Original Article

Flupirtine's anticonvulsant role +/- celecoxib versus diazepam on induced generalized seizures and status epilepticus in mice

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ABSTRACT

Epilepsy represents a personal and major community burden because about one-third of epileptic patients remain resistant to medical treatment. Flupirtine, as a potassium channel opener, can be a rational template for the treatment of epilepsy. To investigate flupirtine +/- celecoxib versus diazepam anticonvulsant role on induced epilepsy in mice. Generalized seizures and status epilepticus were induced by pentylenetetrazole and lithium/pilocarpine, respectively. The latency period to 1st seizure was calculated. A modified racine's scale was used to characterize behavioral and electrographic seizures. Brain GABA and IL-6 levels were measured. Cresyl violet stain and Glial fibrillary acidic protein (GFAP) immunostain were used to assess hippocampal irreversible neuronal damage and gliosis. Diazepam and flupirtine prolonged latency periods to 1st seizure, attenuated behavioral seizures, recorded lower grades of electrographic seizures, and guarded against a decline in brain GABA level. The three drugs used attenuated brain IL-6 level, chiefly in the treated groups of the celecoxib. Irreversible neuronal damage and increased GFAP immunoreactivity witnessed in the hippocampus of both seizure models were markedly mitigated, mostly in diazepam/celecoxib and combined therapy. Diazepam, flupirtine and celecoxib possess anticonvulsant, immuno-modulatory and neuroprotective effects. Diazepam is still superior in controlling seizures and EEG changes, while celecoxib is the least effective. Flupirtine showed almost the same results as diazepam in biochemical parameters in addition to better histopathological results, indicating that it is a promising drug that could contribute to the control of seizures.

Keywords: Flupirtine, Epilepsy, Racine's scale, GABA, IL-6

Introduction

Epilepsy is a neurological disorder characterized by abnormal CNSelectrical activity and recurrent seizures**.** Epilepsy affects 65 million people worldwide, mainly neonates and geriatrics , thus, representing a personal and community major burden [1-3]. About one-third of patients remain resistant to treatment despite substantial increase in the available antiepileptic drugs [4].

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Ligand-gated and voltage-gated ion channels (VGICs) are vital players in epilepsy pathogenesis, providing potential targets for treatment of epilepsy [5].

Potassium channels are abundant in neuronal and glial cell membranes. These channels are currently among the most promising targets for anticonvulsant pharmacotherapy [6]. Flupirtine is a Selective Neuronal Potassium Channel Opener (SNEPCO). It is unique as a non-steroidal, non-opioid, and non-NSAID analgesic. Flupirtine has potent neuroprotective, cytoprotective, anticonvulsant, myorelaxant, and antiparkinsonian potentials. Its pharmacological profile includes numerous cellular targets, such as G protein-regulated inwardly rectifying K channels, Kv7 channels, and $GABA_A$ receptors. There is evidence of additional unidentified mechanisms included in the impacts of flupirtine. Flupirtine is suggested to shift voltage-dependent activation of KCNQ channels towards

hyperpolarized potentials causing an increased neuronal action potentials threshold [7].

CNS is not isolated from the immune system, and increasing evidence suggests it contributes to seizures and epilepsy. Inflammatory processes have been implicated in acute and chronic neurodegenerative conditions such as epilepsy [8]. Cell membrane excitability and synaptic plasticity in the hippocampus were found to be regulated by COX-2, suggesting its critical role in convulsive states. Besides, COX-2 inhibitors act as voltage-gated potassium channel openers that show antiepileptic properties. Moreover, chronic nature of epilepsy makes it liable to be associated with comorbidities requiring treatment with anti-inflammatory drugs. Hence, molecular mediators of inflammation may serve as targets for novel antiepileptic drugs development [9]. This study aimed to determine flupirtine's potential anticonvulsant effect and mechanism alone and combined with celecoxib versus diazepam in mice.

Materials and Methods

Animals

Adult male Swiss albino mice, matched for age and weight were utilized for the study. Mice were kept on standard rat chow with ad libitum access to tap water, maintained at a temperature of ~ 22 °C under a 12 h light/dark cycle and a fixed relative. The investigation is based on the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 2011).

Materials

Mouse GABA & IL-6 brain ELISA kits were purchased from Elabscience biotechnology Co. (Houston, TX, USA). Dimethyl sulfoxide (DMSO 10%) was purchased from Leader Co. (Cairo, Egypt). Diazepam powder was from Hoffmann-La Roche (Basel, Switzerland). Flupirtine powder was purchased from TEVA Pharmaceuticals (Slovakia, Bratislava, Slovak Republic). Celecoxib powder was from Amoun Pharmaceutical Co. (Cairo, Egypt). Lithium powder was bought from Nile Pharma Co. (Cairo, Egypt). Pentylentetrazol (PTZ) powder and Pilocarpine hydrochloride powder were bought from New Test Co. (Alexandria, Egypt).

Experimental design

Mice were randomly assigned into multiple groups of 6 each (n=6). The groups were repeated in the two models; generalized seizures and status epilepticus models. All groups received the corresponding drug/s 30 minutes before induction of convulsions. **Group 1 (control group)** received I.P. normal saline, **Group 2 (vehicle group)** received I.P. DMSO 10%, **Group 3 (diazepam treated group)** received diazepam (10 mg/kg i.p) [10], **Group 4 (flupirtine treated group)** received flupirtine i.p. at three different doses; 10 mg/kg, 25 mg/kg and 50 mg/kg. These doses were chosen according to previous studies [11-13]. The smallest effective dose in controlling behavioral seizures and showing promising biochemical and histopathological results was 2 5mg/kg. It was used in the combined group (7) and was chosen to represent group 4 in comparing the results among other study groups. **Group 5 (celecoxib treated group)** received celecoxib (20 mg/kg i.p.) [14], **Group 6 (combined diazepam/celecoxib treated group)** received combined diazepam (10 mg/kg i.p.) and celecoxib (20 mg/kg i.p.) and **Group 7 (combined flupirtine/celecoxib treated group)** received combined flupirtine (25 mg/kg i.p.) and celecoxib (20 mg/kg i.p.).

Induction of convulsions

Convulsive tests were carried out 30 min after injection of drugs by the two following methods: ***A- Pentylenetetrazole induced generalized seizures:*** PTZ (80 mg/kg i.p.) in normal saline was injected [15]. ***B- Pilocarpine induced status epilepticus:*** Pilocarpine (30 mg/kg i.p.) in normal saline was injected preceded by lithium (127 mg/kg, i.p.) 24 hours before pilocarpine to enhance its action [16]. For eluding side effects of peripheral cholinergic activation, 30 minutes before pilocarpine injection, mice were treated with atropine sulfate monohydrate (1 mg/kg i.p.). Mice in both models were monitored for 3o minutes for the appearance of convulsions.

Pharmacological studies

Latency period to first seizure: Latencies were calculated as the time between PTZ or pilocarpine injections to the onset of seizures [17].

Modified racine's scale [18] for i) characterization of behavioral seizures as follows: stage 0: nil response, stage 1: facial and ear twitching, stage 2: myoclonic jerks without rearing, stage 3: myoclonic jerks with rearing, stage 4: clonic-tonic seizures and turning over into a side position and, stage 5: generalized clonic-tonic seizures and turning over into a back position. ii) characterization of electrographic seizures by electroencephalography (EEG) guided by ADInstruments, (2012). Electrodes were placed under the scalp of the mice under diethyl ether anesthesia, one positive and the other negative on both sides of the skull, with the third reference electrode at the back of the head corresponding to the base of the skull. Shielded, low-weight, flexible cables connecting the electrodes to the input EEG dual bioamplifier (model: ML136, serial: BA1359) were attached to the mice. EEG was recorded on a single channel (channel 1: source channel) with a low-frequency filter (LFF) of 0.1 Hz and high-frequency filter (HFF) of 60 Hz, and a sampling rate of 1kHz [19]. EEG recording continued for 2 hours to record electric brain changes as follows: stage 0: Baseline, stage 1: High amplitude activity or slow waves, stage 2: Spikes, sharp waves, stage 3: Spikes or poly spikes, sharp waves and, stage 4: Spike bursts/spike and wave discharges (a spike burst is defined as a

cluster of high-amplitude and high-frequency spikes, each of which lasts a few seconds to several minutes). EEG was digitized and stored with the use of standard PC-based hardware (ADInstruments). Power lab 4/30 (model: ML866, serial: 430-0835) was used to illustrate the recording diagrams.

Biochemical measurements

Mice were humanely euthanized by decapitation under diethyl ether anesthesia, and their brains were isolated. The right hemisphere of each brain was homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 1000 ×g for 20 min at 4 °C, and the obtained supernatant was then frozen at −80 °C [21] for subsequent assays measuring GABA & IL-6 levels using specific ELISA kits.

Histopathological studies

The left hemisphere of each brain was stabled in 10% formalin and kept for histopathological researches. Tissue was sliced, routinely processed, and embedded in paraffin wax. Then 5 µm coronal sections were cut, mounted, and stained by a- Cresyl violet special stain to detect irreversible neuronal damage at the cellular level hippocampal areas where the pathological changes were greatest [22]. Nuclear pyknosis or loss of nuclear membrane integrity and apoptosis were considered markers for irreversible neuronal damage. The cells were seen at high magnification (200X & 400X). Cresyl violet scoring system was established as follows: 0= no damaged cells, 1= scattered damaged cells <10% of total neurons in the hippocampus, 2 = damaged cells 10% up to 50% of total neurons in the hippocampus and, 3 = damaged cells more than 50% of total neurons in the hippocampus. **b- Glial fibrillary acidic protein (GFAP) immunostain**__to assess gliosis in hippocampal areas where the greatest pathological changes [23]. GFAP is an intermediate filament protein specific for astrocytes. GFAP scoring system was established as follows: 0 = no gliosis, 1 = mild gliosis, 2 = moderate gliosis and, 3 = severe gliosis.

Statistical analysis

Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). They were summarized using mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons between groups were performed via analysis of variance (ANOVA) with multiple comparisons post hoc test [24]. Chi-square ((2) test was carried out to compare the categorical data. An exact test was utilized instead when the expected frequency was less than 5 [25]. P-values less than 0.05 were considered statistically significant.

Percentage of protection against convulsions [20]

$$\frac{\%\ of\ convulsive\ animals\ in\ control\ -\ \%\ of\ convulsive\ animals\ in\ the\ test}{\%\ of\ convulsive\ animals\ in\ the\ control} \quad (1)$$

Results and Discussion

Pentylenetetrazole-induced generalized seizures

Pharmacological studies

Latency period to 1st seizure

There was a significant increase in mean latency period to 1st seizure in diazepam treated group (3) compared to groups (1, 2, 4, 5, 7). There was also a significant increase in the flupirtine treated group with a dosage of 25 mg/kg i.p. (4) compared to groups (1, 2). A significant increase in the mean latency period was also detected in the combined diazepam/celecoxib group (6) compared to all other groups.

Modified racine's scale

1. Characterization of behavioral seizures **(Table 1)**

83.3% of diazepam treated group (3) scored stage 1 and 16.7% scored stage 2. 100% of flupirtine treated group (4) scored stage 2. 100% of combined diazepam/celecoxib group (6) scored stage 0. 66.7% of the combined flupirtine/celecoxib group (7) scored stage 1 and 33.3% scored stage 2.

2. Characterization of electrographic seizures by EEG **(Table 2 and Figure 1)**

66.7% of group 3 recorded stage 0 and 33.3% recorded stage 1. 83.3% of group 4 recorded stage 2 and 16.7% recorded stage 1. 83.3% of group 6 recorded stage 0 and 16.7% recorded stage 1. 66.7% of group 7 recorded stage 2 and 33.3% recorded stage 3.

Percentage of protection against convulsions

Group 3 showed 83.33% protection against convulsions. Group 6 showed 100% protection against convulsions. All other groups showed 0% protection against convulsions.

Biochemical studies

1. Brain GABA level

All treated groups showed a significant rise in brain GABA level compared to control & vehicle groups (1 &2). There was a significant rise in mean brain GABA content in flupirtine treated group (4) compared to celecoxib (5) and flupirtine/celecoxib (7) treated groups. A significant rise was also detected in the combined diazepam/celecoxib group (6) compared to groups (3, 5, 7) **(Figure 2)**.

2. Brain IL-6 level

All treated groups showed a significant reduction in brain IL-6 content compared to control (1) and vehicle (2) groups. There was a significant reduction in mean brain IL-6 content in celecoxib treated group (5) compared to group (3, 4, 6) and also in combined flupirtine/celecoxib group (7) compared to groups (3 & 6) **(Figure 3)**.

Histopathological studies

1. Cresyl violet staining score **(Figure 4)**

The microscopic examination of cresyl violet stained slides of the brain revealed moderate damage with up to 40% damaged neural cells in the hippocampal area (100% scored 2) in both control (1) and vehicle (2) groups. There was mild to moderate damage in the diazepam treated group (3). Only 10% of neural cells showed damage (66.7% scored 1 and 33.3% scored 2). In flupirtine treated group (4), 100% scored 1 with mild damage. As regarding celecoxib treated group (5), 66.7% of the group scored 1 with mild damage affecting less than 10% of the neurons, while 33.3% scored 2. While 100% of diazepam/celecoxib (6) and flupirtine/celecoxib (7) were treated, groups scored 1 (mild damage).

2. Glial fibrillary acidic protein immunostain scoring **(Figure 5)**

The microscopic examination of GFAP immunostained brain slides to detect gliosis in hippocampal areas revealed in the control group (1), 66.7% scored 2. In comparison, 33.3% scored 3. 100% of vehicle group (2) scored 2, and 100% of diazepam treated group (3) scored 1. In flupirtine treated group (4), 100% scored 0 (no gliosis). As regarding celecoxib treated group (5), 66.7% of the group scored 1 with mild gliosis, while 33.3% scored 2. In diazepam/celecoxib (6) and flupirtine/celecoxib (7) treated groups, 33.3% of both groups scored 0 (no gliosis).

Pilocarpine-induced status epilepticus

Pharmacological studies

Latency period to 1st seizure

There was a significant increase in mean latency period to 1st seizure in groups (3, 4 & 6) compared to control (1) and vehicle (2) groups. The increase in mean latency period to 1st seizure in diazepam treated group (3) was significant compared to flupirtine (4), celecoxib (5), and flupirtine/celecoxib (7) treated groups. The same was detected in the diazepam/celecoxib treated group (6) compared to all other groups.

Modified racine's scale

1. Characterization of behavioral seizures **(Table 1)**

83.3% of diazepam treated group (3) scored stage 0 and 16.7% scored stage 1. 50% of flupirtine treated group (4) scored stage 1, and the other 50% scored stage 2. 50% of celecoxib treated group (5) scored stage 1 and the other 50% scored stage 2. 100% of diazepam/celecoxib treated group (6) scored stage 0. 50% of flupirtine/celecoxib treated group 7 scored stage 1, and 50% scored stage 2.

2. Characterization of electrographic seizures by EEG **(Table 2 and Figure 1)**

66.7% of diazepam treated group (3) recorded stage 0 and 33.3% recorded stage 1. 83.3% of flupirtine treated group (4) recorded stage 2 and 16.7% recorded stage 1.50% of celecoxib treated group 5 recorded stage 2 and the other 50% recorded stage 3.100% of diazepam/celecoxib treated group (6) recorded stage 0. 83.3% of flupirtine/celecoxib treated group (7) recorded stage 2 and 16.7% recorded stage 1.

Percentage of protection against convulsions

Group 3 showed 83.3% protection against convulsions. Group 6 showed 100% protection against convulsions. All other groups showed 0% protection against convulsions.

Biochemical studies

1. Brain GABA level

All treated groups showed a significant rise in brain GABA level compared to control (1) & vehicle (2) groups. There was a significant enhancement in mean brain GABA content in the diazepam/celecoxib group (6) compared to flupirtine (4) and celecoxib (5) treated groups **(Figure 2)**.

2. Brain IL-6 level

All treated groups showed a significant reduction in brain IL-6 content compared to control (1) and vehicle (2) groups. There was a significant reduction in mean brain IL-6 content in groups treated with flupirtine (4), celecoxib (5) & flupirtine/celecoxib (7) compared to the diazepam treated group (3). This significant decrease was also seen in the flupirtine/celecoxib treated group (7) compared to diazepam treated groups (3 & 6) **(Figure 3)**.

Histopathological studies

1. Cresyl violet staining score **(Figure 4)**

The microscopic examination of cresyl violet stained brain slides revealed that 66.7% of the control group (1) and 100% of the vehicle group (2) scored 2 with damaged neural cells in the hippocampal area up to 50%. In diazepam treated group (3), there was mild to moderate damage only up to 25% of neural cells (66.7% scored 2 and 33.3% scored 1). In flupirtine treated group (4), 100% scored 1 with mild damage affecting <10% of hippocampal neurons. As regarding celecoxib (5) and flupirtine/celecoxib (7) treated groups, 66.7% scored 1 with mild damage affecting <10% of the neurons while 33.3% scored 2 with moderate damage. In diazepam/celecoxib treated group (6), 100% scored 1 (mild damage).

2. Glial fibrillary acidic protein immunostain scoring **(Figure 5)**

The microscopic examination of GFAP immunostained brain slides to detect gliosis in hippocampal areas revealed that 66.7% scored 2 while 33.3% scored 1 in control (1) and vehicle (2) groups. In diazepam treated group (3), 33.3% scored 0, 33.3% scored 2 and 33.3% scored 2. In flupirtine treated group (4), 33.3% scored 0 (no gliosis) while 66.7% scored 1 (mild gliosis). Regarding the celecoxib treated group (5), 66.7% of the group scored 1 with mild gliosis, and 33.3% scored 2, while in the diazepam/celecoxib treated group (6), 33.3% scored 0, and 66.7% scored 1. In the combined flupirtine/celecoxib treated group (7), 100% scored 1 (mild gliosis).

Table 1. Characterization of Behavioral Seizures of Different Groups according to Modified Racine's Scale

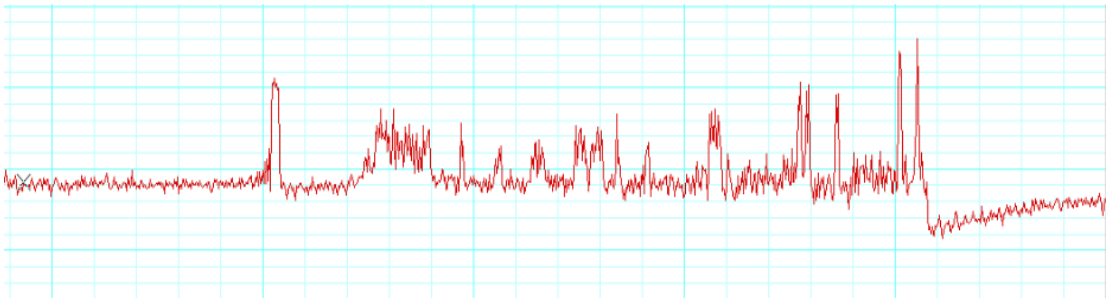
Behavioral seizures															
A-PTZ model															
Groups	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**P value**
Stages	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	
0	0	0	0	0	5	83.3	0	0	0	0	6	100	0	0%	< 0.001
1	0	0	0	0	1	16.7	0	0	0	0	0	0	4	66.7	
2	0	0	0	0	0	0	6	100	0	0	0	0	2	33.3	
3	0	0	1	16.7	0	0	0	0	1	16.7	0	0	0	0	
4	3	50	2	33.3	0	0	0	0	3	50	0	0	0	0	
5	3	50%	3	50%	0	0%	0	0%	2	33.3%	0	0%	0	0%	
B- Pilocarpine model															
0	0	0	0	0	5	83.3	0	0.0	0	0	6	100	0	0	
1	0	0	0	0	1	16.7	3	50	3	50	0	0	3	50	
2	2	33.3	2	33.3	0	0	3	50	3	50	0	0	3	50	
3	4	66.7	3	50	0	0	0	0	0	0	0	0	0	0	
4	0	0	1	16.7	0	0	0	0	0	0	0	0	0	0	
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Table 2. Characterization of Electrographic Seizures by Electroencephalography (EEG) of Different Groups according to Modified Racine's Scale

EEG															
A- PTZ model															
Groups	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**P value**
Stages	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	
0	0	0	0	0	4	66.7	0	0	0	0	5	83.3	0	0	< 0.001
1	0	0	0	0	2	33.3	1	16.7	0	0	1	16.7	0	0	
2	0	0	0	0	0	0	5	83.3	0	0	0	0	4	66.7	
3	1	16.7	1	16.7	0	0	0	0	3	50	0	0	2	33.3	

4	5	83.3	5	83.3	0	0	0	0	3	50	0	0	0	0	
						B- PTZ model									
0	0	0	0	0	4	66.7	0	0	0	0	6	100	0	0	
1	0	0	0	0	2	33.3	1	16.7	0	0	0	0	1	16.7	
2	0	0	0	0	0	0	5	83.3	3	50	0	0	5	83.3	< 0.001
3	1	16.7	0	0	0	0	0	0	3	50	0	0	0	0	
4	5	83.3	6	100	0	0	0	0	0	0	0	0	0	0	

A- PTZ model



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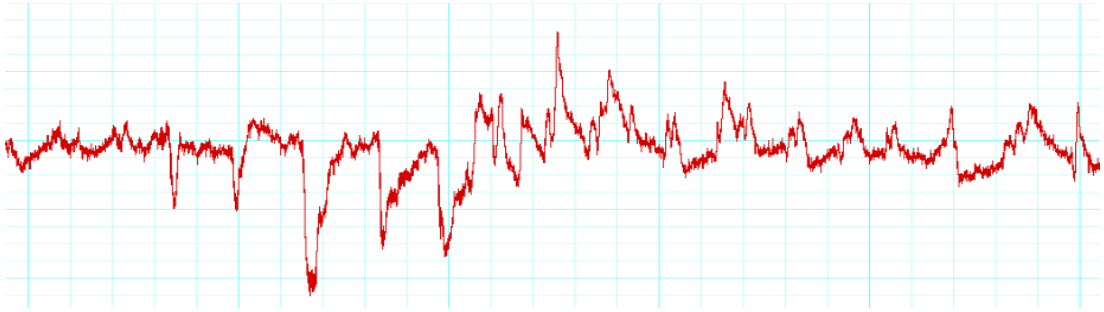

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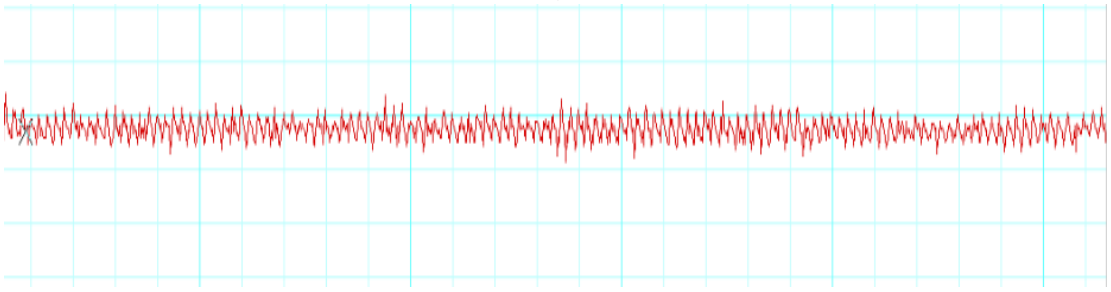

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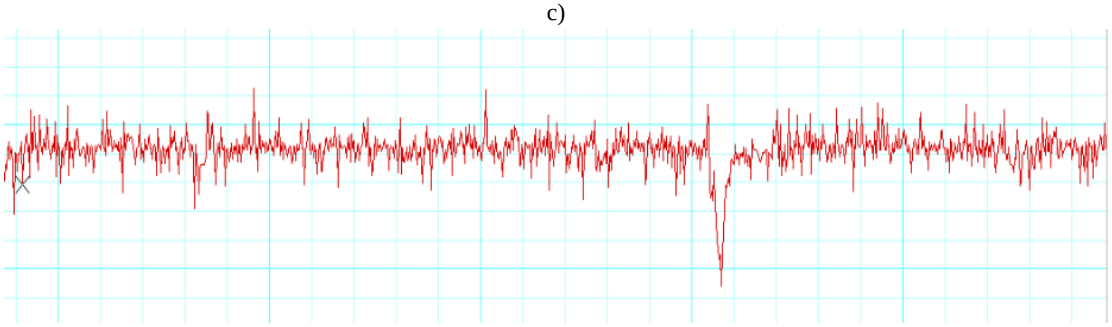

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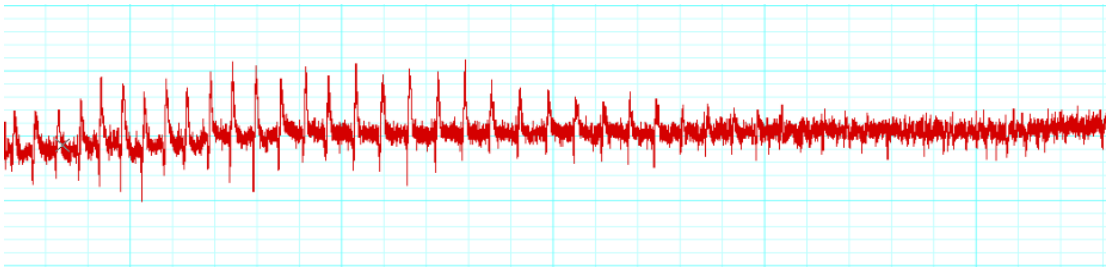

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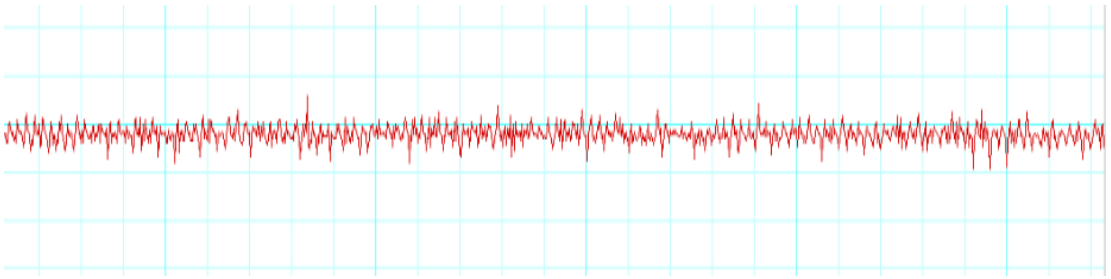

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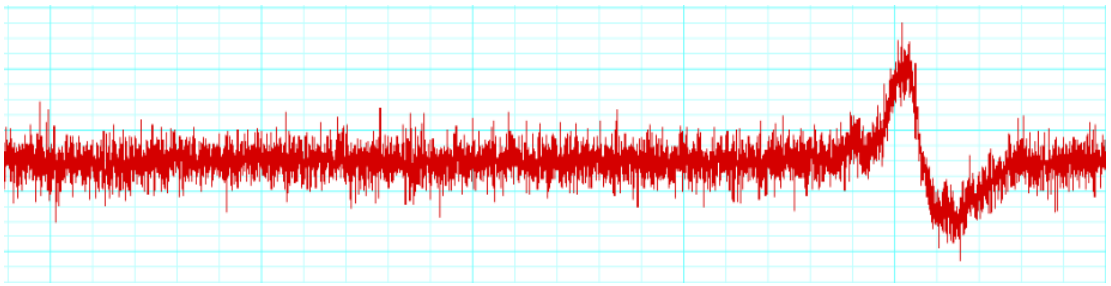
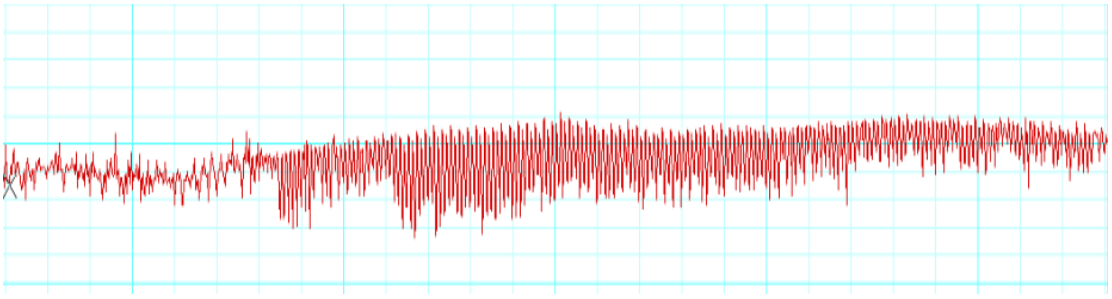


g)

B- pilocarpine model



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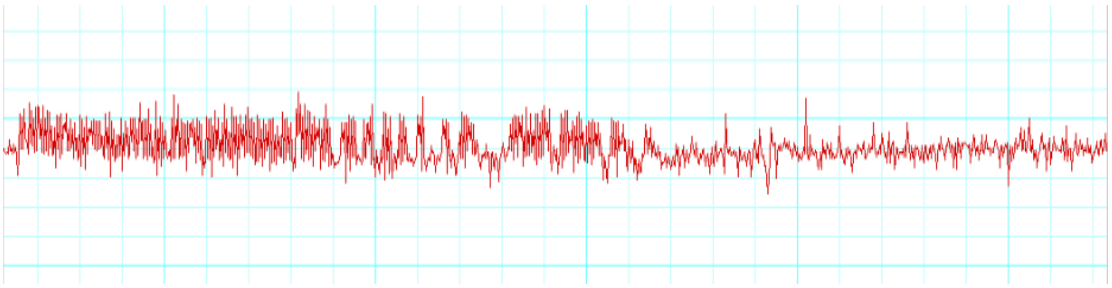

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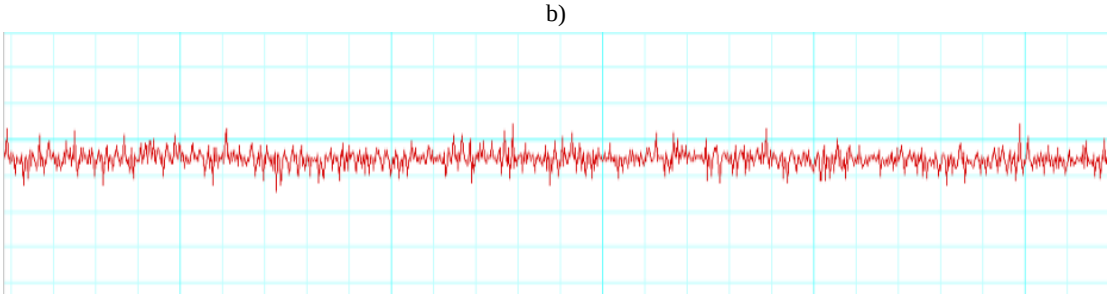

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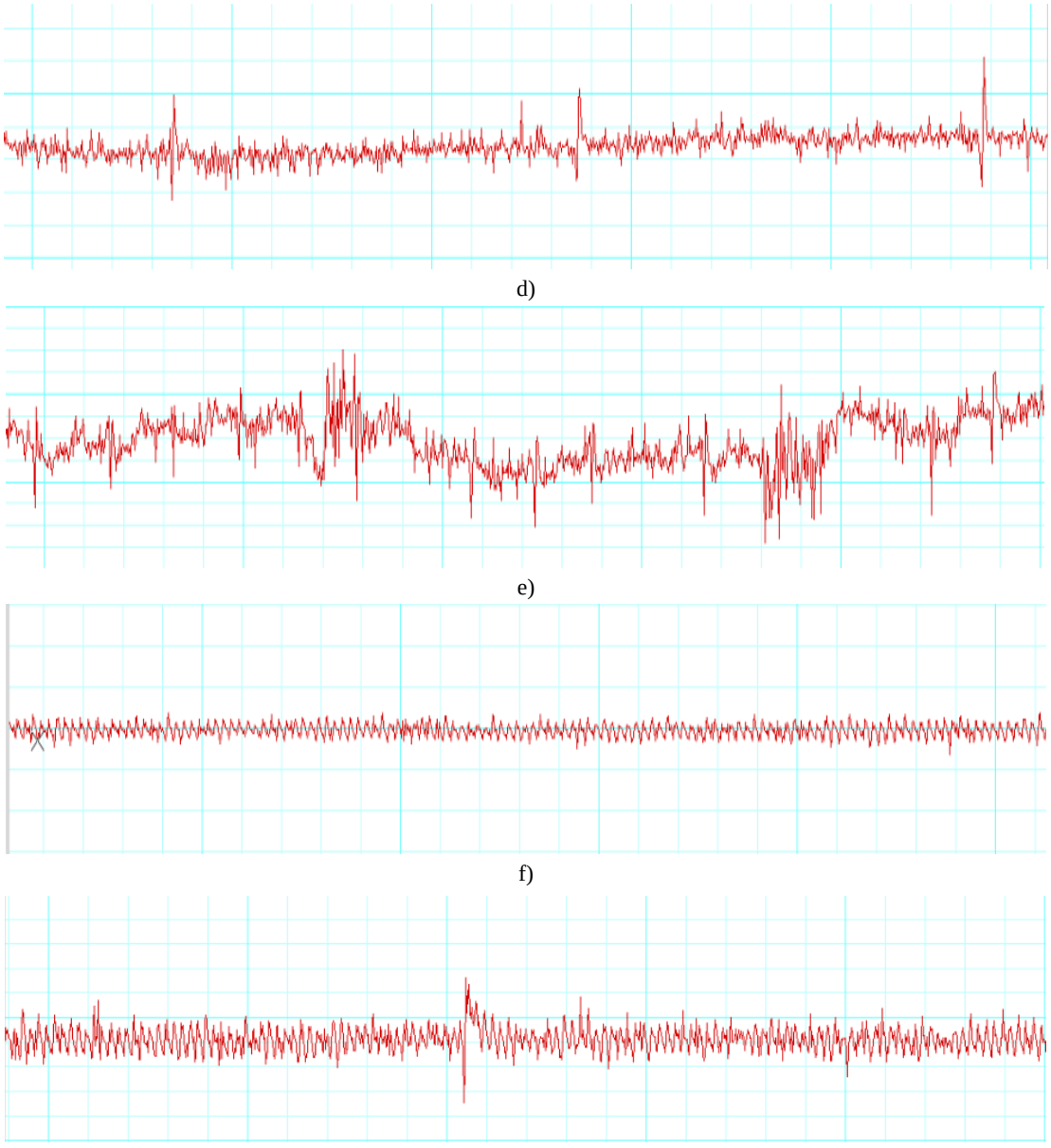


d)

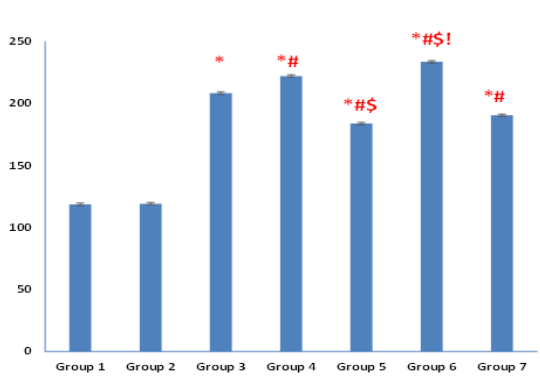
e)

f)

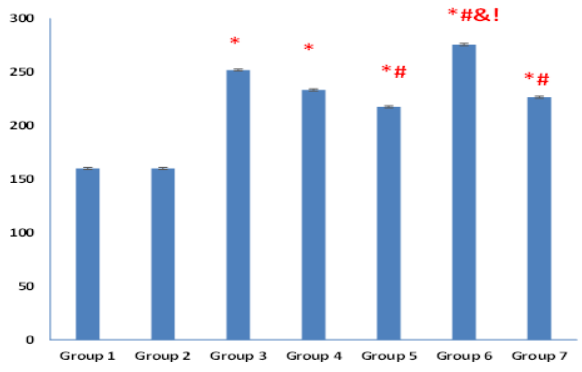
g)

Figure 1. Sample of EEG Recordings of Different Groups:
a) Stage 4 recorded in group 1, b) Stage 4 recorded in group 2, c) Stage 0 recorded in group 3, d) Stage 2 recorded in group 4, e) stage 4 in model A and Stage 3 in model B recorded in group 5, f) Stage 0 recorded in group 6, g) Stage 2 recorded in group 7

GABA (PG/GM TISSUE)



A-PTZ model

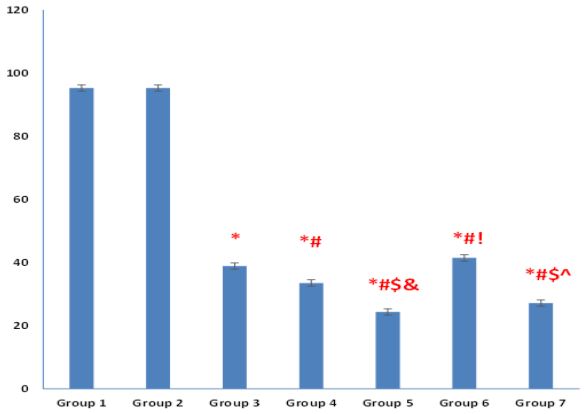


B-Pilo carpine model

Figure 2. Brain GABA Level (pg/gm tissue) of Different Groups (mean ± SEM, n=6)
* P<0.05 vs. control group

# P<0.05 vs. vehicle group
$P<0.05 vs. diazepam group
&P<0.05 vs. flupirtine group
! P<0.05 vs diazepam/celecoxib group
^ P<0.05 vs. flupirtine/celecoxib group

IL-6(PG/GM TISSUE)



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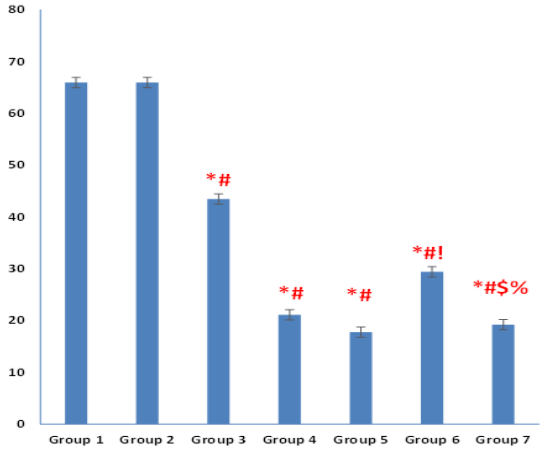
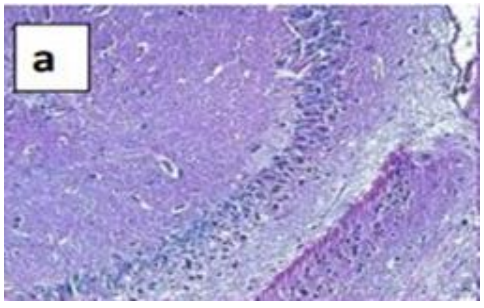


B-Pilocarpine model

Figure 3. Brain IL-6 Level (pg/gm tissue) of Different Groups (mean ± SEM, n=6)
* P<0.05 vs. control group
# P<0.05 vs. vehicle group
$ P<0.05 vs. diazepam group
& P<0.05 vs. flupirtine group
! P<0.05vs.diazepam/celecoxib group
^ P<0.05 vs. flupirtine/celecoxib group

A- PTZ model



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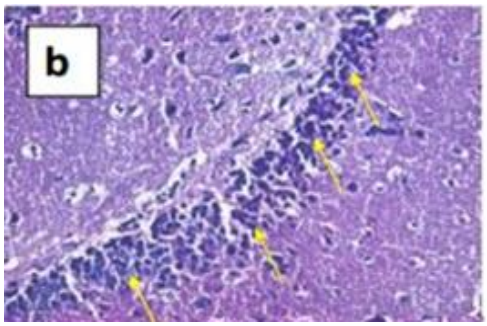

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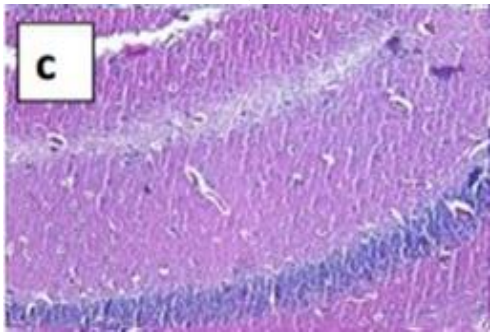

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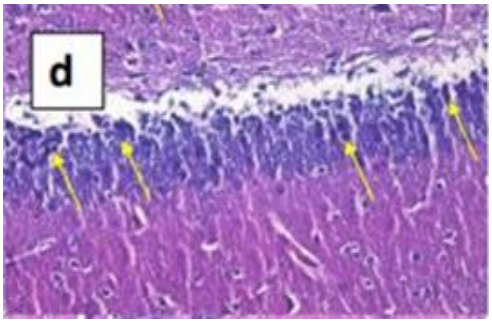

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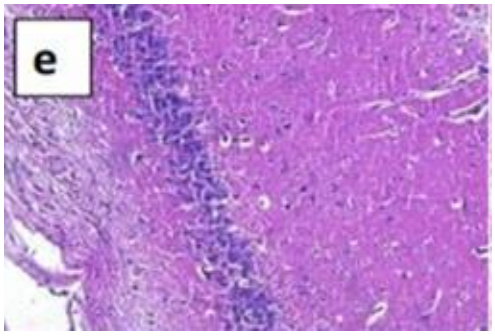

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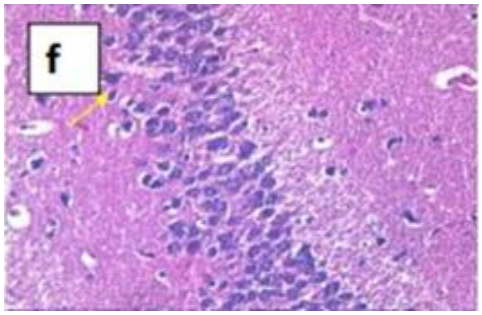

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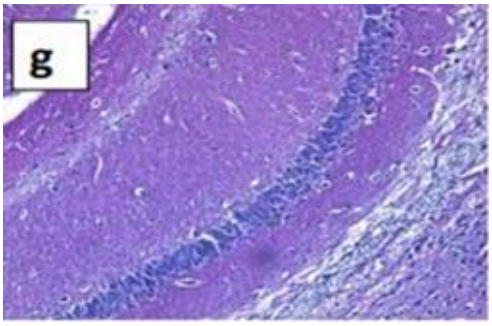

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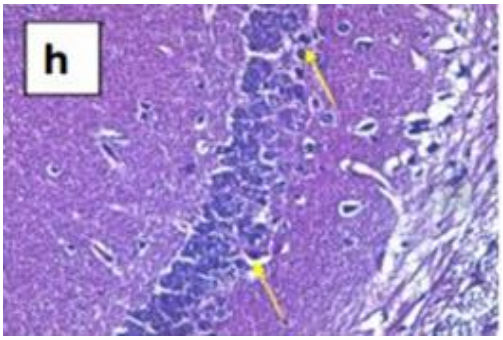

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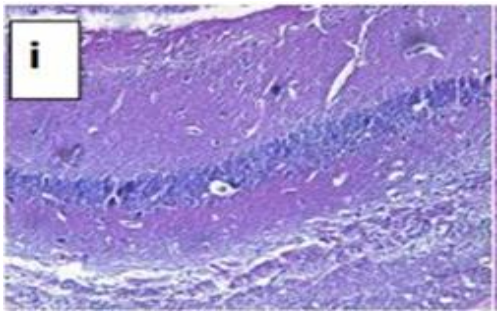

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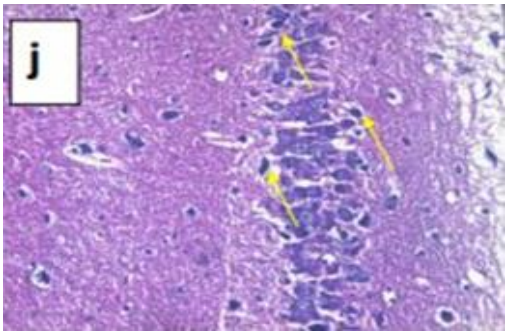

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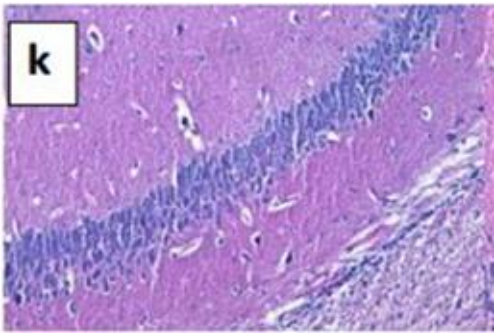

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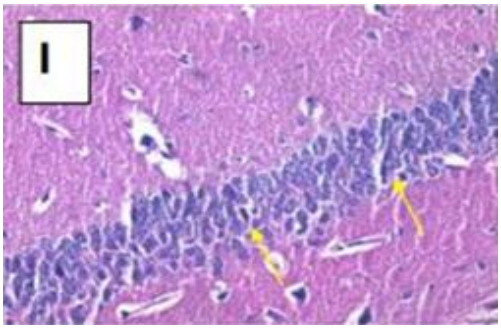

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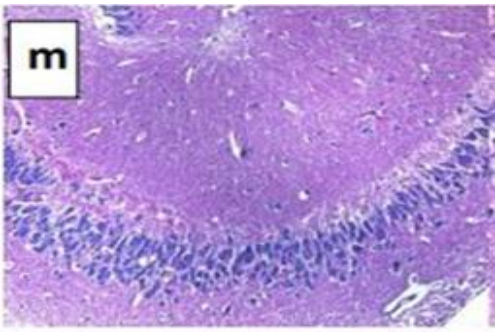

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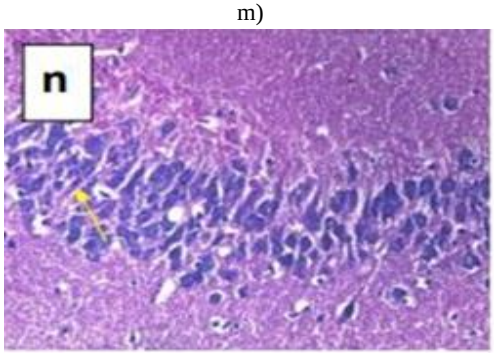

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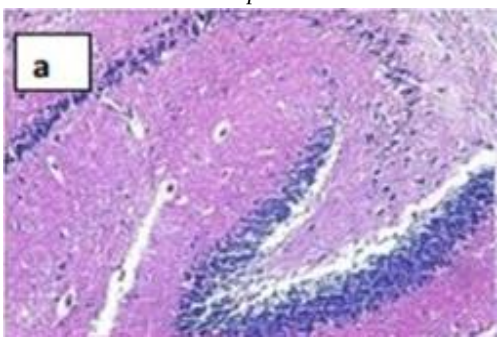
B- Pilocarpine model



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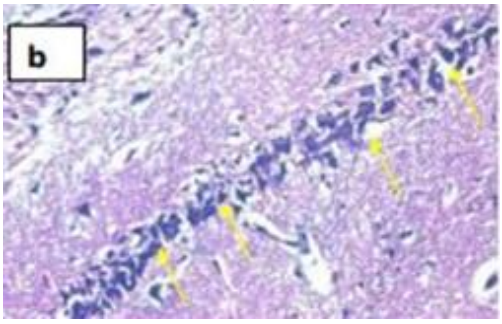

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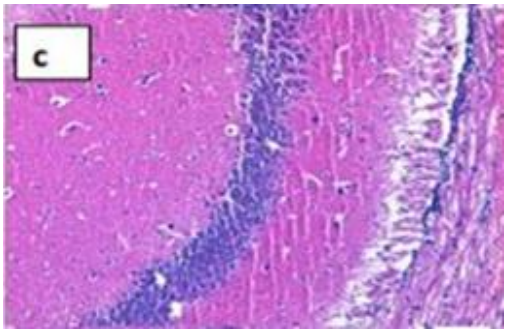

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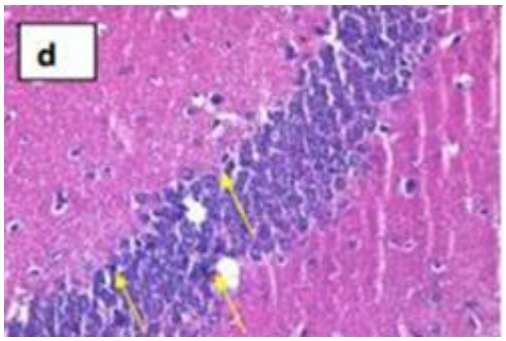

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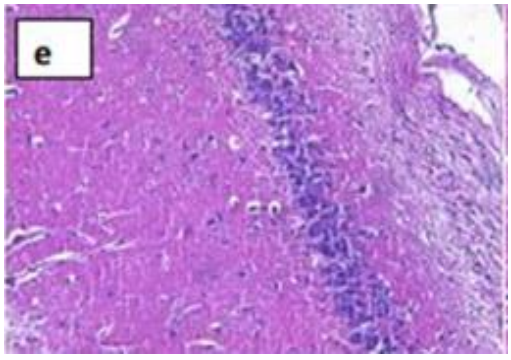

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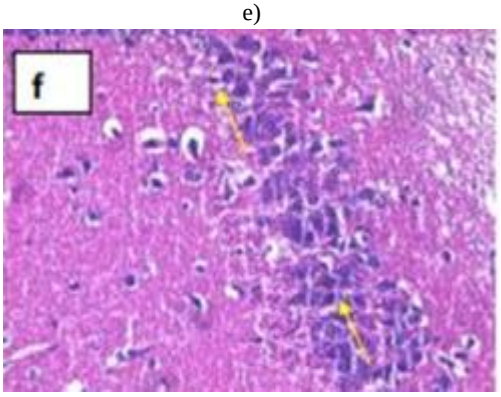

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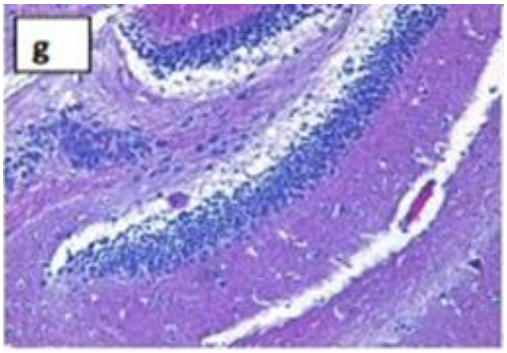

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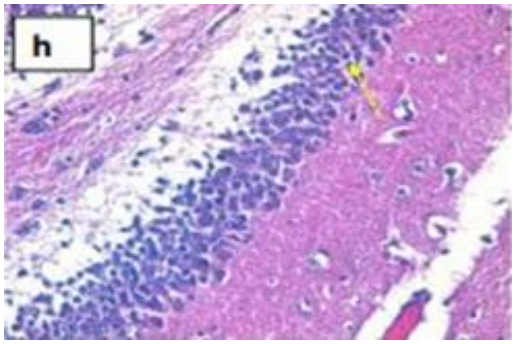

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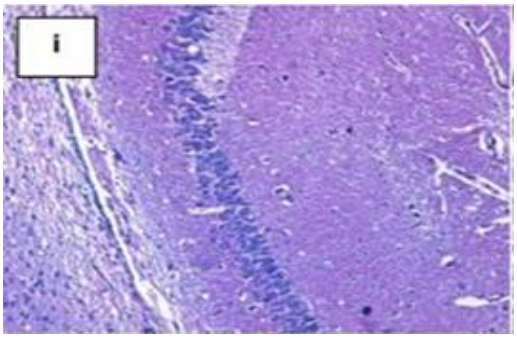

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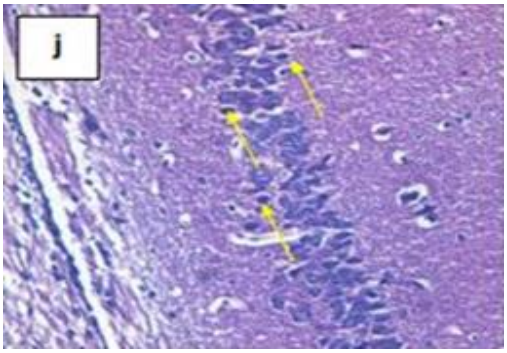

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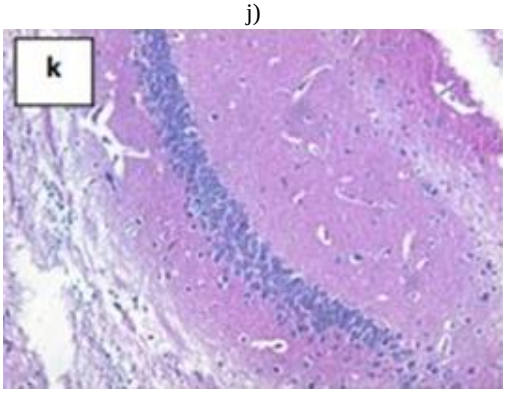

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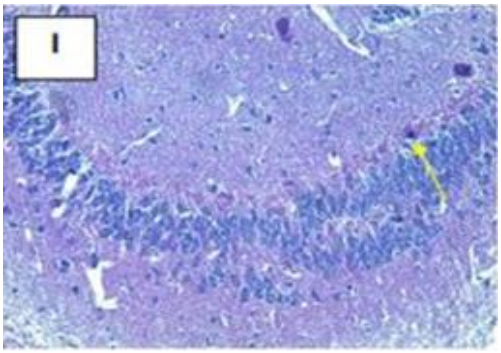

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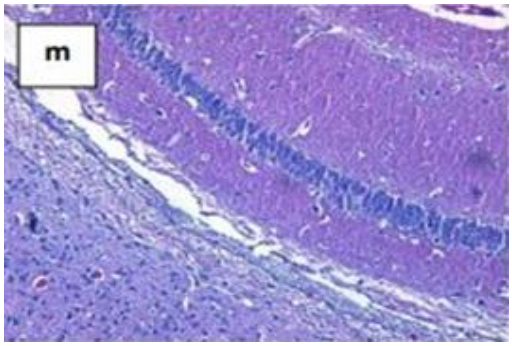

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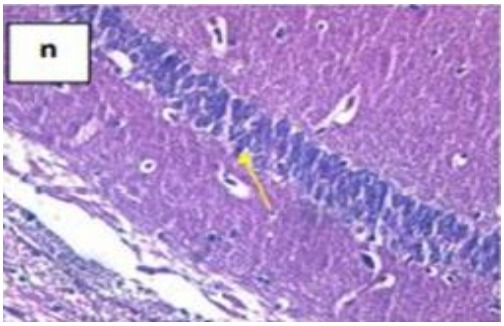
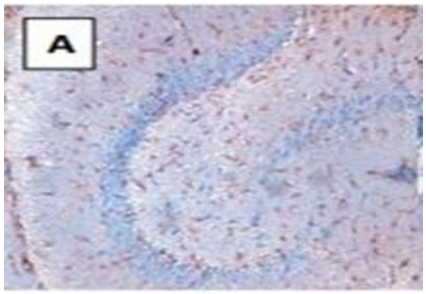


n)

Figure 4. Cresyl violet staining of brain (x200, x400) of: A- Pentylenetetrazole induced generalized seizures. **(a, b) Group 1** damaged cells 40%, score 2. **(c, d) Group** damaged cells 10%, score 2. **(e, f) Group 3** damaged cells <10%, score 1.**(g, h) Group 4** damaged cells <10%, score 1. **(I, j) Group 5** damaged cells 25%, score 2. **(k, l) Group 6** damaged cells < 10%, score 1. **(m, n) Group 7** damaged cells <10%, score 1.

B- Pilocarpine induced status epilepticus. **(a, b) Group 1** damaged cells 60%, score 3 **(c, d) Group 2** damaged cells 10%, score 2. **(e, f) Group 3** damaged cells <10%, score 1. **(g, h) Group 4** damaged cells <10%, score 1. **(I, j) Group 5** damaged cells 25%, score 2. **(k, l) Group 6** damaged cells < 10%, score 1. **(m, n) Group 7** damaged cells <10%, score 1.

A- PTZ model



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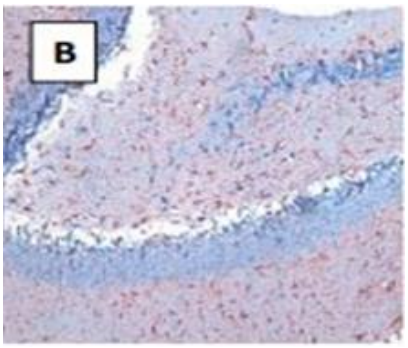

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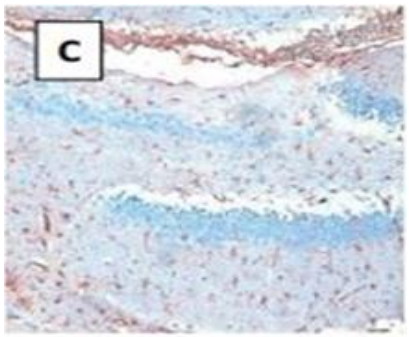

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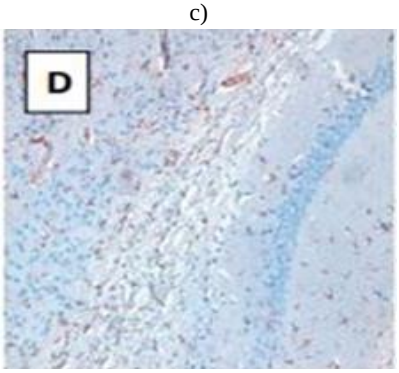

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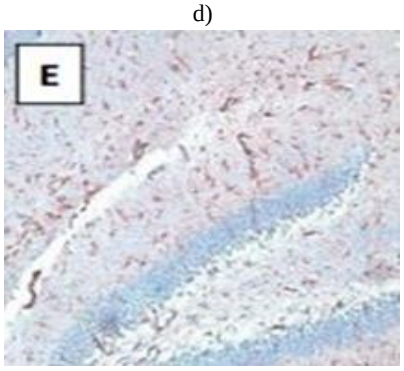

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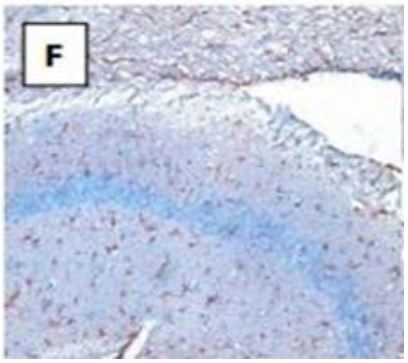

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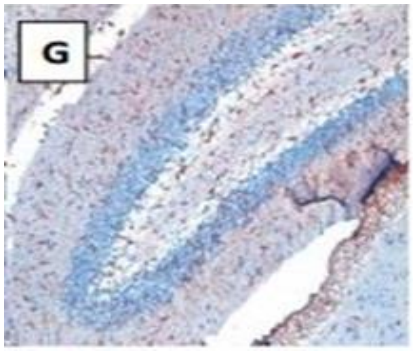
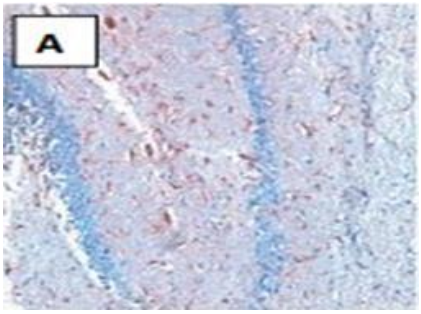


g)

B- Pilocarpine model



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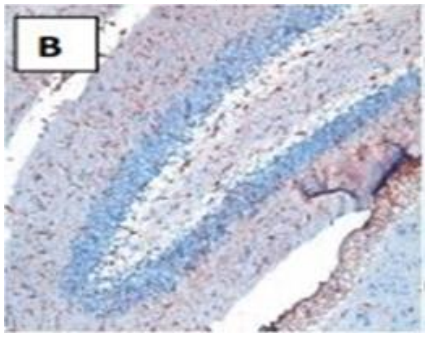

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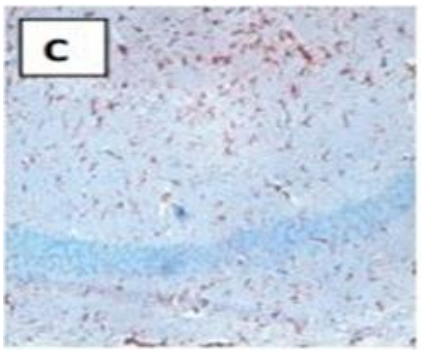

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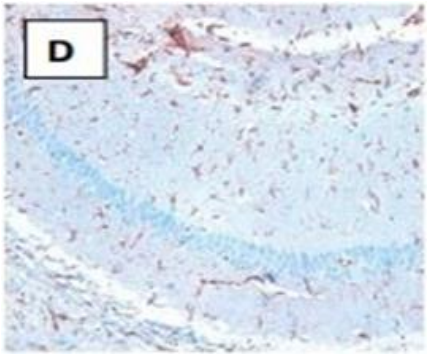

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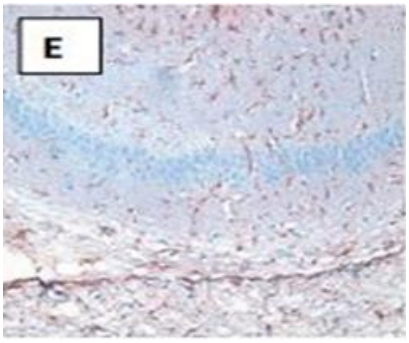

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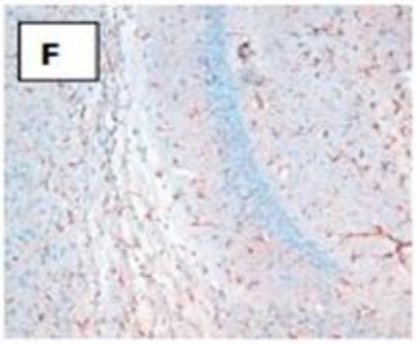

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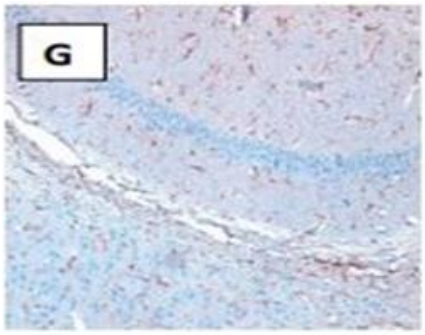

g)

Figure 5. GFAP immunostaining of brain (x200) of:
A- Pentylenetetrazole-induced Generalized Seizures. **(A) Group 1** Moderate Gliosis, Score 2. **(B) Group 2** Mild Gliosis, Score 1. **(C) Group 3** Mild Gliosis, Score 1. **(D) Group 4** No Gliosis, Score 0. **(E) Group 5** and **(F) Group 6** Mild Gliosis, Score 1. **(G) Group 7** Moderate Gliosis, Score 2.
B- Pilocarpine-induced status epilepticus. **(A) Group 1** moderate gliosis, score 2. **(B) Group 2** moderate gliosis, score 2. **(C) Group 3** mild gliosis, score 1. **(D) Group 4** no gliosis, score 0. **(E) Group 5** and **(F) Group 6** mild gliosis score 1. **(G) Group 7** mild gliosis, score 1.

Based on our study, regarding both pentylenetetrazole induced generalized seizures and pilocarpine induced status epilepticus models, flupirtine at a dose of 25 mg/kg was the smallest efficacious dose among the three used doses. Pharmacological, biochemical and histopathological results of this group were statistically significant to control group (1). In pentylenetetrazole induced generalized seizures model, brain GABA and IL-6 levels was statistically significant to group receiving flupirtine 10 mg/kg. Added to these results, in pilocarpine induced status epilepticus model, latency period was statistically significant to group receiving flupirtine 10 mg/kg and brain IL-6 level was statistically significant to group receiving flupirtine 50 mg/kg, which were in accordance with [26, 27], where flupirtine blocked behavioral and electroclinical seizures, increased the latency to first seizure and reduced duration and severity of febrile seizures.

Flupirtine is believed to shift KCNQ channels' voltage dependent activation towards hyperpolarized potentials especially KV7.2, KV7.3 and KV7.5 subunits, increase opening and slow closing of KCNQ channels, thus, resulting in an increased threshold for generating neuronal action potentials [26, 28]. Kv7 potassium channels also counteract the spike after-depolarization generated by recruitment of persistent sodium

currents (INaP), which can lead to bursting. Hence, they act as an epileptic burst firing brake [29]. Retigabine, an analog of flupirtine, also shifted the voltage activation of Kv7 channels to more hyperpolarized potentials [30]. The FDA has approved Retigabine to treat partial epilepsies in adult patients before being withdrawn in 2017 due to retinal abnormalities and skin blue discoloration. Due to the similarity of flupirtine's mechanism with that of retigabine, it is believed that flupirtine also effectively controls seizures of infants with Kv7 encephalopathy [27].

$GABA_A$ receptors have been described as novel sites for flupirtine action. Flupirtine modulates $GABA_A$ receptors currents by enhancing the power of GABA to induce currents without increasing maximal current amplitudes. Flupirtine has also been recognized to potentiate hippocampal GABA responses of the delta subunit but not the gamma subunit-containing $GABA_A$ receptors [31]. However, potentiation of $GABA_A$ receptors is less likely to be flupirtine's anti-seizure mechanism because, 1) GABA is depolarizing during early development, unlike the inhibitory KCNQ channel activity all through the development. 2) Hippocampal neurons indicate very low contents of the delta subunit, through which flupirtine potentiates $GABA_A$ receptor activity. 3) on the contrary to the potentiating impact of flupirtine on $GABA_A$ receptor under normal conditions, flupirtine has been indicated to hinder depolarization-induced release of GABA and glutamate from presynaptic terminals via KCNQ2 activation, prevent 4-AP-induced enhancement in GABA and glutamate contents and reduce new GABA and glutamate synthesis [26, 32].

CNS is not isolated from the immune system. Evidence linking neuroinflammation to epileptogenesis is not limited to IL-1ß. Clinical and experimental pieces of evidence suggest active roles of IL-6 in seizure generation and exacerbation [33] via activating nuclear factor kappa B (NF-κB) transcriptional signaling and inducing COX-2 to synthesize PGE_2. In our study, flupirtine significantly reduced the rise in brain IL-6, thus exerting a neuroprotective role, in accordance with [34, 35] which concluded that retigabine and flupirtine analogs have anti-apoptotic potentials [35, 36].

Additionally, flupirtine was found to reduce acute and improve chronic brain injuries and cognitive deficits as assessed by cresyl violet stain [36, 37]. However, it couldn't specify whether these impacts are because of decreased seizure burden or independent of its impact on seizures.

Indirect antagonism of the NMDA receptor and subsequent glutamate-induced intracellular Ca^{2+} enhancement, upregulation of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), and antioxidant activity through enhanced glutathione contents and decreased reactive oxygen species contents have been considered to be included in the cyto and neuro-protective potentials of flupirtine [7, 37]. NMDA-induced intracellular calcium enhancement results in activation of calpain. Calpain is implicated in STAT6degradation, which in healthy brains inhibits JNK and NF-κB signaling pathways that are critical in the progression of post-ischemic proteolysis and brain cell death. As a consequence of indirect NMDA receptor antagonism, flupirtine at clinically relevant condensations decreases the calcium-dependent calpain activation and restores the STAT6 induced inhibition of JNK and NF-κB pathways proteasomal activity. Increased expression of Bcl-2 will inhibit autophagy and influence proteostasis in by complexing with beclin-1. Flupirtine blocks the decrease in glutathione and induces the expression of Bcl-2 reducing cell toxicity [7].

[38] was in accordance where a single dose of flupirtine attenuated the increase in GFAP caused by SNP in in-vitro studies. Flupirtine functions as an antioxidant not by solely enhancing glutathione contents but also by nullifying the direct influence of nitric oxide (NO) or its derivatives.

In our study, diazepam showed better results than flupirtine in some parameters, mainly pharmacological, almost the same results in biochemical parameters, while flupirtine showed better immunohistopathological results. In contrast to our results, [39] concluded that flupirtine was more influential than phenobarbital, and diazepam, in suppressing neonatal seizures. Pretreatment with diazepam was ineffective at preventing the progression of induced seizures to status epilepticus, while flupirtine potently controlled the induced seizures.

BBB disruption related to seizure occurrence leads to BBB leakage and efflux of administered ASDs. Brain expresses several ATP binding cassette (ABC) efflux transporters, including P-glycoprotein (P-gp), BCRP, MRP1, MRP2, MRP4, and MRP5 [40]. Many pieces of research indicated upregulation of P-gp in the brain and BBB due to seizure activity in rodent models and human patients with refractory epilepsy [41]. P-gp is upregulated by glutamate-mediated NMDA receptor activation in brain capillary endothelial cells. Activation of COX-2/EP receptor signaling during seizure activates a signaling cascade involving the transcription factor, NF-κB leading to increased expression of P-gp. Overexpression of P-gp may cause enhancing the efflux of the prescribed ASD/s, resulting in pharmacoresistance. Since COX-2 serves as a transcriptional regulator of P-gp, inhibiting COX-2 may assist in achieving increased efficacy of prescribed ASDs. P-gp upregulation can be prevented by selective COX-2 inhibitors, including celecoxib, facilitating drug delivery to brain and increasing drug efficiency provided the ASD is a substrate of P-gp [42]. Diazepam is a weak substrate for canine P-gp [43]. In contrast, flupirtine is not a substrate of the efflux transporter P-gp (ABCB1) and MRP2 (ABCC2) [44]. This may explain why celecoxib didn't improve flupirtine effect in our study.

Conclusion

Based on this study, the three used drugs, diazepam, celecoxib, and flupirtine, possess anticonvulsant, immuno-modulatory, and neuroprotective effects. Diazepam is superior in controlling seizures, while celecoxib is the least effective. Diazepam showed better results than flupirtine in some pharmacological parameters, almost the same results in biochemical parameters. In comparison, flupirtine showed better histopathological results indicating that flupirtine is a promising drug that could

contribute to controlling generalized seizures and status epilepticus. Combined diazepam and celecoxib showed comparable results to combined flupirtine and celecoxib and will be a good choice in epileptic patients with comorbidities requiring treatment with anti-inflammatory drugs. Our data highlight the importance of further studies on flupirtine's efficacy as a monotherapy or an add-on treatment on other epilepsy models for reliable screening of potential new epilepsy treatment strategies.

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