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N ACETYL CYSTEINE ALLEVIATES PHENYTOIN INDUCED BEHAVIORAL ABNORMALITIES IN RATS

G.R. Saraswathy*[1], E. Maheswari [2], T. Santhrani [3], J. Anbu [1]

Department of Pharmacology, Faculty of Pharmacy, M.S. Ramaiah University of Applied Sciences [1], Bangalore, Karnataka, India
Department of Pharmacy Practice, Faculty of Pharmacy, M.S. Ramaiah University of Applied Sciences [2], Bangalore, Karnataka, India
Division of Pharmacology, Institute of Pharmaceutical Technology, Sri Padmavathi Mahila Visvavidyalayam (Women's University) [3], Tirupati, Andhra Pradesh, India

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Correspondence to Author:

G.R. Saraswathy
Department of Pharmacology, Faculty of Pharmacy, M.S. Ramaiah University of Applied Sciences, Bangalore, Karnataka, India

E-mail:
saraswathypradish@gmail.com

ABSTRACT: NAC being an antioxidant combats oxidative stress induced by phenytoin, thereby hypothesized to reduce phenytoin induced behavioral abnormalities. The influence of N acetyl cysteine (NAC) supplementation on phenytoin induced behavioral abnormalities was investigated. Male Wistar rats were divided into 5 groups and each group received vehicle (0.2% CMC), Phenytoin (20mg/Kg), Phenytoin co administered with three graded doses of NAC (50, 100, 200 mg/kg) in 0.2% CMC for 45 days. Motor coordination, exploratory behavior, memory and spontaneous motor activity were evaluated by Rota rod, Hole board, Elevated plus maze and Actophotometer respectively. On day 45, regional brain lipid peroxidation, acetylcholinesterase (ACh E) activity and histopathological studies were performed after euthanasia. In addition, pharmacokinetic and pharmacodynamic drug interactions between phenytoin and NAC were also studied. Long term administration of phenytoin induced behavioral abnormalities, elevated regional brain malondialdehyde (MDA) and ACh E activity, also revealed congestion in addition to damaged cells in brain regions. NAC significantly prevented phenytoin induced behavioral abnormalities, oxidative stress and reversed the histopathological abnormalities. There were no significant differences in the serum levels of phenytoin and the degree of protection offered by phenytoin in NAC supplemented groups revealing that there were no pharmacokinetic and pharmacodynamic interactions between phenytoin and NAC. This study demonstrates that NAC is effective in preventing phenytoin induced behavioral abnormalities and oxidative stress in rats without altering the serum phenytoin levels and its therapeutic effect. This suggests the potential of adjuvant NAC therapy in alleviating behavioral disturbances induced by chronic phenytoin therapy.

INTRODUCTION: Epilepsy is characterized by periodic recurrent seizures, which varies from a brief lapse of attention or muscle jerks to severe and prolonged convulsions [1].

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The goal of antiepileptic drug (AED) therapy is to achieve freedom from seizures with no or minimal side effects. Even with the advent of new generation AEDs, it is known that significant number of epileptic patients still struggle with adverse effects [2].

Phenytoin is a most common and effective AED prescribed for a prolonged period to achieve seizure control in all types of generalized as well as partial seizures and status epilepticus [3].

Phenytoin therapy is reported to induce cognitive dysfunction [4] and cerebellar degeneration resulting in ataxia, nystagmus and slurred speech [5].

Brain is vulnerable to free radical damage owing to its rich polyunsaturated fatty acid (PUFA) and iron content, high oxygen consumption and scanty availability of antioxidant enzymes [6]. Iron is necessary for brain development while the iron ions form reactive oxygen species (ROS) that rearranges the double bonds of PUFA, producing degradation products like lipid alkoxyl, peroxyl radicals and lipid hydroperoxides [7]. ROS influences gene expression leading to neuronal death [8]. High oxidative metabolism in catecholamine rich areas like basal ganglia makes neurons susceptible to lipid peroxidation [9].

Phenytoin is bio-activated to reactive intermediates that generate ROS. This results in oxidative stress leading to free radical attack on neural cells that contributes calamitous role to neuro-degeneration. Free radicals damage proteins and DNA, induce inflammation and subsequent cellular apoptosis [10]. Any mutation in DNA leads to impaired ATP generation and perturbed oxidative phosphorylation cascade that may further lock the neuronal function. Free radicals have been reported for their great contribution to neuronal loss in cerebral ischemia, seizure disorders, schizophrenia, Parkinson's disease and Alzheimer's disease [11, 12].

N-acetylcysteine (NAC), is an acetylated derivative of the amino acid L-cysteine. NAC is a powerful antioxidant and potential therapeutic agent in the treatment of cancer, heart disease, myoclonic epilepsy and other diseases characterized by oxidative damage. NAC enhances glutathione-S-transferase activity thereby increases the synthesis of reduced glutathione (GSH) [13], which in turn alleviates oxidative stress. NAC was observed to reverse the memory deficit and oxidative stress caused by hypoxic exposure in rats [14]. NAC also was reported to offer neuroprotection against aluminum induced cognitive dysfunction and oxidative damage [15].

The therapeutic or toxic effects of phenytoin depend on its serum concentration. Pharmacokinetic interactions like alterations in drug absorption, bioavailability, metabolism and excretion often make drug combinations impractical and drug levels are to be monitored frequently to optimize the dosage for individual patients with epilepsy. The serum levels of phenytoin were estimated at the end of the study period after the steady state of the drug was achieved to study if there were any pharmacokinetic interactions between phenytoin and NAC. Pharmacodynamic interference of NAC upon antiepileptic protection offered by phenytoin was also studied.

Cognition and other behavioral parameters in epileptic individuals may be influenced by several factors, including basic neuropathology as well as the frequency, severity and the type of seizures. Thus, in the present investigation, epilepsy was not induced in the experimental animals which permit assessment of the effects of phenytoin on behavioral parameters without added complexities of the disease state (epilepsy).

Our work is a preliminary and pioneering study to assess the ameliorative effect of NAC against phenytoin induced behavioral abnormalities. Phenytoin and its metabolites are reported to induce oxidative stress in brain regions via free radical generation leading to behavioral abnormalities. Thus it is worthwhile to explore the protective effect of NAC against phenytoin induced behavioral abnormalities. The influence of NAC against phenytoin impaired memory; exploratory behavior, spontaneous motor activity and locomotor activity were studied along with estimation of regional brain lipid peroxidation and acetyl cholinesterase (ACh E) activity. Our hypothesis proposes that antioxidant property of NAC offers protection against phenytoin induced behavioral abnormalities. Antioxidant supplementation is suggested to render an excellent antiepileptic therapy devoid of toxicity which may improve the quality of life in patients under phenytoin treatment.

MATERIALS AND METHODS:

Animals: Adult male albino rats weighing 150-200 g were maintained at room temperature (25 ± 3°C), fed with a rodent lab diet and tap water *ad libitum*. The study protocol was approved by the Institutional Animal Ethical Committee of M.S.

Ramaiah College of Pharmacy, Bangalore, Karnataka, India, Ref. No. 220/abc/CPCSEA.

Study Protocol: The rats were divided into five groups consisting of six animals each. First group served as control and received 0.2% carboxy methyl cellulose (CMC) (p.o) for 45 days. Second group received 20mg/Kg phenytoin dissolved in 0.2% CMC (p.o) for 45 days. Third, fourth and fifth group received 50, 100 and 200 mg/kg of NAC in 0.2% CMC (p.o) respectively 1 h prior to administration of 20mg/Kg phenytoin for 45 days.

Parameters to assess behavioral abnormalities: The behavioral parameters were analyzed 2hrs after the administration of NAC and phenytoin. Memory, motor co-ordination, locomotor activity and exploratory behavior were assessed on 0, 15th, 30th and 45th day. On 45th day behavioral tests were carried out, 3 h after phenytoin administration (steady state concentration) and phenytoin with NAC supplementation the animals were subjected to maximal electro shock (MES) induced convulsions to compare the degree of protection offered by phenytoin in phenytoin treated group and groups subjected to combination of phenytoin and antioxidants. Immediately after MES the animals were decapitated under ether anesthesia, blood was collected from retro-orbital plexus to estimate the serum levels of phenytoin. The brains were quickly removed and differentiated into cortex, mid brain, medulla, pons and cerebellum and were subjected to assessment of the extent of lipid peroxidation and acetylcholinesterase (ACh E) activity.

Motor co-ordination test: Motor co-ordination test was conducted in rats using a Rota-Rod apparatus (Inco-Ambala, India). The animals were screened for motor co-ordination and the animals which stayed on the rotating rod without falling for 120 sec were chosen for the study. Each animal was placed on the Rota rod and the time taken by the animal to fall down was noted [16].

Test for locomotor activity: Spontaneous motor activity was monitored using Actophotometer. Each animal was subjected to an adaptation period of 2-5 minutes after which their locomotor activity was assessed for 5 minutes. Increase in count was regarded as CNS stimulant activity. Decrease in count was considered as CNS depressant activity [16].

Test for memory impairment: Elevated plus maze test was used for the assessment of memory. The elevated plus maze consists of two closed arms and two open arms forming a cross, with a quadrangular center and has a height of 50 cm. The rats were placed individually at the end of one open arm facing away from central platform and the time it took to move from the open arm to either of the enclosed arms (transfer latency) was recorded on the day of acquisition trial. Transfer latency is the time taken by the rats to move from one end of the open arm to enclosed arm. The rat was allowed to move freely in the plus maze regardless of open and closed arms for 10 sec after the measurement of transfer latency. The rat was then gently taken out of the plus maze and was returned to its home cage. On the test day, the transfer latency test was performed in the same manner as in the acquisition trial [17].

Test for alertness (Exploratory Behavior): 0.5m^3 wooden board with 16 holes (3 cm in diameter) was employed for the study. Each rat was placed individually on the board for a period of 6 minutes. In first 2 minutes the animal was allowed for acclimatization and then the number of head dipping performed in the next 4 minutes was noted for each animal [18].

Assessment of oxidative stress in brain tissues: The brain samples were quickly removed, cleaned with chilled saline, dissected into cortex, midbrain, medulla, pons and cerebellum, were stored at −40°C.

Estimation of lipid peroxidation in brain regions: The extent of lipid peroxidation in tissues was assessed by measuring the level of MDA according to the method of Ohkawa, et al., (1979). Briefly, 1ml (10%) tissue homogenate was added to the reaction mixture containing 1 ml of trichloro acetic acid (15%) and 2 ml of thiobarbituric acid (0.38%). The reaction mixture was heated for 60 min at 90^{o}C, cooled and centrifuged at 6900 rpm for 15 min. The absorbance of supernatant was measured at 532 nm against blank, which contained all reagents except homogenate.

MDA was quantified and expressed as µmol of MDA per mg of wet tissue [10].

Estimation of Acetylcholine Esterase activity in brain regions: Acetylthiocholine iodide was used as a synthetic substrate for the assay of ACh E, replacing the natural substrate acetylcholine (ACh). This enzyme hydrolyses the substrate to yield acetate and thiocholine. The free thiol group of thiocholine reacts with 5, 5'-dithio-bis-nitrobenzoic acid (DTNB) (Ellman's reagent) included in the assay mixture, producing the yellow 4-nitrothiolate anion. The release of this yellow anion is measured at 412 nm.

The reaction mixture (2mL final volume) contained 100mM potassium phosphate buffer, pH 7.5 and 1mM DTNB. The method is based on the formation of the yellow anion, 5,5'-dithio-bis-acid nitrobenzoic, measured by absorbance at 412nm during 2-min incubation at 25°C. The enzyme was pre-incubated for 2min. The reaction was initiated by adding 0.8mM acetylthiocholine iodide [19].

Histopathological investigation on brain tissues: Brain tissues were dissected out carefully and were kept in 10% formalin solution prepared with normal saline. Tissues were stained using Hematoxylin and Eosin stain [20].

Maximal electroshock induced seizures (MES): Electroconvulsions were induced by ear electrodes (current intensity-150 mA, duration - 0.2 sec). The animals were observed for tonic hind limb extension i.e., the hind limbs of animals outstretched 180° to the plane of the body axis [17].

Estimation of plasma phenytoin concentration by HPLC method:

Chromatographic conditions: Mobile phase consisting of methanol: water: glacial acetic acid (67: 33: 1 v/v/v) was prepared and mixed thoroughly, degassed and was used for the HPLC analysis. 1.0 ml per minute flow rate was maintained throughout the analysis. The eluent was monitored using a UV-VIS detector set at 230 nm and sensitivity was set at 0.001 a.u.f.s.

Preparation of standard graph: Standard solutions: Stock solution of 100 µg/ml of phenytoin was prepared in methanol and diluted with methanol to the required concentration. The solutions were stored at –4ºC. For standard graph 2, 4, 6, 8, 10, 12, 14, 16, 18 20 µg/ml of pure phenytoin was used.

Plasma extraction: To each 100 µl of plasma sample, 25 µl of internal standard (100 µg/ml carbamazepine solution) was added and extracted with 1.7 ml of ethyl acetate, vortexed for 1 min and centrifuged at 13,000 rpm for 8 min. The supernatant was evaporated to dryness, the residue was reconstituted with 100 µl of mobile phase, vortexed for 1 min. and 20µl was injected onto C18 column. The retention times were 4.49 min. and 5.15 min. for phenytoin and carbamazepine respectively. The peak area obtained at different concentrations of the drug was plotted against the concentrations of the drug [21].

Statistical analysis: The results were expressed as mean ± SEM of each group. One way analysis of variance (ANOVA) followed by the Tukey's post hoc test was used to assess the differences among treatment groups. Statistical analysis was performed using GraphPad Instat software. $p<0.05$ was considered significant.

RESULTS: There were no significant differences in transfer latency, exploratory activity, motor coordination and spontaneous motor activity between control, phenytoin alone and phenytoin with NAC pretreated groups on 0 day of the study.

Effect of N Acetyl Cysteine on phenytoin induced memory impairment: The retention transfer latencies increased from 34.5±2.23 sec (0 day) to 124.66±1.82 sec (45^{th} day) ($p< 0.001$) in phenytoin treated animals. Co-administration of NAC in all the three doses significantly reduced the transfer latency from 15^{th} day till 45^{th} day. The values decreased from 124.66±1.82 sec in the phenytoin treated group to 96.5±1.47 sec ($p< 0.001$), 85.16±1.7 sec ($p< 0.001$) and 72.0±1.12 sec ($p< 0.001$) in NAC 50, 100 and 200 mg/Kg co-administered groups respectively on 45^{th} day of the study. NAC at all the three doses produced significant reversal of phenytoin induced memory impairment in a dose dependent fashion (**Fig. 1**).

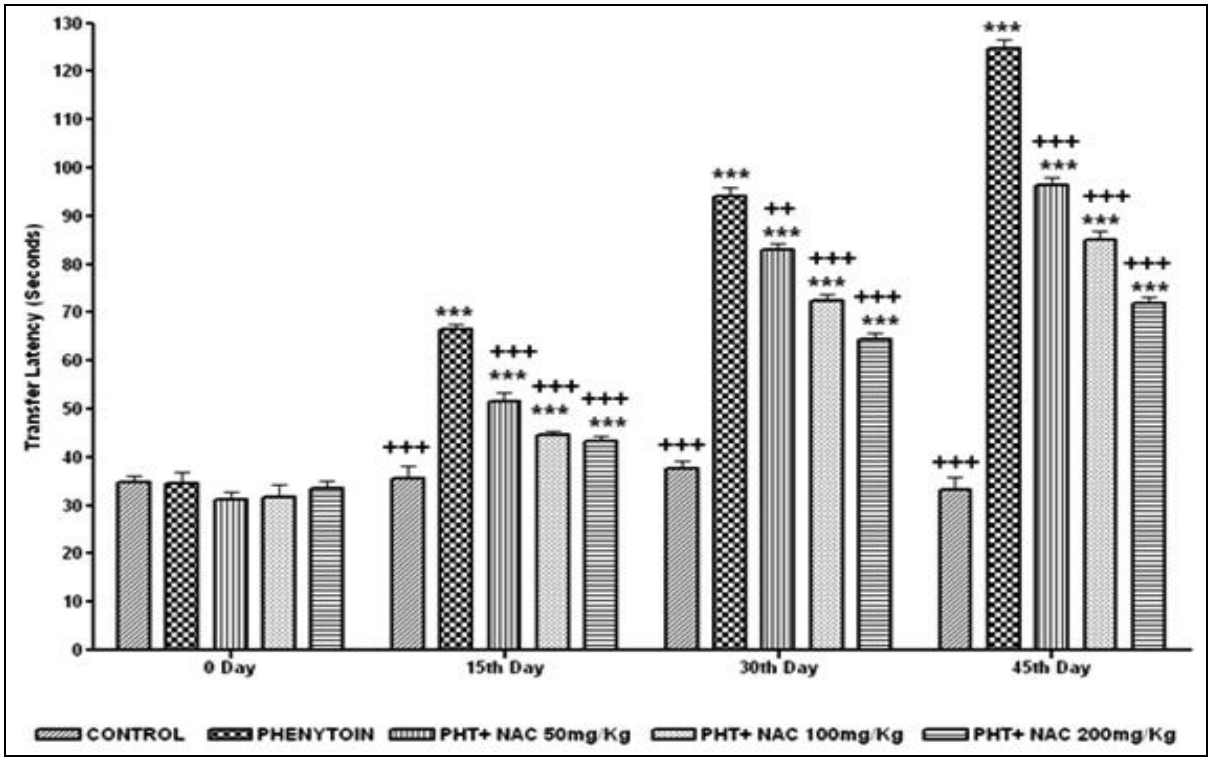

FIG. 1: EFFECT OF NAC ON PHENYTOIN INDUCED MEMORY IMPAIRMENT. Influence of Phenytoin and Phenytoin + NAC (50, 100 and 200 mg/Kg) on transfer latency using elevated plus maze on 0th, 15 th, 30 th and 45 th day. Values are expressed as means± SEM of 6 rats, Statistical analyses used was Tukey test. ***(p< 0.001), **(p< 0.01), *(p< 0.05) Vs Control group +++(p< 0.001), ++(p< 0.01), +(p< 0.05) Vs Phenytoin group.

Effect of N Acetyl Cysteine on phenytoin impaired exploratory activity: The exploratory activity was assessed by the number of head dippings into the holes of the hole board apparatus. The number of head dippings decreased from 24.5±1.5 (0 day) to 2.5±0.76 (45th day) (p< 0.001) in phenytoin treated animals. Co-administration of NAC in all the three doses significantly increased the exploratory movements from 15th day till 45th day. The number of head dippings increased from 2.5±0.76 in the phenytoin treated group to 7.5±0.76 (p< 0.05), 11.166±0.7 (p< 0.001) and 15.33±0.714 (p< 0.001) in NAC 50, 100 and 200 mg/Kg co-administered groups respectively on 45th day of the study. NAC at all the three doses produced significant reversal of phenytoin impaired exploratory behavior in a dose dependent manner (**Fig. 2**).

Effect of N Acetyl Cysteine on phenytoin induced motor in co-ordination: Phenytoin (20 mg/Kg, p.o.) significantly impaired the Rota Rod performance of rats from 120 sec (0 day) to 17.83±0.87 sec on 45th day (p< 0.001). Co-administration of NAC in all the three doses significantly improved the motor coordination from 15th day till 45th day. The values increased from 17.83±0.87 sec in the phenytoin treated group to 55.33±1.54 sec (p< 0.001), 84.83±1.51 sec (p< 0.001) and 91.3±1.86 sec (p< 0.001) in NAC 50, 100 and 200 mg/Kg co-administered groups respectively on 45th day of the study. NAC at all the three doses produced significant reversal of phenytoin impaired motor coordination in a dose dependent fashion (**Fig. 3**).

Effect of N Acetyl Cysteine on phenytoin impaired locomotor activity: Phenytoin 20 mg/Kg, significantly decreased the spontaneous motor activity. The activity count was decreased from 311.66±3.73 (0 day) to 86.5±1.408 (45th day) (p< 0.001). Co-administration of NAC in all the three doses significantly improved the spontaneous motor activity from 15th day till 45th day. The values increased from 86.5±1.408 in the phenytoin treated group to 107±1.65 (p< 0.001), 161.5±1.5 (p< 0.001) and 215.5±1.64 (p< 0.001) in NAC 50, 100 and 200 mg/Kg co-administered groups respectively on 45th day of the study. NAC at all doses produced significant reversal of phenytoin impaired locomotor activity in a dose dependent fashion (**Fig. 4**).

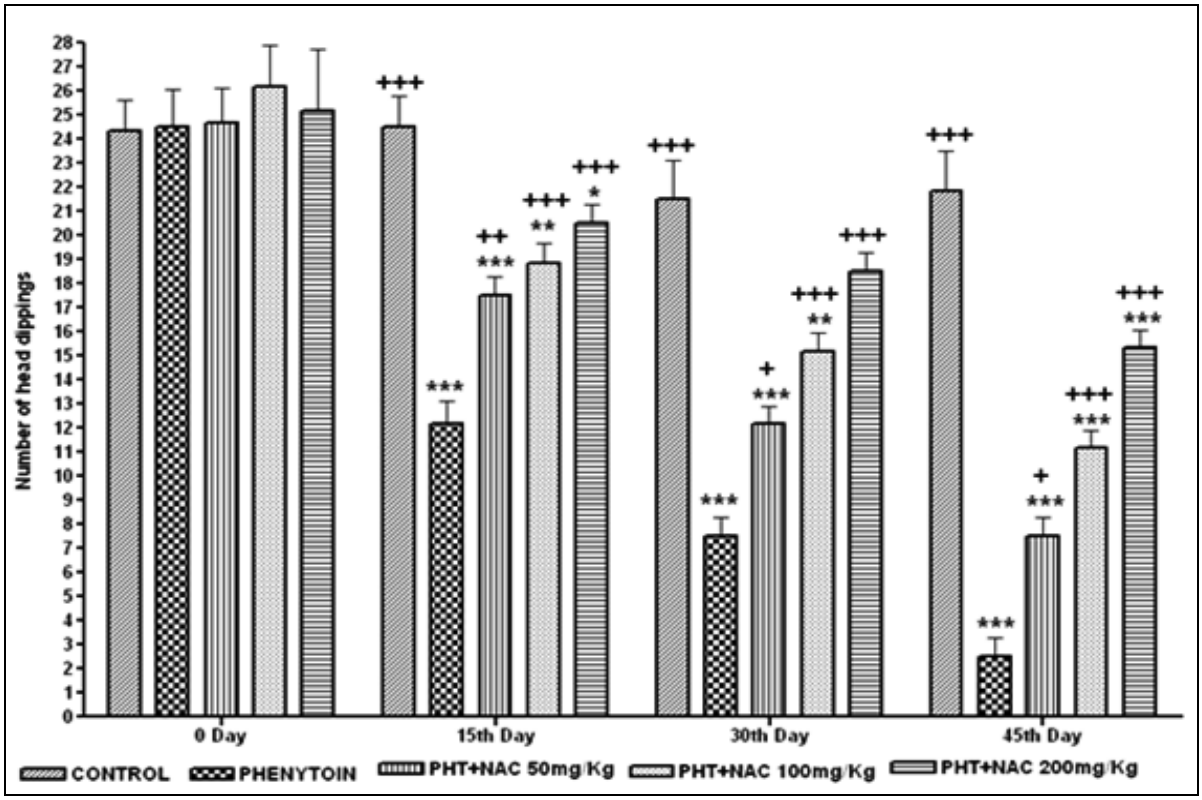

FIG. 2: EFFECT OF NAC ON PHENYTOIN IMPAIRED EXPLORATORY BEHAVIOUR. Influence of Phenytoin and Phenytoin + NAC (50, 100 and 200 mg/Kg) on number of head dippings using hole board apparatus on 0th, 15th, 30th and 45th day. Values are expressed as means± SEM of 6 rats, Statistical analyses used was Tukey test. ***(p< 0.001), **(p< 0.01), *(p< 0.05) Vs Control group +++(p< 0.001), ++(p< 0.01), +(p< 0.05) Vs Phenytoin group.

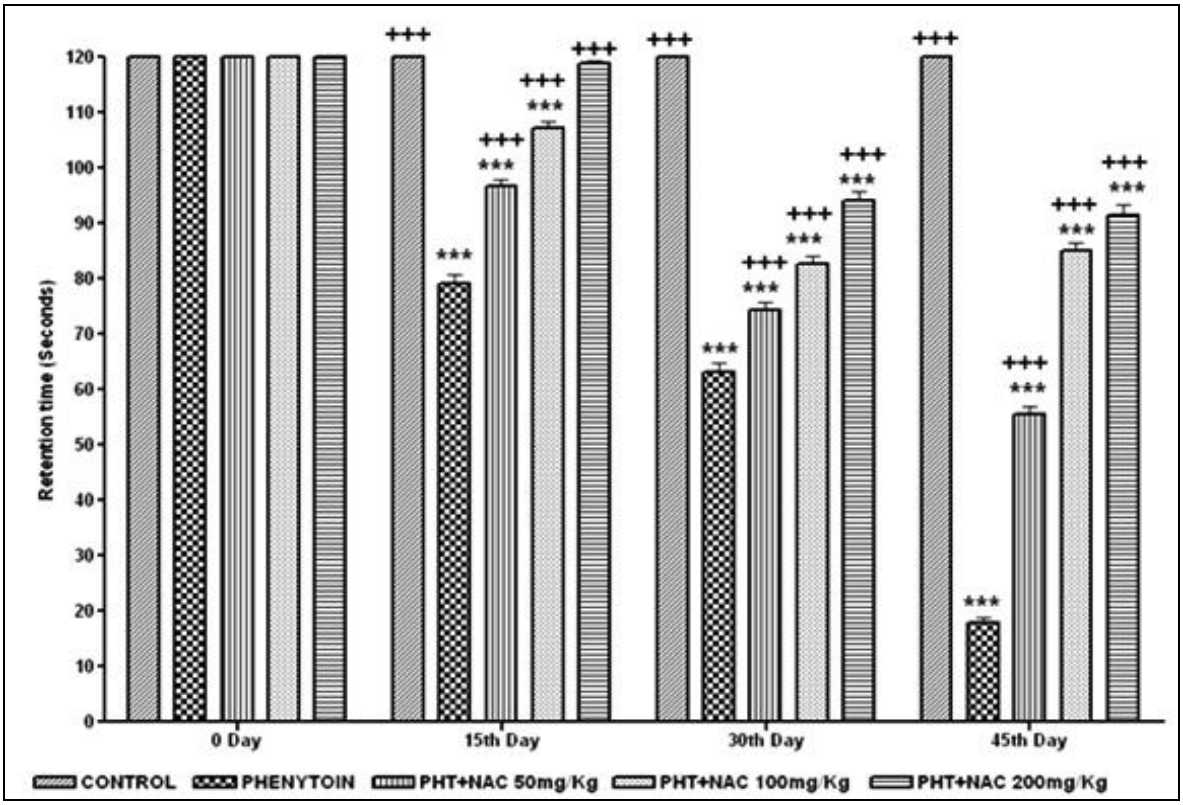

FIG. 3: EFFECT OF NAC ON PHENYTOIN INDUCED MOTOR IN-COORDINATION. Influence of Phenytoin and Phenytoin + NAC (50, 100 and 200 mg/Kg) on retention time using rota-rod apparatus on 0th, 15th, 30th and 45th day. Values are expressed as means± SEM of 6 rats, Statistical analyses used was Tukey test. ***(p< 0.001), **(p< 0.01), *(p< 0.05) Vs Control group +++(p< 0.001), ++(p< 0.01), +(p< 0.05) Vs Phenytoin group.

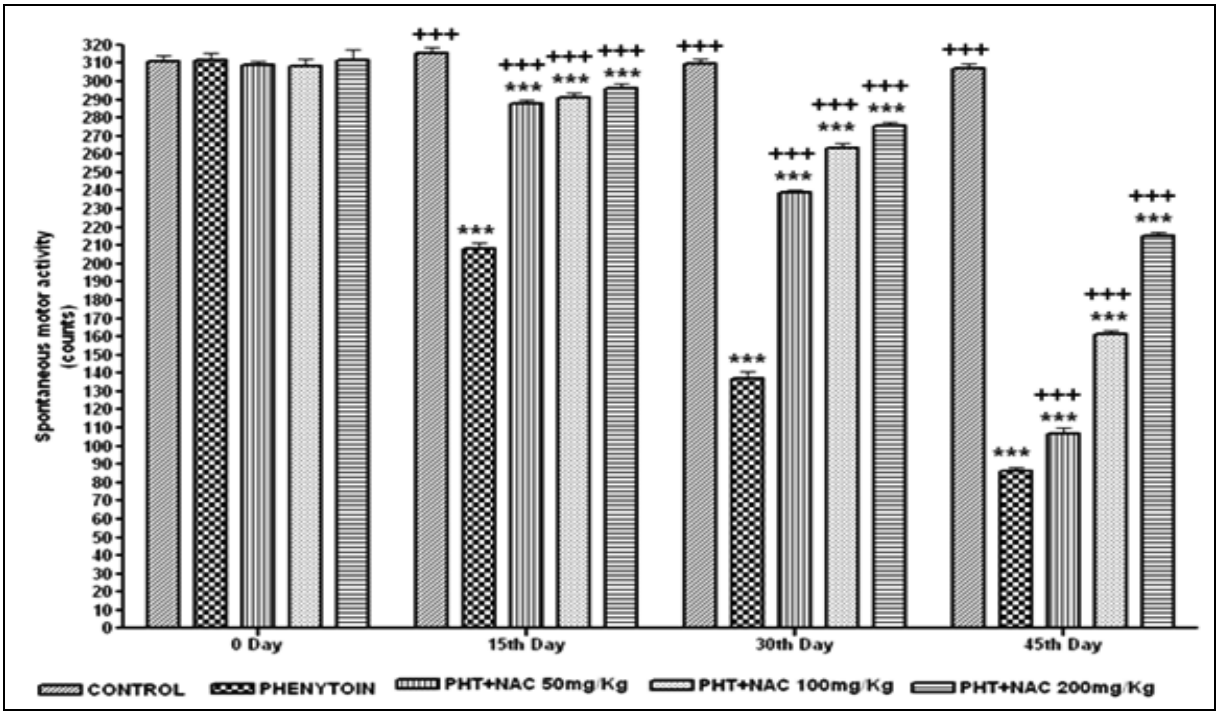

Fig. 4: Effect of NAC on phenytoin impaired locomotor activity. Influence of Phenytoin and Phenytoin + NAC (50, 100 and 200 mg/Kg) on spontaneous motor activity using actophotometer on 0th, 15th, 30th and 45th day. Values are expressed as means± SEM of 6 rats, Statistical analyses used was Tukey test. ***(p< 0.001), **(p< 0.01), *(p< 0.05) Vs Control group +++(p< 0.001), ++(p< 0.01), +(p< 0.05) Vs Phenytoin group

Effect of N Acetyl Cysteine on regional brain MDA levels: Phenytoin showed a significant rise in lipid peroxidation in medulla, pons, midbrain, cerebellum and cortex. NAC significantly reduced (p< 0.001) the lipid peroxidation in all the brain regions dose dependently, but the values did not reach the normal values except in medulla when compared with the control group (**Fig. 5**).

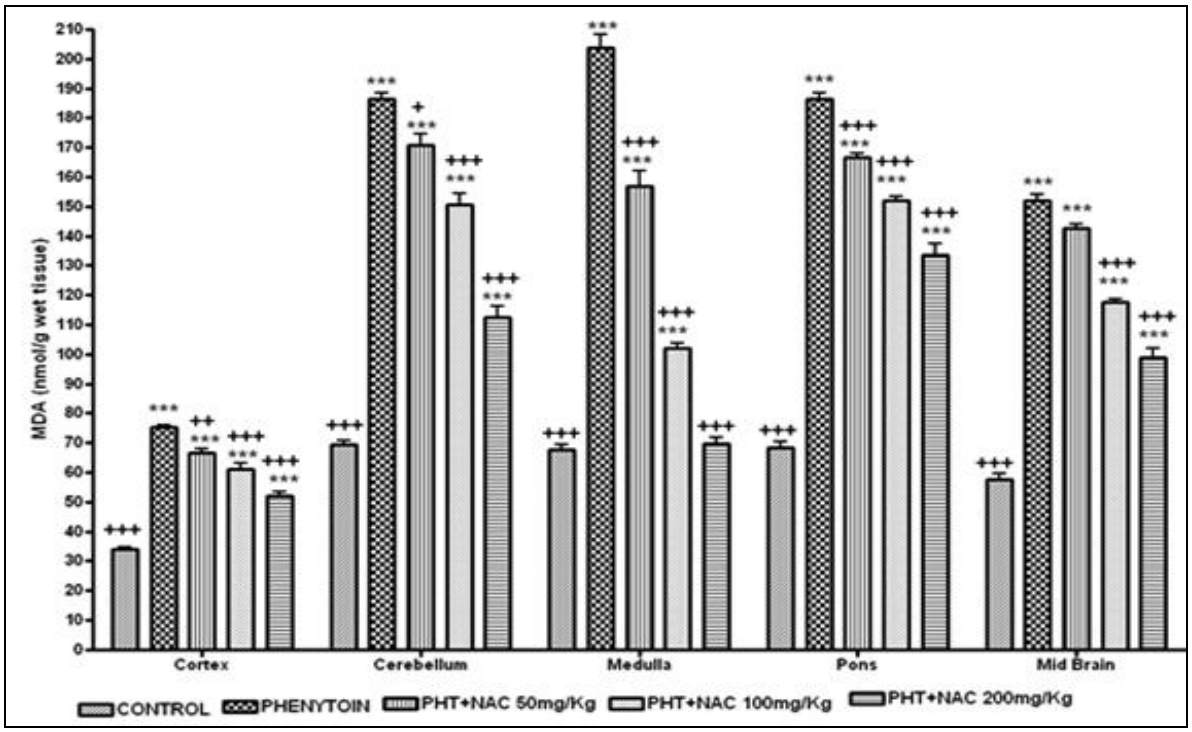

FIG. 5: EFFECT OF NAC ON PHENYTOIN INDUCED ALTERATIONS IN REGIONAL BRAIN LIPID PEROXIDATION. Influence of Phenytoin and Phenytoin + NAC (50, 100 and 200 mg/Kg) on regional brain lipid peroxidation (45th day). Values are expressed as means ± SEM of 6 rats, Statistical analyses used was Tukey test. ***(p< 0.001), **(p< 0.01), *(p< 0.05) Vs Control group +++(p< 0.001), ++(p< 0.01), +(p< 0.05) Vs Phenytoin group.

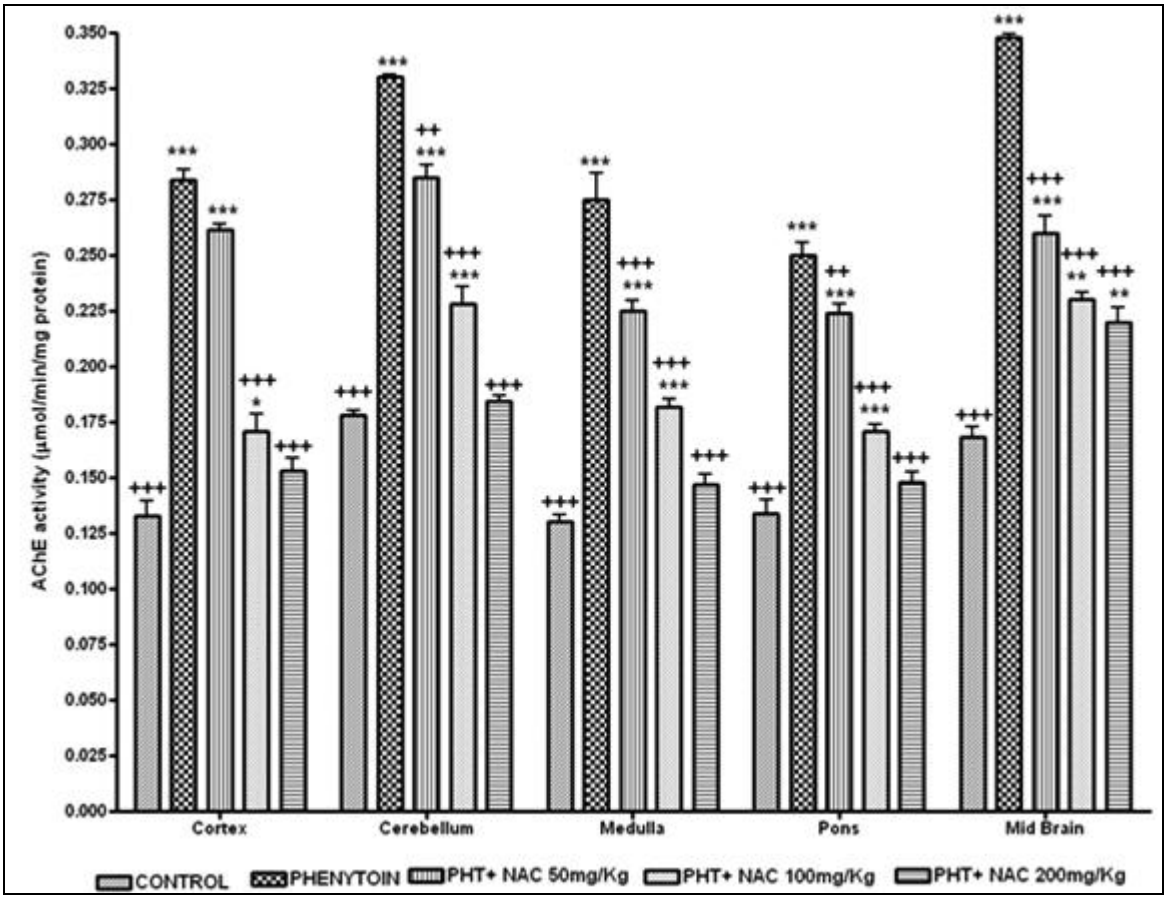

FIG. 6: EFFECT OF NAC ON PHENYTOIN INDUCED ALTERATIONS IN REGIONAL BRAIN ACETYL CHOLINESTERASE ACTIVITY. Influence of Phenytoin and Phenytoin + NAC (50, 100 and 200 mg/Kg) on regional brain acetyl cholinesterase activity (45th day). Values are expressed as means ± SEM of 6 rats, Statistical analyses used was Tukey test. ***(p< 0.001), **(p< 0.01), *(p< 0.05) Vs Control group +++(p< 0.001), ++(p< 0.01), +(p< 0.05) Vs Phenytoin group.

Effect of phenytoin on regional brain histopathology: Fig. 7 illustrates the effect of phenytoin on brain. Control group showed normal brain architecture (**Fig. 7a**). Phenytoin treated group revealed severe necrosis in cortex (**Fig. 7b**).

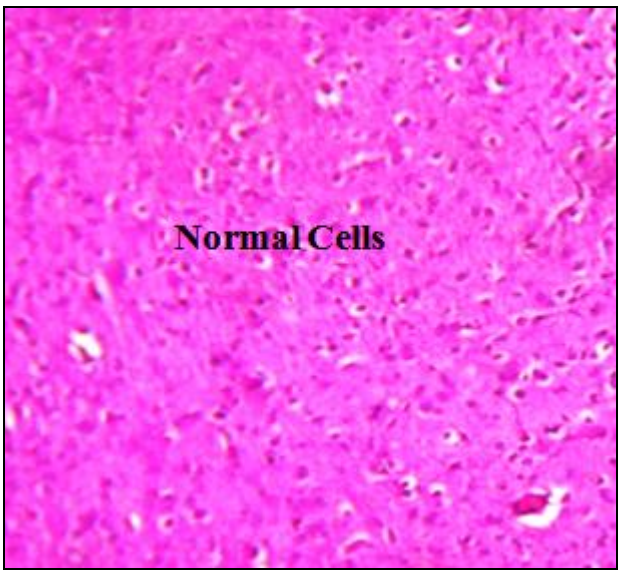

FIG. 7A: CONTROL

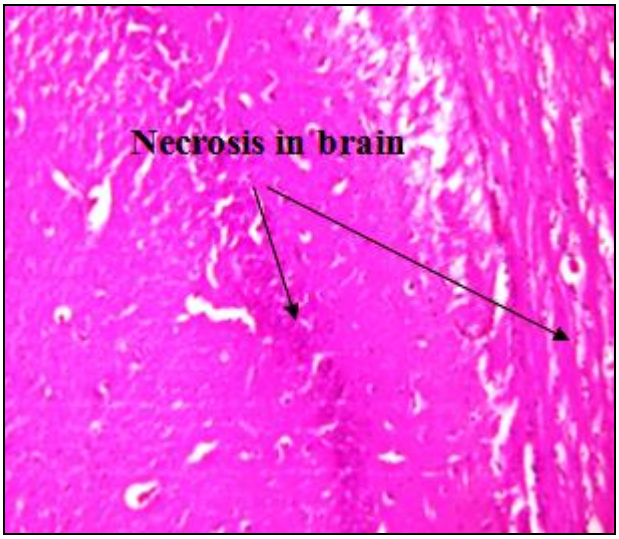

FIG. 7B: PHENYTOIN

FIG. 7: EFFECT OF PHENYTOIN ON REGIONAL BRAIN HISTOPATHOLOGY. Fig. 7 illustrates the effect of phenytoin on the brain. Control group exhibited normal brain architecture (Fig. 7a.). Phenytoin treated group revealed severe necrosis in cortex (Fig. 7b).

Effect of N Acetyl Cysteine on phenytoin induced alterations in brain histopathology: Fig. 8 shows the effect of NAC on phenytoin induced histopathological changes in rat brain. Phenytoin supplementation with NAC (50 mg/Kg) showed mild necrosis (**Fig. 8a**), while both the doses of NAC (100 and 200 mg/Kg) showed normal brain parenchyma (**Fig. 8b. and 8c**).

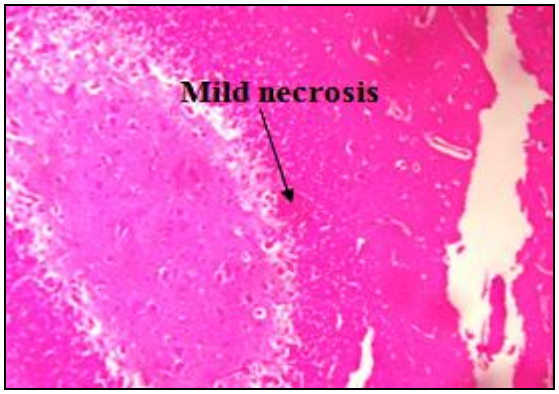

FIG. 8A: PHENYTOIN + NAC (50 mg/Kg)

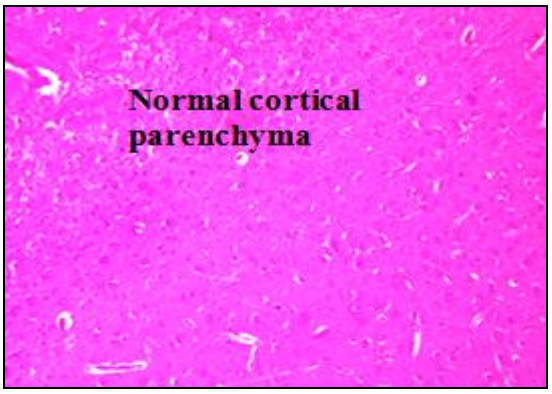

FIG. 8B: PHENYTOIN + NAC (100 mg/Kg)

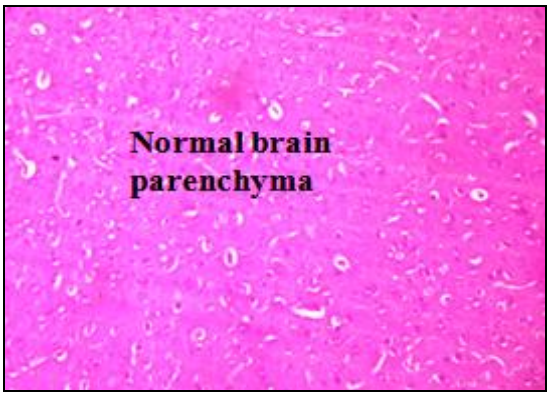

FIG. 8C: PHENYTOIN + NAC (200 mg/Kg)

FIG. 8: EFFECT OF NAC ON PHENYTOIN INDUCED ALTERATIONS IN BRAIN HISTOPATHOLOGY. Fig. 8 shows the effect of NAC on phenytoin induced histopathological changes in rat brain. Phenytoin supplementation with NAC (50 mg/Kg) showed mild necrosis (Fig. 8a.), while both the doses of NAC (100 and 200 mg/Kg) showed normal brain parenchyma (Fig. 8b. and 8c.).

Influence of NAC on pharmacodynamic effect of phenytoin: Phenytoin as well as phenytoin supplemented with NAC (50, 100 and 200 mg/kg) offered same degree of protection (100%) against MES induced convulsions in rats.

Effect of NAC on serum phenytoin levels: There was no significant difference in the serum concentration of phenytoin treated group as compared to the groups co-administered NAC 50, 100 and 200 mg/kg along with phenytoin. The serum phenytoin levels were 15.740 ±1.8, 16.230±1.6, 14.230±1.4, 15.720±1.5 µg/ml in the groups treated with phenytoin and NAC 50, 100 and 200 mg/kg along with phenytoin respectively. All these values were within the normal therapeutic range (10-20 µg/ml) of phenytoin.

DISCUSSIONL Phenytoin significantly impaired the motor-coordination, cognition, exploratory behavior and spontaneous motor activity. Phenytoin also significantly increased the regional brain lipid peroxidation and ACh E activity along with appreciable degeneration in the brain regions as confirmed by the histopathological investigations.

Cognitive impairment is associated with epilepsy, the underlying pathology as well as AED treatment result in poor cognitive function [22]. Since cognition is affected by a number of factors in epileptic patients, it is logical to evaluate the effect of AEDs on memory and cognitive function in experimental animals without any additional complexities of the disease. Phenytoin is reported to affect the learning and memory [23].

In the present study, memory function was assessed by elevated plus maze [24]. Prolongation of transfer latency indicates the impairment of learning and memory. Phenytoin (20 mg/Kg) was reported to substantially impair the memory of (prolonged the transfer latency) non-epileptic rats in elevated plus maze paradigm, indicating the risk of this drug in impairing cognition even in healthy individuals also.

Our results coincide with previous studies in which, learning and memory was impaired by majority of the conventional AEDs in non-epileptic rats [25] and in healthy volunteers [26].

Phenytoin was reported to affect the exploratory behavior [27], induce sedation and decrease the wakeful state of the rats.

Hole board test assesses the exploratory behavior/alertness of the rodents. Phenytoin appreciably decreased the exploratory behavior/alertness as observed by the decrease in number of head dippings in the holes of the hole board. Rota rod apparatus reveals the motor coordination of rodents. Phenytoin impaired the Rota rod performance of rats indicating the incidence of muscle weakness and motor in co-ordination, which was believed to be an attributing factor for phenytoin induced ataxia [23, 28]. Actophotometer explores the spontaneous motor activity. Phenytoin significantly reduced the spontaneous motor activity, indicating the CNS depressant property of the drug.

In experimental animals severe oxidative stress was reported with chronic phenytoin treatment [10, 29]. In epileptic patients, phenytoin treatment decreased the activities of endogenous antioxidants like SOD (superoxide dismutase), glutathione reductase, glutathione peroxidase, Vitamin C, Vitamin E and increased the TBARS (thiobarbituric acid reacting substances). The results of the present study also illustrated an increase in oxidative stress in the phenytoin treated rats, as indicated by increase in MDA (malondialdehyde) levels in different regions of brain. MDA an end product of lipid peroxidation is a biochemical marker to determine the degree of lipid peroxidation which indicates the extent of neuronal damage in various brain regions [23].

In the present study, phenytoin increased regional brain lipid peroxidation. Memory is maintained by groups of neurons present in hippocampus, cerebral cortex, cerebellum and mid brain. The cerebral cortex is the part of the brain involved in many higher level tasks such as language, memory and consciousness. Memory of learned motor sequences (motor subroutines) seems to be stored in the supplementary motor area which is called the "executive centre" of the brain. Cerebellar learning critically involves the cerebellar cortex, while the cerebellar nuclei play a more critical role in long term memory storage [30] and thus memory is consolidated in the cerebellum [31].

In the present study, phenytoin increased the lipid peroxidation in cerebral cortex, cerebellum, mid brain, pons and medulla oblongata. Increased lipid peroxidation in different brain regions was observed to cause peroxidative injury to the neuronal membranes and macromolecules, alter neurotransmitters, disrupt key neuronal functions and perturb motor function [32]. Neuronal damage induced by phenytoin in brain regions was believed to be responsible for memory impairment, motor in co-ordination, sedation, ataxia and loss of exploratory drive.

Cholinergic neurons and their projections are widely distributed throughout the CNS with an essential role in regulating many vital functions such as learning, memory, cortical organization of movement and cerebral blood flow [33]. This cholinergic activity is in turn regulated by ACh E, which hydrolyses the neurotransmitter acetylcholine (ACh) in the synaptic cleft of cholinergic synapse and neuromuscular junctions [34].

ACh E is an important enzyme for cholinergic neurotransmission and there are strong indications for its essential role in regulating many vital functions as well as neuro-behavioral processes [35].

Deutsch, (1971) evaluated the role of the cholinergic synapse in the storage and retrieval of new information. Cognitive decline in aging and dementia is related to a decrease in cholinergic function [36]. The effects of cholinergic antagonists and lesions of cholinergic nuclei are often related to cognitive deficits similar to those observed in aging and dementia [37].

Numerous pharmacological studies evaluated the effects of cholinergic antagonists and choline-mimetics on learning and memory performance [38]. The cholinergic muscarinic antagonist scopolamine is most widely used to induce amnesia in experimental subjects. ACh E inhibitors enhance the availability of ACh in the synaptic cleft and reverse the scopolamine induced memory deficit. Many studies have shown that there is a relation between the decrease in cognitive functions and markers of the cholinergic system in senile dementia.

Cognitive functions are highly dependent on central cholinergic neurotransmission. Although other neurotransmitters were known to be involved in learning and memory performance, acetylcholine plays a vital role in in storage and retrieval of memory. Decline in the cholinergic system underlies the cognitive deficits of dementia [39] and ACh E levels are reported to be high in AD. Melo, et al., (2003) studied the involvement of oxidative stress in the enhancement of ACh E activity. It was observed that amyloid beta-peptide enhanced ACh E activity mediated via oxidative stress [40].

In new referrals with epilepsy, patients receiving phenytoin performed consistently poorer on memory tasks than those untreated. Intellectual dulling and impaired memory has been observed in patients receiving phenytoin. Investigations on the effect of phenytoin on learning, memory and psychomotor functions revealed that both acute and chronic administration of phenytoin considerably impaired learning and memory [25]. It was reported that phenytoin decreased brain ACh levels [41]. Phenytoin's impairing effects on learning and memory are attributed to enhanced ACh E activity in brain. For an optimum antiepileptic therapy, it is desirable to have an absolute seizure control without cognitive impairment.

Since central cholinergic system plays an important role in learning and memory and as phenytoin reduced ACh concentration in brain regions, the drug was reported to induce serious memory impairment [41]. We have measured the ACh E activity in different brain regions as a marker enzyme for cholinergic function. Our results were on par with the previous reports which also revealed that phenytoin at therapeutic doses increased ACh E activity in the brain regions of the rats. The rats also showed poor performance in the elevated plus maze test indicating memory impairment. It was believed that phenytoin via oxidative stress enhanced the ACh E activity and thereby depleted the levels of ACh in brain regions resulting in subsequent memory impairment.

Brain sections of phenytoin treated rats showed damaged cells and congestion in periventricular region and cortex, which substantiates phenytoin induced apoptosis in cortex and periventricular region.

Phenytoin via oxidative stress induced the damage in rat brain, which in turn resulted in adverse behavioral abnormalities.

Since phenytoin induced oxidative stress was considered to be the etiologic factor for deterioration of behavioral parameters, the present investigation explored the antioxidant potential of NAC to combat phenytoin induced oxidative damage and behavioral abnormalities.

NAC was reported to prevent apoptotic death in neuronal cells and protect synaptic mitochondrial proteins from oxidative damage in aged mice [42]. NAC was observed to preserve the nigrostriatal cells against oxidative damage and is considered to provide a neuroprotective therapeutic strategy against Parkinson's disease [43]. Oxidative stress is reported in depressed patients and in animals subjected to stress. NAC was found to possess significant antidepressant like activity in forced swimming induced depression without affecting the exploratory behavior [44]. NAC was found to be safe and effective against depressive symptoms in bipolar disorder [45].

NAC was observed to selectively inhibit acute fatigue of rodent skeletal muscles stimulated at low tetanic frequencies, improve performance of human limb muscle during fatiguing exercise. The study confirmed that oxidative stress plays a crucial role in the fatigue process and recommended an antioxidant NAC therapy against fatigue [46]. NAC was reported to offer protective effect against hypoxia-induced cytotoxicity in primary hippocampal culture. Supplementation with NAC resulted in a significant fall in ROS generation followed by inhibition of DNA strand breaks, indicating the neuroprotective effect of NAC during hypoxia in primary hippocampal culture [47].

Rats exposed to cadmium showed increased TBARS levels in hippocampus, cerebellum and hypothalamus along with increased serum urea and creatinine levels. NAC was reported to decrease lipidperoxidation as well as oxidative stress and improved memory along with reduction in serum urea and creatinine levels [48]. NAC treatment conferred protection against lead induced oxidative damage in cerebral region by arresting the lipid peroxidative damage [49].

Hypercholesterolemia is found to deplete the GSH content of cerebral tissues [50]. NAC administration decreased the lipid peroxidation products and enhanced the GSH content in the brain of hypercholesterolemic rats, thus offer neuroprotection in hypercholesterolemia [51]. Aluminum is a potent neurotoxin involved in the initiation and progression of various cognitive disorders like AD by inducing oxidative stress. NAC significantly improved memory retention in tasks, attenuated oxidative damage and ACh E activity in aluminum treated rats [15].

In the present study, NAC was observed to decrease phenytoin induced lipid peroxidation in brain regions and thereby improve the phenytoin affected cognitive function in a dose dependent fashion. In addition, NAC also decreased the regional brain ACh E activity, which might be another mechanism by which it reversed the phenytoin induced memory impairment. Phenytoin treatment adversely induced motor incoordination in rats and NAC effectively ameliorated the motor coordination and improved the Rota Rod performance in those rats affected by phenytoin.

The present study also reported that NAC improved the alertness as well as spontaneous motor activity and reversed the phenytoin induced depression. NAC offered protection against phenytoin induced histopathological damages in brain regions. NAC (100 and 200 mg/Kg) showed normal brain parenchyma evidencing the degree of protection offered by the above antioxidant against phenytoin induced brain damage.

Pharmacodynamic study was carried out to evaluate whether NAC supplementation hinders the therapeutic efficacy of phenytoin. In the present study, administration of phenytoin (20 mg/Kg for 45 days) produced 100% protection against MES induced seizures.

Co-administration of NAC with phenytoin also offered the same degree of protection against MES induced convulsions. This finding suggests that antioxidant supplementation with phenytoin did not reduce the therapeutic effect of phenytoin, revealing that there was no pharmacodynamic interaction between phenytoin and the selected antioxidants.

The serum levels of phenytoin were estimated at the end of the study period well after the steady state (3 h after administration of phenytoin) of the drug was achieved. The serum levels relate to the therapeutic or toxic effects of phenytoin. Moreover, the serum levels of AEDs have been reported to provide an important parameter in judging behavioral abnormalities induced by anticonvulsant drugs. It was evident from the findings of the present study that the serum levels of phenytoin were not different in the groups supplemented with NAC as compared to phenytoin alone treated group. This suggests that NAC does not alter the serum phenytoin concentration. This finding reveals that NAC did not alleviate the behavioral abnormalities by reducing serum phenytoin levels.

NAC offered protection against phenytoin induced behavioral abnormalities. The neuroprotective potential of NAC is due to its antioxidant property and its ability to increase the intracellular GSH. Though the behavioral abnormalities were reversed, the values did not reach normal even with higher dose of NAC, indicating the involvement of other mechanisms in addition to oxidative stress in phenytoin induced behavioral abnormalities.

Phenytoin therapy is found to be associated with oxidative stress induced behavioral abnormalities in experimental animals and epileptic patients undergoing phenytoin therapy. NAC, by virtue of its antioxidant property scavenges free radicals and alleviates behavioral abnormalities induced by phenytoin, which supports the hypothesis of our study.

The degree of protection offered by NAC against phenytoin induced toxicities in rats was found to be satisfactory. The limitation of the study is that it was not done in epileptic patients under phenytoin therapy. This investigation may find an excellent scope in framing a superior antiepileptic treatment strategy if extended on epileptic patients undergoing long term phenytoin therapy.

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REFERENCES:

1. Wells BG, Dipiro J, Terry S, Hamilton C: Neurologic Disorders, Pharmacotherapy Handbook. Mc Graw-Hill, sixth edition 2006.
2. Brodie MJ and Dichter MA: Antiepileptic drugs. New England Journal of Medicine 1996; 334:168-175.
3. McNamara JO: The Pharmacological Basis of Therapeutics. Goodman and Gilman's Mc Graw-Hill, tenth edition 2006.
4. Pandhi P and Balakrishnan S: Cognitive dysfunction induced by phenytoin and valproate in rats: effect of nitric oxide. Indian Journal Physiology and Pharmacology 1999; 43:378-382.
5. Luef G, Chemelli A, Birbamer G, Aichner F and Bauer G: Phenytoin overdosage and cerebellar atrophy in epileptic patients: clinical and MRI findings. European Neurology 1994; 34:79-81.
6. Skaper SD, Floreani M, Ceccon M, Facci L and Giusti P: Excitotoxicity, Oxidative Stress, and the neuroprotective potential of melatonin. Annals of the New York Academy of Sciences 1999; 890:107-118.
7. Gutteridge JMC and Halliwell B: The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochemical Sciences 1990; 15:129-135.
8. Gilgun-Sherki Y, Rosenbaum Z, Melamed E and Offen D: Antioxidant Therapy in Acute Central Nervous System Injury: Current State. Pharmacological Reviews 2002; 54:271-284.
9. Ohkawa H, Ohishi N and Yagi K: Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. Analytical Biochemistry 1979; 95:351-358.
10. Winn LM, Kim PM and Nickoloff JA: Oxidative stress-induced homologous recombination as a novel mechanism for phenytoin-initiated Toxicity. Journal of Pharmacology and Experimental Therapeutics 2003; 306:523-527.
11. Bayani U, Ajay VS, Paolo Z and Mahajan RT: Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Current Neuropharmacology 2009; 7:65-74.
12. Gilgun-Sherki Y, Melamed E and Offen D: Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 2001; 40:959-975.
13. De Vries N and De Flora S: N-Acetyl-l-cysteine. Journal of Cellular Biochemistry Supplement 1993; 17F:270-277.
14. Jayalakshmi K, Singh B, Kalpana B, Sairam M, Muthuraju S and Ilavazhagan G: N-acetyl cysteine supplementation prevents impairment of spatial working memory functions in rats following exposure to hypobaric hypoxia. Physiology and Behavior 2007; 92:643-650.
15. Prakash A and Kumar A: Effect of N-Acetyl Cysteine against Aluminium-induced cognitive dysfunction and oxidative damage in rats. Basic and Clinical Pharmacology and Toxicology 2009; 105:98-104.
16. Sharma AC and Kulkarni SK: Evaluation of learning and memory mechanisms employing elevated plus maze in rats and mice. Progress in Neuro-Psychopharmacology and Biological Psychiatry 1992; 16:117-125.
17. Kulkarni SK: Hand book of experimental pharmacology. Vallabh Prakashan, third edition 1999.
18. Takeda H, Tsuji M and Matsumiya T: Changes in head-dipping behaviour in the hole-board test reflect the anxiogenic and/ or anxiolytic state in mice. European Journal of Pharmacology 1998; 350:21-29.
19. Ellman GL, Courtney KD, Andres V and Feather-Stone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 1961; 7:88-95.
20. Li Y, Powers C, Jiang N and Chopp M: Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. Journal of Neurological Sciences 1998; 156:119-132.
21. Chen LC, Chou MH, Lin MF and Yang LL: Effects of Paeoniae radix, a traditional Chinese medicine, on the pharmacokinetics of phenytoin. Journal of Clinical Pharmacy and Therapeutics 2001; 26:271-178.
22. Vermeulen J and Aldenkamp AP: Cognitive side-effects of chronic anti-epileptic drug treatment: a review of 25 years of research. Epilepsy Research 1995; 22:65-95.
23. Thaakur S and Pushpa KB. Influence of spirulina on phenytoin induced selected behavioural abnormalities and regional brain lipid peroxidation in rats. International Journal of Neurodegeneration and Neuroprotection 2008; 4:263-272.
24. Kumar A, Seghal N, Naidu PS, Padi SS and Goyal R: Colchicines-induced neurotoxicity as an animal model of sporadic dementia of Alzheimer's type. Pharmacological Reports 2007; 59:274-283.
25. Vohora D, Pal SN and Pillai KK: Protection from phenytoin-induced cognitive deficit by Bacopa monniera, a reputed Indian nootropic plant. Journal of Ethnopharmacology 2000; 71:383-390.
26. Meador KJ, Loring DW, Moore EE, Thompson WO, Nichols ME, Oberzan RE, Durkin MW, Gallagher BB and King DW: Comparative cognitive effects of phenobarbital, phenytoin and valproate in healthy adults. Neurology 1995; 45:1494-1499.
27. Bala Krishnan S, Bhargava VK and Padhi P: Effect of nimodipine on the psychomotor dysfunction induced by phenytoin rats. Indian Journal of Pharmacology 1998; 30:299-305.
28. Bhuller Y, Jeng W and Wells PG: Variable in vivo embryo protective role for ataxia telangiectasia mutated against constitutive and phenytoin enhanced oxidative stress in knockout mice. Toxicological Sciences 2006; 93:146-155.
29. Sobaniec H, Sobaniec W, Sendrowski K, Sobaniec S and Pietruska M: Antioxidant activity of blood serum and saliva in patients with periodontal disease treated due to epilepsy. Advances in Medical Sciences 2007; 52:204-206.
30. Christian KM and Thompson RF: Long-term storage of an associative memory trace in the cerebellum. Behavioral Neuroscience 2005; 119:526-537.
31. Fliessbach K, Trautner P, Quesada CM, Elger CE and Weber B: Cerebellar contributions to episodic memory encoding as revealed by fMRI. NeuroImage 2007; 35:1330-1337.
32. Markesbery WR: Oxidative stress hypothesis in Alzheimer's disease. Free Radical Biology and Medicine 1997; 23:134-147.
33. Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG and Lockridge O: Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience 2002; 110:627-639.
34. Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Correa M, Rosa MM, Rubin MA, Schetinger MRC and Morsch VM: Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in

streptozotocin-induced diabetic rats. European Journal of Pharmacology 2009; 610:42-48.
35. Pari L and Murugavel P: Diallyl tetrasulfide improves cadmium induced alterations of acetylcholinesterase, ATPases and oxidative stress in brain of rat. Toxicology 2007; 234:44-50.
36. Deutsch JA: The cholinergic synapse and the site of memory. Science 1971; 174:788-794.
37. Dawson GR, Heyes CM and Iversen SD: Pharmacological mechanisms and animal models of cognition. Behavioural Pharmacology 1992; 3:285-297.
38. Molchan SE, Martinez RA, Hill JL, Weingartner HJ, Thompson K, Vitiello B and Sunderland T: Increased cognitive sensitivity to scopolamine with age and a perspective on the scopolamine model. Brain Research Reviews 1992; 17:215-226.
39. Blockland A: Acetylcholine: a neurotransmitter for learning and memory?. Brain Research Reviews 1996; 21:285-300.
40. Melo JB, Agostinho P and Oliveira CR. Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. Neuroscience Research 2003; 45:117-127.
41. Domino EF and Olds ME: Effects of D-amphetamine, scopolamine, chlordiazepoxide and diphenylhydantoins on self-stimulation and brain acetylcholine. Psychopharmacologia 1972; 23:1-16.
42. Martinez M, Hernandez AI and Martinez N: N-Acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria. Brain Research 2000; 855:100-106.
43. Martinez M, Martinez N, Hernandez AI and Ferrandiz ML. Hypothesis: can N-acetylcysteine be beneficial in Parkinson's disease?. Life Sciences 1999; 64:1253-1257.
44. Ferreira FR, Biojone C, Joca SR, Guimarães FS: Antidepressant like effects of N-acetyl-L-cysteine in rats. Behavioral Pharmacology 2008; 19:747-750.
45. Berk M, Copolov DL, Dean O, Lu K, Jeavons S, Schapkaitz I, Anderson-Hunt M and Bush AI: N-acetyl cysteine for depressive symptoms in bipolar disorder a double-blind randomized placebo-controlled trial. Biological Psychiatry 2008; 64:468-475.
46. Reid MB, Stokic DS, Koch SM, Khawli FA and Leis AA: N-acetylcysteine inhibits muscle fatigue in humans. Journal of Clinical Investigation 1994; 94:2468-2474.
47. Jayalakshmi K, Sairam M, Singh SB, Sharma SK, Ilavazhagan G and Banerjee PK: Neuroprotective effect of N-acetyl cysteine on hypoxia-induced oxidative stress in primary hippocampal culture. Brain Research 2005; 1046:97-104.
48. Goncalves JF, Fiorenza AM, Spanevello, RM, Mazzanti CM, Bochi GV, Antes FG, Stefanello N, Rubin MA, Dressler VL, Morsch VM and Schetinger MRC: N-acetylcysteine prevents memory deficits, the decrease in acetylcholinesterase activity and oxidative stress in rats exposed to cadmium. Chemico-Biological Interaction 2010; 186:53-60.
49. Nehru B and Kanwar SS: N-acetylcysteine exposure on lead induced lipid peroxidative damage and oxidative defense system in brain regions of rats. Biological Trace Element Research 2004; 101:257-264.
50. Gokkusu C and Mostafazadeh T: Changes of oxidative stress in various tissues by long-term administration of Vitamin E in hypercholesterolemic rats. Clinica Chimica Acta 2003; 328:155-161.
51. Sevgi E, Semsi A, Serpil B and Erol C: The effect of NAC on brain tissue of rats fed with high cholesterol diet. Turkish Journal of Biochemistry 2008; 33:58-63.

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