

Pharma Sci 2020- Effect of Calcium Channel Blockers on the Seizures, Oxidative Stress, and Histoarchitecture in the Rats- Azeezia Institute of Medical Sciences and Research, India

K. Saniya, B. G. Patil¹, C. Madhavrao², K. G. Prakash

Department of Anatomy, Azeezia Institute of Medical Sciences and Research, Kollam, Kerala, ¹ Department of Anatomy, Shri B M Patil Medical College, BLDE University, Vijayapura, Karnataka, ² Department of Pharmacology, All India Institute of Medical Sciences, Mangalagiri, Andhra Pradesh, India

Introduction

In spite of availability of considerable number of antiepileptic drugs, many patients continue to have seizures that are refractory to treatment defying our understanding and approaches of epilepsy. Many old drugs continue to be evaluated for newer indications. Before such experiments, it is prudent to understand the pathological basis of epilepsy considering many newer understandings. Many of the currently used antiepileptic drugs are shown to inhibit calcium channel activity. Theoretical considerations and few animal model studies have suggested that calcium channel antagonists may play a role as anticonvulsants. These drugs are postulated to inhibit the positive inward burst firing activating wide range of neurons leading to seizures. To support such theoretical considerations, few animal model studies and clinical studies have shown that nimodipine has anticonvulsant property. Combination of calcium channel blockers was shown to have mixed effects. Diltiazem enhances the nimodipine's antiseizure effects. Flunarizine inhibits nimodipine's effects. During the last decade of the 20th century, there was a heightened interest in the evaluation of calcium channel blockers for epilepsy in animal model. However after the introduction of gabapentin, topiramate, tiagabine, levetiracetam, and zonisamide, the interest in the evaluation of monotherapy for epilepsy has waned. However, the evaluation has continued as add-on therapy in both animal model and in clinical trials.

Many animal models have been developed over the previous two decades for evaluation of the novel antiepileptic drugs. The maximal electroshock (MES) model remains as an important gatekeeper for such evaluation, in spite of the fact that it failed in

levetiracetam efficacy. Brain inflammation and brain immune responses play a key role in epilepsy according to recent studies. Oxidative stress mediated by free radicals leading to changes in neuronal structure and function is regarded as possible mechanism of epileptogenesis. Even though the anticonvulsant effects of diltiazem, nimodipine, and flunarizine were previously evaluated, the studies have not concentrated on the neurotransmitter levels and antioxidant effects of these drugs in the rat brain. The objective of the present study was to determine the anticonvulsant effects of these drugs in rat MES model. The study also evaluates the effects on oxidative stress and neurotransmitter levels in rats pretreated with these drugs in MES model. The other objective of the study was to evaluate the acute histological changes in different parts of rats' brain.

Materials and Methods

Experimental animals

Experiments were conducted with 30 inbred Wister rats of 3–4 weeks old. All rats were obtained from animal house, BLDEU's Shri B M Patil Medical College, Vijayapura, Karnataka, and KMCH College of Pharmacy, Coimbatore, Tamil Nadu. Rats were group-housed in cages of three with ad libitum to food and water. The temperature was maintained at 22°C–25°C with relative humidity of 41.55%. A 12:12, light: dark cycle was followed during the experiment. The experiment was carried out during 1200–1400 h. Water was withdrawn 8 h before and during the experiments.

Institutional Animal Ethics Committee, BLDEU's Shri B M Patil Medical College, Vijayapura, Karnataka

(with CPCSEA, India registered) (approval letter number: 32/16, dated January 16, 2016), and also Institutional Animal Ethics Committee, KMCH College of Pharmacy, Coimbatore, Tamil Nadu (approval letter number: KMCRET/PhD/05/16-17, dated February 22, 2016), approved the study before the start of the study.

Evaluation of anticonvulsant activity-maximum electroshock model

The rats were pretested prior to the drug administration for the electroshock sensitivity. Convulsions were induced using electroconvulsimeter(Techno India Ltd.). MES stimulation was given using transauricular(ear-clip) electrodes from the apparatus. The intensity of MES was at 150 mA for 0.2 s, with constant voltage stimulators of 250 V. At this intensity and duration, all the control group rats exhibited tonic hind limb extension. Only those rats that consistently exhibited the tonic hind limb extension in three trials on three separate days were used for the study

During and after the MES, the duration of flexion, duration of tonic hind limb extension, and duration of clonus (in seconds) were noted. Abolition of hind limb extension and reduction (or absence) of the clonus duration after the drug administration were considered as an anticonvulsant effect of the test drug.

Dissection of brain and processing of the two hemispheres

All rats were anesthetized using thiopental sodium (50 mg/kg) after 24 h. All rats were sacrificed by cervical decapitation. The brain was dissected out of the cranial cavity. Each brain was hemisectioned along the longitudinal fissure into right and left halves. One hemisection was homogenized and used for estimation of neurotransmitters and oxidative stress markers. Another hemisection was fixed with formalin and used for histological study.

Estimation of brain neurotransmitters

Hemisections of the brain tissue dissected were homogenized in 5 mL HCl-butanol for 1 min using a manual glass homogenizer. The unbroken cells were

removed by centrifugation at 2000 rpm for 10 min. An aliquot supernatant phase (1 ml) was removed and added to centrifuge tube containing 2.5 ml heptane and 0.31 ml HCl of 0.1 M. After 10 min of vigorous shaking, the tube was centrifuged under the same conditions as above in order to separate the two phases, and the overlaying organic phase was discarded. The aqueous phase (0.2 ml) was taken for gamma-aminobutyric acid (GABA), glutamate (GLU), serotonin (5-HT), and dopamine (DA) assay.

Dopamine assay

About 0.2ml of aqueous phase of tissue was mixed with 0.05ml 0.4 M HCl and 0.1 ml of sodium acetate buffer (pH 6.9). This mixture was oxidized with 0.1 ml iodine solution (0.1 M in ethanol). After 2 min, the oxidation was stopped with addition of 0.1 ml Na₂SO₃ solution. The mixture was heated to 100°C for 6 min. Spectrophotometric measurements were taken at 350 nm after the reaction mixture cools down to room temperature.

Serotonin and glutamate assay

About 0.2 ml of aqueous phase of tissue was mixed with 0.25 ml of o-phthalaldehyde. This mixture was heated at 100°C for 10 min. After heating, the mixture forms a chemical that re-emits light upon excitation (fluorophore)[10]. Spectrophotometric measurements were taken at 410 nm for 5-HT and 515 nm for GLU after the reaction mixture cools down to room temperature.

Gamma-aminobutyric acid assay

About 0.1 ml of aqueous extract of the tissue was mixed with 0.2 ml of 0.14 M ninhydrin solution in 0.5 M carbonate-bicarbonate buffer (pH 9.95) kept in a water bath at 60°C for 30 min. This mixture was oxidized with oxidizing agent, 5 ml of copper tartrate reagent (0.16% disodium carbonate, 0.03% copper sulfate, and 0.0329% tartaric acid). After 10 min, fluorescence at 430 nm in a spectrophotometer was recorded.

Enzymatic antioxidant activity

Estimation of reduced glutathione

To 250 μ L of tissue homogenate, 1 mL of 5% trichloroacetic acid (TCA) was added and centrifuged at 3000 g for 10 min at room temperature and supernatant was collected. About 1.5 ml of 0.2 M phosphate buffer was added to the supernatant and mixed well. About 250 μ L of 0.6 mM of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) was added to the above mixture, and the absorbance was measured at 412 nm. A standard graph was plotted using glutathione (GSH)-reduced solution (1 mg/mL), and GSH content present in the tissue homogenates was calculated by interpolation. Amount of GSH was expressed as μ g/mg protein.

Lipid peroxidation assay

To 100 μ L of the tissue homogenate, 2 mL of (1:1:1 ratio) thiobarbituric acid reagent (thiobarbituric acid 0.37%, 0.25 N hydrochloric acid, and 15% trichloroacetic acid) was added and mixed. The above content was incubated in a boiling water bath for 15 min, cooled, and centrifuged at 3500 rpm for 10 min at room temperature. The pink color developed was estimated at 535 nm against a reagent blank, in a spectrophotometer. Lipid peroxidation (LPO) was expressed as nmol of MDA/mg protein.

Myeloperoxidase assay

Diluted homogenized latent samples were mixed with monoclonal antibody to myeloperoxidase (MPO) and incubated. The MPO-monoclonal antibody complex was labeled with a biotin-linked marker. The biotin-avidin complex with covalently linked alkaline phosphatase was prepared. With the addition of 4-nitrophenylphosphate 9pNPP, MPO was enzymatically measured by reading the microplate at 450 nm.

Histopathological evaluation

Hemisectons of the brain were fixed with 10% formalin for 48 h. Paraffin blocks were prepared. 5- μ m-thick sections were prepared and processed for histopathological and immunohistochemical studies.

Hematoxylin- and eosin-stained slides showing regional changes in the frontal lobe, temporal lobe,

basal ganglia hippocampus, and cerebellum were evaluated for histoarchitectural changes.

Immunohistochemistry

5- μ m-thick pretreated sections were placed on L-lysine slides. For heat shock protein (HSP) antigen retrieval, the slides were immersed in sodium citrate 0.1 M. Slides were preheated at 750 W microwave oven for 7 min. The mouse antibody to HSP70 kD (Bio SB, Bio Sciences For the World, CA 93117, USA) was diluted in 1:100 phosphate-buffered saline. The slides covered with antibody were placed in a solution jar containing buffer. The slides were covered with horse radish peroxidase and incubated for 10 min and washed with deionized water and buffer periodically. This was followed by washing with buffer solution 3 times. Then, slides were placed in DAB (3,3'-diaminobenzidine) solution for 10 min and later washed with buffer [15]. Hematoxylin counterstained the slides. All slides were evaluated for HSP70 immunohistochemistry by pathology expert.

Statistical analysis

The data were expressed as mean \pm standard deviation. Comparison between the groups was done by one-way ANOVA, followed by post hoc Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

MES-induced seizures

Following the ear electrode stimulus, an immediate tonic seizure with hind limb extension was observed in all animals of Group I and Group II. There were no signs of toxicity in the control groups. The positive control group which was administered phenytoin did not show the phase of clonic seizures. The latency of onset of flexion, extension, and clonic seizures is tabulated in Table 2. Rats pretreated with diltiazem, nimodipine, and flunarizine showed a statistically significant reduction in the duration of hind limb extension phase and clonic seizures. The total duration of the seizures was also significantly lower and comparable to phenytoin pretreated rats. All rats in all the groups survived the experiments indicating the doses used during the study was not lethal.

Oxidative stress markers

Estimation of GSH, LPO, and MPO indicated the level of oxidative stress in the groups. There was a significant ($P < 0.05$) increase in the reduced GSH from the homogenized samples of the rat brain from diltiazem and nimodipine. The change indicates a greater neutralization of free radicals. There was a significant increase in LPO and MPO levels ($P < 0.001$) in the diltiazem and nimodipine groups. These changes indicate better oxidative stress outcomes in rat brains pretreated with diltiazem and flunarizine.

Neurotransmitter estimation

DA levels were significantly decreased in the phenytoin pretreated group ($P < 0.001$). Similar significant changes were noted in the nimodipine (levels increased) and flunarizine groups (levels decreased). However, the diltiazem group did not show any change in DA levels.

There was no significant difference between the GABA level of the control and phenytoin groups. Diltiazem and flunarizine

Discussion

Calcium channels, both L type and T type, have been increasingly implicated in epileptogenesis. It is postulated that calcium channel antagonists have antiseizure and neuroprotective roles. Many previous studies have demonstrated such antiseizure actions of calcium channel blockers in rat MES model.

Nifedipine (in doses of 10 mg/kg), amlodipine (in doses of 1–4 mg/kg), felodipine (in doses of 5–10 mg/kg), verapamil (in doses of 5 mg/kg), [5] flunarizine (in doses of 5 mg/kg), nicardipine (in doses of 5 mg/kg), nimodipine (in doses of 5–10 mg/kg), and diltiazem (in doses of 5–10 mg/kg) have been shown to have antiseizure actions in animal models. In the present study as well, diltiazem, nimodipine, and flunarizine were proven to have anti-seizure actions.

Rats pretreated with diltiazem and flunarizine have shown better oxidative stress outcomes. There are indirect evidences that improving the oxidative stress status in patients with epilepsy may prolong the interseizure duration. T-type calcium channel blockers

were shown to have neuroprotective role in animal models. Flunarizine effect on oxidative stress among migraine patients is clinically proven. The changes in the present study indicate better oxidative stress outcomes in rat brains pretreated with diltiazem and flunarizine.

DA levels in several rodent model studies have shown differential effects in epileptogenesis. Drugs binding to D1 receptors are proven to have proconvulsant effects. D2R-mediated cAMP-dependent canonical pathway has been implicated in D2 receptor mechanisms of DA. In the present study, differential D1 and D2 receptor-mediated epileptogenic actions may be implicated for decreasing and increasing levels of DA documented with different calcium channel blockers.

In our study, there was a significant increase in GABA in diltiazem and flunarizine pretreated rats. This implies that both these drugs exhibit their antiepileptic action by enhancing neuronal inhibitory actions of GABA. A similar increase in GABA levels was recorded in a study evaluating the antiepileptic action of Shilajit in experimental rats.

In the present study, all the three-drug pretreated rat groups showed a significant decrease in GLU levels. This implies that diltiazem, nimodipine, and flunarizine bring about reduction in excitotoxic damage of the epileptogenic stimulus. A similar decrease in the brain GLU levels was recorded during evaluation of anticonvulsant and neuroprotective role of anise oil (*Pimpinella anisum*) in rat brain.

5-HT levels were significantly reduced in all the groups. This along with documented reduction in the seizure duration indicates that serotonergic pathways contribute to the antiepileptic action of the diltiazem, nimodipine, and flunarizine. However, the exact mechanism of action cannot be ascertained by the estimation of total 5-HT. A similar reduction in the 5-HT levels was reported in a study evaluating the antiepileptic action of *Culcasia falcifolia* flower extract. There are previous studies documenting the cerebral edema, vacuole formation, cell clustering, and intracytoplasmic inclusion granules after acute administration of phenobarbital and gabapentin. In the present study as well, the vasogenic edema was

documented in the cerebellum and areas of basal ganglia. Apoptosis marker, HSP, was positive in temporal lobe sections of rats pretreated with nimodipine in the present study. However, a direct causal relationship of nimodipine-induced neuronal apoptosis cannot be implicated with these results.

Limitations of the study

The use of multiple animal models nullifies the shortcomings of each other and achieves better clinical correlations in humans. Dose variations during MES model would have provided minimum effective dose (ED50) of the drugs. The use of standard antiepileptic drug in each group would have evaluated the additive role the calcium blockers. Currently, only calcium channel blockers are used, which are rarely clinically used as standalone therapy for epilepsy. Concomitant estimation of aspartate levels in the rat brain was not done. Estimation of aspartate, GLU, and GABA levels would have given overall neurotransmitter environment of the brain. Only total 5-HT levels were measured in the present study from rat brain hemisections. However, 5-HT affects epilepsy through complex mechanisms involving 5-HT receptors. The overall levels of 5-HT convey limited information about the mechanisms involved. Quantification of ACh, 5-HT, GABA, GLU, and DA from the specific regions of the brain would have resulted in more precise understanding of the antiseizure effect of the three calcium channel blockers used in the study. Overall GSH was estimated in the current study. However, estimation of GSH peroxidase and GSH reductase would have led to a better understanding of oxidative stress environment.

Conclusions

Diltiazem (20 mg/kg), nimodipine (20 mg/kg), and flunarizine (10 mg/kg) have shown anticonvulsant action in Wistar rats in MES model. GSH, LPO, and MPO changes indicate better oxidative stress outcomes in rat brains pretreated with diltiazem and flunarizine. There were histoarchitectural changes such as cerebral edema, vacuole formation, and intracytoplasmic granules with all three calcium channel blockers in acute phase.

Acknowledgments

The authors thank the animal house in-charge of BLDEU's Shri B M Patil Medical College, Vijayapura, Karnataka, and also the animal house, KMCH College of Pharmacy, Coimbatore, Tamil Nadu.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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