FETAL MALFORMATIONS AND OXIDATIVE STRESS IN WOMEN WITH EPILEPSY

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DECLARATION

I, Deepa D., hereby declare that I had personally carried out the work depicted in the thesis entitled "**Fetal malformations and oxidative stress in women with epilepsy**" under the direct supervision of **Dr. Sanjeev V. Thomas,** Professor, Department of Neurology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help sought and acknowledged.

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This is to certify that Mrs. Deepa D., Senior Research Fellow in the Department of Neurology of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled **"Fetal malformations and oxidative stress in women with epilepsy"** was carried out under my direct supervision.

Dr. Sanjeev V. Thomas

The Thesis Entitled

FETAL MALFORMATIONS AND OXIDATIVE STRESS IN WOMEN WITH EPILEPSY

Submitted By

DEEPA D.

For the Degree of Doctor of Philosophy

of

Sree Chitra Tirunal Institute for

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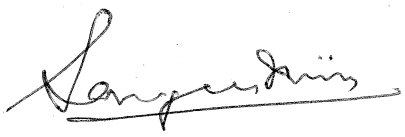
Evaluated and Approved By



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Deepa D.

To my husband, Baji

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ABBREVIATIONS

AED	Antiepileptic drug
AO	Antioxidants
CAT	Catalase
CLB	Clobazam
CLZ	Clonazepam
CBZ	Carbamazepine
CT	Computerised tomography
EDTA	Ethylene diamene tetra acetic acid
EEG	Electro encephalogram
FR	Free radical
GBP	Gabapentine
GE	Generalized epilepsy
GR	Glutathione reductase
GSH	Glutathione
ISP	Isoprostane
KREP	Kerala registry of epilepsy and pregnancy
LTG	Lamotrigine
LRE	Localisation related epilepsy
MDA	Malondialdehyde
MRI	Magnetic resonance imaging
OXB	Oxcarbazepine
PHT	Phenytoin
PB	Phenobarbitone
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RT	Room temperature.
SD	Standard deviation
SOD	Superoxide dismutase

TAO	Total antioxidant capacity
TPM	Topiramate
TGB	Tigabine
VPA	Valproate
WWE	Women with epilepsy

SYNOPSIS

FETAL MALFORMATIONS AND OXIDATIVE STRESS IN WOMEN WITH EPILEPSY

INTRODUCTION

Epilepsy affects an estimated 5 million people in India, and approximately 46% of them are women. It is estimated that there are over 2.5 million women with epilepsy (WWE) in India, with up to 52% of them being in the reproductive age group. 50% of the 80,000 WWE in Kerala come under the age group of of 20-50. The risk of birth defects in the offsprings is one of the major concerns for women with epilepsy and their family members. About 10% of offsprings of WWE have one or more major congenital malformations. Exposure to antiepileptic drugs (AEDs) during pregnancy is potentially harmful to a foetus. AEDs are the main stay of treatment and most WWE will require continued use of AEDs during pregnancy. If untreated, they may get frequent seizures, which could lead to more complications to the mother and her unborn child. Many AEDs, during their metabolism generate reactive metabolites eliciting systemic toxicity. Several AEDs, such as phenobarbitone (PB), phenytoin (PHT), carbamazepine (CBZ), valproic acid (VPA), lamotrigine (LTG) have been shown to have teratogenic potential in human and animal studies. The first step towards prevention of such malformations would be to ascertain the precise mechanism of teratogenesis. Though the different AEDs have different chemical composition, metabolism and mechanism of action, it had been observed that the morphological characteristics of malformations observed with these AEDs share

much in common and are often indistinguishable. This suggests that there exists a common pathway for the teratogenicity and the malformations are caused by a common mechanism. The list of suggested mechanisms for AED teratogenicity is long and diverse. AEDs are known to be metabolized to free radical (FR) intermediates. AEDs increases oxidative stress and excess oxidative stress may be one of the mechanisms that contribute to teratogenicity.

This study has therefore been designed with the following objectives.

OBJECTIVES

1) To compare the oxidative stress status and antioxidant profile of WWE (pregnant and non-pregnant) with that of healthy Controls (pregnant and non-pregnant).

2) To ascertain any association between the oxidative stress status and fetal outcomes in WWE (congenital malformations, abortions, neonatal death, intra-uterine death and other defects).

MATERIALS AND METHODS

This study was carried out in the Kerala Registry of Epilepsy and Pregnancy (KREP) in Sree Chitra Tirunal Institute for Medical Sciences and Technology. Women with active epilepsy on AEDs and good control of seizures were included in the study. Excluded from the study were those women with other concomitant disorders and who were taking antioxidants or other medications. Control group included age and socio-economic status matched healthy women without epilepsy and other disorders and not taking any medications. Clinical and demographic details about the patient were extracted from their medical records. The current status of all details required for the study gathered through personal interview. Dietary questionnaire was filled

up to know their antioxidant intake. Malformation of the baby was screened at the time of neonatal evaluation or at three months of age when they came for neonatal ultrasound and echo testing as part of Registry protocol. 5 ml random blood samples were drawn by venous puncture from all women.

Indicators of Oxidative stress measured are Malondialdehyde (MDA) and Isoprostanes (ISP). The total antioxidant capacity (TAO), Superoxide dismutase (SOD), catalase(CAT),glutathione reductase(GR) and Glutathione (GSH)were also measured as antioxidant profile.The concentration of MDA in serum was quantitated spectrophotometrically by Thiobarbituric acid (TBA) method. This technique is based on the capacity of reaction of TBA with MDA to form an adduct that absorbs light at 535 nm. A standard curve of MDA bis-(dimethylacetal) was used to estimate the concentration values in the sample . TAO, CAT, ISP and GSH content were measured in serum using commercial kits by Cayman chemical, USA. The GR activity in serum and SOD activity in whole blood were determined by using commercial kits of Randox Laboratories Ltd,UK.

STATISTICAL ANALYSIS

Data are expressed as mean (SD). Statistical comparison among different groups was performed using independent t test. The differences of $p< 0.05$ were considered significant.

RESULTS

Study group consisted of WWE taking antiepileptic drugs (n=79) which include pregnant (n=14) and non-pregnant (n=65) cases. Control group included normal healthy women(n=30) which included pregnant(n=10) and non pregnant (n=20)

subjects. In the group of epilepsy 32 women had fetal malformations and 17 women had abortions or still birth. In WWE (N=65; generalized epilepsy = 32, localization related epilepsy = 33) all subjects were on AEDs, 54 monotherapy and 11 on polytherapy.CBZ, PHT,VPA, PB are the most commonly used drugs .

Comparison of oxidative stress in different groups.

1.Normal Healthy Control group Vs epilepsy group.

Both indicators of oxidative stress Malondialdehyde (3.31±0.90 Vs 2.42±0.51) and Isoprostane (15.63±4.71 Vs 10.77±4.08) were significantly increased in WWE taking AEDs compared to controls. Their levels of SOD (147.73±42.24 Vs 175.81±42.61) and Glutathione (0.98±0.99vs 1.55±1.3) were significantly lower than those of controls. No changes are seen in TAO,GR &CAT.

a.Polytherapy:

Within the epilepsy group,WWE who were on polytherapy showed high oxidative stress when compared to monotherapy group indicated by the increased Malondialdehyde (3.89±0.91 Vs 3.22±0.87) level. No significant changes are seen in antioxidant profile.

b.Effect of different drugs:

WWE taking CBZ, PHT, PB and VPA showed significant increase in oxidative stress when compared to controls.When compared to controls, WWE exposed to CBZ showed significant reduction in SOD & GSH. PHTexposure showed significant reduction in SOD only. No significant changes are seen in antioxidant profile of WWE taking VPA and PB.

c.Type of epilepsy:

MDA levels or AO enzymes within the WWE did not show any correlation according to types of epilepsy-GE vs LRE.

2.Effect of Pregnancy

MDA levels were significantly elevated during pregnancy for the WWE (5.48±1.34 vs 3.31±0.90) as well as healthy controls (5.29±0.67 Vs 2.42±0.51). TAO (4.39±1.65 vs 2.43±0.92) was elevated and GR (29.11±5.70. vs 42.71±9.08) was significantly reduced in pregnancy for healthy women and no change in antioxidant profile is seen in WWE when compared with their non pregnant states.

3.Fetal malformation

The subgroup of WWE who had infants with congenital malformations had higher levels of MDA (3.48±0.87 vs 2.42±0.51) when compared to Controls. No significant changes are seen in antioxidant profile.

4.Abortions, still birth..

The subgroup of WWE who had abortion,stillbirth... showed high MDA (3.48±0.64 vs 2.42±0.51) and Isoprostane (17.77±2.96 Vs 10.77±4.08) levels . Their levels of SOD (136.65±34.85 Vs 175.81±42.61) and Glutathione (0.79±1.37 vs 1.55±1.3) were significantly lower than those of controls. No changes are seen in TAO,GR & CAT.

CONCLUSION

Women with epilepsy exposed to AEDs have higher oxidative stress and reduced antioxidant activity when compared to normal women. For WWE, exposure to polytherapy and exposure to certain specific AEDs was associated with increased oxidative stress. The AEDs particularly CBZ and PHT had altered the antioxidant profile in them. Pregnancy was associated with increased oxidative stress in both healthy women and WWE. Nevertheless,there was no increase in the total antioxidant capacity during pregnancy for WWE when compared to women without epilepsy.Unlike what is observed in the non-epilepsy groups,the respose to the increased demand of those exposed to AEDs is different from that of unexposed group.It appears that AEDs alter capacity to handle oxidative stress particularly during pregnancy. This may leads to teratogenicity. WWE had higher oxidative stress particularly the group with fetal malformations or abortions when compared to healthy women.

The observataions in this study point to a possible causal relationship between AED usage, elevated oxidative stress and fetal malformation or abortions. Oxidative stress can be one of the mechanisms for teratogenicity of AEDs.

Thus oxidative stress is found to be an important link between AED usage and fetal malformations in WWE. This opens an opportunity to modify oxidative stress and there by reduce the risk of malformation by the administration of antioxidants during pregnancy. Further studies are required to ascertain whether reduction of oxidative stress would prove the hypothesis.

Chapter 1

INTRODUCTION

Epilepsy is the most common serious neurological disorder. It is estimated that there are over 50 million people with epilepsy in the world and 5.5 million people with epilepsy live in India (Sridharan et al., 1999). It has a prevalence of about 0.5%, and a cumulative lifetime prevalence of 3% (Hauser et al., 1993). Epilepsy covers a range of different conditions with varying etiology. It is estimated that there are over 2.5 million women with epilepsy (WWE) in India (Sridharan et al., 1999), with up to 52% of them being in the reproductive age group. In Kerala state, 75% of the 80,000 WWE come under the age group of 10-50, and 50% come under the age group of 20-50 (Radhakrishnan et al., 2004). Exposure to antiepileptic drugs (AEDs) during pregnancy is potentially harmful to the fetus. The risk of birth defects in the offsprings is one of the major concerns for WWE and their family members.

1.1.Background of the study.

About 6 – 10% of the offsprings of WWE have one or more major congenital malformations, most probably due to the teratogenic effects of antiepileptic drugs that are used during pregnancy (Thomas et al., 2001). It has been reported that around eight lakhs babies with malformations are born annually in India due to diverse causes (Verma et al., 2002) and anti epileptic drugs contribute to a substantial proportion of them. Pharmacotherapy is the main stay of treatment of epilepsy. Most of the WWE will require continued use of AEDs during pregnancy. If treatment is discontinued, they are likely to get frequent seizures, which by itself could lead to more complications to the mother and her unborn child. Trauma is the leading cause of nonobstetric deaths in pregnant women with epilepsy, and the risks of maternal seizures to the fetus include intracranial hemorrhage, suppression of fetal heart rate, premature delivery, and miscarriage (Kimford et al., 2004).

Phenobarbitone (PB), phenytoin (PHT), carbamazepine (CBZ), valproic acid (VPA), lamotrigine (LTG), clobazam(CLB), clonazepam(CLZ) etc are the commonly used antiepileptic drugs and all of them have been shown to have teratogenic potential in studies in humans and other animals (Morrow et al., 2006, Meador et al., 2006., Finnel et al., 1991).

1.1.1.Similarity in different AED embryopathy

Phenobarbitone has been associated with the same major and minor abnormalities and dysmorphic features as with PHT. PHT exposure in utero has been associated with a pattern of abnormalities known as fetal hydantoin

syndrome (FHS), consisting of major anomalies such as cleft lip and palate and cardiac anomalies and pre and postnatal growth retardation (microcephaly and mental retardation) and minor anomalies such as midfacial hypoplasia with a snub nose, a broad nasal bridge, ocular hypertelorism, an arched upper lip, hypoplasia of distal phalanges and nails (Hansen et al., 1986, Dansky et al., 1991). Due to the large overlap in the pattern of teratogenicity between PHT and PB, the term "Hydantoin-Barbiturate Embryopathy" was proposed (Majewski et al.,1981). There is a striking similarity between the malformation pattern of CBZ and fetal hydantoin syndrome (Buehler, 1987). Due to a great deal of overlap between the drug-specific syndromes for different AEDs, a term "fetal antiepileptic drug syndrome"(FADS) has been proposed to more appropriately describe these malformations as opposed to a specific syndrome for each individual drug (Finnell et al., 1997).

1.1.2.Final common pathway

Though the different AEDs have different structure, chemical composition, metabolism and mechanism of action, it had been observed that the malformations observed with these AEDs share much in common and are often indistinguishable . This suggests that there exists a common pathway for the teratogenicity and the malformations are caused by a common mechanism (Vorhees, 1987, Danielsson et al.,1997).

Despite the widespread use of AEDs and knowledge of their teratogenicity, the precise mechanism by which the AEDs mediate malformations in fetus is uncertain. The list of suggested mechanisms for AED teratogenicity is long and

diverse. AEDs are known to be metabolized to free radical intermediates (Kubow et al., 1989). It had been shown in animal models that treatment with agents which reduce free radical formation reduces phenytoin induced teratogenicity and inhibition of enzymes which detoxify free radicals increases phenytoin induced teratogenicity (Wong et al., 1989). AEDs increase oxidative stress and excess oxidative stress may be one of the mechanisms that contribute to teratogenicity (Liu et al., 1994, Liu et al., 1995, Azarbayjani F., 2001).(figure 1)

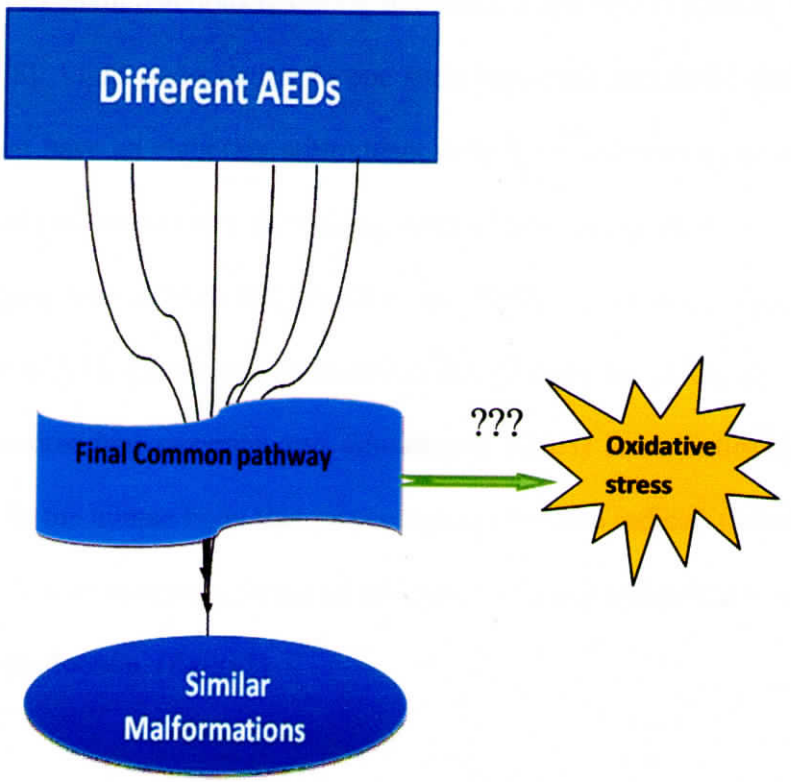


Figure 1. Similarity in different AED embryopathy

1.1.3.Oxidative stress and its role in teratogenesis

Oxidative stress is defined as the imbalance between free radical damage (e.g. the oxidation of lipids) and antioxidant protection. In women with epilepsy, AEDs, during their metabolism, produce toxic intermediate compounds like Arene oxides, Epoxides and Free radicals. Free radicals are atoms or molecules carrying odd number of electrons at outer atomic or molecular orbitals. Free radicals are unstable and highly reactive. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecules causing a cascade of chain reactions resulting in cellular damage and diseases (Valentine et al., 1998). Lipid peroxidation is one such important metabolic pathway. Living organism have an elaborate antioxidant defense mechanism consisting of many enzymes and antioxidant molecules. Antioxidants act at each of these steps for scavenging free radicals (Halliwell et al., 1997). Superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GP) are the major antioxidant enzymes and Glutathione (GSH) is an antioxidant molecule present in the human body that protect against the free radical toxicity. Oxidative stress is a consequence of reduced efficiency of these antioxidants or excess free radical production. (figure2)

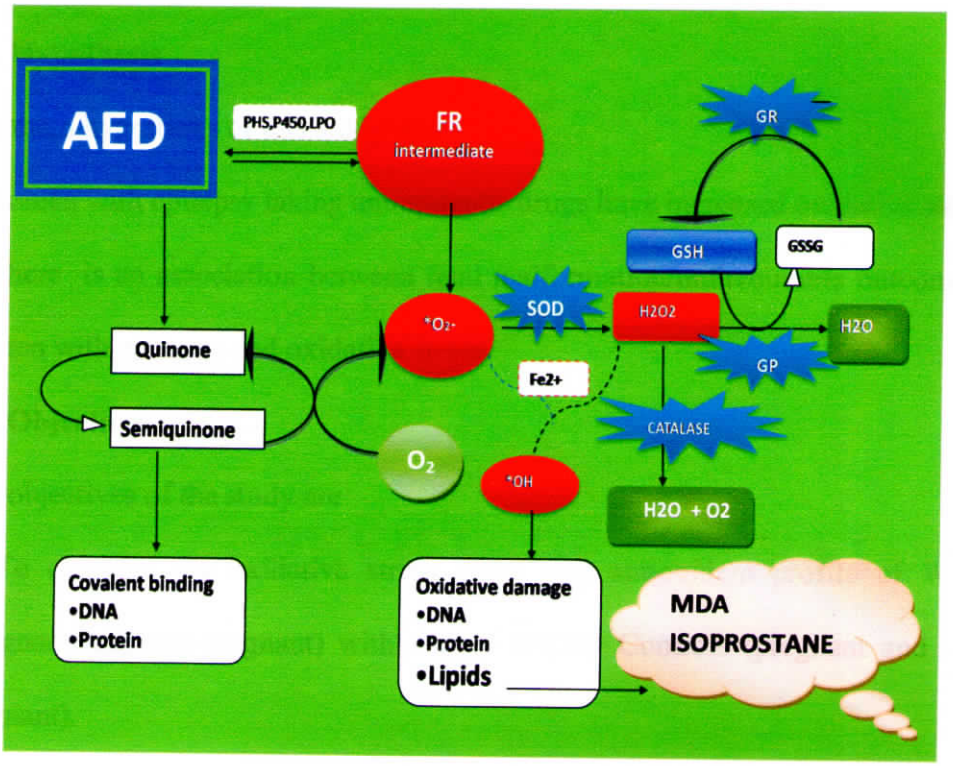


Figure 2. AED induced oxidative stress

1.2.Need and significance of the study.

There are a large number of women with epilepsy in India and about half of them belong to the reproductive age group. About 6-10% of children of women with epilepsy have malformations, most probably due to the teratogenicity of antiepileptic drugs that are used during pregnancy. The precise mechanism by which the antiepileptic drugs mediate malformations in the fetus is uncertain. The first step towards prevention of fetal malformations would be to ascertain the precise mechanism of teratogenesis. Oxidative stress is supposed to be one of the mechanisms leading to antiepileptic drug teratogenicity. This study was therefore been designed with the objectives to prove the following hypothesis.

1.3. Hypothesis

The main hypothesis of this study was

1.Women with epilepsy taking antiepileptic drugs have increased oxidative stress.

2. There is an association between fetal malformation/unfavourable outcome in women with epilepsy and oxidative stress.

1.4. Objectives

The objectives of the study are

1) To compare the oxidative stress status and antioxidant profile of WWE (pregnant and non-pregnant) with that of healthy Controls (pregnant and non-pregnant).

2) To ascertain any association between the oxidative stress status and fetal outcomes in WWE (congenital malformations, abortions, neonatal death, intra-uterine death and other defects).

Chapter2

REVIEW OF LITERATURE

2.1.Epilepsy

The word epilepsy is derived from the Greek verb- epilamvanein ("to be seized, to be taken hold of, to be attached"). The terms convulsive disorder, seizure disorder and cerebral seizures are synonymous with epilepsy; they all refer to recurrent paroxysmal episodes of brain dysfunction manifested by stereotyped alterations in behaviour. Epilepsy is a neurological condition characterized by recurrent seizures, which are clinical manifestations of abnormal electrical discharges in the cerebral cortex. According to Indian literature the term for epilepsy is 'Apasmara' which referes to loss of memory or loss of consciousness. The ancient physicians belonging to Ayurvedic tradition were probably aware of the core dysfunction in epilepsy viz. alteration in sensorium. Epilepsy is not a homogenous entity, but may vary widely in its forms, aetiology and severity. An epileptic seizure (a fit) is caused by a transient, excessive and abnormal discharge

of nerve cells. Seizures are sudden, transitory, and uncontrolled episodes of brain dysfunction resulting from abnormal discharge of neuronal cells with associated motor, sensory or behavioural changes. The abnormal discharges may involve a small part of the brain only (a partial or focal seizure) or much more extensive area in both hemisepheres (generalized seizures). The symptoms of an epileptic seizure reflect the activation of the part of the brain affectd by this excessive activity. The abnormal discharges may vary in site, extent and severity and this explains the wide diversity of clinical forms that a seizure might take (Shorvon SD., 1988).

Epilepsy can occur at any age but is most frequently seen in very young and the elderly. Epilepsy affects an estimated 5.5 million people in India, its point prevalence is about 5-10 cases per 1000 persons. The number of new cases of epilepsy each year would be close to half a million. In most countries worldwide, the prevalence of active epilepsy ranges from 4 to 10 per thousand population (Sridharan&Murthy, 1999, Radhakrishnan et al., 2000). The incidence of epilepsy ranges from 40 to 70 per 100,000 in most developed countries and from 100 to 190 per 100,000 in developing countries (Sridharan, 2002). The incidence of epilepsy is highest under the age of 2 and over the age of 65. The Greek philosopher Hippocrates (460-377 BC) was the first to recognize that epilepsy is a disorder of the brain. Many famous persons including Alexander the Great, Julius Caesar and Vincent Van Gogh, actor Danny glover and professional rugby player Dean Ryan have had epilepsy. Specific epileptic syndromes have been identified by their characteristic seizure types, pattern of seizure recurrence, age

of onset, associated neurologic and other clinical signs, EEG findings, presence or absence of familial occurrence and prognosis.

2.1.1.Classification of epileptic seizures:

Galen (AD 175)- classified epileptic seizure as idiopathic or symptomatic according to its origin. Tissot in the mid 18th century, not only classified epileptic seizure but distinguished between epileptic seizure and the underlying causative conditions that we now call epilepsies.The currently accepted classification was put forward by International League Against Epilepsy, ILAE (1981) and it is based on phenomenology and EEG Findings.

Table 1. International League Against Epilepsy, ILAE Classification of Epileptic Seizures	
I. Partial (focal) Seizures	
	A. Simple Partial Seizures(consciousness not impaired)
	B. Complex Partial Seizures(with impairment of consciousness)
	C. Partial seizures evolving to secondary generalized seizures.
II. Generalized Seizures	
	A. Absence seizures
	B. Myoclonic seizures
	C. Clonic seizures
	D. Tonic Seizures
	E. Tonic - clonic seizures
	F. Atonic seizures
III. Unclassified epileptic seizures	

2.1.2.Classification of the epilepsies and epilepsy syndromes.

The diagnosis of epilepsy is based on not only the main seizure type, but also on the probable etiology, age of onset, neurological signs and investigation results. Many types of epilepsy are combinations of different kinds of seizures. In an attempt to encompass a broader range of clinical features than is possible in a classification of seizure type, the ILAE published in 1985, and revised in 1989 a Classification of Epilepsies and Epileptic Syndromes. An epileptic syndrome is defined as an epileptic disorder characterized by a cluster of signs and symptoms customarily occurring together.

Epilepsies are classified into two major categories based upon presumed site of origin as Localisaton Related Epilepsy (LRE) or Generalized epilepsy (GE). LRE is the tendency to have recurrent partial seizure (with or without secondary generalization). About 80% of adult onset and 60% of childhood epilepsies are localization related. Generalised epilepsies are tendency to have recurrent generalized seizure.

Epilepsies are divided into those with a non specific aetiology and those with a specific identifiable cause. On the basis of presumed etiology that is whether the cause is known or unknown syndromes are further divided into 3 subgroups : primary or idiopathic, secondary or symptomatic, cryptogenic.

The International League Against Epilepsy, ILAE Classification of Epilepsies and Epileptic syndromes is given in table 2.

Table2. The International League Against Epilepsy, ILAE Classification of Epilepsies and Epileptic syndromes.	
1.Generalized Epilepsies and syndromes	
Idiopathic	Benign neonatal familial convulsions Benign myoclonic epilepsy in infancy
	Childhood absence epilepsy
	Juvenile absence epilepsy
	Juvenile myoclonic epilepsy
	Epilepsy with generalized tonic clonic seizures on awakening.
Cryptogenic or symptomatic	West's syndrome
	Lennox-Gastaut syndrome
	Epilepsy with myoclonic-astatic seizures
Symptomatic	Early myoclonic encephalopathies
	Early infantile encephalopathy with burst suppression
2.Localization related Epilepsies and syndromes	
Idiopathic	Benign childhood epilepsy with centrotemporal spikes
	Childhood epilepsy with occipital paroxysms.
Symptomatic	Epilepsia partialis continua
Cryptogenic	Symptomatic focal epilepsy with unknown aetiology
	Special syndromes-situation-related syndromes
3.Epilepsies and syndromes undetermined whether focal or generalized	
	Neonatal seizures
	Severe myoclonic epilepsy in infancy
	Epilepsy with continuous spike-waves during slow wave sleep
	Acquired epileptic aphasia(Landau-Kleffner syndrome)
4.Special syndromes	
	Febrile convulsions
	Isolated seizures or isolated status epilpeticus
	Seizures occuring only when there is an acute metabolic or toxic event due to factors such as alcohol, drugs, eclampsia, non-ketotic hypergylcinaemia.

Idiopathic epilepsies are mostly of genetic etiology. They are characterized by normal intelligence, age-specific onset of epilepsy, no obvious structural abnormalities of the brain and a normal background activity of the EEG. They have characteristic epileptiform discharges in the EEG. Idiopathic epilepsies are generally benign in the sense that they are not associated with brain lesions, neurologic abnormalities other than seizures or mental impairment and that they tend to be self-limited or respond readily to AEDs. Genetic factors are important, and manifestations are typically age related.

Symptomatic epilepsies have a physical cause, for example, injury to the brain at birth, head trauma, brain tumor, or metabolic disorder. The term remote symptomatic epilepsy is applied to epilepsies that result from long standing static lesion such.

Cryptogenic epilepsies refer to LRE or GE in which no underlying cause has been identified yet, a microscopic structural cause is suspected. When epilepsies are presumably symptomatic but currently of unknown specific etiology, they are termed cryptogenic.

2.1.2.1.Unclassified epilepsy

In clinical practice, about one third of cases are unclassifiable, even after a detailed account from the patient and witness.

2.1.2.2.Epidemiological classification of epilepsy:

From Epidemiological perspective, epilepsy is classified in to active and inactive. Person are classified to have active epilepsy, if they had atleast one seizure in the

past five years. Inactive epilepsy (epilepsy in remission) refer to persons with epilepsy who did not have any seizures in the past five years.

2.1.2.3.Status epilepticus

It is a condition in which epileptic seizures continue, or are repeated without regaining consciousness, for a period of 30 minutes or more. This is the maximal expression of epilepsy, and often requires emergency therapy. There are physiological and neurochemical changes which distinguish status eplepticus from ordinary seizures.

2.1.3.Seizure precipitants

Are those circumstances that precede the onset of an epileptic attack and are considered by both patient and neurologists to be a possible explanation for why seizure happened, when it did happen or not earlier. These include seizure inducing factors which are of environmental or endogenous origin and produces transient lowering of the seizure threshold . Seizure precipitating or triggering factors involve chemical or physiologic stimulation capable of precipitating a seizure. Common seizure precipitants include sleep depravation, sudden awakening, fatigue and exercise, alcohol, missed AEDs, drugs lowering seizure threshold, metabolic factors, hyperventilation and fever (Shorvon SD., 2000).

2.1.4.Epileptogenesis

Most, if not all, forms of epilepsy develop over a defined time period. That is, at some point of time, the brain functions normally but either after a specific development sequence or injury. A new state develops in which the neuronal

circuits become hyperexcitable, leading to spontaneous recurrent seizures. This process is termed epileptogenesis.

There are two essential physiologic elements each represent the net effect of many complex interacting processes. The first is an abnormality of cellular excitability, which might be termed 'neuronal deregulation', arising from mechanisms that affect membrane depolarisation and repolarisation. Second is a network defect which derives from mechanisms underlying the development of aberrant neuronal integration, abnormal synchronizations of neuronal populations and propagation of the epileptic discharges within neural pathways.

Different brain areas are responsible for different aspects of epileptic phenomenology. The irritative zone is the area of the cortex that generates interictal EEG spikes. Ictal onset zone is the area of cortex that initiates seizures. Epileptogenic lesion is the pathologic substrate of the epilepsy. Seizures can arise within, adjacent to or even sometimes distant from an epileptogenic lesion. Symptomatogenic zone is that portion of the brain responsible for producing the first clinical ictal symptoms or signs. Functional deficit zone is the cortical area or areas exhibiting focal non-epileptic dysfunction. Epileptogenic zone is the total area of brain that is necessary and sufficient to generate seizures and that must be removed to abolish seizures.

2.1.4.1.Cell signalling mechanisms:

The epilepsies are a complex group of disorders whose common features is a tendency for hyperexcitability to develop in one or another region of the Central nervous system. A difference in electrical potential exists across the plasma

membrane that surrounds all cells. When stimulated, excitable cells are able to produce action potentials which are the brief reversals of the electrical potential across their plasma membranes. Neurons, cardiac and smooth muscle cells, some endocrine cells-beta cells of pancreas, plasma membrane of oocytes(for a brief time) are excitable cells. The transmembrane potential that exists across an excitable cell when in an unstimulated state is called the resting potential.

2.1.4.2.Control of neuronal excitability

The behaviours of nerve cells is determined by receptor and voltage gated ionic channels and by more or less permanently open leak channels. Resting potentials arise because of the difference in the concentrations of ions between the inside and outside of the cell and because the cell membrane has different permeabilities for these ions.

Ions can flow through the channels in an inward or outward direction. An inward current depolarises the membrane and is carried either by movement of positive charges into the cells or negative charges outside the cell. Outward current drives the membrane potential in hyperpolarizing direction and are carried either by cation currents out of or anion currents into the cell. Leak and appropriately voltage gated conductance determines the resting membrane potentials of the cell, a state in which inward and outward currents balance each other. Receptor gated ionic channels mediate the information traffic between cells. Voltage gated channels regulate the membrane potential, influence the integrating properties of the dendrites and the discharge mode of a cell and are responsible for the generation and propagation of action potentials at the presynaptic terminals.

They influence calcium loading of the terminal which is a pre requisite for transmitter release. For such currents to flow an electrochemical gradient must be provided which depends on the transport processes across the neuronal membrane.

The extracellular fluid that bathes cells is essentially a dilute solution of sodium chloride (Na+Cl-). The intracellular solution, in contrast, has quite a high concentraion of potassium ions (K+) that are balanced by a variety of anions to which the cell membrane is completely impermeable. These include organic acids, sulfates, phosphates, some aminoacids and some proteins. The cell membrane is permeable to K+. Basis of the resting potential is due to the passive efflux of K+ from the cell, down its electro chemical gradient. Differences in ion concentration are maintained by the sodium potassium ATPase pump which pumps sodium(Na+) out and K+ into the cell. (Figure 3)

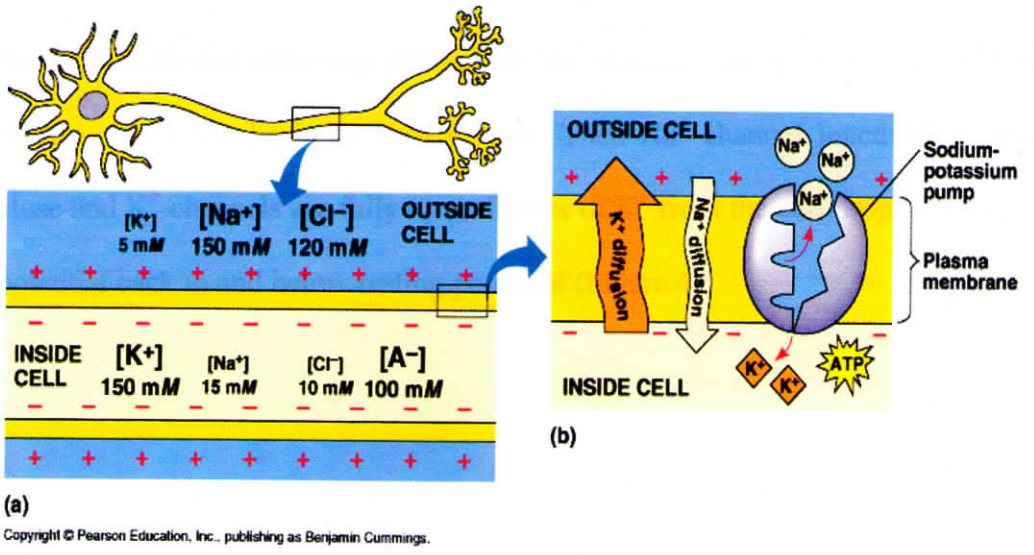


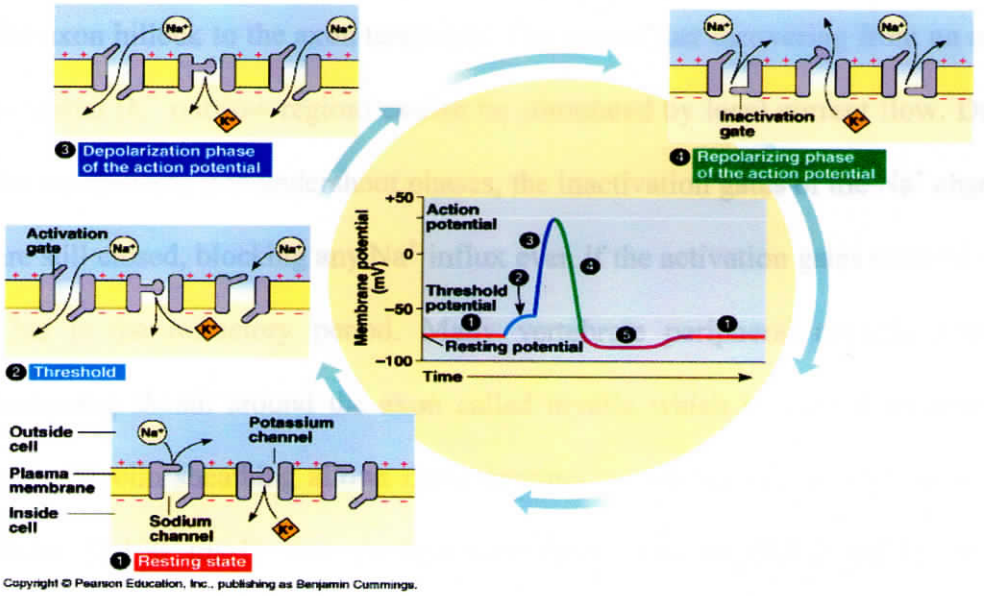
Figure 3. Ionic concentraions across neuronal membranes and sodium potassium ATP ase.

Sodium potassium ATPase transport three Na+ out of the cell in exchange for only two K+ ions. Hence these ATPase imposes a hyperpolarizing drive on the membrane potential and there by drives the membrane potential in a negative direction.

2.1.4.3.Mechanism of seizure genesis

Most neurons have membrane potential more positive than –90 mV, this is because channels permeable to Na + and Cl- are opened under resting conditions. A stimulus opens activation gate of some Na^+ channels depolarizing membrane potential. A depolarizing potential that reaches a critical level called the threshold potential (or firing threshold) will trigger an action potential or nerve impulse. If threshold potential is reached, more Na^+ channels open, triggering an Action potential. Unlike the graded potentials that get larger with increasing stimulus strength, the action potential is an all-or-none response. Action potential size is independent of the stimulus and reaches a value of about +40 mV. Above threshold potential activation gates of all Na^+ channels are open. K^+ channels are mostly closed but begin to slowly open. Then Na^+ channel inactivation gates close and K^+ channels are fully open. Efflux of K^+ from the cell drops membrane potential back to and below resting potential (Figure 4).

Figure 4.The Action potential.



During a seizure, resulting from activation of Na+ and K+ channels, Na+ accumulates with in the neurons and K+ in the extracellular space. This leads to activation of the electrogenic sodium pump and thence to a hyperpolarising drive. This drive contributes to the termination of seizures and is responsible for after hyperpolarization that follows a single seizure. When sodium pump lose their efficacy, after hyperpolarisation becomes smaller and this loss may underlie episodes of status epilepticus.

2.1.4.4..Propagation of the Action Potential

The action potential is regenerated all along the axon like a series of relay stations. Localized flow of current from the region undergoing an action potential depolarizes the adjacent membrane. Voltage gated Na^+ channels in the adjacent membrane respond by opening their activation gates. A new action potential is triggered in the adjacent membrane. This sequence is repeated down the length of the axon. Action potentials do not decay in strength as they are conducted down

the axon. Propagation of the action potential only moves in one direction, from the axon hillock to the axon terminals. The region just recovering from an action potential (K^+ outflow region) cannot be stimulated by local current flow. During the repolarizing and undershoot phases, the inactivation gates of the Na^+ channels are still closed, blocking any Na^+ influx even if the activation gates were to open. This is the refractory period. Many vertebrate peripheral neurons have an insulating sheath around the axon called myelin which is formed by Schwann cells. Myelin sheathing allows these neurons to conduct action potentials much faster (7-100 ms-1) than in non-myelinated neurons (0.5-2 ms-1). Myelin sheathing is interrupted by bare patches of axon called nodes of Ranvier where ion channels are concentrated. Action potentials jump from node to node without depolarizing the region under the myelin sheath called saltatory conduction. Myelin sheathing improves the ability of electrical charge to flow far enough down the axon to reach the next node. Axon terminals contain small storage sacs called synaptic vesicles. Vesicles contain neurotransmitter molecules. Information is transmitted from the presynaptic neuron to the postsynaptic cell. Chemical neurotransmitters cross the synapse, from the terminal to the dendrite or soma. The synapse is very narrow, so transmission is fast. An action potential causes voltage-gated calcium (Ca^{2+}) channels to open; Ca^{2+} ions flood in. Ca^{2+} causes vesicle membrane to fuse with presynaptic membrane. Vesicle contents empty into cleft by exocytosis releasing neurotransmitters from the presynaptic membrane. Neurotransmitter diffuses across synaptic cleft. They bind to receptors within the postsynaptic membrane, altering the membrane potential. Depending on the type of ion channel which opens, the postsynaptic cell

membrane becomes either depolarized or hyperpolarized. Ions will tend to follow the concentration gradient from high to low concentration, and the electrostatic gradient towards the opposite charge. Opening of ion channels which leads to depolarization makes an action potential more likely, hence "excitatory Post synaptic potentials"EPSPs. Opening of ion channels which leads to hyperpolarization makes an action potential less likely, hence "inhibitory post synaptic potentials"IPSPs.

Two types of transmission occur in Nervous system –electrical and chemical. Electrical transmisson synapses, which are gap junctions between adjuscent neurons. Gap junctions are arrays of paired hexameric ion channels called connexons. Chemical transmission contributes vast majority.

2.1.5.Neurotransmitters

Aminoacids (glutamate, aspartate, gama amino butyrate-GABA, glycine), monoamines/ catecholamines (acetyl choline, dopamine, norepinephrine, epinephrine, serotonin), peptides, opioids (dynorphins, endorphins, enkephalins), tachykinins, hormmones (cholecystokinin, somatostatin)

2.1.5.1.Fast Neurotransmission

Two excitatory amino acids that are thought to be the most likely neurotransmitter candidates are L-glutamate and L-aspartate. Both compounds act as agonists at all subtypes of ionotropic glutamate receptors. Alpha amino 3 hydroxy 5 methyl 4 isoxazole propionate receptor- AMPA/Kainate receptors mediate most fast glutamate neurotransmission. These are ligand gated ion channels, transmembrane proteins, with an intrinsic ion channel and recognition

site for glutamate on the extracellular face that projects in to the synaptic cleft. Binding of glutamate to this site causes the protein to change shape, opening the ion channel. The channel is selective for both Na+ and K+. The movement of these ions down their respective concentration gradients simultaneously through several hundred glutamate receptors causes the excitatory post synaptic potential. The duration of excitatory post synaptic potentials in the Central Nervous System is largely regulated by a fast desensitisation, whereas the duration of inhibitory post-synaptic potentials is regulated by GABA uptake into presynaptic terminals and glia.

The most important inhibitory ligands are Gama aminobutyrate -GABA and glycine; they open a Cl- permeable channel. Most interneurons use GABA as do many motor pathways with in the brain. This produces inhibitory post synaptic potential by hyperpolarization which carries the membrane potential away from the threshold for firing action potentials. In response to the release of GABA, the permeability of Cl- increases. When the membrane potential of the neuron is more positive than the reversal potential Cl- enters the cell, making it more negative inside, ie, the cell hyperpolarizes. GABA-A recetors are responsible for all fast GABA transmission.

2.1.5.2.Slow Neurotransmission

It is mediated by G-protein linked receptors. Binding of ligand to receptor causes liberation of a guanosine 5'-triphosphate-GTP bound form of G protein which activates its targets. There are several different forms of G protein families with their own particular targets. Gs proteins activate adenylyl cyclase, which converts

ATP to cyclic AMP, a second messenger molecule. This activates protein kinase A which phosphorylates its target protiens. Gi proteins inhibit adenylyl cyclase. Gq proteins activate phospholipase C which cleaves a membrane phospholipid to generate two second messenger molecules- diacylglycerol(DAG) which activates protein kinase C and inositol triphosphate (IP3) which mobilizes calcium from internal stores to increase the cytoplasmic Ca2+ concentration, which activates calcium dependent protein kinases. Effects are slow and account for slow depolarisations or hyperpolarsations. Activation of these receptors often produces long lasting effects in that phosphorylation of an ion channel persists until the channel is used. The activation of these receptors leads to different cellular effects that can be either excitatory or inhibitory. This depends on the type of G protein activated, the intracellular second messenger and the target in a given cell as well as in a given neuronal network. (Smith PEM et al., 2002, Engel J et al., 2002, Longstaff., 2002)

2.1.6.Experimental models of epilepsy

Animal models for seizures and epilepsy have played a fundamental role in our understanding of the physiological and behavioural changes associated with human epilepsy. A diversity of animal models are available for the study of epilepsy and these models have a proven history in advancing our understanding of basic mechanisms underlying epileptogenesis and have been instrumental in the screening of novel antiepileptic drugs. In vivo animal models have been categorized into models of seizures and those of epilepsy. Since human epilepsy is defined by the appearance of multiple spontaneous recurrent seizures, induction of acute seizure activity alone without chronic epileptiform behavior is

considered a model for seizures and not epilepsy (Engel J., 1992). Kindling is a phenomenon whereby repetitive, focal application of initially subconvulsive electrical stimulation ultimately results in intense partial and generalized convulsive seizures (Sarkisian., 2001).

2.2.Antiepileptic drugs (AEDs)

Antiepileptic drugs remain the mainstay of treatment of epilepsy. With the appropriate treatment, more than 70% of people could remain seizure free. Surgery may help a small number of people with epilepsy whose seizures do not respond to medication.

Table 3 shows the history of antiepileptic drug therapy. .

Table 3.History of Antiepileptic Drug Therapy	
Bromides	1857
Phenobarbital	1912
Phenytoin	1940
Primidone	1954
Ethosuximide	1958
Carbamazepine	1974
Clonazepam	1975
Valproate	1978
Felbamate, Gabapentine	1993
Lamotrigine	1995
Topiramate,Tiagabine	1997
Leveteracetam	1999
Oxcarbazepine, Zonizamide	2000

2.2.1.Mechanism of action of AEDs.

The AEDs can be grouped according to their main mechanism of action, although many of them have several actions and others have unknown mechanisms of action. The main groups include sodium channel blockers, calcium current

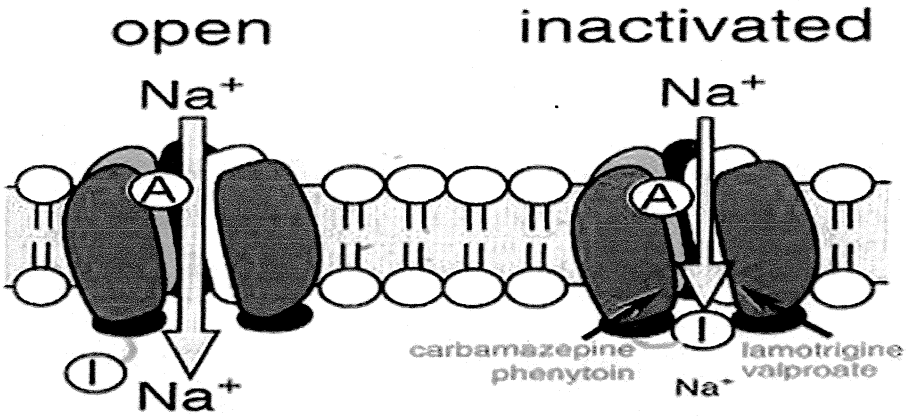
inhibitors, gamma-aminobutyric acid (GABA) enhancers, glutamate blockers, carbonic anhydrase inhibitors, hormones, and drugs with unknown mechanisms of action.

2.2.1.1.Sodium currents/channels

During an action potential, these channels exist in the active state and allow influx of sodium ions. Once the activation or stimulus is terminated, a percentage of these sodium channels become inactive for a period of time known as the refractory period. With constant stimulus or rapid firing, many of these channels exist in the inactive state, rendering the axon incapable of propagating the action potential. AEDs that target these sodium channels prevent the return of these channels to the active state by stabilizing the inactive form of these channels. In doing so, repetitive firing of the axons is prevented.(figure5)

Eg-Carbamazepine, Phenytoin, Oxcarbazepine, Lamotrigine, Zonisamide.

Figure 5. Antiepileptic drug-enhanced sodium channel inactivation.



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Some antiseizure drugs prolong the inactivation of the Na+ channels, thereby reducing the ability of neurons to fire at high frequencies.

2.2.1.2.Calcium channels

Calcium channels exist in 3 known forms in the human brain: L, N, and T. These channels are small and are inactivated quickly. The influx of calcium currents in the resting state produces a partial depolarization of the membrane, facilitating the development of an action potential after rapid depolarization of the cell. They function as the "pacemakers" of normal rhythmic brain activity. This is true particularly of the thalamus. T-calcium channels have been known to play a role in the 3 per second spike-and-wave discharges of absence seizures. AEDs that inhibit these T-calcium channels are particularly useful for controlling absence seizures.(figure 6) Eg- ethosuximide, dimethadione,valproate

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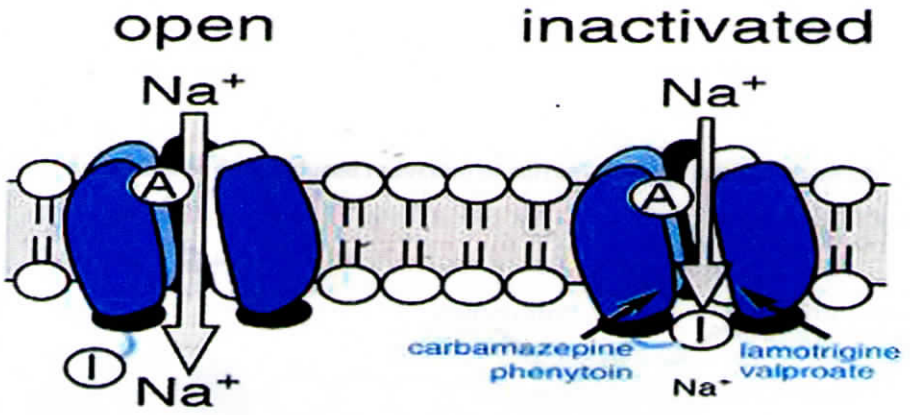

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Figure 6. Antiepileptic drug induced reduction of current through calcium channels

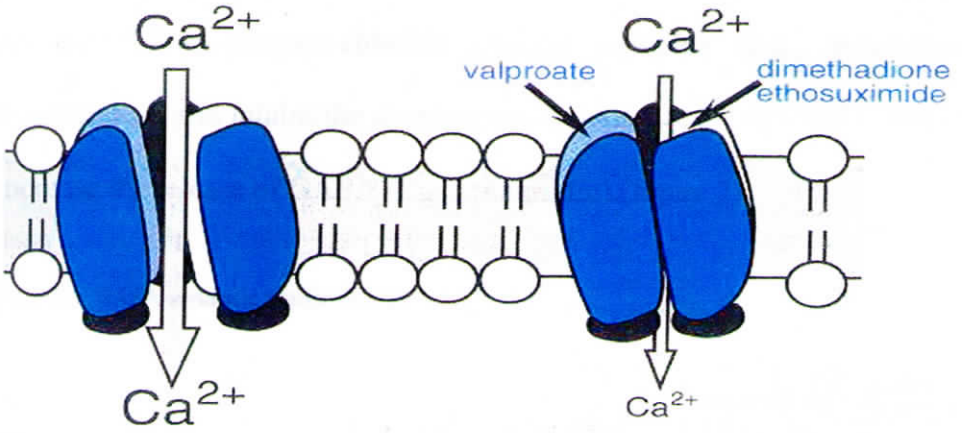


Figure 6 downloaded from
www.pharmacy.utah.edu/medchem/.../**anticonvulsants_2006.ppt**

Some antiepilepticdrugs reduce the flow of Ca2+ through T-type Ca2+ channels, thus reducing the pacemaker current that underlines the thalamic rhythm in spikes and waves seen in generalized absence seizures.

2.2.1.3.GABA-A receptors/channels

When GABA binds to a GABA-A receptor, the passage of chloride, a negatively charged ion, into the cell is facilitated via chloride channels. This influx of chloride increases the negativity of the cell (ie, a more negative resting membrane potential). This causes the cell to have greater difficulty reaching the action potential. GABA is produced by decarboxylation of glutamate mediated by the enzyme glutamic acid decarboxylase (GAD). Some drugs may act as modulators of this enzyme, enhancing the production of GABA and down-regulating glutamate.

Some AEDs function as an agonist to this mode of chloride conductance by blocking the reuptake of GABA (ie, tiagabine) or by inhibiting its metabolism mediated by GABA transaminase (ie, vigabatrin), resulting in increased accumulation of GABA at the postsynaptic receptors. The drug may act directly on the GABA-receptor-chloride channel complex (e.g., benzodiazepines, barbiturates), and inhibit the metabolism of GABA (e.g., vigabatrin, valproate) or increase the release of GABA (e.g., gabapentin).(figure 7)

Figure7. GABA synaptic transmission

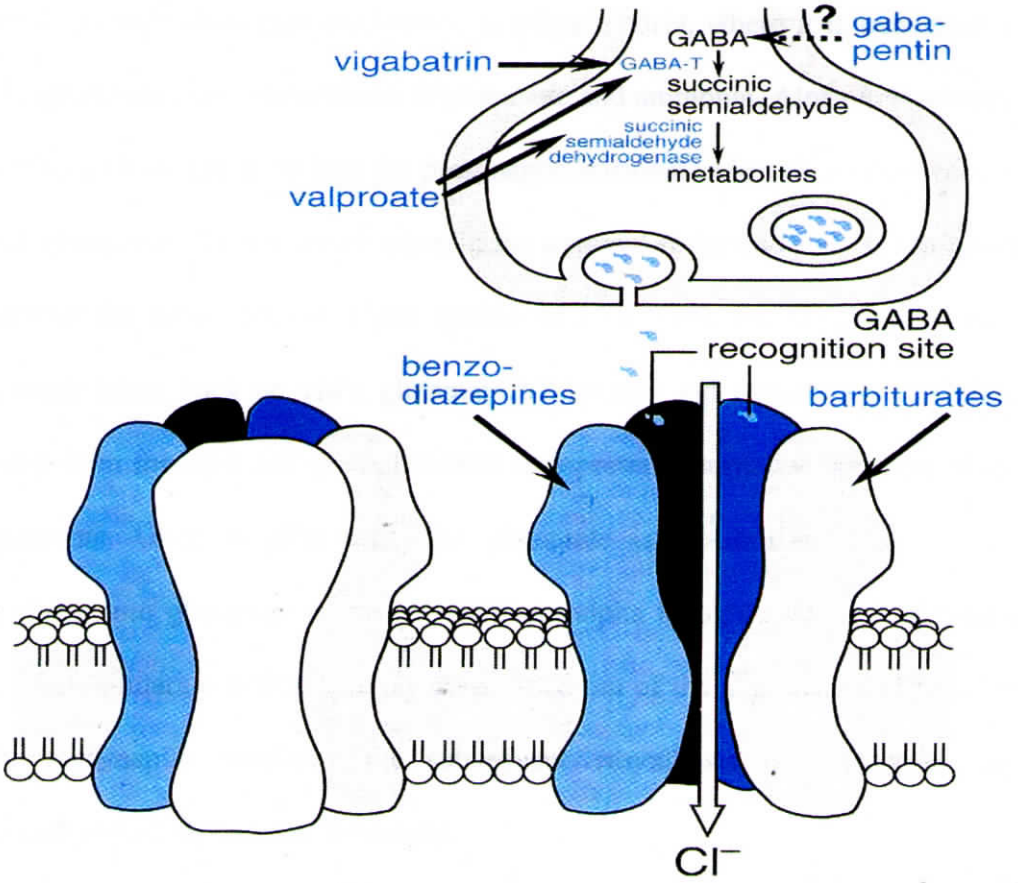


Figure 7. downloaded from
www.pharmacy.utah.edu/medchem/.../**anticonvulsants_2006.ppt**

2.2.1.4.Glutamate receptors

Glutamate receptors bind glutamate, an excitatory amino acid neurotransmitter. Upon binding glutamate, the receptors facilitate the flow of both sodium and calcium ions into the cell, while potassium ions flow out of the cell, resulting in excitation. AEDs that modify these receptors are antagonistic to glutamate .

Glutamine and alpha-ketoglutarate are major precursors for glutamate which is subsequently packaged into vesicles for future release into the synaptic cleft. Glutamine is taken up into the presynaptic terminal via an active, Na dependent uptake protein. It is then transported to mitochondria, where it is converted via phosphate-activated glutaminase to glutamate and ammonia. Alpha keto-glutarate is also actively taken up into the presynaptic terminal, where it is transaminated into glutamate. The glutamate anion in the terminal is then actively taken up into vesicles for future release. Upon release into the cleft, the glutamate either is actively taken back up via a glutamate transporter and repackaged or diffuses away form the cleft and glial glutamate transporters internalize the extracellular glutamate. Once in glial cells, the glutamate is metabolised via glutamine synthase into glutamine or metabolised into alpha ketoglutaate. This glutamine and keto glutarate is then actively transported out of the glial cells and back into the presynaptic terminals for subsequent resynthesis of glutamate. e.g., phenobarbital, topiramate, felbamate.

2.2.1.5.Carbonic anhydrase inhibition

Inhibition of the enzyme carbonic anhydrase increases the concentration of hydrogen ions intracellularly and decreases the pH. The potassium ions shift to

the extracellular compartment to buffer the acid-base status. This event results in hyperpolarization and an increase in seizure threshold of the cells. Acetazolamide has been used as an adjunctive therapy in refractory seizures with catamenial pattern (ie, seizure clustering around menstrual period). Topiramate and zonisamide also are weak inhibitors of this enzyme; however, this is not believed to be an important mechanism for their antiseizure efficacy.

2.2.1.6.Sex hormones

Progesterone is a natural anticonvulsant that acts by increasing chloride conductance at GABA-A receptors and attenuates glutamate excitatory response. It also alters messenger RNA for glutamic acid decarboxylase, GAD and GABA-A receptor subunits. On the other hand, estrogen acts as a proconvulsant by reducing chloride conductance and acting as an agonist at NMDA receptors in the CA_1 region of the hippocampus.

2.2.2.AED Pharmacokinetics

Pharmacokinetics is the quantitative description of what happens to a drug when it enters the body, including drug absorption, distribution, metabolism and elimination / excretion.

2.2.2.1. Absorption

This is determined by route of intake. Most AEDs are available for oral administration, although some have formulations that are also available for intravenous, intramuscular or rectal administration.

Oral Absorption: Most AEDs undergo complete or nearly complete absorption when given orally. Most often, administration of AEDs with food slows

absorption and can help avert peak dose related side effects. Calcium containing antacids may interfere with phenytoin absorption. Gabapentin is absorbed by a saturable amino acid transport system and does not get absorbed after a certain dose.

Intramuscular Administration: Fosphenytoin may be administered intramuscularly if intravenous access cannot be established in cases of frequent repetitive seizures.

Rectal administration: Diazepam (available as a rectal gel) has been shown to terminate repetitive seizures and can be administered by family members at home.

Intravenous administration: This route is used for emergencies. Phenytoin, fosphenytoin, phenobarbital,diazepam, lorazepam and valproic acid are available as IV preparations .

2.2.2.2. Distribution

Following absorption into the bloodstream, the drug is distributed throughout the body. Lipid solubility and protein binding affect Central nervous system, CNS availability. Drugs can displace others from albumin and protein binding and is responsible for many pharmacokinetic interactions between AEDs. An example of this is the interaction between phenytoin and valproic acid. If valproic acid is added to a patient who is already taking phenytoin, the phenytoin is displaced from albumin binding sites, resulting in a higher free fraction and toxicity.

2.2.2.3. Metabolism

Most AEDs are metabolized in the liver by hydroxylation or conjugation. These metabolites are then excreted by the kidney. Some metabolites are themselves active (carbamazepine, oxcarbazepine, primidone). Gabapentin undergoes no metabolism and is excreted unchanged by the kidney. Most AEDs are metabolized by the P450 enzyme system in the liver. Different AEDs either induce or inhibit certain isoenzymes of this system and can result in changes of the pharmacokinetic properties of different medications. In general enzyme inducers decrease the serum concentrations of other drugs metabolized by the system and enzyme inhibitors have the opposite affect.

Valproic acid is metabolized by a combination of conjugation by uridine glucuronate (UDP)-Glucuronyltranferase (UGT) via conjugation and by mitochondrial beta-oxidation.

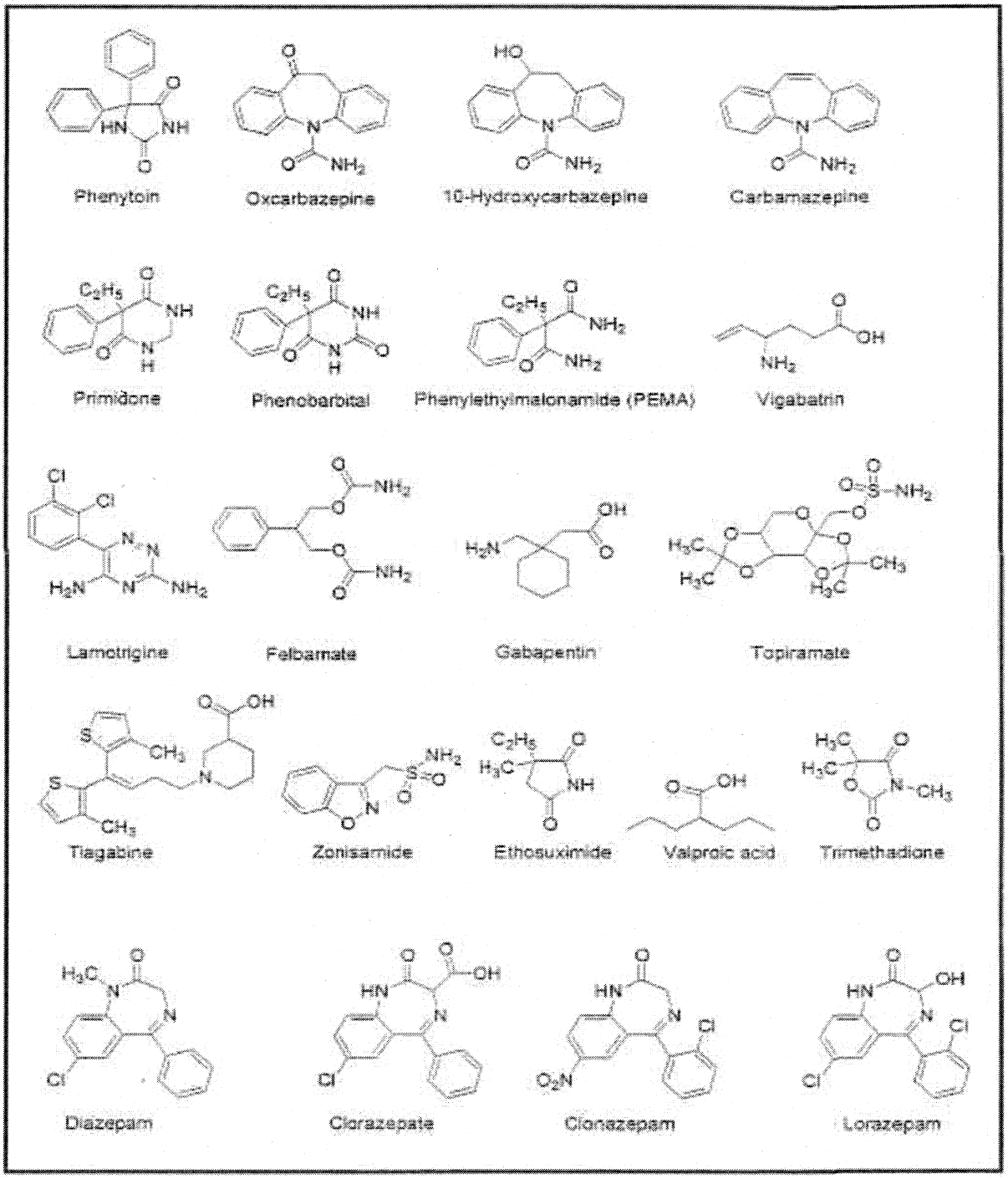
A major player contributing to various forms of drug toxicity is the cytochrome P-450 (cyt P-450) family of enzymes. This system evolved to oxidize xenobiotics (foreign compounds, such as drug molecules) in order to prepare them for excretion after conjugation to a highly water-soluble moiety, such as glucuronic acid. In order to form the watersoluble glucuronide by the so-called Phase II reaction, the lipid-soluble drug molecule must have a functional group suitable for conjugation, such as a hydroxyl group, conferred on the drug by the Phase I reaction catalyzed by cyt P-450. The above reaction can be used to describe the metabolism involving the hydroxylation of phenytoin (PHT), carbamazepine (CBZ), and others before those drugs can be glucuronidated by a Phase II reaction involving UDP-glucuronyltransferase. Even though we conveniently

describe the above reaction (Phase I) as mediated by cyt P-450 in one step, it actually takes place in two steps . In the case of drugs containing an aromatic ring, this step often involves the production of a highly reactive arene oxide, which can potentially interact with biological macromolecules to create adverse outcomes.

2.2.2.4. Elimination

Drug elimination rate is usually expressed as the biological half-life and is defined as the time required for the serum concentration to decrease by 50% following absorption and distribution. This changes for some drugs based on serum concentration e.g. phenytoin has a longer half-life at high serum levels. The half-life also determines the dosing frequency required for a drug to be maintained at a steady state in the serum. Most drugs are eliminated by the kidneys and dosage adjustments are required in cases of renal impairment.

Figure 8. Structure of AEDs. -



2.2.3.Metabolism of different AEDs

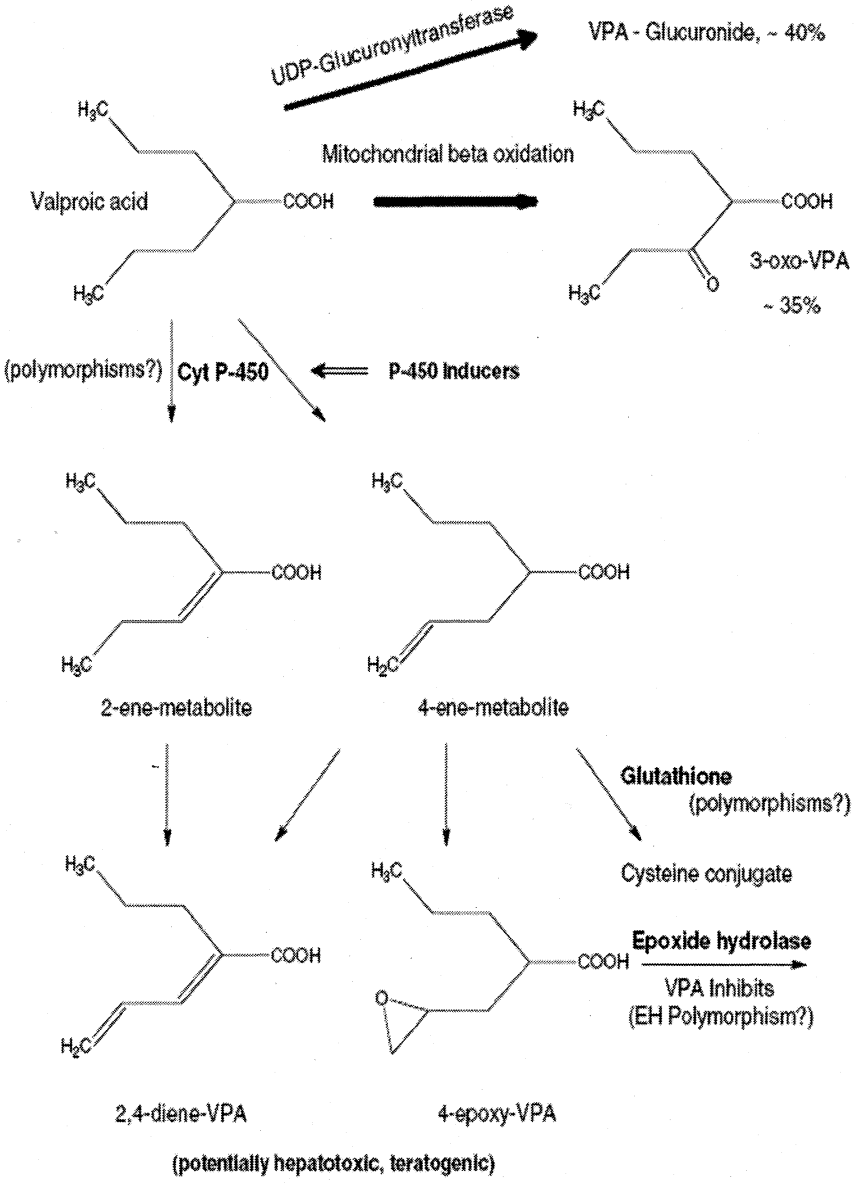
2.2.3.1.Valproic Acid

Valproic acid (VPA) is an established antiepileptic drug with a simple chemical structure but an unusually broad spectrum of action. As a branched chain carboxylic acid, VPA is extensively metabolized by the liver via glucuronic acid conjugation, mitochondrial beta- and cytosolic omega-oxidation to produce multiple metabolites. At least 10 metabolites have been identified. The major urinary metabolite is 2-propyl-3-keto-pentanoic acid. Other minor metabolites identified are the hydroxylated or their dehydrated products, and 2-propylglutaric acids. All of these metabolites are excreted as glucuronides.

Carnitine is an amino acid derivative that is an essential cofactor in the transport of long-chain fatty acids across the mitochondria for beta oxidation, and the removal of potentially toxic acylcoenzyme-A metabolites from the inner aspect of mitochondrion as acylcarnitines. It is synthesized from the essential amino acids, methionine and lysine. VPA inhibits the biosynthesis of carnitine by decreasing the concentration of alpha-ketoglutarate and may contribute to carnitine deficiency. Carnitine supplementation may increase the beta-oxidation of VPA, thereby limiting cytosolic omega-oxidation and the production of toxic metabolites that are involved in liver toxicity and ammonia accumulation. (Lheureux & Hantson.,2009)

Normal metabolism of valproic acid and the pathways that result in the generation of toxic derivatives are shown in figure 9 . Sites where genetic polymorphisms can modify toxicity are shown.

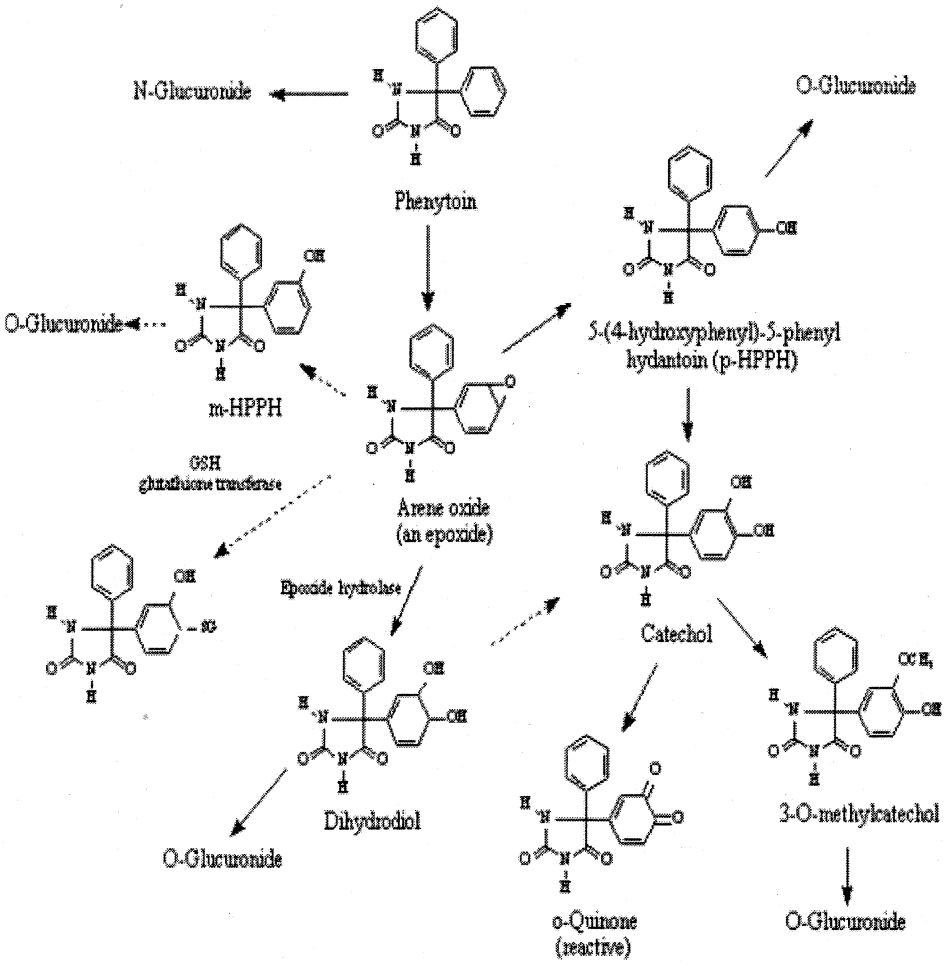
Figure 9. Metabolism of valproic acid.



2.2.3.2.Phenytoin and Phenobarbital

The principal metabolic pathway of Phenytoin and Phenobarbital in human is aromatic hydroxylation, catalyzed by the cytochrome P-450 isozymes (CYP2C9 and CYP2C19). The reactive intermediate, arene oxide is deactivated by either epoxide hydrolase to dihydrodiol or by the action of glutathione (GSH) and glutathione transferase. The pathways of phenytoin metabolism are depicted below.

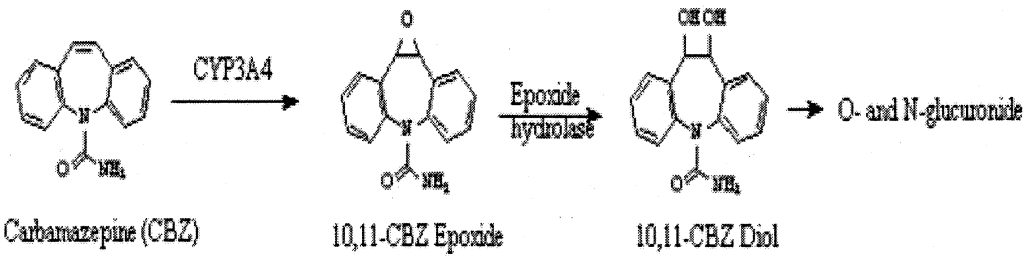
Figure 10. Metabolism of phenytoin



2.2.3.3.Carbamazepine

The major metabolic pathway of carbamazepine (CBZ) is the formation of the stable 10,11-CBZ epoxide by cytochrome P-450 isozyme CYP3A4. This reactive metabolite is further deactivated by the action of epoxide hydrolase to give inactive 10,11-CBZ-diol that is excreted as glucuronides. Several other minor metabolites have also been identified with CBZ. These are derived from the reactive intermediate, arene oxide by CYP2C9/19. Further metabolic conversions of this intermediate lead to the formations of 2(3)-hydroxy-CBZ, CBZ-2, 3-diol, CBZ-catechol, and CBZ-o-quinone in a similar manner as in phenytoin.

Figure 11. Metabolism of carbamazepine.

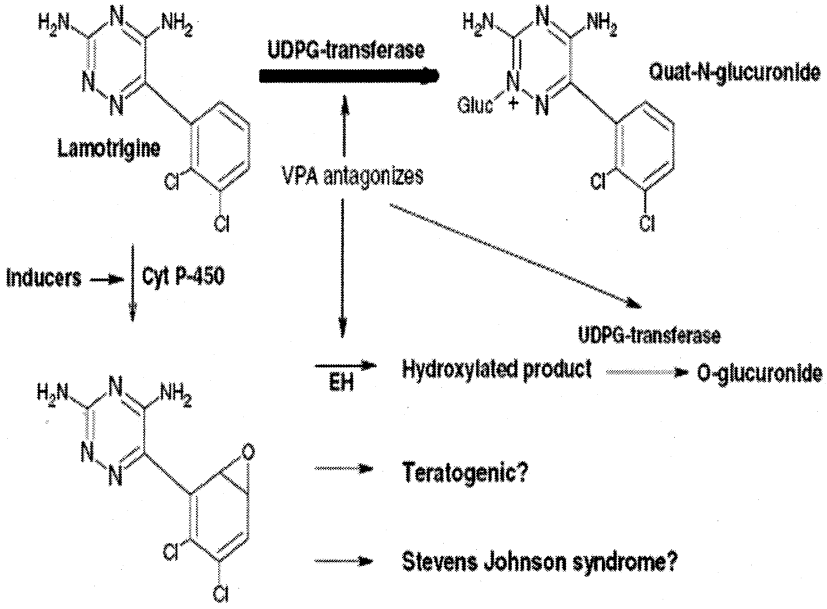


2.2.3.4.Lamotrigine

Lamotrigine, an antiepileptic drug of the phenyltriazine class, is metabolized predominantly by glucuronidation. The major inactive urinary metabolites isolated are a 2-N-glucuronide (76%) and a 5-N-glucuronide (10%). The aromatic ring is deactivated by the presence of chlorine atoms toward aromatic oxide formation. Coadministration of lamotrigine with valproic acid greatly enhances the incidence of idiosyncratic reactions. It is conceivable that in the presence of valproic acid (an inhibitor of glucuronidase enzyme) the concentration of the

reactive arene oxide intermediate may be increased due to the reduced capacity of glucuronidase to metabolize lamotrigine (Shorvon SD., 2000, SankarR., 2007).

Figure12. Metabolism of Lamotrigine.



2.3. Fetal Malformations

Malformations refer to major abnormalities that require surgical intervention within the first year of life or are likely to result in significant impairment and disability. Minor congenital anomalies are defined as those which do not have functional or cosmetic importance, but sometimes may signify an underlying genetic disorder.

Etiologically, malformations can be caused by one of the following factors (Debdas AK., 2005).

1. chromosomal abberation

2. genetic defects
3. teratogenic/environmental forces
4. unknown factors.

2.3.1. Malformations due to chromosomal aberrations.

50 % of embryonic deaths, 5-7% of fetal losses, 6-11 % of stillbirths and neonatal deaths, and 0.9% of live borns have chromosomal abnormalities. The frequency of their occurrence may increase with maternal age,eg.trisomy 21. Chromosomal abnormalities may be numerical alterations and structural alterations.

(a)Numerical chromosomal abberation (aneuploidy)- The most obvious or easily recognized chromosomal abnormalities are numerical. In these, the affected individual may have trisomy (an extrachromosome),or monosomy (missing chromosome) or polyploidy (abnormal number of haploid chromosomal) complement. Eg: Trisomy 13,18, 21.

(b) Structural chromosomal aberrations -These results from breakage and fusion of chromosome segments. It can occur in the form of deletion, inversion, translocation.

2.3.2Malformations due to genetic defects.

This may be due to single gene defect or multiple gene defect. Of these single gene defects are more recognizable, and possibly curable by genetic engineering, and hence its importance.

Single-gene (Mendelian) disorders occur in approximately 1% of newborn infants. The distribution of recognized single gene traits is as follows.

2.3.2.1.Autosomal dominant disorders

In autosomal dominant disorders affected people are heterozygous for an abnormal allele, which they transmit to 50% of their offspring. Age of onset on many of these is variable and severity also varies. Some autosomal dominant disorders are not fully penetrant, ie, not all persons who inherit the gene have recognizzble signs or symptoms of the disorder. Not uncommonly autosomal dominant disorders first appear in a family as a new mutation. The common example of autosomal dominant disorder are achondroplasia, adult polycystic kidney disease, marfan syndrome, polyposis of the colon and neurofibromatosis.

2.3.2.2.Autosomal recessive (AR) disorders

AR disorders occur most commonly in persons whose parents are healthy but both carry the same recessive gene. The risk to the offspring of such parents is 25% in each pregnancy, and these disorders most commonly occur with no family history. People who are related to each other or from the same ethnic background, are more likely to carry the same recessive genes. This is why consanguinity increases the risk of AR disorders. The parents who are first cousine run increased risk of even having children with severe abortion. In Phenylketonuria, PKU- phenylalanine hydroxylase deficiency,. Phenyl alanine readily crosses the placenta and high maternal serum level can result in pregnancy loss and it can also induce birth defects such as microcephaly with mental retardation and cardiac defects.

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2.3.2.3.X-linked disorders

Most are recessive, with homozygous males affected and heterozygous females unaffected. A female carrier will transmit the mutant gene to 50% of her offspring, so half of her sons will be affected and half of her daughters will be carriers. An affected male will pass the mutant **x** chromosomes to all of his daughters and to none of his sons. Eg.Colour blind ness, hemophilia A and Duchenne muscular dystrophy.

2.3.2.4.Mitochondrial gene disorders.

The mitochondria of cells contain their own genome, distinct and separate from the nuclear DNA containing 46 chromosomes. Because all of the mitochondria in a zygote are obtained from the cytoplasm of oocyte, all mitochondrial genes are inherited from the mother. Mitochondrial mutations will affect those organs with high energy requirements which depend on mitochondrial activity, such as brain and muscle. A number of neuromuscular and neurodegenerative disorders occur due to mitochondrial inheritance.

2.3.2.5.Multifactorial inheritance

Isolated birthdefects such as club foot, cleft lip, cardiac anomalities and neural tube defects are among the most common genetic abnormalities and are inherited through a combination of genetic and environmental factors. Genetic factors predispose the organism to one or more morphogenic errors which may be amplified by the environment and chance and result in abnormal development.

Malformations affecting different organ system are listed below.

Table4. Malformations affecting different organ systems	
Cardio Vascular System malformations	Atrial septal defect (ASD), Ventricular septal defect (VSD), Patent ductus arteriosus (PDA), Tetralogy of Fallot
Gastro intestinal tract, GIT malformations	Cleft lip and Cleft palate, Oesophageal atresia, Exomphalos and gastroschisis , Congenital diaphragmatic hernia, Hypertrophic pyloric stenosis, Omphalocele .
Central Nervous System (CNS) malformations	Neural tube defects (NTD), Anencephaly, Spina bifida, Hydrocephalus .
Limb defects	Telepes; Congenital dislocation of hip; Achondroplasia.
Respiratory System	Choanal atresia, Cystic adenomatous malf-ormation of lung
Renal defects	Cystic kidney disease, Congenital hydronephrosis
Genital defects	Hypospadias, Indeterminate sex

2.4.Epilepsy and Pregnancy

Around one in 200 pregnancies are thought to be exposed to AEDs. Women of childbearing age with chronic medical conditions are concerned about hazards from drug exposure during pregnancy and lactation is unlikely to cause birth defects. Major effects occurring within first 8 weeks is embryopathy, major effects caused after 8 weeks is fetopathy. Although teratogens may increase the risk for birth defects, they do not necessarily cause problems in all cases. Exposure to a teratogen may increase this risk, but nothing can eliminate the risk. Higher incidence of birth defects are seen among infants exposed to AEDs than

among control infants. Frequency of congenital defects increased when in utero exposure involved two or more AEDs- Polytherapy is an increased risk factor. Congenital malformations associated with AED exposure during pregnancy present as a wide range of anomalies affecting many different body systems including the central nervous system, the gastrointestinal tract and the cardiovascular system. Infants of mothers with epilepsy who did not take AEDs during pregnancy had no increased risks for birth defects. (Holmes et al., 2001, Barret et al., 2003).

2.5.Teratogenic drugs

Teratogen is any agent that acts during embryonic or fetal development to produce a permanent alteration of form or function. A teratogen is any agent, chemical, substance, or exposure that may cause birth defects to the developing fetus. The critical period for causation of malformation by any teratogen is the period of rapid organogenesis. Most medications pose little or no risk to the fetus, when consumed in therapeutic doses. The effect of teratogens depends upon the timing of exposure. The first trimester of pregnancy is the critical period of organ and limb development in the fetus. The fetal brain develops throughout pregnancy and can be affected at any time. Exposure to a teratogen during 5-10 weeks after last menstrual period, or between 20-55 days from the date of ovulation is critical.

The incidence of mental retardation is increased in children of mothers with epilepsy, but not in children of fathers with epilepsy (Majewski et al., 1981). Multiple factors could contribute to the observed differences, but animal studies

suggest that AEDs play a role. Teratogen can cause an effect only after a certain level of exposure is reached. There are a variety of teratogens that are relatively common. Some examples are listed in the table below.

Table5 . Teratogens	
Social drugs	Alcohol,cocaine,cigarettes
Medications	ACE inhibitors-analapril,captopril(cause renal tubular dysgnesis, pulmonary hypoplasia)
	Anticonvulsants (causes all common major malformations), Hormones-androgen, danazol(cause genito urinary system malformations), Warfarin (crosses placenta causes midline CNS dyspasia), Antimicrobials-Tetracycline,streptomycin
	Thalidomide , chemotherapy, drugs for psychiatric disorders, lithium ,vaccines
Environmental agents	Organic solvents, chemicals, lead,anaesthetic gases, organic mercury.
Infectious diseases	Rubella, cytomegalovirus, genital herpes, toxoplasmosis, chicken pox.
Others	Diabetes, radiation, hyperthermia.

2.6.Malformation pattern of diiferent AEDs

2.6.1.Phenytoin

PHT exposure in utero has been associated with a pattern of abnormalities known as fetal hydantoin syndrome (FHS), consisting of minor anomalies as midfacial hypoplasia with a snub nose, a broad nasal bridge, ocular hypertelorism and an arched upper lip, and hypoplasia of distal phalanges and nails. Major anomalies such as cleft lip and palate and cardiac anomalies and pre and postnatal growth retardation (microcephaly and mental retardation) have also been associated with in utero exposure to PHT in humans (Hanson., 1986; and Finnell., 1991, Azarbayjani., 2001).

2.6.2.Carbamazepine

Carbamazepine (CBZ) is also teratogenic causing an increased incidence of craniofacial and limb development in exposed children (Hiilesmaa et al., 1981, Bertollini et al., 1987) and even experimental animals (Paulson et al., 1979; Vorhees et al., 1990). There is a striking similarity between the malformation pattern in children exposed to CBZ and fetal hydantoin syndrome, and they share the same teratogenic mechanism (Buehler., 1987).

2.6.3.Phenobarbital

Barbiturates have also been associated with the same major and minor abnormalities and dysmorphic features as with PHT. These include congenital heart defects, facial clefts, craniofacial abnormalities and growth deficiency . Due to the large overlap in the pattern of teratogenicity between PHT and PB, the term "Hydantoin-Barbiturate Embryopathy" was proposed (Majewski etal., 1981).

2.6.4.Valproate

VPA has been reported to be teratogenic. A fetal valproate syndrome has been described which is characterized by posterior neural tube defects and a distinctive facial appearance featuring upward slanting palpebral fissures, epicanthic folds, and posteriorly rotated ear (Vorhees., 1987). There have been increasing evidence that antenatal exposure to VPA increases the risk of fetal malformations by 1.5 to 2 times. Recent data from the Australian Pregnancy registry shows that dosages higher than 800mg per day is particularly harmful to the fetus.

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2.7. Mechanisms of teratogenicity of AEDs.

A variety of factors may contribute to the teratogenic risks of antiepileptic drugs in children of women with epilepsy. e.g., AEDs, seizures during pregnancy, seizure type, heredity, maternal age/parity, socioeconomic status (Janz.,1975). However, data from animals and humans suggest that AEDs play an important role in this regard. AEDs are established as human teratogens based on the following factors. The association of in utero exposure to an increased incidence of specific major malformations, AED-induced dysmorphogenic syndromes, dose-response relationship (i.e., both dose and numbers of AEDs), and evidence that genetic pharmacological differences in metabolism are correlated with the occurrence of congenital malformations (Finnell et al., 1991). AED s and are potentially be teratogenic. Despite the wide spread use of AEDs and knowledge of their teratogenicity, the mechanism by which they damage the developing embryo is still not fully understood. There are several postulates on the mechanism of teratogenesis.

The list of suggested mechanisms for teratogenicity is long and diverse. The important theories are discussed below.(table6).

Table 6 . Various teratogenic mechanisms.	
1	Toxic intermediary metabolites-free radicals and epoxides.
2	Alterations in folate and methionine metabolism
3.	Histone deacetylase deficiency
4	Ischemia/hypoxia
5	Apoptotic effects of neurotransmitters
6	Neuronal suppression
7	Alteration in thyroid status with lower levels of thyroxine and thyrotropine
8	Interaction with glucocorticoid receptors (Goldman et al., 1987)
9 ·	Vitamin K metabolism (Yerby., 1987, Ramsay et al., 1991)

2.7.1 Toxic Intermediate Metabolites:

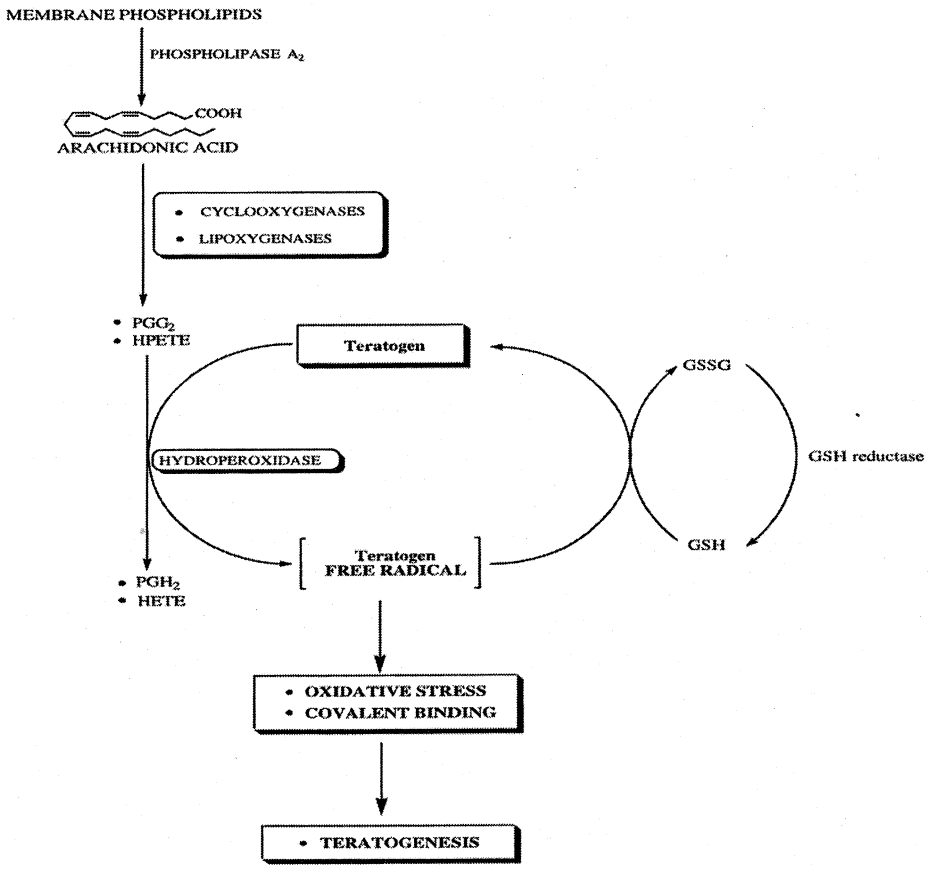
The fetotoxicity of some AEDs may be mediated not by the parent compound but by toxic intermediary metabolites (Parman et al.,1995). Two possible toxic intermediates include free radicals and epoxides.

2.7.1.1.Free radicals:

Metabolism of AEDs to free radical intermediates may be responsible for teratogenicity. AEDs are co-oxidized to free radical intermediates which bind proteins . If a patient who is exposed to AEDs has a deficiency in the activity of an enzyme that normally inactivates free radicals, then the level of free radicals will increase along with malondialdehyde levels. As a result, both maternal and infant malondialdehyde levels should be related to neurodevelopmental outcome. Multiple mechanisms are involved in the formation and detoxification of free radicals. Genetic differences in elimination, bioactivation, detoxification,

cytoprotection, and repair may alter free radical effects on neurodevelopment as a result of in utero AED exposure. The teratogenicity of PHT can be due to the formation of free radicals of the drug during the normal metabolism of free arachidonic acid to prostaglandin G2 (PGG2) and H2 (PGH2). Two different enzymes are involved in this conversion, namely cyclooxygenase (conversion of arachidonic acid to PGG2) and hydroperoxidase (conversion of PGG2 to PGH2). The reduction step catalyzed by hydroperoxidase could utilize a number of drugs as co-factors and convert them to free radicals. The drug radical formed in these reactions could attack a wide range of targets in the developing organism and initiate oxidative stress (Parman et al.,1995). Figure13.

Figure13. Oxidative stress as a mechanism of teratogenicity.



2.7.1.2.Epoxides

Studies have suggested that the teratogenicity of AEDs might be dependent on bioactivation of these compounds to reactive epoxides. The theory relates the existance of a phenyl substituent, which is needed for the formation of epoxide, as a pre requisite for structurally related AEDs. Some AEDs are metabolized to arene oxide intermediates (epoxides) which are highly reactive and can covalently bind fetal nucleic acids. These epoxides are thought to be the primary teratogenic agent for phenytoin and perhaps carbamazepine. Arene oxides are detoxified by epoxide hydrolase, and inhibition of this enzyme in an animal model leads to an increase in malformations (Martz et al., 1977).

2.7.2. Alterations in folate and methionine metabolism

Folic acid is an essential vitamin used in single carbon metabolism, including nucleotide synthesis and DNA methylation (Hansen et al., 1985). Since humans cannot synthesize folic acid, they are dependent on dietary sources. Dietary folate should be monoglutamated and reduced in order to be able to enter the circulation. Many enzymes are involved in this process, among which folylpolyglutamate hydrolase, 5,10-methylene tetrahydro folate reductase and dihydrofolate reductase are the most important. Rapidly growing and developing embryos have an increased requirement for folate. An abnormal pattern of folate metabolism would result in a decreased rate of DNA synthesis and gene methylation, with deleterious effects on the developing embryo (Finnell et al. 1991). Phenytoin, Phenobarbial, Vaproate etc can interfere with folate metabolism (Meadow., 1968, Dansky et al., 1989). Patients undergoing AED

therapy usually develop folate deficiency (Kishi et al.,1997) and folate deficiency has been related to the impaired development of offsprings born to mothers both in humans (Smithells et al., 1976) and experimental animals(Hansen et al., 1985). Valproate and Carbamazepine are shown to increase the risk of neural tube defect by increasing oxidative stress and deranging folate metabolism (Scaria et al., 2005).

2.7.3.Ischemia/hypoxia

AEDs cause embryonic cardiac arrythmia, resulting in hypoxia reoxygenation damage (Danielson et al., 1995). Phenytoin induced congenital defects in animals resemble the effects of ischemia, and hyperbaric oxygen can reduce phenytoin malformations. The similarity of ischemia and phenytoin effects may be because ischemia induces free radicals. No studies have been conducted in humans to confirm or refuse the hypothesis.

2.7.4.Alterations in Vitamin K metabolism

Maternal use of AEDs during pregnancy causes a coagulation defects in about 50% newborns (Mountain et al., 1970). Phenytoin induced hemorrhage is Vitamin K responsive (Deblay et al., 1982). Phenytoin crosses placenta and induces fetal microsomal enzymes resulting in increased oxidative degradation of vitamin K, leading to its deficiency (Keith et al.,1979). Prothrombin needs to be carboxylated before it is functional, which is a vitamin K dependent process. The drug induced deficiency would result in the formation of descarboxylated non

functional prothrombin and other vitamin K dependent clotting factors which can not prevent hemorrhage (Howe et al., 1995)

2.7.5.Histone deacetylase deficiency

VPA is able to inhibit the enzyme class of histone deacetylases (HDACs), proteins with a fundamental impact on gene expression and therefore possible molecular targets of VPA-induced signaling cascades (Eikel et al., 2006). Inhibition of histone deacetylase activity on specific embryonic tissues shows a new mechanism for teratogenicity. Histone deacetylase (HDAC) and histone acetyltransferase (HAT) are enzymes that influence transcription by selectively deacetylating or acetylating the ε-amino groups of lysine located near the amino termini of core histone proteins. Chromatin acetylation correlates with transcriptional activity (euchromatin), whereas deacetylation correlates with gene silencing. HDACs are also involved in the reversible acetylation of non-histone proteins (Eyal et al., 2004). Altered HDAC and/or HAT activities are present in many types of cancers.

2.7.6. Apoptotic Effects of Neurotransmitters:

Animal studies of the adverse effects of in utero alcohol exposure on the fetal brain have suggested NMDA antagonism and GABA agonism which lead to an abnormal reduction in neurons (Ikonomidou et al., 2000). Since some AEDs affect these receptors, their adverse effects on neurodevelopment may be produced by this action. Evidence for this theory should be revealed by

contrasting the neurodevelopmental effects of AEDs which do or do not affect these receptors.

2.7.7. Neuronal Suppression:

AEDs suppress neuronal irritability and as a consequence impair neuronal excitation. Reduction of neuronal excitation in utero might alter synaptic growth and connectivity during the early stages of neurodevelopment resulting in long-term deficits in cognition and behavior.

2.7.8.Genetic susceptibility to teratogenicity of AEDs

The fact that not all infants of epileptic mothers undergoing AED therapy are born with congenital birth defects emphasises the importance of fetal genotype and its interacton with various environmental factors (Hansen et al., 1983). Genetic predisposition in susceptibility of embryonic heart to the arrythmogenic effects of PHT is one suggested explanation (Danielsson et al., 1992). Genetic predisposition may also be related to the content and potency of antioxidant enzymes in capturing and detoxifying the harmful reactive species. There is evidence that reactive oxygen species, generated in the embryo, may be directly responsible to teratogenic effects, such as cleft palate (Azarbayjani., 2001).

2.8.Free radicals

Free radicals are highly reactive chemical entities capable of independent existence and contain one or more unpaired electrons in their outer most orbits . Humans are constantly exposed to FRs created by internal cellular metabolisms

and by external sources from the environment . Under certain conditions can be highly toxic to the cells. Excess oxygen is toxic, which was half a century ago proposed to be due to the formation of oxygen radicals. An oxygen radical is an oxygen-centred radical.

FR are generally unstable and try to become stable, either by accepting or donating an electron. They can act as an oxidant, act as a reductant, or react with another radical or with a non-radical . This enables the FRs to participate in auto catalytic chain reactions.

2.8.1.Production of free radicals:

FR are formed in side our body by Physiological (Natural), Pathological stimuli . Physiological stimuli include Normal respiration with in the mitochondria, Oxidation of foods and endogenous compounds, many fundamental metabolic processes such as the biosynthesis of steroid hormones, bile acids, eicosanoids and unsaturated fattyacids, Peroxisomal degradation of fatty acids, Transportation of substances for energy production and cytochrome P450 enzyme system.

Transition metals – Iron, Fe2+, Copper, Cu+ in the body when are in free form act as FR. Fe2+ can take part in electron transfer reactions with O_2. Body cells-phagocytic cells, neutrophils and macrophage by enzyme systems produces •NO, $•O_2$ and H_2O_2. Myeloperoxidase in the phagocyte cytoplasm produces Hypochlorous acid, HOC1- by activated neutrophils.

Pathological Stimuli that form free radicals include exposure to alcohol, insecticides, radiation, excessive amounts of sunlight-Ultraviolet rays, high fat

diet or eating fried foods, strenuous exercise, tobacco smoke, metabolism of drugs like CCl3, emotional stress...

There are two major types of free radical species-reactive oxygen species (ROS), and reactive nitrogen species (RNS). Reactive oxygen species are radicals derived from oxygen which represent the most important class of radical species generated in living systems Eg: superoxide O_2•-, Hydrogen peroxide H_2O_2, hydroxyl OH•.

Reactive nitrogen species include Nitric oxide (NO) . NO potently relaxes arterial and venous smooth muscles and, less strongly, inhibits platelet aggregation and adhesion. Peroxynitrite .ONOO- is a powerful oxidant, able to damage many biological molecules, and can decompose at acid pH to release small amounts of OH.

2.8.2.Actions of Free radicals

They act on the cell membranes and membranes of different organelle of cells and cause cell injury and death by oxidative reactions and they are called oxidants.

2.8.2.1.Actions of FRs on Carbohydrates-

Hydroxyl radicals,OH. react with Carbohydrates by randomly abstracting Hydrogen atom from Carbon atom, producing Carbon centered radicals,C.. This leads to chain breaks in important molecules like hyaluronic acid in a process involving intermediates such as ROO. In the synovial fluid surrounding joints, an accumulation and activation of neutrophils during inflammation produces

significant amounts of oxyradicals. This accounts for a significant decraease in the synovial fluid.

2.8.2.2.Actions of FRs on Lipids -

The poly unsaturated fatty acid, PUFA of cell membrane are more vulnerable for this injury. By lipid peroxidation FR increases the permeability of cells, leading to calcium influx and altered P^H of the cell. PUFAs are abundant in cellular membranes and in Low density Lipoprotiens, LDL.

A FR prefers to steal electrons from the lipid membrane of a cell, initiating a FR attack on the cell known as lipid peroxidation. ROS target the carbon-carbon double bond of polyunsaturated fatty acids. The carbon-centered FR stabilize by molecular rearrangement called conjugated diene (CD). The CD then very easily reacts with oxygen to form a peroxy radical. This steals an electron from another lipid molecule in a process called propagation. This process then continues in a chain reaction. The major aldehyde product of lipid peroxidation are malondialdehyde and 4-hydroxy-2-nonenal (HNE).(Figure14)

Isoprostanes,Iso-P are eicosanoids produced by the random oxidation of arachidonyl-containing lipids by oxygen radicals. Increased Iso-P levels in urine or plasma have been found in patients with a variety of disease conditions , as well as in smokers.

Figure 14 Lipid peroxidation

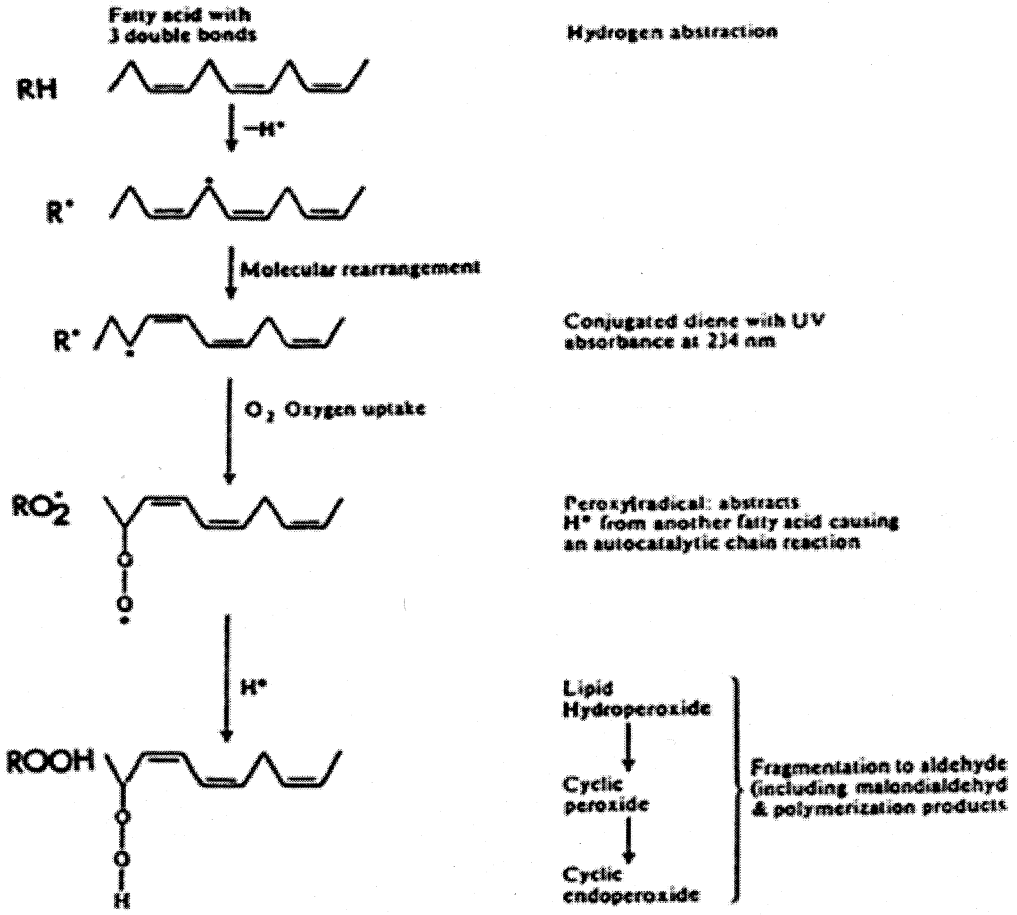


Fig 1. Lipid peroxidation.

2.8.2.3.Actions of FRs on Proteins –

The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues are susceptible to oxidation by the action of ROS/RNS. Protein carbonyl groups, generated by many different mechanisms is a good measure of ROS-mediated protein oxidation.

2.8.2.4.Actions of FRs on DNA -

FRs cause fracturing on the cell nucleus resulting in single strand DNA damage. This oxidative injury may be lethal – Leading to cell death and ultimately removed by phagocytosis. Sub lethal which may result in Increased cell permeability. The most extensively studied DNA lesion is the formation of 8-OH-G (8-hydroxyguanosine). Mechanism of oxidative stress induced cell damage is depicted in figure15.

Figure 15 Mechanisms of oxidative stress induced cell-damage.

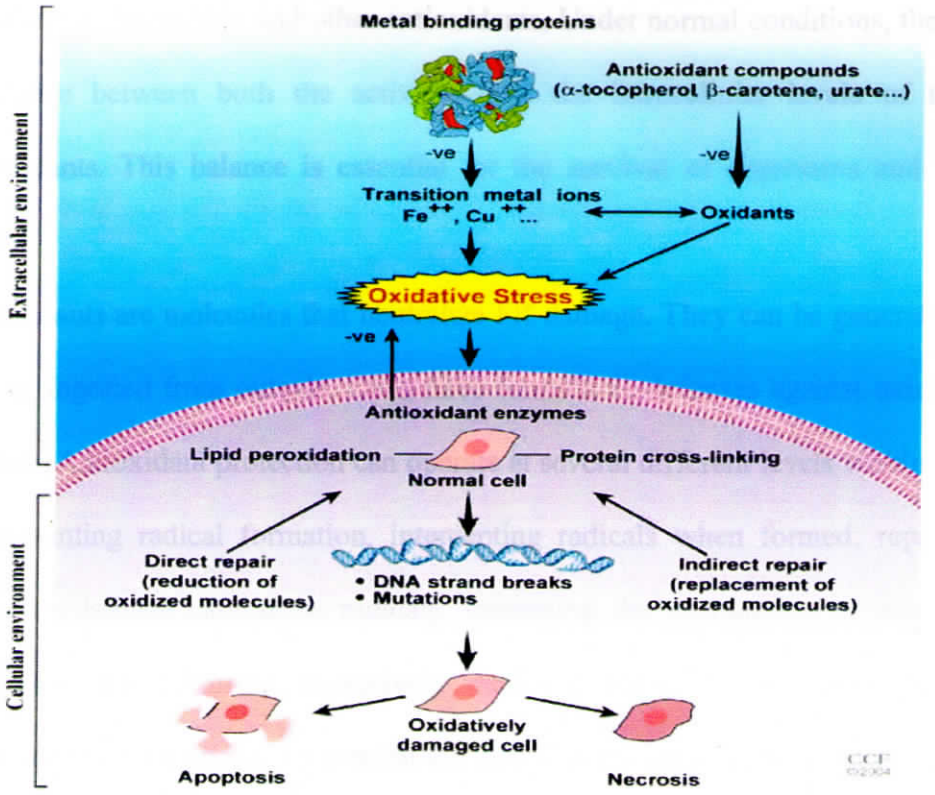


Figure 1
Mechanisms of oxidative stress-induced cell damage.

Figure 15. Downloaded from http://www.rbej.com/content/3/1/28.

2.9.Antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms. Defence mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health.

Antioxidants are molecules that neutralize FR damage. They can be generated in vivo or ingested from outside. Cells have formidable defenses against oxidative damage. Antioxidant protection can operate at several different levels within cells by preventing radical formation, intercepting radicals when formed, repairing oxidative damage caused by radicals, increasing the elimination of damaged molecules, not repairing excessively damaged molecules to minimize the introduction of mutations. To combat the injurious effects of FRs our body has its own system of 'in vivo' Antioxidants'. All cells have intracellular antioxidants which are very important for protecting all cells from oxidative stress at all times. In normal healthy state a balance is maintained between FRs & AOs. Moreover we can as well supplement these from outside (in vitro Antioxidants).

In Vivo Antioxidants: - These are binding proteins. They keep the free ions of plasma in a binding form, so prevent oxidation injury. Eg.-Transferrin, Ceruloplasmin

Endogenous molecules- glutathione (GSH), sufhydryl groups, alpha lipoic acid, CoQ 10, thioredoxin .

GSH is very important as an intracellular antioxidant. GSH has been found to be low in many disease states indicating oxidative stress and inadequate antioxidant activity to "keep up" with the free radicals. GSH in the nucleus maintains the redox state of critical protein sulfydryls that are necessary for DNA repair and expression. Too high concentration of GSSG may damage many enzymes oxidatively. The main protective roles of glutathione against oxidative stress are: (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathionetransferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) glutathione is able to regenerate the most important antioxidants, Vitamins C and E, back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate .

Superoxide dismutase,SOD functions as an antioxidant by catalyzing the conversion of superoxide radicals to hydrogen peroxide, which can subsequently

be reduced to water by catalase, thus converting a very powerful free radical ($\bullet O_2$) to free water. There are three isoforms of SOD that dismutate superoxide anions to H_2O_2.

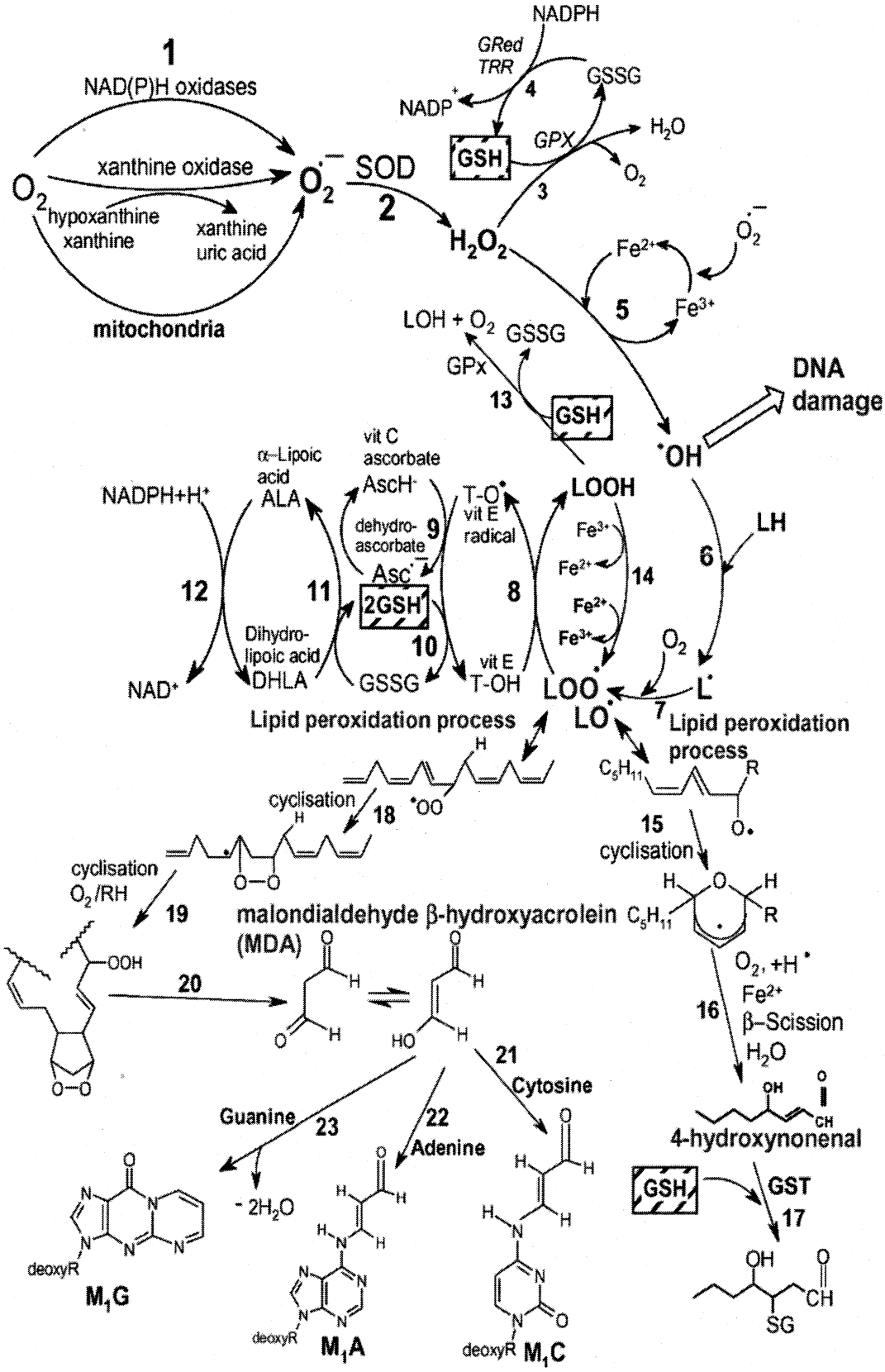
Catalase is an iron-containing enzyme found within peroxisomes. It is responsible for the conversion of H_2O_2.into water and oxygen.

Essential nutrients - vitamin C, vitamin E, selenium, N-acetyl cysteine (NAC)

Food sources: Green & yellow vegetables, Herbs--Turmeric, Garlic, Grape, Tea, Berries, Carrot, Spinach, Broccoli, Red Meat, Kidney, Liver & Lipoic Acid, Dietary compounds: bioflavonoids, proanthocyanidans.

Different pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C,lipoic acid) in the management of oxidative stress are depicted in the following figure (Valko et al., 2007).

Figure 16. summary of various pathways of ROS.



Reaction 1: The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain.

Reaction 2: Superoxide radical is dismutated by the SOD to hydrogen peroxide.

Reaction 3: H_2O_2 is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor.

Reaction 4: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (GR) which uses NADPH as the electron donor.

Reaction 5: Some transition metals (e.g. Fe2+, Cu+ and others) can breakdown H_2O_2 to the reactive hydroxyl radical (Fenton reaction).

Reaction 6: The hydroxyl radical can abstract an electron from polyunsaturated fatty acid(LH) to give rise to a carbon-centred lipid radical (L•).

Reaction 7: The lipid radical (L•) can further interact with molecular oxygen to give a lipid peroxyl radical (LOO•). If the resulting lipid peroxyl radical LOO• is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17).

Reaction 8: The lipid peroxyl radical (LOO•) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E (T-O•).

Reaction 9: The regeneration of Vitamin E by Vitamin C: the Vitamin E radical (T-O•) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbate monoanion, AscH−) leaving behind the ascorbyl radical (Asc•−).

Reaction 10: The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical (T-O•) is reduced by GSH.

Reaction 11: The oxidised glutathione (GSSG) and the ascorbyl radical (Asc•−) are reduced back to GSH and ascorbate monoanion, AscH−, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to _-lipoic acid (ALA).

Reaction 12: The regeneration of DHLA from ALA using NADPH.

Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. Lipid peroxidation process:

Reaction 14: Lipid hydroperoxides can react fast with Fe2+ to form lipid alkoxyl radicals (LO•), or much slower with Fe3+ to form lipid peroxyl radicals (LOO•).

Reaction 15: Lipid alkoxyl radical (LO•) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide.

Reaction 16: Six-membered ring hydroperoxide udergoes further reactions (involving_-scission) to from4-hydroxy-nonenal.

Reaction 17: 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione S-transferase).

Reaction 18: A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical.

Reaction 19: This radical can then either be reduced to form a hydroperoxide or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide.

Reaction 20: Formed compound is an intermediate product for the production of malondialdehyde.

Reactions 21, 22, 23: Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M1C, M1A and M1G, respectively.

2.10.Oxidative stress

Under normal conditions body maintains an equilibrium between its own FR's and Antioxidants. When this equilibrium breaks, a state called oxidative stress arises with in, due to increased FR formation or decreased AO system.

Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/ reperfusion, other diseases and ageing (Dalle-Donne et al., 2006, Jenner., 2003,

Sayre et al., 2001). Oxidative stress increases with age and therefore it can be considered as an important causative factor in several neurodegenerative diseases, typical for older individuals.

2.11. Investigations of Oxidative stress

When chemists began their attempts to study free radicals, they found it difficult to measure steady-state concentrations because of the extremely short half-life of these chemical species. Only technique that can detect Free radicals directly is Electron spin resonance(ESR). Free radical can be measured using spin resonance and spin trapping methods. Exogenous compound with high affinity for FR (Xenobiotics) are utilized in the spin techniques. The compound and radical form a stable entity that can easily be measured. Molecules which could "trap" free radicals were called spin traps.When the free radical molecule comes in contact with the spin trap molecule, it sticks to the spin trap molecule, forming a larger, more stable molecule that can be measured by ESR spectroscopy. Nitroso and nitrone spin traps are the most popular nitroxide radicals. The most used radical trap for the study of oxygen-centered free radicals is 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in biochemical and biological systems. The molecule alpha Phenyl t-Butyl Nitrone (PBN) is used for hydroxyl radical and the superoxide ion.

2.11.1. Luminescence/Fluorescence

Low level chemi luminescence can be detected from many cells and tissues . The total photon count from tissues and organs increases in response to oxidative stress. Luminol and lucigenin are used to increase the output of light.

2.11. 2.Fingerprinting/foot printing

Reactive oxygen species,ROS andReactive nitrogen species,RNS react with DNA, proteins, lipids and certain low molecular mass antioxidants . The products generated can be regarded as fingerprints/footprints of oxidative attack. The extent of lipid oxidation can be determined by measuring measurement of substrate loss (losses of unsaturated fatty acids), quantification of lipid peroxidation products - primary and secondary end products and by measurement of hydrocarbon gases . Assays of potential use to quantify damage of oxidative stress in Vitro and in Vivo include malondialdehyde (MDA), 4-hydroxy 2-trans-nonenal(HNE), Alkanes, F_2-Isoprostanes, Fatty acid analysis, Conjugated dienes, Lipid hydroperoxides, Plasma antioxidants, Total antioxidant activity, Products of DNA damage 8-hydroxy deoxy guanosine, protein carbonyls, Small molecules - glutathione, ascorbate, uric acid... Plasma antioxidants include enzymes Glutathione peroxidaseGP, Glutathione reductase GR, Super oxide dismutase SOD, Catalase etc.

Malondialdehyde, MDA is a marker of the ROS-mediated damage to lipids and an indicator of oxidative stress. Over the past decade, the isoprostanes , has been used extensively to quantify lipid peroxidation in association with risk factors for various diseases(Jason et al., 2005). The isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals.

2.12. Oxidative stress and epilepsy.

The brain is particularly vulnerable to oxidative damage because of its high oxygen utilisation, its high content of oxidisable polyunsaturated fatty acids, and the presence of redox-active metals (Cu, Fe). With regard to epilepsy, there are several biologically plausible mechanisms through which free radicals can lead to neuronal hyperexcitability and thus epileptogenicity. These include abnormal activation of N-methyl-D-aspartate (NMDA) receptors with consequent changes in neuronal activity, adverse changes in patterns of synaptic transmission, increase in the activity of excitatory amino acids such as glutamate and diminution of inhibitory influences on membrane activity . Interictal discharges are the manifestation of the sum of depolarising paroxystic displacements, which generate a pathological entrance to Ca2+ to the neurons, that can potentially unchain excitotoxic damage and activate numerous intercellular signalling pathway (Costello et al., 2004, Lopez et al., 2007)

2.13.Oxidative stress and pregnancy

Oxidative stress influences the entire reproductive span of women's life and even thereafter (i.e. menopause). It has been suggested that the age-related decline in fertility is modulated by Oxidative stress. It plays a role during pregnancy and normal parturition and in initiation of preterm labor. Lipid metabolism changes during pregnancy .The anabolic phase of early pregnancy encourages lipogenesis and fat storage in preparation for rapid fetal growth in late pregnancy (Kaaja., 1998, Toescu et al, 2004.). ROS are a double-edged sword and they serve as key signal molecules in physiological processes but also have a role in pathological processes involving the female reproductive tract. Pregnancy is a condition

exhibiting increased susceptibility to oxidative stress. Pregnancy is characterized by dynamic changes in multiple body systems resulting in increased basal oxygen consumption and in changes in energy substrate use by different organs including the fetoplacental unit (Casanueva et al., 2003, Agarwal et al ., 2005).

2.14. Oxidative stress and antiepileptic drugs

The prime objective of therapy of epilepsy is complete suppression of all seizures without impairment of central nervous system,CNS functions. All antiepileptic drugs are teratogenic. Many AEDs, during their metabolism generate reactive metabolites with the capability of covalent binding to macromolecules such as proteins or other vital bio molecules and hence eliciting systemic toxicity (Graf et al., 1998,Niketic et al., 1995). AEDs are known to be metabolized to free radical (FR) intermediates (Kubow et al., 1989). Treatment with agents which reduce Free radical formation reduces Phenytoin teratogenicity and inhibition of enzymes which detoxify FR increases PHT teratogenicity in animal models (Wong et al., 1989). AEDs increases oxidative stress and excess oxidative stress may be one of the mechanisms that contribute to teratogenicity (Liu et al., 1994,1995, Azarbayjani., 2001).

Chapter 3

MATERIALS AND METHODS

3.1.Settings of the study

The study was conducted in the Kerala Registry of Epilepsy and Pregnancy (KREP), which is functioning at the R.Madhavan Nayar Centre for Comprehensive Epilepsy Care, Department of Neurology in Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. Duration of the study was 3 years from a period of 2005-2008.

3.2.Kerala Registry of Epilepsy and Pregnancy

This registry, started in 1998 has over 1400 women with active epilepsy. They are enrolled in the preconception stage or during early pregnancy and are followed through pregnancy and delivery until the babies are six years old. All patients are followed up according to a standard protocol. Their general medical and neurological findings, laboratory data (EEG, CT Scan, MRI and other investigations) are available in the clinical records. All pregnant WWE are screened for any fetal malformations with antenatal ultrasonography, serum alpha fetoprotein estimation. Their use of antiepileptic drugs, other medications, folic

acid and other vitamins, and exposure to other teratogens including ionizing radiation and seizure burden are updated on a monthly basis. At birth all new borns are examined for any congenital malformation and are further screened with an echocardiography and abdomen ultrasonography at three months of age. Infants are further examined at one year and six years of age also. The registry has an elaborate screening mechanism to ascertain any untoward fetal outcome and document the various exposures. The follow up in the registry was excellent and the drop out rate was only less than five per cent. Thus this registry provided an excellent population of well characterized WWE with known fetal outcome. We recruited our subjects with epilepsy from this pool of patients.

3.3.Design

The present study was a cross sectional analysis aimed at estimating and comparing parameters of oxidative stress in different groups of women. Different groups included in the study were given in table 7.

Table7 Different groups of subjects recruited for Comparison of oxidative stress

Group	Code
Control group	C
Control group-pregnant state	CP
Women with epilepsy	E
Women with epilepsy-pregnant state	EP
Women with epilepsy –with unfavourable pregnancy outcome.	EM
Women with epilepsy –with normal pregnancy outcome.	E_{NM}

3.4.Sample recruitment and inclusion criteria

3.4.1.Women with epilepsy

WWE enrolled in the KREP and taking antiepileptic drugs within the age group of 18-35 yrs were sent letters by giving appointment. Consecutive WWE satisfying the inclusion and exclusion criteria as listed below were recruited in to the study.

3.4.1.1 Inclusion criteria for epilepsy group

1. They should be enrolled in the KREP.
2. Their age should be between 18 – 35 years.
3. Women should have active epilepsy as defined by the ILAE classification.
4. They should have completed follow up in the pregnancy registry at the time of recruitment.
5. All medical and laboratory results should be available and the pregnancy outcome should have been confirmed.

3.4.1.2.Exclusion criteria for epilepsy group

1. Women with other concomitant disorders
2. Those who were taking any antioxidants.
3. In order to exclude the impact of a recent seizure on oxidative stress, we excluded all WWE who had a seizure within one week of the blood sampling.

3.4.2. Control group

Control women were selected from volunteers, staff and also by house visit who were matched for age and socioeconomic status.

The comparison group of women without epilepsy in the pregnant state was selected from the antenatal clinics of Sree Avitam Tirunal Hospital (SATH), which is the women and Children hospital of Medical College, Trivandrum, a premier centre that caters to a large proportion of pregnant women in southern Kerala.

Informed consent was obtained from all.

3.4.3 Groups

3.4.3.1. Pregnancy vs non pregnancy

In order to study the effect of pregnancy on the oxidative stress we had recruited women in pregnant state under the control arm as well as epilepsy arm. Women in the first trimester of pregnancy only were recruited for this purpose. Care was taken to match the cases and controls for gestational period and maternal age.

3.4.3.2. Unfavourable pregnancy outcome

The main objective in this study was to ascertain any correlation between oxidative stress status and risk of fetal malformation. Accordingly we had recruited a cohort of women who had infants with malformations or other unfavourable outcomes such as abortions or intrauterine death. This cohort was identified from the pregnancy registry records and were invited to participate in this study.

3.5.Methods

In keeping with the objectives, the experimental protocol was designed as follows. All subjects were personnally interviewed to collect details on medical history, family history and life style using a standardised proforma. In order to exclude the impact of a recent seizure on oxidative stress, we excluded all WWE

who had a seizure within one week of the blood sampling. Women in the comparison group were without epileptic and other concommittant disorders.

A Dietary questionnaire which include dietary details was filled up to compare the diet intake and intake of antioxidants.

3.6.Blood sampling

5 ml random blood samples were drawn by venous puncture from all subjects.

3.7.Biochemical assay details

Indicators of Oxidative stress measured are malondialdehyde and isoprostane. The total antioxidant capacity (TAO) and antioxidant enzymes superoxide dismutase(SOD), catalase(CAT), and glutathione reductase (GR) and glutathione content(GSH) were also measured as antioxidant profile.Malondialdehyde was measured using Thiobarbituric acid assay method. Isoprostane was done using competative Enzyme Immuno Assay technique using assay kits of Cayman chemicals. SOD and GR assayed using assay kits of Randox Lab using spectrophotometric methods. CAT,TAO and GSH were measured using assay kits of Cayman chemicals by spectrophotometric methods using microplates. All investigations were standardised and done in duplicate.

3.8. Data compilation.

All the details and results were written in the proforma. The data were entered in Excel spread sheets and were analysed. Details of the dietary questionnaire was entered in Excel sheet. Calculated the protein and calorie value of each food for the 3 days taken. Calculated the protein and calorie intkake for each day and average is taken.

3.9.Statistical methods

The data are presented as mean ± SD values for each group. Each experimental observation was done in duplicate. The difference between selected means of any two groups were evaluated using independent t test. A level of $p<0.05$ was selected to indicate statistical significance.

3.10 Experimental methodology:

3.10.1 Sample

a. EDTA whole blood.

b. Serum: Blood is collected without using an anticoagulant and allowed to clot for 30 min at 25° C. Serum is separated after the centrifugation of the blood at 2000 g for 15min. at 4° C. Serum sample is stable for 1 month at –80 °C.

3.10.2.Assay of superoxide dismutase (SOD)

Assay of SOD was carried out using commercially available kit(Randox-RANSOD Catalog no: SD 125) (Woolliams et al., 2000, Suttle N.F.,1986, Arthur J.R., 1985)..

3.10.2.1.Assay Principle:

The role of superoxide dismutase (SOD) is to accelerate the dismutation of the toxic superoxide radical (O2.), produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-indophenyl)-3-(4-nitophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay.

$$\text{Xanthine} \xrightarrow{\text{XOD}} \text{uric acid} + O_2.$$

$$\text{I.N.T} \xrightarrow{O_2.} \text{formazan dye}$$

OR SOD

$$O_2. + O_2. + 2\,H+ \longrightarrow O_2 + H_2O_2.$$

3.10.2.2.Reagents:

1.Mixed substrate (Xanthine - 0.05 mmol/l, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride,(I.N.T) -0.025 mmol/l).The contents of one vial of mixed substrate is reconstituted with 20 ml of buffer and is stable for 10 days when stored at +2°C. to +8 °C.

2. Buffer . Contents ready for use.

3.Xanthine Oxidase (80 U/l).

One vial of xanthine oxidase is reconstituted with 10 ml of redistilled water and is stable for 2 weeks when stored at +2°C. to +8 °C.

4.Standard

One vial of standard is reconstituted with 10 ml of redistilled water. Subsequent dilution of this standard is prepared with RANSOD sample diluent. It is recommended that the following dilutions be made to produce a standard curve. All diluted standards are stable for 2 weeks when stored at +2°C. to +8 °C.

Table 8.Preparation of standards- SOD		
Volume of	Volume of standard solution	Sample diluent
S6	Undiluted standard	-
S5	5 ml of S6	5 ml
S4	5 ml of S5	5 ml
S3	5 ml of S4	5 ml
S2	3 ml of S3	6 ml
S1	-	5 ml

Sample Preparation

EDTA whole blood is used as sample. 0.5 ml of whole blood is centrifuged for 10 minutes at 3000 rpm and then the plasma is aspirated. Then the erythrocytes are washed four times with 3 ml of 0.9% NaCl solution followed by centrifugation for 10 minutes at 3000 rpm after each wash.

The washed erythrocytes are then made up to 2.0 ml with cold redistilled water, mixed and left to stand at + 4 °C for 15 minutes. The lysate obtained is diluted with 0.01 mol/l Phosphate buffer pH 7.0, so that the % inhibition falls between 30% and 60%. A 25 fold dilution of lysate is recommended for human samples (final dilution factor=100) .

3.10.2.3.Procedure

Table 9. Assay procedure- SOD			
Wavelength: Cuvette: Temperature: Measurement:		505 nm 1 cm path length 37 °C against air	
Pipette into cuvette:			
	Sample diluent	Standards S2-S6.	Diluted sample
Diluted sample	-	-	0.05 ml
Standard	-	0.05 ml	-
Ransod sample diluent	0.05 ml	-	-
Mixed substrate	1.7 ml	1.7 ml	1.7 ml
Mix well			
Xanthine oxidase	0.25 ml	0.25 ml	0.25 ml
Mix ,read initial absorbance A1 after 30 seconds and start timer simultaneously . Read final absorbance A2 after 3 minutes.			

3.10.2.4.Calculation

$\frac{A2-A1}{3}$ = ΔA/min of standard or sample.

sample diluent rate (S1 rate) = rate of uninhibited reaction = 100%

All standard rates and diluted sample rates must be converted into percentages of the sample diluent rate, and subtracted from 100 % to give a percentage inhibition.

$$100 - \frac{(\Delta Astd/min \times 100)}{(\Delta As1/min)} = \% \text{ inhibition}$$

$$100 - \frac{(\Delta Asample/min \times 100)}{(\Delta As1/min)} = \% \text{ inhibition}$$

Percentage inhibition for each standard is plotted against standard concentration in SOD units/ml and percentage inhibition of sample is used to obtain units of SOD from standard curve.

SOD units / ml of whole blood = SOD units/ ml from std curve X dilution factor.

Normal Ranges- 164-240 U/ml.

3.10.3.Total Antioxidant Assay

Assay of Antioxidant was carried out using commercially available kit(Cayman chemical co. Catalog no; 709001)(Miller N.J & Rice-Evans C.,1993,1997)

3.10.3.1.Assay Principle:

Reactive oxygen species(ROS) are produced as a consequence of normal aerobic metabolism. Unstable free radical species attack cellular components causing damage to lipids, proteins, and DNA, which can initiate a chain of events resulting in the onset of a variety of diseases. Living organisms have developed complex antioxidant systems to counteract ROS and to reduce their damage. These antioxidant systems include enzymes such as superoxide dismutase, catalase and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin, and an array of small molecules including ascorbic acid, alpha tocopherol, beta carotene, reduced glutathione, uric acid and bilirubin.

The sum of endogenous and food derived antioxidants represents the total antioxidant activity of the system. The cooperation among different antioxidants provides greater protection against attack by reactive oxygen species or nitrogen species, than any single compound alone. Thus the overall antioxidant capacity may provide measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids.

Aqueous and lipid soluble antioxidants are not separated in this protocol, thus the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid etc. are assessed. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2'-Azino-di-(3-ethylbenzthiazoline sulphonate) to ABTS *+ by metmyoglobin. The amount of ABTS *+ produced can be measured by reading the absorbance at 750 nm. Under the conditions used, the antioxidants in the sample cause suppression of the absorbance at 750 nm to a degree, which is proportional to their concentration. The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water soluble tocopherol analogue, and is quantified as millimolar trolox equivanlents.

3.10.3.2.Reagents

1.Anti oxidant Assay Buffer: 3 ml assay buffer is diluted with 27 ml HPLC grade water. This diluted assay buffer contains 5 mM potassium phosphate , pH7.4, containing 0.9% Sodium chloride and 0.1% glucose. It is stable for 6months at 4°C.

2. Antioxidant assay chromogen: One vial of a lyophilized powder of ABTS(2,2'-Azino-di-(3-ethylbenzthiazoline sulphonate) is reconstituted by adding 6 ml HPLC grade water and is stable for 24 hours at 4°C.

3. Anti oxidant assay metmyoglobin: One vial of a lyophilized powder of metmyoglobin is reconstituted by adding 600 µl of diluted Assay buffer and is stable for 1 month at –20° C.

4.Antioxidant assay Trolox. Contain a lyophilized powder of Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid. Reconstitute with 1 ml HPLC water, vortex well.Stable for 24 hour at 4° C.

5. Antioxidant assay Hydrogen peroxide (H_2O_2) : 8.82 M solution of H_2O_2.

Stock H_2O_2 : 10 µl of H_2O_2 is diluted with 990 µl water.

Working H_2O_2 : 20 µl of stock is diluted with 3.98 ml water. Stable for 4 hours at RT.

Sample: Serum is used as sample. Serum is diluted 1: 20 with diluted assay buffer before assay.

Trolox standards : Reconstituted trolox and assay buffer are added to seven glass tubes marked A – G , as follows.

Table 10. Preparation of Trolox standards			
Tube	Reconstituted Trolox µl	Assay Buffer µl	Final concentration. mM Trolox
A	0	1000	0
B	30	970	0.044
C	60	940	0.088
D	90	910	0.135
E	120	880	0.18
F	150	850	0.225
G	220	780	0.330

3.10.3.3.Procedure

In the designated wells on the microplate sample/ trolox standards and other reagents are added as follows-

10 µl Sample / trolox Stds(A-G)
↓

10 µl Metmyoglobin.
↓

150 µl Chromogen
↓
Initiate the reactions by adding 40 µl H_2O_2 (add within 1′)

↓
The plate is covered with plate cover, incubated on a shaker for 5 min. at room temperature and the absorbance is recorded at 750 nm using a plate reader.

3.10.3.4.Calculation

The average absorbance of each standard and sample is calculated.The average absorbance of standards are ploted as a function of the final trolox concentration for a typical standard curve.The antioxidant concentration of the samples is then calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation.

$$\text{Antioxidant (mM)} = \frac{\text{Sample average absorbance} - \text{y intercept}}{\text{slope}} \times \text{dilution}$$

Normal Range- 0.5-2 mM. (Miller N.J et al 1997., Koracevic D et al., 2001).

3.10.4. Catalase Assay

Assay of Catalase was carried out using commercially available kit(Cayman chemical co. Catalog no; 707002.)(Johanson L.H & Borg L.A.,1988, Wheeler C.R et al, 1990.)

3.10.4.1.Assay Principle:

Catalase (CAT) is an ubiquitous antioxidant enzyme that is present in most aerobic cells. CAT is involved in the detoxification of hydrogen peroxide, a reactive oxygen species, which is a toxic product of both normal aerobic metabolism and pathogenic ROS production. This enzyme catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water. CAT also demonstrates peroxidatic activity, in which low molecular weight alcohols can serve as electron donors. While aliphatic alcohols serve as

specific substrates for CAT, other enzymes with peroxidatic activity do not utilize the substrates.

catalase

$$2H_2O_2 \rightarrow O_2 + 2H_2O \text{(catalytic activity)}$$

$$H_2O_2 + AH_2 \rightarrow A + 2H_2O \text{ (peroxidatic activity)}$$

The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purplad) as the chromogen. The assay can be used to measure Catalase activity in serum.

3.10.4.2. Reagents:

1.Assay Buffer: 1 ml of assay buffer is diluted with 9 ml HPLC water to get 100 mM potassium phosphate, pH 7.0 and is stable for 2 months at 4° C.

2. Sample Buffer: 1 ml Sample buffer is diluted with 9 ml HPLC grade water to get 25mM potassium phosphate , pH 7.5, containing 1 mM EDTA and 0.1% BSA. It is stable for 2 months at 4° C.

3. Formaldehyde standard : 4.25 M formaldehyde. Reagent is ready to use.

4.Catalase Control :This vial contains a lyophilized powder of bovine liver CAT and is used as a positive control.

Stock Catalase control: One vial is rconstituted with two ml diluted sample bufferand is stable for 1 month at –20° C.

Working Catalase control: 100 µl of stock is diluted with 1.9 ml diluted Sample buffer and is stable for 30 min.

5. Potassium hydroxide (KOH) : 4 ml cold water in ice is added to KOH, vortexd to yield a 10 M solution . Stable for 3 months at 4° C.

6. Methanol.

7.Hydrogen peroxide,H_2O_2 :This vial contains an 8.82 M solution of H_2O_2. 40 µl of H_2O_2 is diluted with 9.96 ml of HPLC grade water and is stable for 2 hours.

8.Purpald chromogen:This vial contains a solution of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole in 0.5 M HCl. The reagent is ready to use as supplied.

9. Potassium periodate :This vial contains a solution of potassium periodate in 0.5 M potassium hydroxide. The reagent is ready to use.

Sample:Serum: Dilution of sample is done just before assay.

10.Formaldehyde standards : 10 µl of formaldehyde standard is diluted with 9.99 ml diluted sample buffer to obtain a 4.25 mM formaldehyde stock solution. Formaldehyde stock solution and sample buffer are added to seven glass tubes A – G , as follows-

Table 11. Preparation of formaldehyde standards.

Tube	formaldehyde µl	Sample buffer µl	Final concn. µM formaldehyde
A	0	1000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

3.10.4.3.Procedure

To all designated wells of microplate the following solutions are added as indicated-

100 µl diluted Assay Buffer.

↓

30 µl Methanol.

↓

20 µl std / sample / control

↓

Initiate the reactions by adding 20 µl H_2O_2 (workg solution, add within 1′)

↓

cover the plate with plate cover and incubate on a shaker for 20 min at Room temperature

↓

30 µl of KOH and 30 µl of Purpald chromogen .

↓

cover the plate with plate cover and incubate on a shaker for 10 min. at Room temoerature.

↓

10 µl Potassium periodate

↓

cover the plate with plate cover and incubate on a shaker for 5 min. at Room temperature

↓

Read the absorbance at 540 nm using a microplate reader.

3.10.4.4.Calculation

The average absorbance of each standard and sample is calculated. The average absorbance of standard A is subtracted from itself and all other standards and samples. The corrected absorbance of standards is plotted as a function of final formaldehyde concentration (μM).

The formaldehyde concentration of the samples are calculated using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

$$\text{Formaldehyde (}\mu\text{M). } = \frac{\text{Sample absorbance} - \text{y intercept}}{\text{slope}} \times \frac{0.17}{0.02}$$

The catalase activity of the sample is calculated using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1 nmol of formaldehyde per minute at 25°C.

$$\text{CAT activity} = \frac{\mu\text{M of sample}}{20 \text{ min}} \times \text{sample dilutio} = \text{nmol/min/ml}$$

3.10.5. Glutathione Assay

Assay of Glutathione was carried out using commercially available kit(Cayman chemical co. Catalog no; 703002.) (Tietze E.,1969, Eyer P et al.,1986, Baker M.A et al., 1990).

3.10.5.1. Assay principle

Glutathione (GSH) is a tripeptide (Gama glutamylcysteinylglycine) widely distributed in both plants and animals. GSH serves as a nucleophilic co-substrate

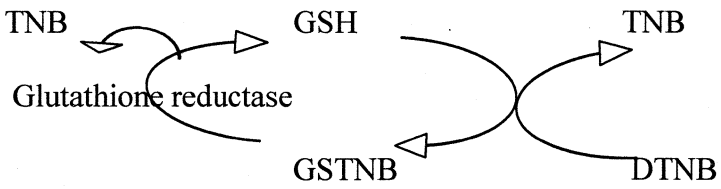
to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides. GSH is also involved in aminoacid transport and maintenance of protein sulfhydril reduction status. Concentration of GSH ranges from a few micromolar in plasma to several millimolar in tissues such as liver.

This assay method utilizes a carefully optimized enzymatic recyclic method, using glutathione reductase, for the quantification of GSH. The sulfhydryl group of GSH reacts with DTNB (5,5'-diithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow coloured 5-thio-2-nitrobenzoic acid,TNB. The mixed disulfide,GSTNB-between GSH and TNB that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of GSH in the sample.

GSH is easily oxidized to the disulfide dimer GSSG. GSSG is produced during the reduction of hydroperoxides by glutathione peroxidase.GSSG is reduced to GSH by glutathione reductase and it is the reduced form that exists mainly in biological systems. Because of the use of glutathione reductase in the assay kit, both GSH and GSSG are measured and the assay reflects total glutathione.

Glutathione reductase

GSSG ⟹ 2GSH



3.10.5.2.Reagents:

1 .MES Buffer: dilute 1:1 with HPLC grade water before use.

2. GSSG Standard: Ready to use.(Stable for 6 months at 4°C.

3. Cofactor mixture: one vial is reconstituted with 0.5 ml water..(Stable for 2 weeks at 0-4 °C.)

4. Enzyme Mixture: one vial is reconstituted with 2 ml diluted MES buffer .1 . (Stable for 2 weeks at 0-4°C).

5. DTNB: One vial is reconstituted with 0.5 ml water. (used with in 10min.)

6. Additional Reagents Needed: Metaphosphoric acid and Triethanolamine.

Sample: serum.

Deproteination of samples:

MPA Reagent : Dissolve 1 g metaphosphoric acid in 10 ml . (stable for 4 hours at 25◦ C)

Procedure:Equal volume of MPA reagent and sample is mixed well by vortexing, allowed to stand at room temperature for 5min., centrifuged at 2000 g for 2min and the supernatant is separated and can be stored for 6 months at –20◦ C.

TEAM Reagent: 4 M solution of triethanolamine.

531 µl TEA is diluted with 469 µl water.(Stable for 4 hrs at 25◦ C)

Just before assay 5oµl of TEAM reagent is added to one ml of supernatant and mixed..(Any dilution of the sample done at this stage is with MES buffer).

Standards : Different concentrations of GSSG standards in MES buffer are prepared as follows-

Table 12. Preparation of GSSG standards

Tube	GSSG µl	MES buffer µl
A	0	500
B	5	495
C	10	490
D	20	480
E	40	460
F	80	420
G	120	380
H	160	340

Preparation of Assay cocktail:

Contains 5.625 ml of MES buffer,0.225 ml reconstituted cofactor mixture, 1.05 ml reconstituted Enzyme mixture, 1.15 ml water and 0.225 ml reconstituted DTNB. (stable for 10 min.)

3.10.5.3. Procedure:

In the designated wells on the microplate , samples/ standards and other reagents are added as follows

50 µl Sample / Standards

↓

150 µl fresh assay cocktail

↓

incubate in dark on an orbital shaker

↓

measure the absorbance at 25min. at 405 or 414 nm using a micro plate reader.

3.10.5.4.Calculation

The average absorbance of each standard and sample is calculated. Substracted the absorbance value of the standard A from itself and all other values of both standards and samples. This corrected absorbance values of each standard is plotted as a function of the concentration of total GSH in micro M. Calculate the values of Total GSH for each sample from the standard curve.

$$\text{Total GSH} = \frac{\text{absorbance at 405 nm} - \text{y intercept} \times \text{dilution}}{\text{slope}}$$

Normal Range- 0-16 µM

3.10.6.Isoprostane Assay:

Isoprostane was estimated by Competitive Enzyme Immunoassay using commercially available kit (Cayman chemical company. Catalog no; 516351.) (Pradelles P et al.,1985, Maclouf J et al., 1987).

3.10.6.1.Assay principle:

The isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals. Isoprostanes appear as artifacts in tissue and plasma samples, which have undergone oxidative degradation during prolonged, or improper storage. They also appear in the plasma and urine under normal conditions and are elevated by oxidative stress. At least one of the isoprostanes, 8-isoprostane has been shown to have biological activity. It is a potent pulmonary and renal vasoconstrictor and has been implicated as a causative mediator of hepatorenal syndrome and pulmonary oxygen toxicity.

This assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase (AchE) conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane specific rabbit antiserum binding sites. Because the concentration of the 8-isoprostane tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-isoprostane tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-isoprostane in the well. This rabbit antiserum-8-isoprostane (either free or tacer) complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent, which contains the substrate to AchE, is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-isoprostane

tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well during the incubation.

3.10.6.2.Reagents:

1. Enzyme Immuno Assay (EIA) Buffer : The contents of vial is diluted with 90 ml of ultrapure water.

2. Wash buffer: 1ml of wash buffer is diluted to 400 ml with ultrapure water and 0.2 ml of Tween 20 is added.

3. 8-isoprostane standard: 100 µl of the standard is diluted with 900 µl ultra pure water. The concerntration of this bulk standard solution is 5 ng/ml. 8 test tubes are taken and numbered as1-8. Aliquot 900 µl of EIA buffer to tube 1 and 750 µl EIA buffer to tubes 2-8. 100 µl of the bulk standard is transfered to tube 1 and mixed thoroughly. This is serially diluted by removing 500 µl from tube 1 and placing in tube2. This process is repeated up to tube 8.

4. 8-Isoprostane AchE Tracer : Reconstituted one vial with 6ml EIA buffer.

5. 8-Isoprostane antiserum : The vial is reconstituted with 6 ml EIA buffer.

6. Ellman's reagent : One vial is reconstituted with 20 ml ultrapure water.

Sample: EDTA plasma.

3.9.6.3.Procedure :

Table 13. Assay procedure -Isoprostane				
Well	EIA buffer	Std/sample	Tracer	Antibody
Blank	-	-	-	-
TA, Total Activity	-	-	5µl	-
NSB, Non specific binding	100µl	-	50µl	-
Bo, Maximum binding	50µl	-	50µl	50µl
Std/sample	-	50µl	50µl	50µl
Cover the plate and incubate for 18 hours at 4◦C. ↓ Empty the wells and rinse five times with wash buffer.				
Ellman's reagent	200 µl	200 µl	200 µl	200 µl
Keep in orbital shaker in the dark. The assay develops in 90-120 minutes.(when Bo wells equals 0.3 A.U). ↓ Read the plates between 405-420 nm.				

3.10.6.4.Calculation:

Calculate the average of the absorbance readings from the NSB wells, the average of the absorbance readings from the Bo wells and subtract the NSB average from the Bo average for corrected Bo or corrected maximum binding. The corrected Bo divided by the actual TA will give the % bound. Plot %B/Bo for standards 1-8 versus 8-isoprostane concentration (pg/ml). The %B/Bo value for each sample is calculated .The concentration of each sample is calculated by identifying the %B/Bo on the standard curve and reading the corresponding values on the X-axis.

3.10.7.Estimation of Malondialdehyde(MDA)

Thiobarbituric acid method (Beuge JA,1978)

3.10.7.1.Reagents:

1. MDA standard: stock standard - 1,1,3,3-tetramethoxy propane – 8 micro litre/100 ml distilled water.

2. Working standard- 1 ml stock diluted to 100 ml.

3. Reagent – Dissolve 3g trichloroacetic acid, 75mg thiobarbituric acid in 19.58ml distilled water and 0.42ml of concentrated HCl, using magnetic stirrer(prepare freshly).

Sample: Serum

3.10.7.2.Procedure:

Malondialdehyde was estimated by thio barbituric acid assay. 1 ml of serum is combined with 2 ml of TCA-TBA-reagent and mixed thoroughly. The solution is heated for 15 minutes in a boiling waterbath. After cooling the mixture is centrifuged at 1000 X g for 10 minutes and the absorbance is recorded at 535 nm in a spectrophotometer. Malondialdehyde (MDA;tetramethoxy propane) is used as standard. Stock solution of standard is prepared by making up 8.3 microliter of MDA in 100 ml double distilled water. The stock solution is diluted1 :100 with double distilled water to make a working standard. The amount of malondialdehyde is expressed in nmol/ml.

3.10.7.3.Calculation

Malondialdehyde = absorbance of test/absorbance of standard X 4 nmol/ml.

Chapter 4

RESULTS

4.1. Descriptives

4.1.1.Epilepsy vs non epilepsy

We recruited 65 WWE (32 with generalized epilepsy and 33 localisation related epilepsy) in this study. Their mean age was 28.9± 4.7 years. All subjects were on AEDs, 54 monotherapy and 11 on polytherapy. The number of subjects on various AEDs (monotherapy exposure is given in bracket) were as follows: carbamazepine 27(17), valproate 22(21), phenytoin 8(6), phenobarbitone 11(5), lamotrigine 3(2), oxcarbazepine 2(2), topiramate 1(1), clobazam 5(0), clonazepam 1(0), tigabine 1(0) and gabapentine 1(0).

Control group consisted of 20 women without epilepsy (mean age 27.8± 3.2 years) or other concomittant disorders. The mean age was comparable for both groups.(table14, 15)

Table 14. Comparison of age between WWE and Controls.

Parameter	WWE	Controls	p
N	65	20	
Age mean(SD)	29.1(4.8)	27.8(3.2)	0.322

Table 15. AED therapy	
AED therapy	N (monotherapy)
Monotherapy	54 (54)
Polytherapy	11 (0)
Carbamazepine,CBZ	27(17)
Valproate,VPA	22(21)
Phenobarbitone,PB	11(5)
Phenytoin,PHT	8(6)
Lamotrigine,LTG	3(2)
Oxcarbazepine,OXB	2(2)
Clobazam,CLB	5(0)
Topiramate,TPM	1(1)
Clonazepam,CLZ	1(0)
Tiagabine,TGB	1(0)
Gabapentine,GBP	1(0)

4.1.1.1.Diet and folate supplementation

Six of WWE were on folic acid, 5mg/day. No subject was taking any dietary antioxidant supplements or any other medications in both groups. Regarding the dietary details, protein and calorie intake are comparable in both groups. (Table 16)

Table 16.Dietary details			
Group	WWE Mean (SD)	Control Mean (SD)	p
Protein (gm/day)	45.59 (11.7)	47.2(13.9)	0.94
Calorie (Cal/day)	1767.1(556.97)	1681.86 (587.5)	0.81

4.1.2.Pregnancy

We recruited 14 WWE (mean age 25.5 ± 3.1 years) and 10 control women (mean age 22.9± 1.4 years) who were pregnant. The duration of pregnancy was between 4-16 weeks after last menstrual period. All the women in the pregnant state were on folic acid and WWE were taking 5mg/day and control women were taking 0.5 mg/day. All the WWE were taking folic acid from the preconceptional period as

per the protocol of the Kerala Registry of Epilepsy and Pregnancy. Healthy controls had started folic acid only after confirmation of pregnancy.

4.1.3.Fetal Malformation/unfavourable outcome

Women with epilepsy recruited for this comparison can be grouped into two-group E_{NM} and EM. There were 43 women in Group EM with unfavourable outcome. They were 32 women with fetal malformations and 17 women with abortions in previous pregnancies. Group E_{NM} consisted of 22 women with normal pregnancy outcome. Of the 32 children with malformations, 18 had congenital heart diseases, five with central nervous system defects , four renal defects, two cleft lip and palate, hypospadias, umbilical hernia and multiple congenital anomalies one each.(Table17). They were also compared with healthy controls,C.

Table 17. **Malformations**	
CHD (ASD,VSD,PDA)	18
CNS defects (Hydrocephalus, Spinabifida, Meningocoele , Craniosynostosis , microcephaly).	5
Renal defects (hydronephrosis, pyelonephrosis)	4
Cleft lip and palate	2
Hypospadias	1
Umbilical hernia	1
Multiple congenital anomalies	1

Number and characteristics of different groups included in the study were summarised in the following table.(table 18)

Table 18. Different groups in the study						
Group	E	EM	E_{NM}	C	CP	EP
N	65	43	22	20	10	14
Epilepsy	Yes	Yes	Yes	No	No	Yes
N	65	43	22			14
AEDS	Yes	Yes	Yes	No	No	Yes
N	65	43	22			14
Unfavourable	Yes	Yes	No	No	No	No
outcome, N	43	43				
Pregnancy	No	No	No	No	Yes	Yes
N					10	14
Non pregnancy	Yes	Yes	Yes	Yes	No	No
N	65	43	22	20		
Folate	Yes	No	Yes	No	Yes	Yes
N	6		6		10	14

4.2.Free radical metabolism end products and antioxidant profile

4.2.1.Epilepsy vs non epilepsy

i. MDA

The mean concentration of MDA was found to be significantly higher in WWE exposed to AEDs when compared to control.(Table 19, Figure 16)

ii. ISP

The mean concentration of ISP also found to be significantly higher in WWE exposed to AEDs when compared to control.(Table 19, Figure17)

Table 19: Comparison of WWE and control group for indicators of oxidative stress (Malondialdehyde - MDA and Isoprostane –ISP).			
Indicators of oxidative stress	WWE Mean (SD)	Control Mean (SD)	P
N	65	20	-
MDA (nmol/ml)	3.33 (0.91)	2.42 (0.51)	0.000
ISP (pg/ml)	15.90 (4.7)	10.77 (4.08)	0.020

Figure 17.Comparison of MDA(nmol/ml) in WWE and Control groups. Group E had significantly higher levels of MDA.

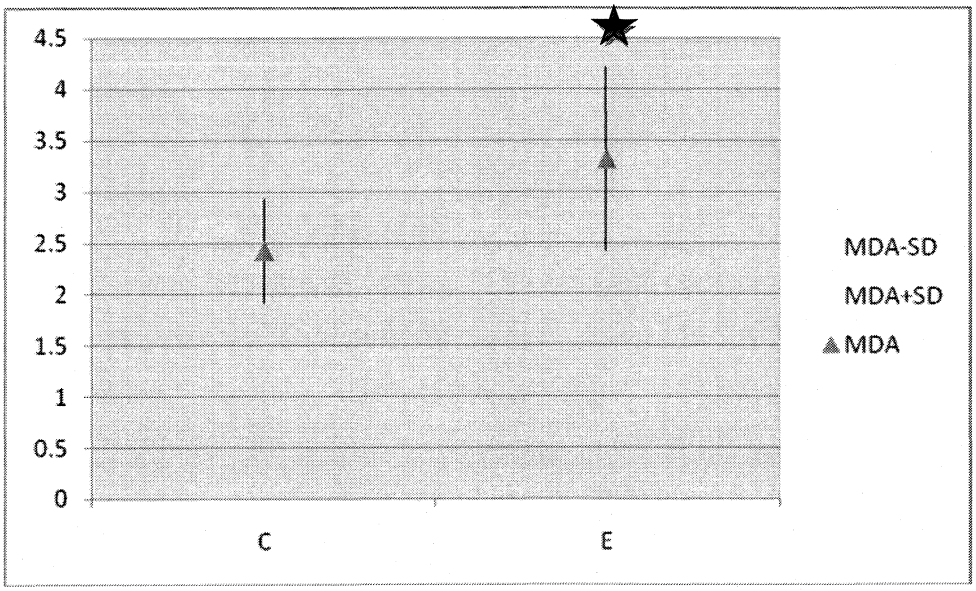
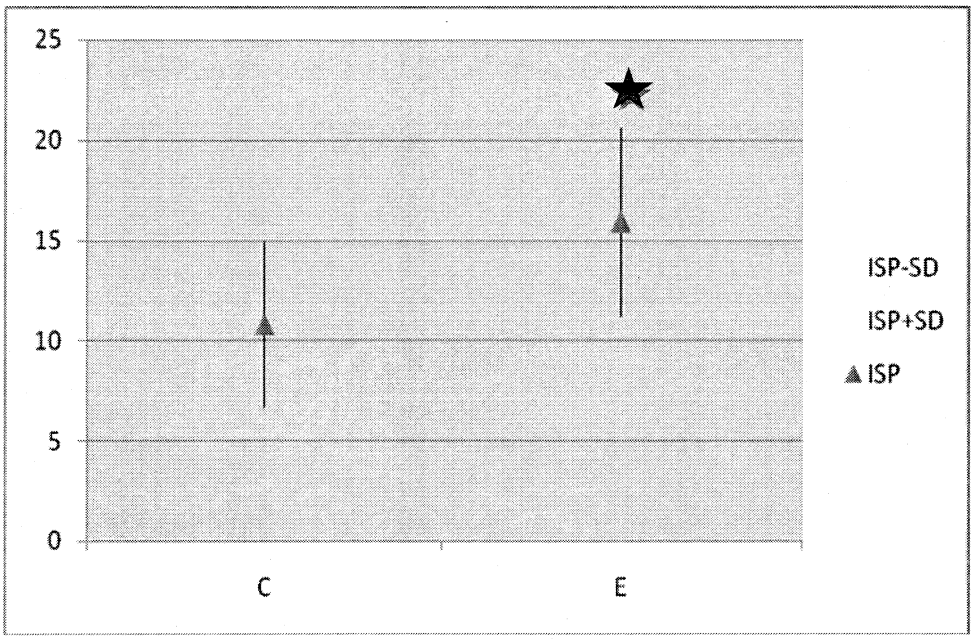


Figure 18. Comparison of ISP(pg/ml) in WWE and Control groups. Group E had significantly higher levels of ISP.



iii. AO profile

Their levels of antioxidant enzyme SOD and GSH content were significantly lower than controls. No significant changes are seen in TAO,CAT and GR .(Table20)

Table 20: Antioxidant profile in WWE and control group.

Parameter	WWE Mean (SD)	Control Mean (SD)	p
N	65	20	-
TAO (mM)	2.33(0.68)	2.43(0.92)	0.603
SOD (U/ml)	146.82(42.65)	175.81(42.61)	0.009
GR (U/l)	41.46(10.18)	42.71(9.08)	0.624
CAT (nmol/min/ml)	2.54(2.81)	2.62(2.04)	0.906
GSH (micro M GSH)	0.98(0.98)	1.55(1.3)	0.037

SOD=Superoxide dismutase, GR=Glutathione reductase, CAT=Catalase, GSH=Glutathione P<0.05 is significant

Correlations with

i. Type of epilepsy

There were 32 women with generalized epilepsy and 33 localisation related epilepsy in this study. Oxidative stress showed no significant change in any of the parameters regarding GE and LRE .(Table21)

Table 21. Comparison of oxidative stress in generalised epilepsy and localisation related epilepsy.

Parameter	GE Mean (SD)	LRE Mean (SD)	p
N	32	33	-
MDA (mol/ml)	3.36 (0.98)	3.31 (0.85)	0.56
TAO (mM)	2.43 (0.69)	2.23 (0.67)	0.88
SOD (U/ml)	148.95 (46.7)	144.75 (38.99)	0.79
GR (U/l)	41.63 (8.56)	41.45 (11.98)	0.98
CAT (nmol/min/ml)	2.85 (3.2)	2.24 (1.87)	0.62
GSH (micro M GSH)	1.1 (0.91)	0.97 (1.06)	0.51

MDA=malondialdehyde,TAO=total antioxidant status, SOD=Superoxide dismutase, GR=Glutathione reductase, CAT=Catalase, GSH=Glutathione.

ii. AEDs used

There were 22 women using valproate, 27 were using carbamazepine, 8 were using phenytoin,11 on phenobarbitone and 5 on clobazam. All the drugs compared showed a significant increase in MDA. But their antioxidant profile were different.

Carbamazepine showed significant reduction in SOD and GSH. Phenytoin showed a significant reduction in SOD only. TAO, GR,CAT showed no significant changes in these groups. No significant changes are seen in any of the antioxidant profile in the case of valproate, phenobarbitone and clobazam.(Table22, 23)

Table 22. Comparison of oxidative and antioxidative parameters of different drugs in WWE.						
Group	MDA (mol/ml)	TAO (mM)	SOD (U/ml)	GR (U/l)	CAT (nmol/min /ml)	GSH (micro M GSH)
NO DRG N=20	2.42(0.51)	2.43(0.92)	175.81(42.61)	42.71(9.08)	2.62(2.04)	1.55(1.3)
VPA N=22	3.43(0.96)*	2.39(0.83)	152.04(41.94)	38.01(7.19)	3.16(3.99)	1.37(1.38)
PHT N=8	3.45(0.9)*	2.13(0.53)	120.69(39.3)*	41.62(10.5)	1.53(0.81)	0.86(0.74)
PB N=11	3.87(0.71)*	2.43(0.51)	143.73(52.42)	40.28(8.19)	2.84(2.32)	1.15(0.6)
CBZ N=27	3.46(0.88)*	2.32(0.66)	140.81(36.52)*	42.86(12.27)	2.39(2.05)	0.79(0.69)*
CLB N=5	3.45(0.95)*	2.14(0.79)	158.58(16.59)	44.0(5.27)	1.58(1.23)	0.98(0.95)

MDA=malondialdehyde,TAO=total antioxidant status, SOD=Superoxide dismutase, GR=Glutathione reductase, CAT=Catalase, GSH=Glutathione.
VPA=valproate, PHT=phenytoin, PB=phenobarbitone, CBZ=carbamazepine, CLB=clobazam.
* significantly different($p<0.05$) from NO DRG group by independent t test.

Table 23.Summary of oxidative stress for different drugs.						
Drug	MDA	SOD	GSH	GR	CAT	TAO
CBZ	↑	↓	↓	_	_	_
PHT	↑	↓	_	_	_	_
VPA	↑	_	_	_	_	_
CLB	↑	_	_	_	_	_
PB	↑	_	_	_	_	_

iii.Monotherapy vs Polytherapy

All the WWE (N=65) were taking antiepileptic drugs and 54 of them were on monotherapy and 11 on polytherapy. Polytherapy group showed a significant increase in MDA level. No changes are seen in antioxidant profiles-TAO, SOD, GR, GSH and CAT..(Table.24)

Table 24. Comparison of oxidative and antioxidative parameters of Monotherapy Vs Polytherapy groups.

Parameter	Monotherapy Mean (SD)	Polytherapy Mean (SD)	P
N	54	11	-
MDA(mol/ml)	3.22 (0.87)	3.89 (0.91)	0.024
TAO (mM)	2.32 (0.69)	2.36 (0.67)	0.86
SOD(U/ml)	147.44 (42.98)	143.76 (42.85)	0.8
GR(U/l)	41.45 (10.56)	41.37 (8.45)	0.98
CAT(nmol/min/ml)	2.62 (3.0)	2.23 (1.62)	0.69
GSH (micro M GSH)	0.94 (1.01)	1.17 (0.80)	0.48

MDA=malondialdehyde,TAO=total antioxidant status, SOD=Superoxide dismutase, GR=Glutathione reductase, CAT=Catalase, GSH=Glutathione. $P<0.05$ is significant.

4.2.2.Pregnancy

i. In Controls

MDA levels were significantly elevated during pregnancy for healthy controls. TAO was elevated and GR was reduced significantly when compared with healthy controls in the non pregnant state. No significant changes were found in other parameters measured SOD,CAT and GSH.(Table 25, figure19, figure20)

ii. In WWE

Pregnancy was associated with higher levels of MDA for the epilepsy group also. In fact the levels were significantly elevated during pregnancy for the WWE. Nevertheless no significant alterations were found in any of the

antioxidant profiles like TAO, SOD, GR, CAT and GSH. (Table 25, figure19, figure20)

Table 25. Comparison of oxidative and antioxidative parameters of Pregnancy with control groups

Parameter	Control gp		WWE	
	Non pregnant	Pregnant	Non pregnant	Pregnant
N	20	10	65	14
MDA (nmol/ml)	2.42(0.51)	5.29(0.67)*	3.33(0.91)	5.48(1.34)@
TAO (mM)	2.43(0.92)	4.39(1.65)*	2.33(0.68)	2.45(0.90)
SOD (U/ml)	175.81(42.61)	174.97(27.91)	146.82(42.7)	135.04(38.29)
GR (U/l)	42.71(9.08)	29.11(5.70)*	41.46(10.18)	40.20(11.73)
CAT (nmol/min/ml)	2.62(2.04)	3.37(1.46)	2.54(2.81)	2.12(1.15)
GSH (micro M GSH)	1.55(1.3)	1.39(1.84)	0.98(0.98)	1.2(1.37)

$p<0.05$ is significant.* significant with control non prg group,
@ significant with WWE non prg group.

Figure 19.Comparison of MDA(nmol/ml) in the pregnant states of WWE and Controls.

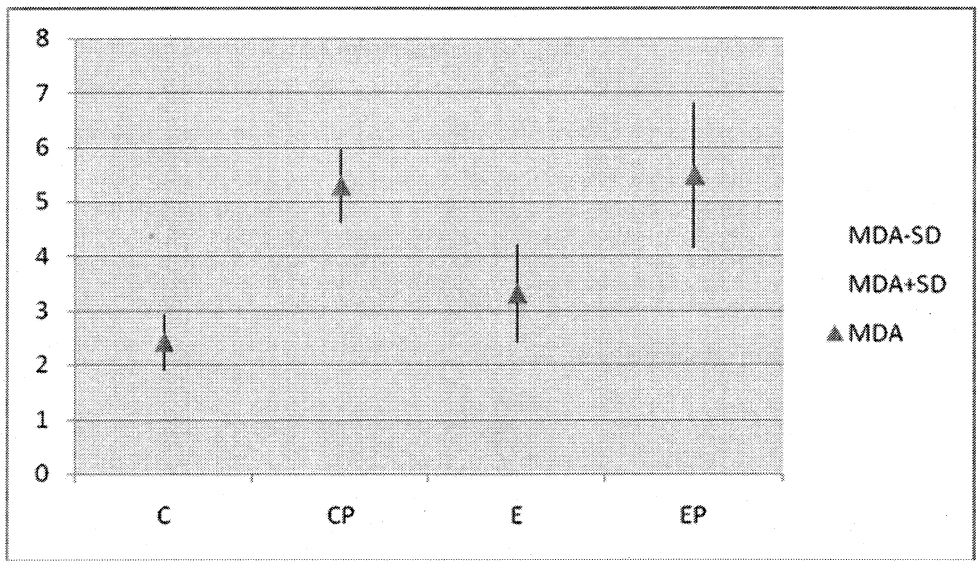
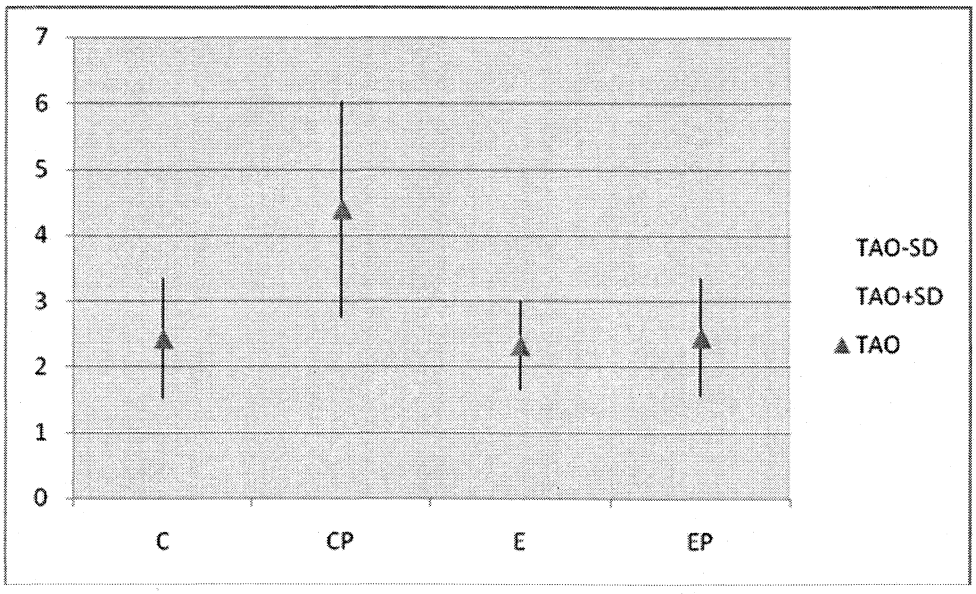


Figure 20. Comparison of TAO during pregnancy of WWE and Controls.



4.2.3.Malformation vs. no malformation

i. MDA & ISP

Group EM had highest levels of MDA and ISP; Group E_{NM} had intermediate levels of MDA and ISP and Group C had the lowest level of MDA and ISP (Table 26,figure 21,figure 22). High MDA levels (> mean + 2SD) was observed in 55.8% of cases in Group EM, 36.4% of cases in Group E_{NM} while none in the Group C had high MDA levels.The difference is statistically significant. (figure23)

ii. AO profile

Regarding antioxidant profile, Group EM had low levels of SOD and Glutathione while Group E_{NM} had low levels of SOD alone when compared to Group C. There were no significant changes with regard to TAO, GR and CAT levels. (Table26) . Antioxidant profile adding all components together, group E_{NM} had low antioxidant profile and is the weakest for group EM(figure 24). A significant proportion of cases in Group EM (14%) had very low SOD levels(< mean – 2 SD) where as none in other groups had very low SOD levels.(figure 25)

Table 26.Comparison of oxidative and antioxidative parameters expressed as Mean (SD) of WWE with fetal malformation or abortion and controls			
Parameter	Control C	Epilepsy E_{NM}	Epilepsy -malformation or Abortion EM
N	20	22	43
MDA(nmol/ml)	2.42(0.51)	3.07(1.02)*	3.46(0.82)*
ISP(pg/ml)	10.77 (4.1)	14.0(5.3)	17.77(3.0)
TAO (mM)	2.43(0.92)	2.31(0.66)	2.34(0.70)
SOD(U/ml)	175.81(42.61)	150.61(35.97)*	144.88(45.97)*
GR(U/l)	42.71(9.08)	37.6(15.67)	41.71(9.90)
CAT(nmol/min/ml)	2.62(2.04)	2.76(4.03)	2.43(1.96)
GSH (micro M GSH)	1.55(1.3)	1.21(1.05)	0.83(0.94)*
MDA=malondialdehyde,TAO=total antioxidant status, SOD=Superoxide dismutase, GR=Glutathione reductase, CAT=Catalase, GSH=Glutathione,P<0.05 is significant.			

Figure 21. Comparison of MDA(nmol/ml) in WWE with unfavourable outcome and WWE with normal outcome and Controls. MDA significantly high for group E_{NM} and EM when compared with C.

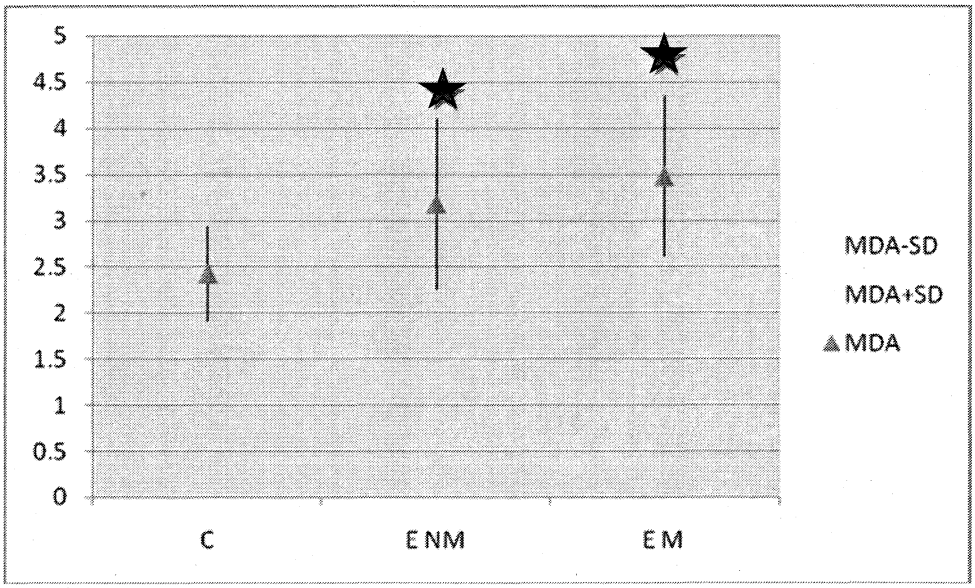


Figure 22. Comparison of ISP(pg/ml) in WWE with unfavourable outcome and WWE with normal outcome and Controls. ISP significantly high for group E_{NM} and EM when compared with Controls.

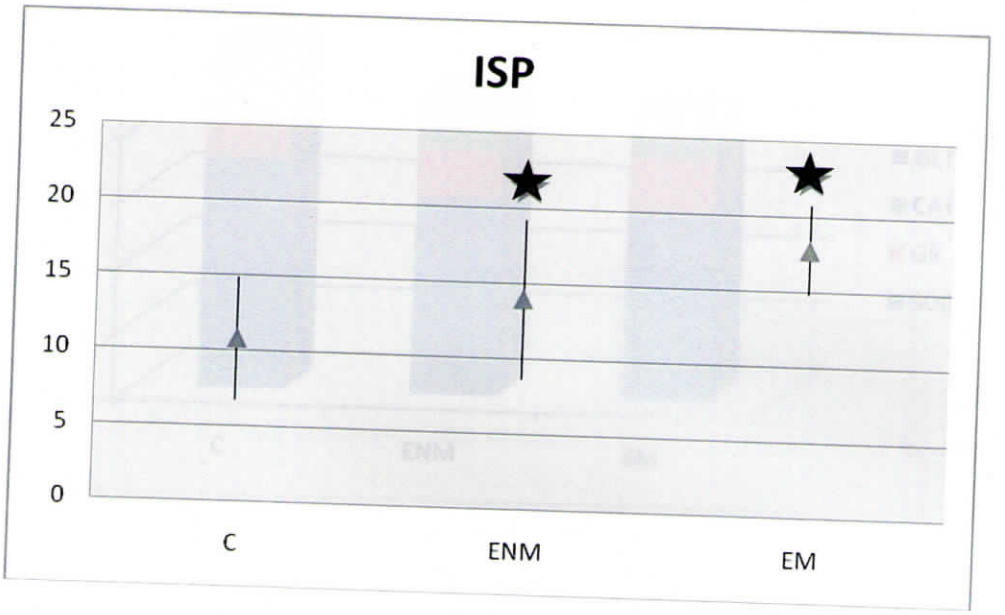


Figure23. Comparison of high MDA(nmol/ml), MDA>mean+2 SD. Significantly high value is seen for EM when compared with E_{NM}.

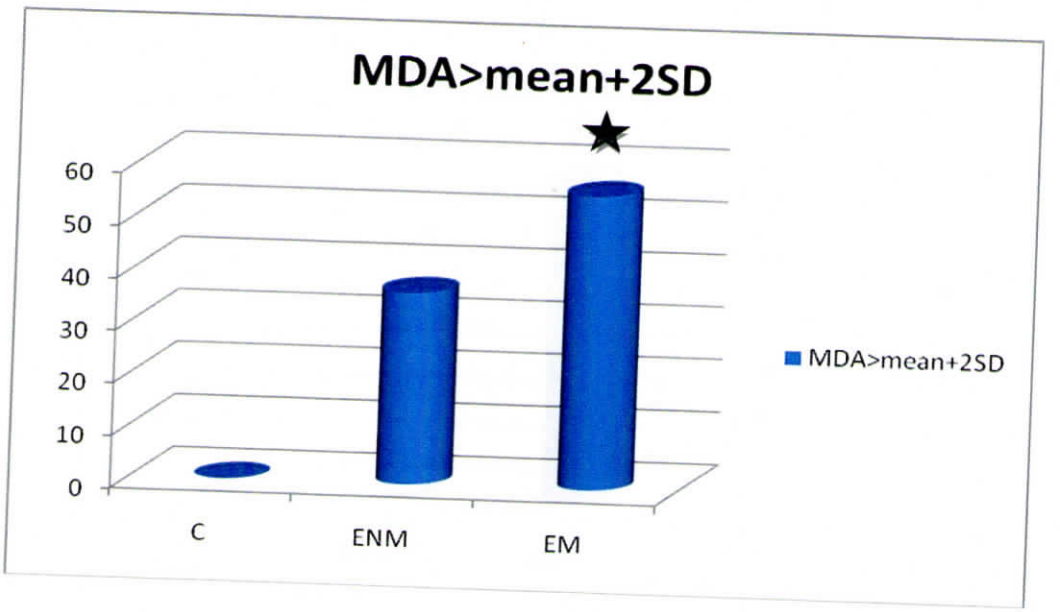


Figure 24. Comparison of Antioxidant profile adding all components togather, group E_{NM} had low antioxidant profile and is the weakest for group EM

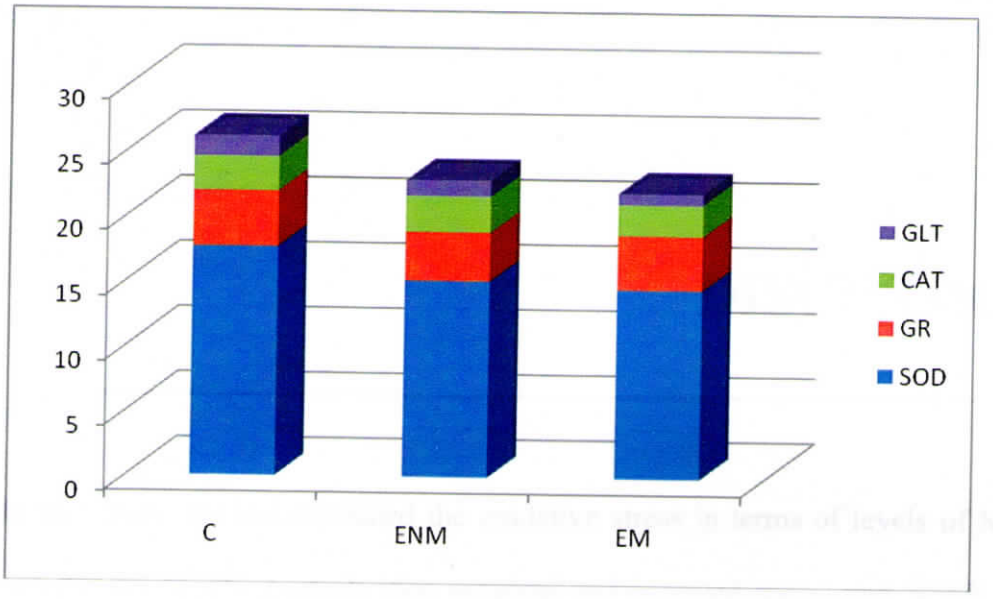
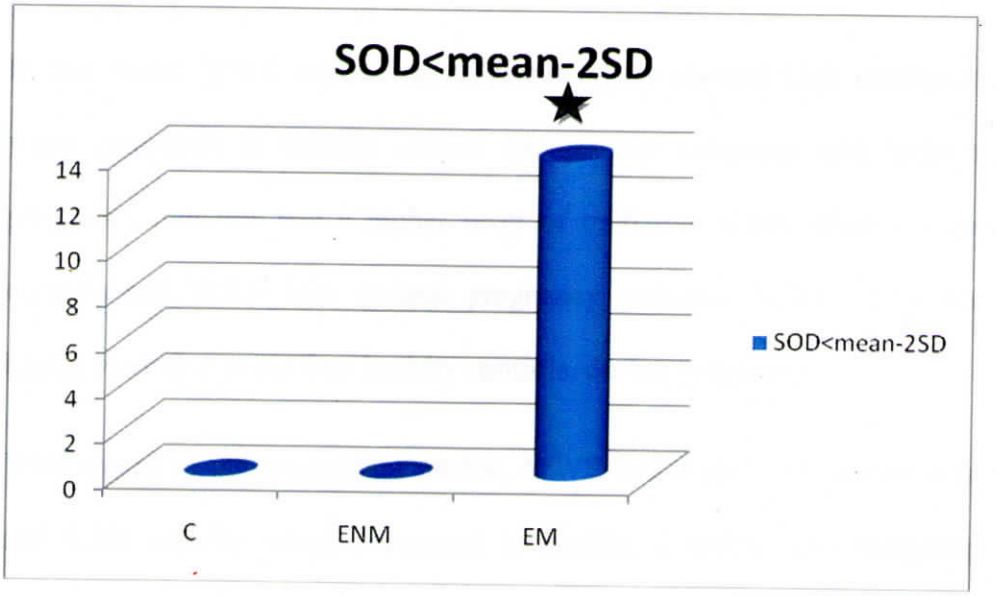


Figure 25. Comparison of low SOD(U/ml), SOD<mean - 2 SD. Significantly low value is seen for EM when compared with E_{NM}



Chapter 5

DISCUSSION

In this study, we had estimated the oxidative stress in terms of levels of MDA and ISP for healthy controls (non pregnant and pregnant states) and WWE (non pregnant and pregnant states). The nonpregnant WWE were drawn from those with normal pregnany outcome and those with abnormal pregnancy outcome. We also compared these groups for antioxidant profile including TAO, SOD, CAT, GR and GSH.

In this study, WWE exposed to different AEDs showed high oxidative stress when compared to healthy control group. The subgroup with unfavourable pregnancy outcome had a higher level of oxidative stress, when compared to controls and WWE with normal pregnancy outcome. WWE responded with higher oxidative stress than healthy controls, during pregnancy.

With regard to the antioxidant profile, WWE had significant reduction in SOD and GSH activity when compared to healthy controls. The subgroup with unfavourable pregnancy outcome had significant reduction in SOD and GSH and

the group with normal pregnancy outcome showed significant reduction in SOD only when compared with healthy controls. Healthy controls responded by significant increase in their TAO and reducing GR, where as no significant alteration is seen in antioxidant profile in WWE during pregnancy.

Epilepsy, not being a metabolic disorder and epileptogenesis are unlikely to elicit hike in FR production or deficiency of antioxidants directly. Earlier report suggests that there were no significant differences in the parameters of oxidative stress measured between WWE without antiepileptic drug therapy and healthy female controls (Liu et al.,1997). WWE included in this study had good control of seizures and none of them had seizures for at least one week prior to blood collection. Hence it is unlikely that the oxidative stress profile was influenced by epilepsy or occurrence of recent seizures. Intake of natural antioxidants and other dietary supplements are comparable for both groups (those with epilepsy and healthy controls). Both groups were well matched for sociodemographic characteristics and age. The detailed inventory of diet and drug history did not reveal any difference between these two groups. Hence the differences in the oxidative stress and antioxidant profile observed in the two groups are unlikely to be related to nutritional or environmental factors. Under these circumstances, the increase in oxidative stress seen in WWE has to be attributed to their exposure to AEDs.

It is intriguing that the antioxidant profile of WWE did not reveal a consistent pattern although all of them had excess oxidative stress. All WWE exposed to any of the AEDs showed significant reduction in SOD and Glutathione content.

However only some of the other antioxidant enzymes were down regulated. The increase in the oxidative stress can be due to the low activity of antioxidant enzymes and glutathione content. SOD showed a significant reduction in activity in WWE exposed to AEDs leading to an increase of superoxide radical. SOD is the first enzyme involved in the antioxidant defence of living tissues. It lowers the steady state levels of superoxide. Since SOD levels are low, the H_2O_2 formed by dismutation of superoxide radicals will not increase. H_2O_2 is further decomposed by CAT enzyme and the actitivity of CAT showed no significant alteration in WWE . Also a significant depletion of GSH was found in WWE exposed to AEDs. Metabolic disposition of different AEDs include formation of intermediate compounds which are teratogenic and detoxification of these compounds actively involve antioxidant system, mainly GSH (Sankar., 2007). It had been demonstrated that oxidative stress induces an efflux of GSSG and thereby decreasing the GSH activity of red blood cell (Manoharan et al.,2004). It can be postulated that the excess ROS generated during the metabolism of AEDs rapidly depletes the SOD and GSH content and thereby compromising the organisms ability to detoxify ROS. This in turn leads to considerable oxidative damage including teratogenesis.

WWE with fetal malformation or abortion(unfavourable pregnancy outcome) showed much higher oxidative stress and lower antioxidant profile when compared to the controls and WWE who had normal pregnancy outcome. WWE with unfavourable outcome showed significant reduction in SOD and GSH content where as WWE with normal outcome showed significant reduction in

SOD only. SOD levels showed significant reduction in WWE with unfavourable outcome when compared with women having normal outcome. Higher oxidative stress in WWE with malformation or abortion can be due to the decreased antioxidant profile. The antioxidant pathways that protect the tissues from damage are complex and multifactorial. The susceptibility of cells to oxidative stress is a function of the over all balance between the degree of oxidative stress and the antioxidant defence capability. WWE having unfavourable outcome had low antioxidant profile when compared with WWE having normal outcome. The increase in oxidative stress and low antioxidant profile can be a reason for their unfavourable outcome.

Our study with these positive findings can prove the hypothesis that women with epilepsy has increased oxidative stress and it has a relation with unfavourable outcome in women with epilepsy.

Pregnancy was associated with increased oxidative stress in both healthy women and WWE. Nevertheless, there was no increase in the total antioxidant capacity during pregnancy for WWE when compared to women without epilepsy. Healthy women respond to the excess oxidative stress induced by pregnancy by enhancing their total antioxidant capacity. In contrast to this, there was no such compensatory increase in adaptive response was observed in women exposed to AEDs during pregnancy. It appears that AEDs interfere with the capacity to handle oxidative stress particularly during pregnancy and thereby leads to teratogenicity.

Polytherapy was associated with higher oxidative stress when compared with monotherapy group. But no changes are seen in the antioxidant profile between the two groups. Polytherapy, however, is more likely to have an adverse effect on the foetus and is associated with higher risk, and also more severe malformations(Barrett et al.,2003).

In this study, we had opportunity to examine the effect of various AEDs on oxidative stress and antioxidant profile. All commonly used AEDS - carbamazepine, phenytoin, valproate, clobazam, phenobarbitone – showed significant increase in oxidative stress when compared with controls who were not exposed to any AEDs. But changes in their antioxidant profile is different. Carbamazepine showed significant reduction in SOD and GSH, phenytoin showed significant reduction in SOD only, no changes are seen for other drugs compared. The different drugs showed different response to the increased oxidative stress. Nevertheless, no firm conclusions can be drawn as the numbers under individual drugs for different groups were small.

Data in literature regarding AED induced oxidative stress is conflicting. Some of the AEDs are known to increase oxidative stress in human studies. Studies by Mahle et al., 1997, Sudha et al., 2001, Ayciecek et al., 2007, Hameed et al., 2004, Liu et al.,1997, Turkdogan H et al., 2002 revealed increased oxidative stress in persons with epilepsy who were taking AEDs, as observed in our study. The results of a study by Martinez-Ballesteros et al., showed that the serum lipid peroxidation level was higher in epileptic patients treated with valproic acid and presented a linear relationship with drug plasma levels. A recent study by

Aycicek et al.showed that total peroxide levels, which is a marker of oxidative stress, were elevated in PB treated epileptic children.

In contrast to these findings, there are other reports where there was no increase in oxidative stress in response to exposure to AEDs. Sobaniec et al., 2006 reported a differential response to AEDS. They observed that MDA levels were significantly lower in children with epilepsy exposed to CBZ, where as it was high for those children were exposed to VPA. Cengiz et al., reported no change in lipid peroxidation product MDA for epileptic children taking VPA and CBZ, but their GSH was significantly reduced and GP was significantly increased when compared with controls. Yis et al., 2009 showed that the levels of malondialdehyde were significantly lower and activity of superoxide dismutase was insignificantly higher in patients with newly diagnosed epilepsy. During treatment with valproic acid, lipid peroxidation increased but did not reach pathological levels. There was a positive correlation between superoxide dismutase activity and duration of valproic acid treatment.

With regard to antioxidant profile also there is much variation in the findings. Persons exposed to CBZ had low SOD activity in erythrocytes (Niketic et al., 1995). On the contrarary, the SOD level for those exposed to VPA or CBZ was similar to that of controls in another study (Kurekci et al., 1995). A mild elevation of serum SOD activity was found in patients recieving carbamazepine monotherapy (Liu et al., 1998).

Verrotti et al.,2002 had reported that neither epilepsy itself nor the antiepileptic drugs sodium valproate or carbamazepine altered the serum levels of GP and SOD in children with these agents for 1 year.

GR activity was elevated in adult patients who received phenobarbital for one year (Sudha et al, 2001). Also GR is reported to be reduced (Cotariu et al., 1992) or unchanged (Pippenger et al., 1989) in erythrocytes of children receiving valproate.

Much of these differences in the observations are due to methodological issues. The antioxidant profile is measure with diverse techniques and tissue samples. Most of the studies had been on small numbers without adequate power to demonstrate any significant differences. The exposure to AEDs had been to variable periods of time and the dosage had not been standardized. It is also possible that there may ethnic variation in the metabolic pathways and antioxidant response.

Most antiepileptic drugs are known to cause oxidative stress which can be a reason for their teratogenicity. VPA is teratogenic in most animal species and a daily dose of 1000 mg or more and/or polytherapy are associated with a higher teratogenic risk. Also several other AEDs potentiate the teratogenic effects of VPA.(Ornoy A.,2009, Devi et al., 2008). Lamotrigine inhibits presynaptic voltage-gated sodium channels and reduces the presynaptic release of glutamate in pathological states.(Tufan et al., 2008) Hepatotoxicity induced by Aromatic antiepileptic drugs - carbamazepine, phenytoin and phenobarbital has been attributed to a defective detoxification by the epoxide hydrolase and

accumulation of arene oxides leading to oxidative stress.(Santos et al.,2008). VPA treatment developed obesity and showed increased oxidative stress and decreased antioxidant markers in epileptic children.(Verrotti et al., 2008)

Observations on oxidative stress and antioxidant profile of our control subjects are in good agreement with previously reported studies and they are within the normal ranges given by the assay kits. Only TAO showed slight increase in control subjects (2.42 Vs 2.0 mM trolox equivalent) than the reported normal range by the assay kit procedure. The normal range given in the assay procedure was quoted from two studies conducted at Yugoslavia and London. Variation in results may be due the difference in indian population. Opara et al reported the mean Trolox equivalent antioxidant capacity of the control group as 2.7 ± 0.45mM.

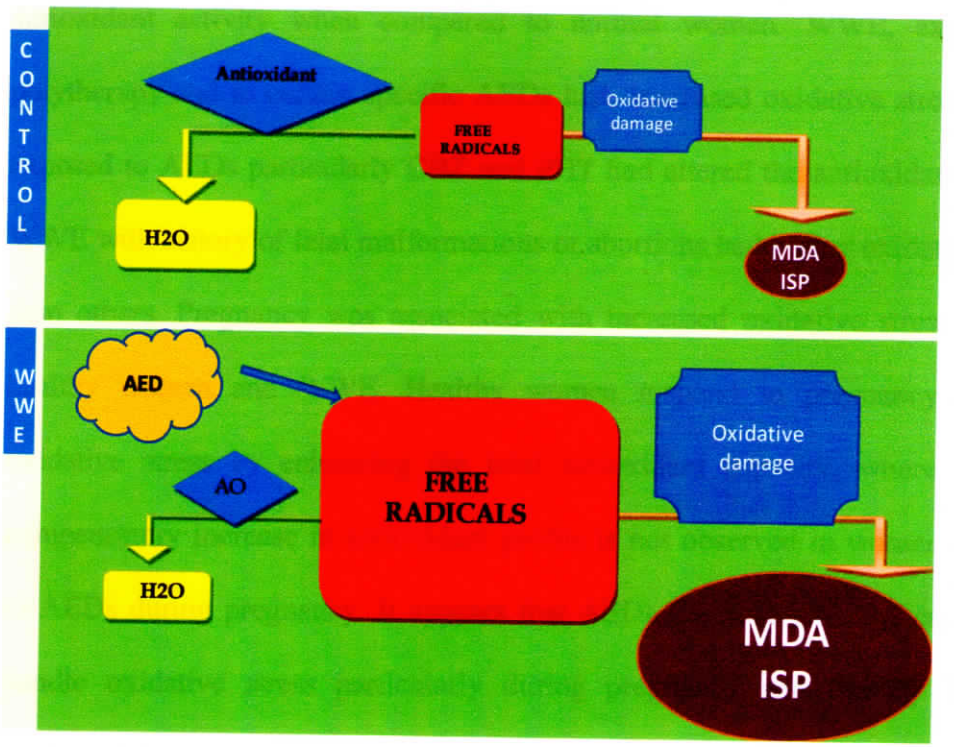
5.2.Power of the test.

We had looked at the power of individual tests to identify significant differences. Since there had been no normative data on these tests for this population, we could not carry out this test before commencing the study. We observed that variation was rather narrow for the MDA and ISP where as it was quite wide for the antioxidant enzyme profile in normal subjects. Based on the sample sizes taken 20 (healthy subjects) and 65(for the epilepsy group), the obtained mean and SDs for each variables, the power of the test is estimated (for α=.05). See table 27.

Table 27. Power of the test	
Variable	Estimated Power (%)
TAO	7.0
MDA	**99.8**
SOD	**83.8**
GR	12.7
CAT	6.5
GSH	**61.4**

The power analysis showed that there was very high power to detect any significant difference in MDA, SOD and GSH levels. The variation for the other enzymes were so wide that for the given sample size it had only low power.

Figure 26. Summary of results of oxidative stress in WWE and controls



Chapter 6

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS.

Women with epilepsy exposed to AEDs have higher oxidative stress and reduced antioxidant activity when compared to normal women. WWE, exposed to polytherapy and to certain specific AEDs had increased oxidative stress. Those exposed to AEDs particularly CBZ and PHT had altered the antioxidant profile. WWE with history of fetal malformations or abortions had higher oxidative stress than others. Pregnancy was associated with increased oxidative stress in both healthy women and WWE. Healthy women respond to pregnancy induced oxidative stress by enhancing the total antioxidant capacity, where as such compensatory increase in antioxidant profile is not observed in women exposed to AEDs during pregnancy. It appears that AEDs interfere with the capacity to handle oxidative stress particularly during pregnancy and thereby leads to teratogenicity.

The observations in this study point to a possible causal relationship between AED usage, elevated oxidative stress and fetal malformation or abortions. Our study indicates that oxidative stress is possibly an important link between AED usage and fetal malformations in WWE. This opens a possible opportunity to modify oxidative stress and there by reduce the risk of malformation by the administration of antioxidants during pregnancy. Further studies are required to ascertain whether reduction of oxidative stress would prove the hypothesis.

Chapter 7

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