**ABSTRACT**

Phenytoin is the mostly used drug for the seizures. However question regarding safety and efficacy of this drug make it particularly compelling to identify adjunct therapy that could boost therapeutic benefit. Study found that one adjunct therapy is beta carotene. The main aim of present study was to evaluate potentiation of antiepileptic activity of Phenytoin by beta carotene in mice against maximal electroshock induced (MES) induced convulsions. In these methods all the animals were divided into sixteen groups & each group consists of six animals. Shock 50mA for 0.2 sec was applied for all groups of mice. Testing drugs doses were randomised within groups of animal in such way that each group was required during a given test series. The results revealed that beta carotene significantly potentiated efficacy of phenytoin but did not exert antiepileptic effect on its own. Alone beta carotene at the dose of 20mg/kg p.o. significantly reduced elevated MDA level in serum. Concurrent administration of phenytoin and beta carotene on serum MDA activity was not differed with alone beta carotene at the dose of 20mg/kg. It is concluded that beta carotene potentiates the antiepileptic activity of phenytoin.

**KEYWORDS:** Phenytoin, Beta carotene, MES, Seizures.
INTRODUCTION

Epilepsy is one of the oldest recorded neurological disorders, with at least 3000 years of written history.\[^1\] It is the most common neurological condition, affecting 1-2\% of the population worldwide and can profoundly affect many aspects of quality of life.\[^2\] The incidence of epilepsy in developed countries is approximately 50 per 100,000 while that of developing countries is 100 per 100,000 affecting people of all ages, races, and social groups.\[^3\]

Epilepsy is the term used for a group of disorders characterized by recurrent spontaneous seizures and involves hyperexcitable neurons. It is assumed that there is an imbalance between inhibitory GABA-mediated and excitatory glutamate-mediated neurotransmission.\[^4\] It is commonly associated with the brain dysfunctions leading to several behavioral comorbidities.\[^5\]

The seizure activity during epilepsy decreases the antioxidant defense mechanism in the brain and increases the amount of free radicals, which further induces the oxidative stress. Free radicals (FR) can be defined as molecules or molecular fragments that contain one or more unpaired electrons.\[^6\] These free radicals were involved in causation of lipid peroxidation, brain edema and epilepsy, including coma and death.

Several antiepileptic medications are employed to manage epilepsy. However, they exhibit serious side effects, such as depression, ischemia, impaired cognition and motor disability. Additionally, 20–30\% of those afflicted have seizures that are resistant to treatment with the currently available antiepileptic drugs.\[^7\]

Oxidative stress is both the cause and the consequence of impaired functional homeostasis that characterizes human aging.\[^8\] The oxidative stress is the most prominent mechanism in the development and progression of epilepsy and other diseases, including Alzheimer’s disease, chronic degenerative diseases, stroke, rheumatoid arthritis, diabetes and cancer.\[^2\]

Reactive oxygen species (ROS) are spontaneously generated in cells during metabolism and are capable of causing oxidative damage when produced in excessive quantities.\[^16\] ROS have been implicated in the pathogenesis of a wide variety of human diseases\[^17\] that alter the function of genetic apparatus and can oxidize and damage nucleic acids, proteins and lipids, which are the major components of cell membranes\[^15\] and also, lead to cell death.\[^18\]
Oxidative stress is caused by excessive production of reactive oxygen (ROS) species such as hydroxyl radical (HO’), superoxide anion radical (‘), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), peroxyl radicals (HOO’) and high amounts of nitric oxide (NO’) and its derivative reactive nitrogen species (RNS).\cite{2}

Peroxidation of membrane polyunsaturated fatty acids produces toxic malondialdehyde (MDA), which compromises membrane lipid, matrix dynamics and results in the progression of seizures.\cite{22} A significant increase in MDA levels and a decrease in GSH levels indicate antioxidant-oxidant imbalance.\cite{26}

Antioxidants play a major role in protection against molecular oxidative damage and may have therapeutical relevance in neurodegenerative diseases.\cite{28}

The selection of an antiepileptic drug for treatment is predicated on its efficacy for the specific type of seizures, tolerability and safety. Though a large number of newer antiepileptic drugs (AEDs) are available, treatment is still not satisfactory.\cite{26}

Phenytoin, another standard antiepileptic drug, mainly produces its antiepileptic effect by blocking sodium channels which reduces cell excitability.\cite{22}

MES-induced convulsion model causes facilitation of Ca\textsuperscript{2+} and another positive ion like Na\textsuperscript{+} into the cells and the blockade of them can prevent MES-induced tonic extension. Potentiation of GABA receptor may offer protection against MES-induced seizures.\cite{39} Seizures are induced in rats by delivering electroshock of 150 mA for 0.2 s by means of a convulsiometer through a pair of ear clip electrodes. All established antiepileptic drugs have anticonvulsant activity in at least maximal electroshock seizure (MES) model.\cite{23}

MES-induced seizure can be prevented either by drugs that inhibit voltage-dependent Na\textsuperscript{+} channels such as phenytoin, valproate, felbamate and lamotrigine or by drugs that block glutamatergic receptor such as felbamate.\cite{31} Many neurotransmitter systems other than GABA, such as norepinephrine, dopamine and serotonin (5-HT), are related to the occurrence of electroshock seizure.\cite{32}
MATERIALS AND METHODS

Animals
Swiss Albino mice (20-30 g) were procured from Animal House, Anuradha College of Pharmacy, Chikhli (Dist- Buldhana). They were housed in polypropylene cages and fed on standard pellet diet and water ad libitum. Animal study protocol was approved by Institutional Animal Ethics committee of Anuradha College of Pharmacy, Chikhli (Dist- Buldhana).

Drugs
β-carotene (Dolphin chemicals, Mumbai) and Phenytoin (Ranbaxy, India), ammonium acetate, potassium dihydrogen phosphate, trichloroacetic acid( Merck), Thiobarbituric acid and butylatedhydroxytoluene were used for this study.

Maximal Electroshock (MES) Induced Convulsions
Animals were divided into sixteen groups and each group consist of six animals. They were removed from their home cage, weighed, numbered and treated with phenytoin and beta carotene 90 min. before applying current 50mA for 0.2 sec. Electroconvulsive shock (50 mA for 0.2 sec) was delivered through corneal electrode to induce convulsions to sixteen groups of mice. The various phases of convulsions which were produced like Flexion, Extension, Clonus and stupor. Prior to delivery, current output was checked by multimeter. After the electric stimulation occurrence, the duration of convulsions was noted for 10min.

Blood Collection
Blood was collected by venipuncture into EDTA from each mouse of each group. After centrifugation (3500 rpm for 10 min), plasma was taken as a sample.

Estimation of lipid peroxidase (MDA)
To 0.5 ml plasma, 2.5 ml of 20 mg/dl trichloroacetic acid was added and the tube was left to stand for 10 min at room temperature. After centrifugation at 3500 rev./min for 10 min, the supernatant was decanted and the precipitate was washed once with 0.05 mM sodium ACD. Then 2.5 ml of 0.05 M sulphuric acid and 3.0 ml of 0.2 mg/dl TBA in 2 M sodium sulfate are added to this precipitate and the coupling of lipid peroxide with TBA was carried out by the heating in a boiling water bath for 30 min. After cooling in cold water, the resulting chromogen was extracted with 4.0 ml of n-butyl alcohol by vigorous shaking. Separation of
the organic phase was faciliated by centrifugation at 3000 rev/min for 10 min and its absorbance was determined at the wavelength of 530 nm.

Statistical Analysis
All data were expressed as mean, ± SD and statistically analyzed by One Way ANOVA followed by dunnett’s test. P**< 0.01 were considered to be statistically significant.

RESULTS
Maximal electroshock (MES) induced convulsions
Treatment with beta carotene (5, 10, 20 mg/kg p.o.) in the absence of phenytoin did not significantly decrease duration of convulsions and mortality rate. Moreover when animals were treated with lowest dose of phenytoin (5mg/kg p. o.) either alone or in combination with beta carotene (5,10 and 20mg/kgp.o.) neither duration of convulsions nor mortality rate differed from control groups.

When animals were treated with a dose of 10 mg/kg of phenytoin, no significant decrease in duration of convulsions and protection of mortality rate was found. When this dose of phenytoin was combined with high dose of beta carotene (20mg/kg p.o.), animals showed a significant reduction in duration of convulsions and protection of mortality rate as compared to control groups (P<0.01) and as compared to 10mg/kg of phenytoin without subsequent beta carotene (P<0.01) and toxicant control group.

The highest dose of Phenytoin 20mg/kg p.o. significantly decrease in duration of convulsions and protection of mortality rate as compared to toxicant control group and all tested groups. Beta carotene did not significantly modify this effect at any dose tested when compared to control group. The results are given in table 1.

Effect of phenytoin and beta carotene on oxidative stress in seizure induced by MES
Treatment with phenytoin (5, 10,20 mg/kg p.o.) and beta carotene (5 & 10 mg/kg p.o.) and in combination of each other did not significantly reduce the MDA level in plasma but beta carotene at the dose of 20mg/kg p.o. could significantly reduce the MDA level in plasma as compared to toxicant control group. The results are given in table1.
Table Number:-1 Effect of phenytoin (5 mg/kg, 10mg/kg & 20mg/kg) and β carotene against MES induced convulsions and oxidative stress.

<table>
<thead>
<tr>
<th>Phenytoin (mg/kg)</th>
<th>β carotene (mg/kg)</th>
<th>Duration of convulsions (sec)</th>
<th>Mortality Rate (n=6)</th>
<th>MDA Level (nmol/ml)</th>
<th>Phenytoin (mg/kg)</th>
<th>β carotene (mg/kg)</th>
<th>Duration of convulsions (sec)</th>
<th>Mortality Rate (n=6)</th>
<th>MDA Level (nmol/ml)</th>
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<tr>
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<td>38±0.33</td>
<td>6</td>
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<td>0</td>
<td>36±0.29</td>
<td>6</td>
<td>5.5±0.33</td>
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</tr>
<tr>
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<td>5</td>
<td>32±0.28</td>
<td>6</td>
<td>5.3±0.47</td>
<td>5</td>
<td>29±0.46</td>
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<td>6</td>
<td>5.0±0.46</td>
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<td>29±0.43</td>
<td>6</td>
<td>4.9±0.47</td>
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</tr>
<tr>
<td></td>
<td>20</td>
<td>31±0.31</td>
<td>6</td>
<td>4.1±0.41**</td>
<td>20</td>
<td>28±0.23</td>
<td>6</td>
<td>4.0±0.52**</td>
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</tr>
<tr>
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<td>5.6±0.51</td>
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<tr>
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<td>20</td>
<td>nil**</td>
<td>0</td>
<td>4.1±0.52**</td>
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</table>

Values are mean ± SD (n=6). P** < 0.01 were considered to be statistically significant.
DISCUSSION

Epilepsy is a chronic disorder of central nervous system with a prevalence varying between 3 and 6/1000 of population it is a collective term for a group of chronic convulsive disorders.

Currently available anticonvulsant drugs like Phenytoin is able to efficiently control epileptic seizure in almost all patients but undesirable side effects from its therapeutic dose render treatment difficult so that a demand for reducing dose of these drugs by antioxidants may help reducing side effects.

The results of the present study indicate that potentiation of antiepileptic activity of phenytoin by antioxidants such as beta carotene against maximal electroshock (MES) induced epileptic seizure in mice. GABA is the major inhibitory neurotransmitter in the brain while glutamic acid is an excitatory neurotransmitter in the brain. The inhibition of GABA neurotransmitter and the enhancement of the action of glutamic acid have been shown to be the underlying factors in epilepsy. Our study shows that the phenytoin (10mg/kg) in combination with antioxidant such as beta carotene (20mg/kg) protected mortality rate against seizures induced by maximal electroshock and also decreased the duration of convulsions.

Maximal electroshock produced seizures in all the animals used. Antiepileptic drug like Phenytoin that block MES-induced tonic extension are known to act by blocking seizure spread. Moreover, MES induced tonic extension can be prevented by drugs (Phenytoin) that inhibit voltage-dependent Na+ channels, or by drugs (felbamate) that block glutaminergic excitation mediated by the N-methyl D-aspartate (NMDA) receptor. In the present study Phenytoin at the dose 5mg/kg p.o. and 10mg/kg p.o. and also antioxidants such as beta carotene at all doses did not significantly reduce duration of convulsions and protect mortality rate. But at the dose of 20mg/kg p.o of phenytoin could significantly reduce duration of convulsions and protect mortality rate as compared to toxicant control animals and Phenytoin treated animals at the dose of 5mg/kg and 10mg/kg p.o. in combination with beta carotene at all doses did not significantly reduce duration of convulsions and protect mortality rate. Phenytoin at the dose of 10mg/kg p.o. and in combination with beta carotene (20mg/kg) significantly reduced duration of convulsions and protected mortality rate. Effects seems as that of alone phenytoin 20mg/kg p.o. This shows antioxidants can be useful in reducing dose of phenytoin and thus its side effects.
Reactive oxygen species and particularly free radical induced lipid peroxidative tissue damage have been implicated in the pathogenesis of various diseases. Lipid peroxidation is assessed indirectly by the measurement of the secondary products such as malondialdehyde. MDA is a three – carbon low molecular weight aldehyde and spontaneous breakdown product of peroxides that can be produced from free radical attack on polyunsaturated fatty acids.[2] The analysis of MDA by the thiobarbituric acid assay has been widely employed over the many years in biological systems for the assessment of lipid peroxidation. It is a spectrophotometric assay, based upon heating of the sample under acidic conditions to form the adduct of MDA-TBA.[18]

Due to MES, the biochemical changes occur in the blood such as increase in the level of lipid peroxidation and reduction in glutathione level were reported earlier also.[4] The role of oxidative stress in electroconvulsive therapy–related effects is not well studied. A study has determined oxidative stress parameters in blood serum after a single electroconvulsive seizure or multiple electroconvulsive seizures to mice. In our MES study, After administration of phenytoin at the dose of 5, 10 and 20mg/kg p.o. and antioxidants such as beta carotene (5 & 10 mg/kg) and in combination of each other did not reduce MDA level as compared to toxicant control animals. But beta carotene (20 mg/kg p.o.) could significantly reduce MDA level. The biochemical changes which were measured in this study are in confirmation with the results obtained in the earlier reports.

CONCLUSION
In conclusion this study has demonstrated that phenytoin at the dose of 20 mg/kg p.o. has significant anticonvulsant action against MES induced seizure, but phenytoin at the dose of 5 mg/kg and 10 mg/kg p.o. and beta carotene (5 and 10 mg/kg) alone and in combination of each other could not significantly reduce duration of convulsions and protect mortality rate induced by MES, while phenytoin at the dose 10 mg/kg p.o. in combination with β carotene (20 mg/kg) significantly reduce duration of convulsions and protect mortality rate induced by MES as compared to phenytoin alone at the dose of 10 mg/kg. β carotene may be used as an add on therapy with phenytoin, may provide a greater effectiveness against epilepsy.

MES induced convulsions produced an increased lipid Peroxidation in plasma of the mice, Treatment with β carotene ( 20 mg/kg p.o.) significantly decreased plasma MDA activity, which is increased by MES, thereby suggesting that these drugs act positively on lipid peroxidation. Combination of β carotene with phenytoin at the dose of 10 mg/kg p.o. may be
promising for the treatment of epilepsy also the dose of phenytoin can be reduced. It is concluded that reduced dose of phenytoin may decrease the side effect of it also this dose is effective to control epilepsy and oxidative stress.

From the study, it may be speculated antioxidants potentiate antiepileptic activity of phenytoin by reducing oxidative stress (MDA level) in MES induced seizure. However, studies with other models of epilepsy and in combination with different conventional antiepileptic drugs on experimental animals and human beings would be needed with other antioxidants to substantiate the present work.

REFERENCES


