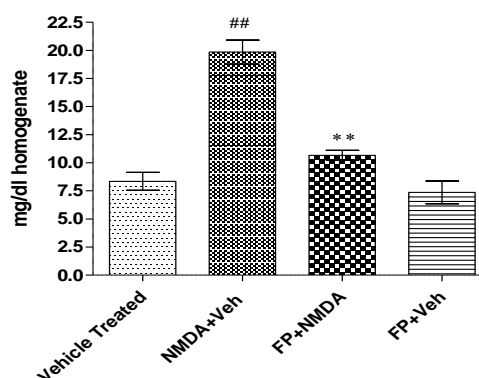


Figure 2: Effect of flupritine on lipid peroxidation in NMDA induced rats.

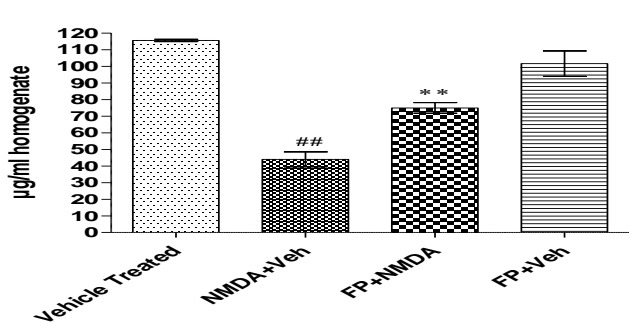


Figure 3: Effect of flupritine on glutathione content in NMDA induced rats.

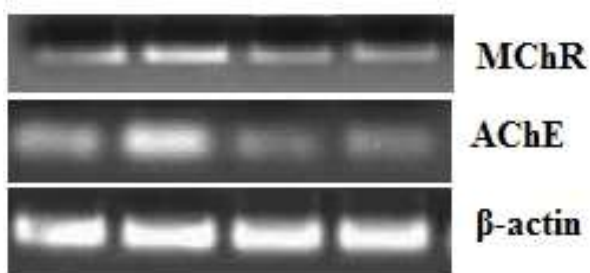


Figure 4: Lane 1: Normal control; Lane 2: NMDA + Vehicle; Lane 3: NMDA + FP and Lane 4: Vehicle + FP.

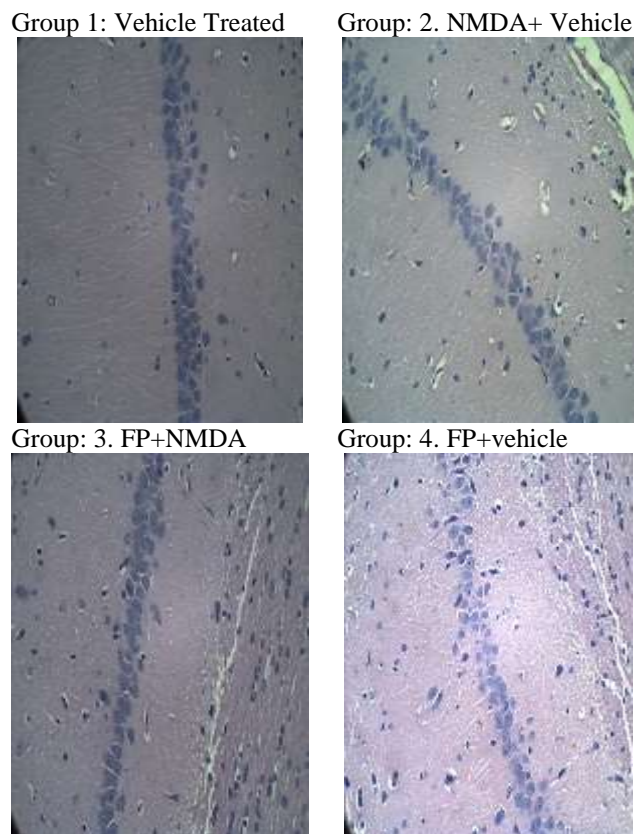


Figure 5: Histo pathological Changes.

Table 1: Anxiety, depression and exploratory behaviour - open field exploratory test.

Treatment	No.of squares crosses	Immobility period (Sec)	Rearing	Grooming	Fecal pellet
Vehicle treated	79.67±10.26	14.33±5.03	6.67±1.23	2.83±0.48	1.00±0.26
NMDA+Veh	50.00±10.43	135.17±25.27	16.67±3.42	7.50±2.55	2.00±0.26
FP+NMDA	73.60±14.33	78.20±17.36	12.00±2.72	4.00±0.87	0.80±0.34
FP+Vehicle	56.67±5.71	59.50±17.83	6.33±0.56	5.17±0.48	1.17±0.17

Table 2: Anxiety and depression - elevated plus maze.

Treatment	No.of Entries		Time Spent		No of head dips	Fecal pellets
	Open arm	Closed arm	Open arm	Closed arm		
Vehicle treated	11.67±1.31	7.50±0.76	344.50±8.40	132.67±8.99	3.67±0.49	1.50±0.34
NMDA+Veh	6.67±0.42	21.50±1.12	101.83±6.72	379.83±4.31	3.33±0.56	3.17±0.48
FP+NMDA	7.00±0.32	24.20±1.86	190.80±23.78	256.40±18.16	2.60±0.37	3.00±0.29
FP+Vehicle	9.33±0.80	15.67±1.41	219.50±31.82	236.83±34.62	3.33±0.80	3.00±0.58

Table 3: Gene expression.

S. No	Gene Marker	Forward primer	Reverse primer
1	β-actin	5'-GACATGGAGAAAATCTGGCA-3'	5'-AATGTCACGCACGATTTCCC-3'
2	AChE	5'GCAGAACTTCACTGACCAAAA3'	5'TCAAAGGAGGGGACTCATA-3'
3	MChR	5'-CTGTACCGTTCATGTGTACTGT-3'	5'-CCGGGCTCGTTTTTCTGT-3'

DISCUSSION

NMDA is an excitatory neuro transmitter. In addition to their physiological functions, mounting evidence has suggested their involvement in excitotoxicity. Cell death triggered by seizure or ischemic stroke is attributed to NMDAR over activation.³²⁻³³ Abnormal NMDAR activity is also associated with neurodegenerative disorders, such as Alzheimer's, Huntington's, and Parkinson's disease.³⁴ In vitro and in vivo studies have suggested that flupirtine antagonizes the neurotoxicity caused by the prion agent PrPSc and lead acetate [Pb(C₂H₃O₂)₂•3H₂O] mediated by NMDA receptors.³⁵ Flupirtine significantly inhibits the neurotoxic effect caused by amyloid β-protein segments in Alzheimer's disease³⁶ and other neurological disorders, such as amyotrophic lateral sclerosis.³⁷ Studies using animal models have also revealed that flupirtine counteracts the effects of retinal and cerebral ischemia.³⁸⁻³⁹ In another study, the long-term flupirtine treatment of chronic pain prevents retinal ganglion cells from degeneration in a non-inflammatory animal model of optic nerve transmission. This result indicated that this drug is a potential candidate and should be further evaluated in terms of its neuroprotective potential.⁴⁰ Different molecular mechanisms may also account for flupirtine-mediated neuroprotection. Our previous study indicated flupirtine has neuroprotective effect of on U373 MG cells, which limits its use in the pain management of brain tumors.⁴¹ This property warrants further studies using animal models and large - scale clinical trials.

This study represents the neuroprotective characteristics of flupirtine against to NMDA induced excitotoxicity in an in vivo system. The findings of this study is discussed here.

Behavioral analysis

In elevated plus maze test, the reduction in number of entries and the time spent in open arm and increased number of entries and the time spent in closed arm in NMDA treated animals are reversed in FP treated animals. In open field exploratory test the effects are like reduced no of square cross and increased immobility time, increased rearing and grooming activity with increased no of fecal pellets in NMDA treated animals. These effects are reversed in FP treated animals. These behavioural changes are significant to the antidepressant and antianxiety effect of FP.

Biochemical analysis

The excitotoxic neurotransmitter, NMDA signals in behaviour, memory, synaptic excitability and plasticity.⁸ Excessive activation of the receptor results in neuronal degeneration. NMDA treated animals showed decrease in SOD, GSH levels and elevated levels in MDA. These changes are the markers in excitotoxicity and confirm the effects of NMDA. These effects are counteracted by FP

in the animals treated with FP as per the changes observed in above biochemical parameters. Thus, NMDAR activation is essential for neuronal survival and physiological functions; excessive activation contributes to pathological changes including cell death.⁴²

mRNA expression of AChE and MChR

The up regulation of AChE and MchR in NMDA treated animals is reversed in FP treated animals showing significant interaction between NMDA and FP.

Histopathology analysis

In histopathological view of hippocampus regions of brain showed no treatment related changes because it is supposed that the doses may not be sufficient to elicit histopathological changes in neurons. This gives way for further exploration to study the effect of the drugs in higher doses.

CONCLUSION

Flupirtine is a centrally acting non-opioid analgesic and also flupirtine exhibits potent cyto and neuro protective potential effects which is exhibited as the effect of FP on chronic administration of NMDA to rats, as a model of excitotoxicity in brain. This drug is a potential candidate and should be further evaluated in terms of its neuroprotective potential. These evidence based studies proved neurodegenerative effect of NMDA and neuroprotective effect of FP in addition to its antidepressant and antianxiety effects.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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