EFFECT OF EPILEPSY AND SLEEP DEPRIVATION ON PLC-β1RECEPTOR GENE EXPRESSION IN BRAIN STEM AND BEHAVIORAL CHANGES IN RATS

A Dissertation Project

Submitted to

Nirma University

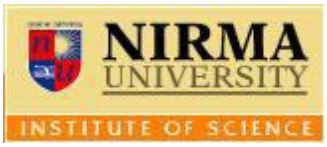
In Partial fulfillment of requirement for

The Degree of

Master of Science

In

Biochemistry



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Abstract:

Epilepsy is one of the most common disorders of the central nervous system characterized by recurrent seizures unprovoked by an acute systemic or neurologic insult. One of every ten people will have at least one epileptic seizure during a normal lifespan, and a third of these will develop epilepsy. Evidences show that epilepsy can disrupt sleep as well as sleep deprivation can increase susceptibility for seizures. Epilepsy was induced in adult Wistar male rat with a single intraperitonial dose of pilocarpine, which is known one of the best models for temporal lobe epilepsy. Rats were partially sleep deprived by modified float over water method. The rats were kept awake for 10 hours daily during their sleep cycle by stand over water method. I investigated the change in feed intake and water consumption in experimental group of rats and alterations in general behaviors in epileptic and sleep of epileptic rats due to sleep deprivation. The pineal gland secrets melatonin into the bloodstream where it modulates the brain stem circuit which plays a key role in influencing the sleep-wake cycle and activate MT1 receptors which then activates PLC-PKC signaling pathway. My last objective was to evaluate the effect of sleep deprivation and epilepsy on PLCß 1 gene expression. A good quality total RNA was isolated which was used for C-DNA preparation but I could not get the amplified product of PLCß 1 gene. The open field test is a good indicator of general exploratory activity which provides simultaneous measures of locomotion, exploration and anxiety. A significant decrease in the exploratory activity in the open field was observed in Control sleep deprived ($p<0.001$), Epileptic ($p<0.001$) and Epileptic sleep deprived ($p<0.001$) showed that epileptic sleep deprived rats show high level of anxiety and depression as compare to epileptic and control sleep deprived, while sleep deprivation in control rats can make their situation worse. “Splash Test” is the indirect measure of body care efficiency. A significant decrease in the number of grooming bouts and increase in duration of grooming was observed in Control sleep deprived ($p<0.001$), Epileptic ($p<0.001$) and Epileptic sleep deprived ($p<0.001$) which can be interpreted as a stress induced attenuation. It has been hypothesized that sleep deprivation represents an oxidative challenge for the brain and that sleep may have a protective role against oxidative damage. Cellular oxidative damage (i.e. Lipid peroxidation) by Peroxidative damage to lipids was determined by measuring Malondialdehyde concentration in Brain Stem, Liver and Kidney by performing Thio Barbituric Acid Reactive Substance enzyme assay spectrophotometrically at 532 nm. Lipid peroxidation was significantly enhanced in Control

sleep deprived ($p<0.001$), Epileptic ($p<0.001$) and Epileptic sleep deprived ($p<0.001$) groups When compared to Control. The increase of reactive oxygen species levels can be because of neurochemical alterations during seizures in epileptic groups. The increased lipid peroxidation in sleep deprived groups indicates that inadequate sleep may activate pathway for reactive oxygen formation ultimately causing changes in membrane permeability.

Key words:

Brain Stem, Epilepsy, Sleep Deprivation, Thio Barbituric Acid Reactive Substance.

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Dr. AMEE K.NAIR

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Surbhi Modi (10MBC007)

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ABBREVIATIONS:

AED- Anti epileptic drugs

BBB- Blood brain barrier

BS- Brain stem

C- Control

C+SD- Control sleep deprived

E+SD- Epileptic sleep deprived

E- Epileptic

TLE- Temporal lobe epilepsy

GPCRs- G protein couple receptor

IED-Interictial epileptiform discharge

ILAE- International league against epilepsy

NREM- Non rapid eye movement

PLC- Phospholipase C

RT PCR- Reverse transcriptase polymerase chain reaction

REM- Rapid eye movement

SRMS-Spontaneous recurrent motor seizure

SE- Status epilepticus

SRS-Spontaneous recurrent seizure

SWC- Sleep wakeful cycle

INTRODUCTION

1. INTRODUCTION

1.1 Epilepsy:

Seizures cause electrical disturbances in the brain affecting one of every ten people during a normal lifespan of which third of these will develop epilepsy. According to a world health organization (WHO) survey epilepsy accounts for 1% of the global burden of disease, equivalent to breast cancer in women and lung cancer in men (Murray et al., 1994). The word Epilepsy is derived from the Greek verb "Epil-ambanein". Epilepsy is a group of neurologic conditions, the fundamental characteristics of which are recurrent, usually unprovoked, epileptic seizures. The International League Against Epilepsy (ILAE) has defined epilepsy as chronic condition of the brain characterized by an enduring propensity to generate recurrent unprovoked seizures, and by the neurobiological, cognitive, psychological, and social consequences of this condition. Two essential epileptogenic factors represent the net effect of many complex interrelated events. The first is an abnormality of cellular excitability that arises from mechanisms that affect membrane depolarization and repolarization. The second is a network defect that derives from mechanisms underlying the development of aberrant neuronal integration, resulting in abnormal synchronization of neuronal populations and propagation of the epileptic discharge within neural pathways (Murray et al., 1994).

1.2 Epidemiology: -

After stoke and dementia, Epilepsy constitutes the 3rd most frequent neurologic disorders encountered in elderly in developed countries. Epilepsy affects 50 million people worldwide, and 80% of them live in the developing world. In developed countries, the age-adjusted incidence of epilepsy ranges from 24 to 53 per 100,000 person-years (Annegers et al., 1999). Studies suggest that the incidence of epilepsy is higher in developing countries which are more than 100/100,000 (Bharucha et al., 2003). A recent meta-analysis of published and unpublished studies puts the overall prevalence rate of epilepsy in India at 5.59 per 1,000 populations, with no statistically different rates between men and women or urban and rural residence (Sridharan et al., 1999). The

worldwide prevalence of active epilepsy is between4 and 10 per thousand populations. Epilepsy is more likely to occur in young children or people over the age of 65 years; however, it can occur at any time. In 2004, the WHO estimated that nearly 80% of the burden of epilepsy worldwide is borne by the resource-poor countries. In developed countries, the lifetime prevalence rate for epilepsy ranges from 3.5 to 10.7 per 1,000 person-years. In recent systematic reviews, the lifetime prevalence rates for active epilepsy varied from 1.5 to 14 per 1,000 person-years in Asia, from 5.1 to 57.0 per 1,000 person-years in Latin America, and from 5.2 to 74.4 per 1,000 person-years in sub-Saharan Africa. A number of studies report the incidence of epilepsy in specific age groups. These include studies of children (Beilmann et al, 1999,), adults (Forsgren et al, 1996) and the elderly. In Western countries, the incidence of epilepsy is higher after the age of 70 years than during the first 10 years of life. Only about 50% of cases of epilepsy start in childhood or adolescence (Olafsson et al, 2005).

1.3 Etiology and risk factors: -

In developed countries, etiology of epilepsy is also age-dependent. In children, about 20% are remote symptomatic, 50% are cryptogenic while 30% are idiopathic. However, in elderly, about 55% is remote symptomatic while 45% are idiopathic/cryptogenic.

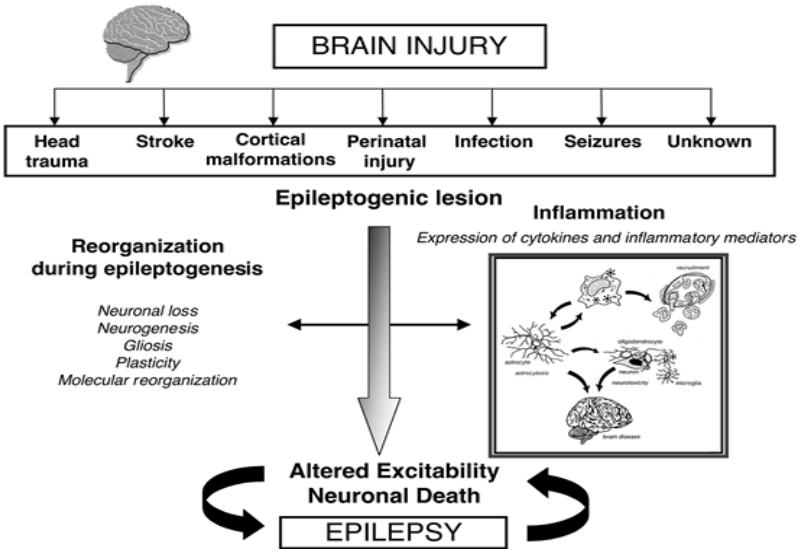


Fig. 1: - Factors contributing to the course of the process leading to epilepsy.

A variety of risk factors are thought to be significant in developing countries. These include neurocysticercosis, other nervous system infections, head trauma, perinatal factors, genetic problems caused by consanguinity, and febrile seizures.

1.4 Imbalance between Excitatory and Inhibitory Neurotransmitters: -

In the resting state a balance exists between excitatory and inhibitory neurotransmitters (Fig.2) Epilepsy is characterized by spontaneous recurrent seizures, caused by focal or generalized paroxysmal changes in neurological functions triggered by abnormal electrical activity in the cortex (Dichter et al., 1994). The major excitatory neurotransmitter is the amino acid glutamate. In the central nervous system (CNS), the disruption of the naturally existing balance between the concentrations of inhibitory and excitatory neurotransmitters is thought to be the main cause of convulsive episodes. GABA deficiency (inhibitory neurotransmission) and the stimulation and modification of either the density or sensitivity of different glutamate receptor subtypes (excitatory neurotransmission) are associated with epilepsy. In contrast, the stimulation of GABA receptors or an increase in positive modulators produces anxiolysis, sedation, anesthesia, myorelaxation and anticonvulsant actions (Silva et al., 2009). The activation of excitatory amino-acid receptors by glutamate or N-methyl-D-aspartic acid has been known to accompany the generation of ROS and reactive nitrogen species, such as superoxide anion radicals, hydrogen peroxide, nitric oxide and peroxide anions, that lead to neuronal damage (Mori et al., 2004).There are several subtypes of glutamate receptors which can be found postsynaptically on excitatory principal cells as well as on inhibitory interneurons, and have been demonstrated on certain types of glial cells. The ionotropic subclasses are the alpha-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA), kainate receptors, and N-methyl-D-aspartate (NMDA). All ionotropic glutamate receptors are permeable to Na^+ and K^+, and it is the influx of Na^+ and outflow of K^+ through these channels that contribute to membrane depolarization and generation of the action potential. but under conditions of local membrane depolarization, Mg^{++} is displaced and the channel becomes permeable to Ca^{++}; influx of Ca^{++} tends to further depolarize the cell, and is thought also to contribute to Ca^{++} mediated neuronal injury under conditions of excessive neuronal activation (such as status epilepticus and ischemia), potentially leading to cell death, a process termed excitotoxicity (Champan, 1998). Experimental

studies using animal epilepsy models have shown that NMDA, AMPA and kainate agonists induce seizure activity, whereas their antagonists suppress seizure activity. The major inhibitory neurotransmitter, GABA, interacts with 2 major subtypes of receptor: $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors are found postsynaptically, while $GABA_B$ receptors are found presynaptically, and can thereby modulate synaptic release. $GABA_A$ receptors are permeable to Cl^- ions; upon activation Cl^- influx hyperpolarizes the membrane and inhibits action potentials. $GABA_B$ receptors are associated with second messenger systems rather than Cl^- channels, and lead to attenuation of transmitter release due to their presynaptically location. The second messenger systems often result in opening of K^+ channels, leading to a hyperpolarizing current. Certain $GABA_B$ agonists, such as baclofen, have been reported to exacerbate hyperexcitability and seizures. Hence seizures are reported to be as a result of imbalance between excitatory and inhibitory neurotransmitters in the brain.

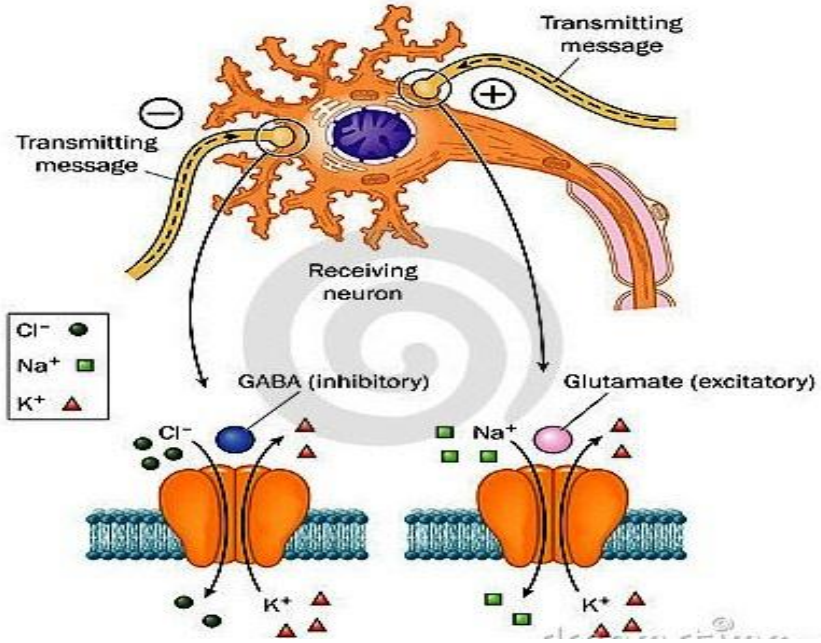


Fig. 2: -Excitatory and inhibitory neurotransmitters in neuronal signaling (Champan, 1998).

1.5 Seizures: -

When nerve cells in the brain fire electrical impulses at a rate of up to four times higher than normal, this causes a sort of electrical storm in the brain, known as seizure (Shridharan et al., 2002).

The bursting of action potential occurs due to very high generation of repetitive action potential. Seizures are of two types:

1. Partial Seizure 2.Generalized Seizure.

Partial seizures (Focal Seizures): -

Partial seizures originate in a small group of neurons that constitute a Seizure focus. Partial Seizures may be divided into Simple and Complex Seizures. This refers to the effect of such a Seizure on consciousness; Simple Seizures cause no interruption to consciousness, whereas Complex Seizures interrupt consciousness to varying degrees. A complex partial seizure may involve the unconscious repetition of simple actions, gestures or verbal utterances, or simply a blank stare and apparent unawareness of the occurrence of the seizure, followed by no memory of the seizure.

Symptoms preceding the onset of a partial seizure are called *auras*. Auras commonly include abnormal sensation such as a sense of fear, a rising feeling in the abdomen, or even a specific odor. The aura is due to electrical activity originating from the seizure focus and thus represents the earliest manifestation of a partial seizure. The time after a partial seizure before the patient return to abnormal neurological function is called the postictal period.

Partial seizures are classified into the following:-

Simple Partial Seizures - consciousness is not impaired

1 With motor signs

2 With sensory symptoms

3 With autonomic symptoms or signs

4 With psychic symptoms

Complex Partial Seizures - consciousness is impaired (temporal lobe or psychomotor seizures)

1 Simple partial onset, followed by impairment of consciousness

2 With impairment of consciousness at onset

Partial Seizures evolving to secondarily generalized seizures

1 Simple partial seizures evolving to generalized seizures

2 Complex partial seizures evolving to generalized seizures

3 Simple partial seizures evolving to complex partial seizures evolving to generalized seizures.

Generalized Seizures: -

Generalized Seizures begin without a preceding aura or focal seizure and involve both hemispheres from the onset. Primarily generalized seizures can be sub-classified into a number of categories, depending on their behavioral effects:-

Absence Seizures: -

Interruption to consciousness where the person experiencing the seizure seems to become vacant and unresponsive for a short period of time (usually up to 30 seconds). Slight muscle twitching may occur. It is classified as:

1 Typical Absence Seizures

2 Atypical Absence Seizures

Myoclonic Seizures:

These Seizures involve an extremely brief (< 0.1 second) muscle contraction and can result in jerky movements of muscles or muscle groups.

Clonic Seizures:

Clonic Seizures are myoclonus that are regularly repeating at a rate typically of 2-3 per second

Tonic–Clonic Seizures: -

Also called as Grand mal. It involve an initial contraction of the muscles (tonic phase) which may involve tongue biting, urinary incontinence and the absence of breathing. This is followed by rhythmic muscle contractions (clonic phase). This type of seizure is usually what is referred to when the term 'epileptic fit' is used colloquially.

Atonic seizures:

It involves the loss of muscle tone, causing the person to fall to the ground. These are sometimes called 'drop attacks' but should be distinguished from similar looking attacks that may occur in narcolepsy or cataplexy.

1.6 Events during seizures:-

1) High-frequency bursts of action potentials and

2) Hyper synchronization of a neuronal population.

All ionotropic glutamate receptors are permeable to Na^+ and K^+, and it is the influx of Na^+ and outflow of K^+ through these channels that contribute to membrane depolarization and generation of the action potential. The NMDA receptor also has a Ca^{++} channel that is blocked by Mg^{++} ions in the resting state, but under conditions of local membrane depolarization, Mg^{++} is displaced and the channel becomes permeable to Ca^{++}; influx of Ca^{++} tends to further depolarize the cell, and is thought also to contribute to Ca^{++} mediated neuronal injury under conditions of excessive neuronal activation such as in *Status Epilepticus*.

These changes are characteristic feature of an epileptic seizure.

- Epilepsy is also known as a seizure disorder because of the tendency is to have recurrent seizures.
- Epileptic seizures vary in severity and frequency, and in the time no diurnal pattern occur.
- While some people may experience no more than two or three seizures during their entire lifetime, others will have several seizures in one day.

1.7 Different types of Epilepsy: -

Just as there are many different kinds of seizures, there are many different kinds of epilepsy. According to the National Institute of Neurological Disorder, the most common types of epilepsy are:

- Absence Epilepsy
- Frontal Lobe Epilepsy
- Occipital lobe Epilepsy
- Parietal lobe epilepsy
- Temporal lobe Epilepsy

Absence Epilepsy:-

People with absence epilepsy have repeated absence seizures that cause momentary lapses of consciousness. These seizures almost always begin in childhood or adolescence, and Childhood absence epilepsy usually stops at puberty. Absence Seizures cause no lasting effect on intelligence or other brain functions.

Frontal Lobe Epilepsy:

Frontal lobe epilepsy is a neurological disorder that is characterized by brief, recurring seizures that arise in the frontal lobes of the brain, often while the patient is sleeping. It is the second most common type of epilepsy after Temporal Lobe Epilepsy and is related to the temporal form by the fact that both forms are characterized by the occurrence of partial (focal) seizures.

Occipital lobe Epilepsy:-

Occipital Lobe Epilepsy localization-related epilepsy in which seizures originate from the occipital lobe. Symptoms commonly include visual abnormalities during seizures.

Parietal Lobe Epilepsy:-

Parietal lobe epilepsies manifest with seizures originating from a primary epileptic focus anywhere within the parietal lobe the symptoms of parietal lobe epilepsy closely resemble those of temporal lobe epilepsy.

Temporal lobe Epilepsy (TLE):-

TLE has been reported to be the most common epilepsy syndrome with focal seizures. TLE is the most common type of partial complex seizure in adulthood (Hauser et al., 1996). Seizures are often associated with auras. TLE often begins in childhood. Research has shown that Repeated temporal lobe seizures cause a hippocampal sclerosis over time. The main features of TLE are:

(i) The localization of seizure foci in the limbic system, particularly in the hippocampus, entorhinal cortex and amygdala (Bartolomei et al., 2005).

(ii) The frequent finding of an “initial precipitating injury” that precedes the appearance of TLE (Mathern et al., 2002).

(iii) A seizure-free time interval following the precipitating injury known as “latent period”.

The hippocampus is important for memory and learning. While it may take years of temporal lobe seizures for measurable Hippocampal damage to occur, this finding underlines the need to treat TLE early and as effectively as possible.

1.8 Different Models of TLE in Animals: -

1.8.1 Pilocarpine model of epilepsy: -

The pilocarpine model was developed by Turski's group In 1983.Seizures elicited by pilocarpine, a muscarinic cholinergic agonist, have been proposed as an animal model resembling some aspects of human temporal lobe epilepsy based on electroencephalographic waves, behavioral and morphological sequelae (Turski et al., 1983). Experiments in cultured Hippocampal neurons have demonstrated that pilocarpine, acting through muscarinic receptors, causes an imbalance between excitatory and inhibitory transmission resulting in the generation of SE (Priel and Albuquerque., 2002).

Some important features of the pilocarpine model are:

(i) The induction of acute *“status epilepticus*” more rapidly than with intraperitonial (i.p.) kainic acid, the other convulsant drug commonly used to reproduce TLE in animals.

(ii) The presence of a latent period followed by the appearance of spontaneous recurrent seizures. (Leite et al., 1990). In addition, animals were motionless for 5–10 min after pilocarpine administration and subsequently displayed oro-facial movements, salivation, eye-blinking, twitching of vibrissae, and yawning (Turski et al., 1983).

The pilocarpine model appears to be highly isomorphic with the human disease, so it has been used in many laboratories since its first description a quarter of a century ago (Turski et al., 1983). This activity persisted up to 45 min. Then, discontinuous seizures were observed 30 min post injection and lasted up to 90–150 min. Pilocarpine-induced SE can be blocked by systemic administration of the muscarinic antagonist atropine (Clifford et al., 1987).

Pilocarpine:-

Pilocarpine is a parasympathomimetic alkaloid, obtained from the leaves of tropical American shrubs from the genus *Pilocarpus*. It is a non-selective muscarinic receptor agonist in the parasympathetic nervous system (Fig 3).

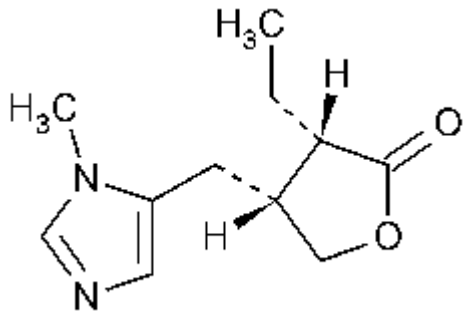


Fig .3: -Structure of pilocarpine (Rang, 2003).

IUPAC name: (3*S*, 4R) - 3-ethyl- 4-((1-methyl- 1*H*-imidazol- 5-yl) methyl) dihydrofuran- 2(3*H*)-one

Formula: $C_{11}H_{16}N_2O_2$

Mol. mass**:** 208.257 g/mol

Half-life: 0.76 hours (5 mg), 1.35 hours (10 mg).

Two models other then pilocarpine are established to induce epilepsy:

1.8.2 The Kainic Acid model of experimental epilepsy: -

When administered systemically or intracerebroventricularly in animal models, kainic acid produces an epilepsy syndrome similar to human temporal lobe epilepsy. Experimental animals treated acutely with kainic acid develop status epilepticus with seizure onset in the hippocampus. After a latent period of days to weeks, these animals begin to exhibit spontaneous, recurrent limbic seizures—the hallmark of the epileptic state. Much like the human syndrome, there are histopathological changes, including cell death in hippocampal sub regions CA1, CA3, and the dentate hilus. (Goldstein, 2008)

1.8.3 Lithium-Pilocarpine Model of experimental epilepsy: -

Lithium chloride (3meq/kg) is administered ip 18–20 h before the injection of pilocarpine (25mg/kg in adult rats). Pilocarpine (pilo) alone or combined with lithium reproduces most clinical and of neuropathlogical features of human TLE with SE. In adult rats, lithium-pilocarpine treatment leads to SE followed by a latent seizure-free phase of a mean duration of 14 –25 days. During this period, neuronal damage develops mainly in the hippocampus, the hilus of the dentate gyrus, the piriform and entorhinal cortices, the amygdala, the neocortex and the thalamus has been observed (V´eronique Andr´e, 2007).

1.9 Sleep: -

Sleep is a natural state of altered consciousness, easily reversible, self-regulating and characterized by a stereotypic posture, decrease in voluntary motor activity and increase in arousal threshold. It is cyclic and associated with various changes in behavior, endocrinal and other physiological functions (Aneja 2005). Melatonin (N-acetyl-5-methoxytryptamine) is a neurohormone that is primarily produced by the pineal gland from tryptophan. The pineal gland then secretes melatonin into the blood stream, where it modulates the brain stem circuit which influence the sleep-wake cycle. Melatonin synthesis increases as light from environment decreases, reaching a maximum between 2 A.M. and 4 A.M.

1.9.1 Stages of sleep: -

Sleep is divided into two main stages: Rapid Eye Movement (REM) and non-REM (NREM) sleep. These stages are characterized by changes measured on instruments such as the electroencephalograph (EEG), which measures changes in electrical signals in the brain; electrooculogram (EOG), which measures eye movements; and the electromyogram (EMG), which measures muscle movements (Carlson, 2001). In humans, REM and NREM sleep alternate in ninety-minute cycles approximately three to six times per night. During the first part of the sleep cycle, REM sleep takes approximately ten minutes of each cycle, but REM sleep periods become longer and closer together as the course of sleep progresses .Non-REM sleep is divided into four stages. As one progresses from stage one to stage four, sleep gets deeper and EEG waves become taller and slower; stages three and four are often grouped together and called slow wave sleep (SWS) (Myers, 1999). During SWS, muscle movements and eye movements are

diminished in comparison to wakefulness, and the EEG is more synchronized, indicating that large portions of brain tissue are firing together. REM sleep is characterized by a desynchronized EEG, a lack of thermoregulation (Rechtschaffen, 1997). However, one part of the brain that does shut off during REM sleep is the part of the hypothalamus that is responsible for temperature regulation. Skeletal muscles are also less active during REM sleep and thus lose muscle tone . This loss enables the muscles to relax during REM sleep.

1.9.2 Sleep Wake Cycle: -

Electrical stimulation of mid brain Reticular Formation causes a state of wakefulness and arousal. This is known as Reticular Activating System. Key components are group of cholinergic neurons. The master clock in mammals is the hypothalamic suprachiasmatic nucleus (SCN). Several neurotransmitter systems with diffuse projections to thalamus and cortex, classically identified as the Ascending Reticular Activating System (Moruzzi et al., 1949) that are essential for inducing and maintaining the thalamocortical system at depolarized state. These are hence called arousal systems (Jones, 2003) which include the serotonergic system of the dorsal raphe nuclei (DR), the noradrenergic system of the locus coeruleus (LC), the histaminergic system of the tuberomammillary nucleus (TM), the orexinergic system of the lateral hypothalamus (LH), the dopaminergic system and the cholinergic system of brain stem laterodorsal tegmental nucleus/pedunculopontine tegmental nucleus (LDT/PPT) and of the basal forebrain (BF). Neurons of the arousal system act in a coordinated fashion to abolish the low-frequency rhythms in the thalamocortical system and promote tonic activity and the appearance of high-frequency oscillations. In contrast to the other arousal systems, which are largely inactive during both SWS and REM sleep, the cholinergic system, which is probably the most important component in EEG activation, is essential also in inducing brain activation during REM sleep (Buzsaki et al., 1989). The arousal systems are in turn reciprocally connected with inhibitory GABAergic cells of the hypothalamic ventrolateral preoptic area (VLPO) (Szymusiak et al., 2008). These cells are most active during transition from waking to sleep and during sleep (Szymusiak et al., 1998) and the balance of mutual inhibition between arousal systems and the sleep-active VLPO underlie the transitions between vigilance states (McGinty et al., 2004). Sleep-wake transitions in this system are modeled by the electrical flip-flop switch concept (Saper et al., 2005). Instead of gradual

transition through intermediate states, the flip-flop switch produces rapid and discrete state transitions.

1.9.3 Significance of Sleep:-

According to the restorative theory, sleep is necessary to the physical health of the body. During sleep, biomolecules that were used up during the day's activities are replenished and cellular damage is repaired (Adam, 1980). Sleep is considered to be important to body restitution, like energy conservation, thermoregulation, and tissue recovery (Maquet 2001). In addition, sleep is essential for cognitive performance, especially memory consolidation (Maquet 2001). Sleep deprivation can adversely affect the brain and cognitive function (Goes et al., 2007). It has been claimed, for instance, that sleep may allow the removal of free radicals accumulated in the brain during wakefulness (Reimund et al., 1995). Moreover, it has been proposed that, during sleep, uridine and glutathione may facilitate the oxidative detoxification of the brain by potentiating GABAergic transmission and inhibiting glutamatergic transmission, respectively (Inoué et al., 1995). Amount of sleep required changes according to age of the person.

Age	Sleep Needs
Newborns (0-2 months)	12-18 hours
Infants (3 to 11 months)	14 to 15 hours
Toddlers (1-3 years)	12 to 14 hours
Preschoolers (3-5 years)	11 to 13 hours
School-age children (5-10 years)	10 to 11 hours
Teens (10-17)	8.5-9.25 hours
Adults	7-9 hours

Source: National Sleep Foundation

Fig. 4:- Amount of sleep required grouped according to age.

1.10 Sleep Deprivation (SD): -

Sleep deprivation is the condition of not having enough sleep. It can be either chronic or acute. Sleep deprivation is a well-established paradigm to verify the deleterious effects of prolonged wakefulness, which ultimately leads to death (Bentivoglio et al., 1997) .Changing urban life style, competition to achieve the targets at the work place, increased study hours and

parental pressure has decreased total sleeping hours from a day in our modern society. Both epidemiological and clinical data suggest that disturbed sleep may contribute to the development of various diseases, e.g. Obesity and type 2 diabetes (Chaput et al., 2006). Sleep loss, instead, seems to activate the sympathetic nervous system, which can lead to a rise of blood pressure (Ogawa et al., 2003) and an increase in cortisol secretion (Spiegel et al., 1999). Immune response may be impaired and metabolic changes such as insulin resistance may occur (Spiegel et al., 2005). People who are exposed to sleep loss usually experience a decline in cognitive performance and changes in mood (Philibert, 2005).

Fig.5: - Effects of sleep deprivation on metabolism and cognitive functions (journal sleep 30).

The general explanation relies on the two-process model of sleep regulation. Cognitive impairments would be mediated through decreased alertness and attention through lapses, slowed responses, and wake-state instability (Fig.5). Attentional lapses, brief moments of inattentiveness, have been considered the main reason for the decrease in cognitive performance during sleep deprivation (Dorrian et al., 2005). The prefrontal vulnerability hypothesis, first proposed by (Horne, 1993) suggests that SD especially impairs cognitive performances that depend on the prefrontal cortex. These include higher functions, such as language, executive functions, divergent thinking, and creativity. The dangers of sleep deprivation are apparent on the road; the American Academy of Sleep Medicine (AASM) reports that one in every five serious motor vehicle injuries is related to driver fatigue, with 80,000 drivers falling asleep behind the wheel

every day and 250,000 accidents every year related to sleep (American Academy of Sleep Medicine. 2 December 2009).

1.11 Effect of sleep deprivation on food water intake:-

Notable effects of sleep deprivation in rats include changes in body temperature, weight loss, and hyperphagia (Rechtschaffen et al., 1989). Thus prolonged, complete sleep deprivation increased both food intake and energy expenditure with a net effect of weight loss and ultimately death (Hasler et al., 2004) Thus, prolonged sleep deprivation induced by the disk-over-water technique has been shown to result in body-weight reduction of 22% and 17% for total and paradoxical sleep deprivation, respectively (Everson et al., 1989). Paradoxically, however, in these studies, the animals increased their food intake by 80% to 100% (Everson et al., 1989). 96 hours of sleep deprivation by the multiple-narrow-platform technique induced a 69% increase in food intake,

1.12 Epilepsy Sleep deprivation and Depression: -

There is an inherent relationship between sleep and epilepsy. Sleep activates the electrical charges in the brain that result in seizures and seizures are timed according to the sleep wake cycle. For some people, seizures occur exclusively during sleep. This is especially true for a particular type of epilepsy known as benign focal epilepsy of childhood. When seizures occur during sleep, they may cause awakenings that are sometimes confused with insomnia. Epilepsy patients are often unaware of the seizures that occur while they sleep. They may suffer for years from daytime fatigue and concentration problems without ever knowing why. In humans, it has been demonstrated that the metabolic activity of the brain decreases significantly after 24 hours of sustained wakefulness. Sleep deprivation results in a decrease in body temperature, a decrease in immune system function Animal studies have shown that sleep is necessary for survival. The normal life span of rats is 2-3 years. However, rats deprived of sleep live for only about 3 weeks (Purves et al., 2008). They also develop abnormally low body temperatures and sores on their tails and paws. Sleep deprivations not only increase the occurrence of interictial epileptiform discharge but also increase the likelihood of occurrence of IED during wake period (Aneja and

Gupta, 2005). Depression is the most frequent psychiatric disorder in epilepsy (Mendez et al., 1986). Depression occurred in as many as 37 percent of people with epilepsy and other work has suggested that that people with depression also have a higher risk of seizures. There is high incidence of depression in patients with epilepsy, with a lifetime prevalence estimated to be between 17% and 43% (Wiegartz et al., 1999). Patients with major depression are six fold more likely to have an unprovoked seizure than the general population (Hesdorffer et al., 2000). Compared to the general population, epilepsy patients are about twice as likely to have depression and are 3.6–5-fold more likely to commit suicide (Nilsson et al., 1997).

1.13 PLC-β1 Receptor:-

PLCB1 is a post-synaptic receptor-activated, G protein-coupled phosphodiesterase. The PLC-PKC signaling pathway is another target when MT1 or MT2 receptors are activated (McArthur et al., 1997). The production of the second messenger molecules diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP3) is mediated by activated phosphatidylinositol-specific phospholipase C enzymes. Phospholipases are a group of enzymes that hydrolyze phospholipids into fatty acids and other lipophilic molecules. Phospholipases are ubiquitously expressed and have diverse biological functions including roles in inflammation, cell growth, signaling and death and maintenance of membrane phospholipids. Phospholipase C catalyzes the production of inositol triphosphate, which leads to activation of calcium channels and increases in intracellular calcium which is released from internal stores via inositol triphosphate receptors (Basheer et al., 2002).Phospholipase C activation leads to activation of protein kinase-c C (Alberts et al, 2002). Protein kinase-c C alters transcription by acting on a number of downstream targets (reviewed in Ventura and Maioli., 2001) including NF-κB (reviewed in Weil and Israel., 2006) NF-κB translocates to the nucleus when one of its binding partners, I-κB, is phosphorylated and detaches from it (Alberts et al., 2002). NF-κB translocation to the nucleus following sleep deprivation occurs almost exclusively in the cholinergic cells of the basal forebrain (Ramesh et al., 2002)

1.14 PLCβ1 and Epilepsy: -

The clinical presentation and evolution of epileptic encephalopathy in a patient, associated with a loss-of-function mutation in the phospholipase C-β 1 gene. Disruption of the PLC-beta 1-mediated signal transduction pathway may lead to a functional cholinergic denervation, which could cause hippocampal remodeling and, in consequence, epileptiform hyper excitability (Bohm et al, 2002).

1.15 PLCβ1in melatonin receptor (MT1) signaling:-

Melatonin affects the phase of circadian rhythms by a direct action on the biological clock that resides within the hypothalamic SCN (Weaver, 1999). Melatonin is known to regulate various physiological functions by activating specific receptors, namely MT1, MT2 andMT3 subtypes (Dubocovich et al., 2003). In the vertebrate retina Melatonin is synthesized and released by photoreceptors and may be implicated in retinomotor responses, rod disc shedding, phagocytosis, etc. (Vanecek, 1998). Accumulating evidence also suggests that, following activation of MT1 receptors, PhospholipaseC (PLC)-dependent pathways could come into play (McArthur et al., 1997). Melatonin has also been reported to influence PLC activity in the SCN (McArthur et al., 1997). Activation of the MT1 receptor induces a transient elevation in cytosolic calcium ion Concentration and in inositol phosphate accumulation (Brydon et al., 1999). Recombinant MT1 receptors were shown to activate potassium ion channels, to promote stimulation of phospholipase C, and to modulate protein kinase-C (PKC) (Fig.6) and phospholipaseA2 (McArthur et al., 1997). PKC activates gene expression of c-fos gene which hyperpolarizes the membrane. Thus MT1 receptor inhibits SCN firing.

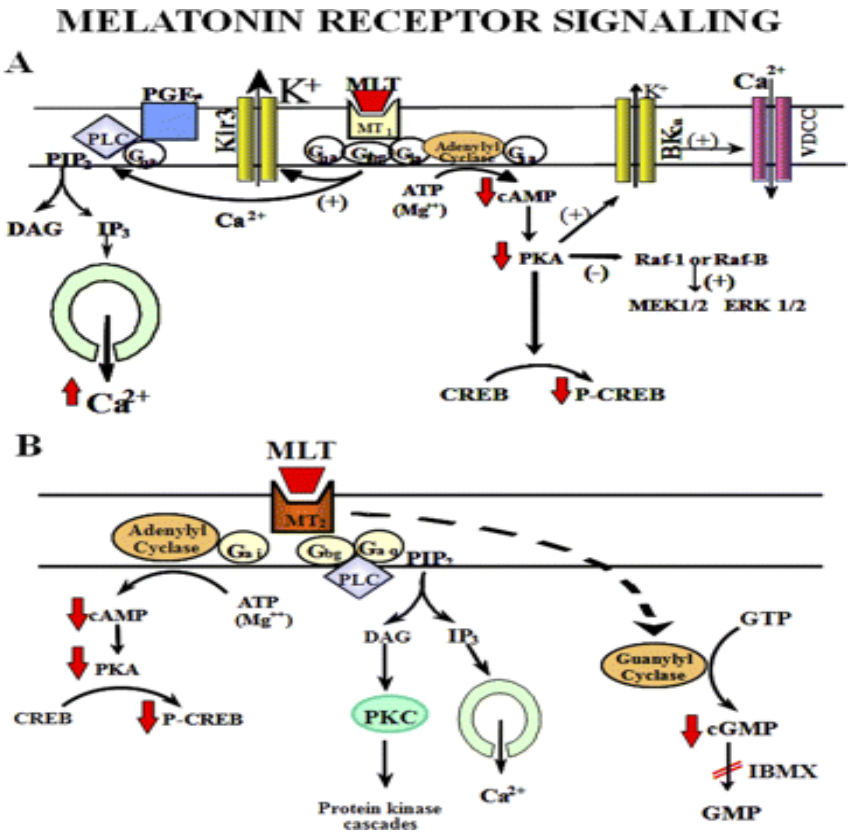


Fig.6: - PLCβ in melatonin signaling (Dubocovich et al., 2001).

1.16 Objectives

1. To induce Sleep Deprivation in control and epileptic group of experimental group of rats.

- Control (C)
- Control+ Sleep deprived (C+SD)
- Epileptic (E)
- Epileptic+ sleep deprived(E+SD)

2. To design specific forward and reverse primer for PLCβ1 gene using different software (i.e. BLAST, Sigma, integrated DNA technology).

3. To check effect of Sleep Deprivation in control and epileptic group of experimental group of rats on food intake and water consumption in experimental group of rats.

4. Thio Barbituric Acid Reactive Substance enzyme assay-To checks the effect of sleep deprivation and epilepsy on ROS formation and lipid peroxidation.

5. To study and compare the gene expression of PLCβ1 Receptor in brain stem of control and experimental groups of rats.

6. Behavior studies- To check effect of epilepsy and sleep deprivation on exploratory behavior and anxiety through Open Field Test and Splash Test.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Chemicals: -

Pilocarpine(Sigma Aldrich), EDTA (Merck Specialists Pvt. Ltd.),Tri reagent (Sigma Aldrich), DEPC water (Himedia Laboratories Pvt. Ltd.), Chloroform (Sisco research Lab, Mumbai), cDNA synthesis kit (Fermentas, USA), PCR Master mix (Fermentas, USA), primers PLC-beta(Sigma Aldrich, Bangalore),Sucrose(Sisco Research Lab, Mumbai), Bromophenol blue (Central Drug House, Mumbai), Agarose (Sisco Research Lab, Mumbai), Ethidium bromide (Central Drug House, Mumbai), DNA ladder (Fermentas, USA).

All biochemicals used in the present study were of analytical grade.

2.2 Animals: -

Adult male Wistar rats of 200-250 kg body weight and age between 10-12 weeks were purchased from Haffkine Biopharmaceuticals Ltd., Mumbai and used for all experiments. They were housed in separate cages under 12-h light and 12-h dark periods and Food and water was given freely before administration of pilocarpine. Animal care and procedures were done according to the Institutional and National Institute of Health Guide lines.

2.3Model induction

2.3.1 Mode of Action of pilocarpine: -

Pilocarpine-induced seizures in rats provide a widely animal model of temporal lobe epilepsy. It is a non-selective muscarinic receptor agonist in the parasympathetic nervous system, which acts therapeutically at the muscarinic acetylcholine receptor M3 due to its topical application

2.3.2 Induction of epilepsy:-

The experiment was done in three sets and each set of animals were divided into the following groups:-

Animal group	**Given treatment**
Control rats (C),	No treatment
Epileptic rats (E),	Atropine + pilocarpine
Control + sleep deprivation (C+SD)	No treatment
Epileptic + sleep deprivation (C+SD)	Atropine + pilocarpine

Each group consisted of 3 animals.

Epilepsy was induced by injecting rats by a single intraperitonial dose of pilocarpine hydrochloride (350 mg/kg body wt ip), preceded 30 min by atropine (1 mg/kg body weight ip) to reduce peripheral effects of pilocarpine (Krishnakumar *et al.*, 2009).

Fig. 7:- Intraperitonial induction of Epilepsy

The body weight, feed and water intake was carried out from the day of pilocarpine treatment

according to their sleep wake cycle to analyze the changes in Body weight, feed and water consumption.

2.4 Sleep Deprivation: -

From the 16th day of pilocarpine C+SD and E+SD groups were partial sleep deprived for 10 hours daily during their sleep cycle using tripod stand in a bucket. In this method for sleep deprivation a bucket was three fourth filled with water and a tripod stand was placed in it and rat was allowed to sit over the tripod stand. As the area of tripod stand was too low, rats were unable to maintain their balance if they fall asleep so they had to awake to maintain their balance. Gentle handling was done in between.

Control sleep deprived rat Epileptic sleep deprived rat

Fig. 8:- Partial Sleep deprivation in control and epileptic group of rats.

2.5 Behavioral studies: - Behavioral studies of all the four experimental groups of rats were carried out. Open field test, Splash test were conducted to assess the levels of anxiety and changes in the exploratory activity .

1. Open Field Test

2. Splash Test

2.5.1. Open Field Test: -

The Open Field Test provides simultaneous measures of locomotion, exploration and anxiety (Walsh & Cummins, 1976).

Open Field Apparatus:-

Animals were placed individually into the center of open field of 43cm (L), 43cm (W) 40cm (H) and allowed to interact with the novel environment. Behavior was tested in a silent room. The investigator sitting approximately 2 m apart from the open field observed and detected the movements of the rats for a total of 5-mins. All open field tests were performed during the dark phase of the day. The test duration was 5 mins.

The behaviors scored included:

1. Line Crossing: Frequency with which the rat crossed one of the grid lines with all four paws.

2. Center Square Entries: Frequency with which the rat crossed one of the red lines with all four paws into the central square **(**Brown et al., 1999).

3. Center Square Duration: Duration of time the rat spent in the central square **(**Brown et al., 1999).

4. Rearing: Frequency with which the rat stood on their hind legs in the maze **(**Brown et al., 1999).

5. Grooming: Duration of time the animal spent licking or scratching itself while stationary **(**Brown et al., 1999).

7. Freezing: Duration with which the animal was completely stationary **(**Brown et al., 1999).

8. Defecation: number of fecal Boli produced.

Control Rat Epileptic Rat

Fig. 9: - Different behavior of control and epileptic rats in open field test

2.5.2 Splash Test: -

Body care (hygienic) behavior in most animal species is a natural adaptation aimed at removal of litter particles, pathogenic microbes, and parasites from fur and skin (Hart , 1990). It entails a sequence of movements (grooming) such as licking the fur and paws, washing the head, and cleaning the genital area (Berridge, 1990).

A typical scale may be as follows-

No grooming	0
Paw licking	1
Nose, Face and head wash	2
Body grooming(fur licking)	3
Leg licking	4
Tail or genital grooming	5

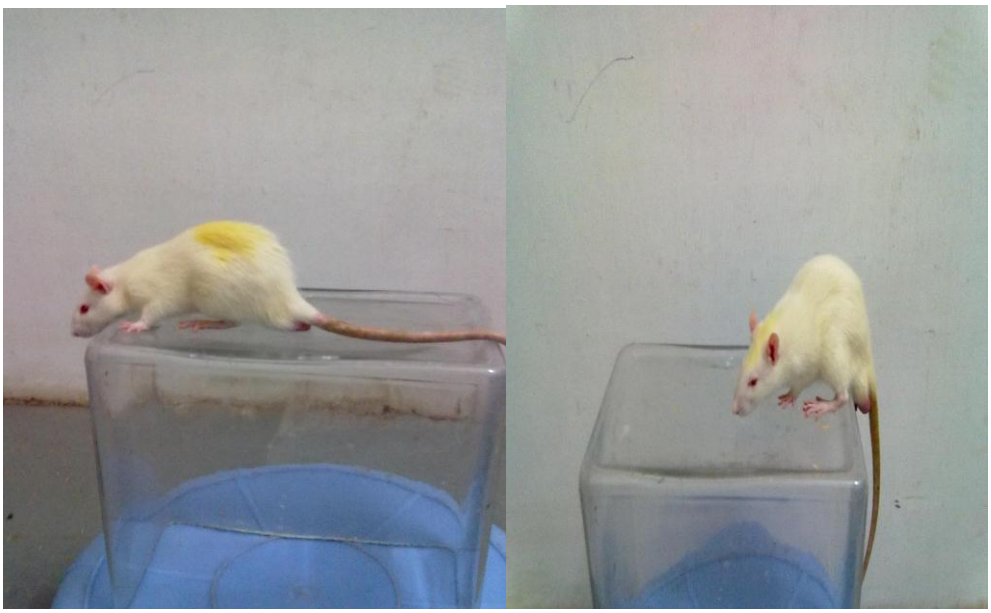
A correct bout is cephalocaudal in direction and follows a (0-1) (1-2) (2-3) (3-4) (4-5)

(5-0).

"Splash Test" is the indirect measure of body care efficiency. The test consists of splashing a 10%sucrose solution on the dorsal coat of an animal and then counting the animal's grooming bouts (Yalcin et al., 2005).

Test set-up:-

10% sucrose solution was squirted on the dorsal surface of control and experimental group of rats. After 5 minutes when the sucrose gets evaporated rats were placed over a chamber and video recording was done to analyze the grooming patterns and duration of grooming.



Control Rat Epileptic Rat

Fig.10: - Control and epileptic rats during Splash Test

2.6 Tissue preparation: -

Rats were sacrificed by decapitation on the 31st day of the experiment. The cerebellum, brain stem, cerebral cortex and hypothalamus from brain and liver, kidney, pancreas, heart and muscle

from body were dissected out quickly over ice according to the procedure of (Glowinski and Iversen 1966). The tissues were stored at –20˚C until assay.

2.7 Total RNA Isolation:

RNA isolation was done by using Tri reagent (Sigma Aldrich). RNA purity was checked at 260/280 in U.V Vis Shimadzu 1800 Spectrophotometer.

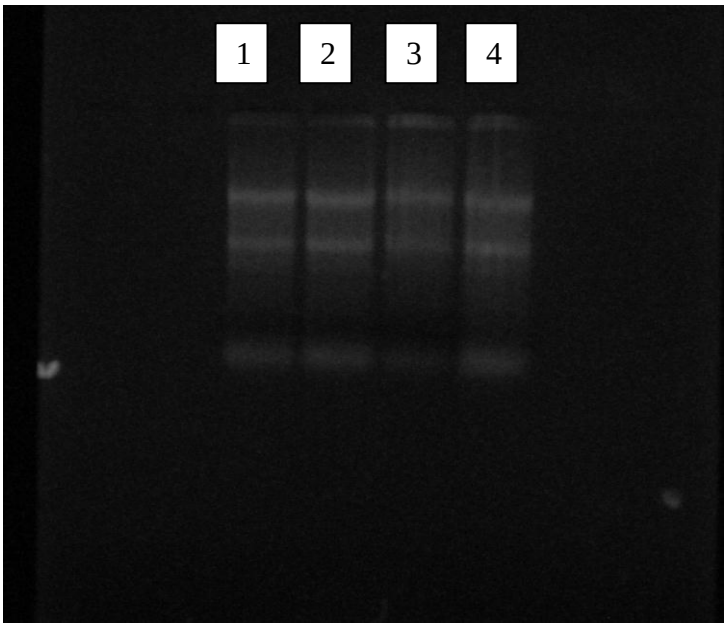


Fig.11:- RNA isolation from brain tissue

1. C 2. C+SD 3. E 4.E+SD

2.8 Thio Barbituric acid reactive substance assay: -

Malondialdehyde (MDA), the by-product of lipid peroxidation forms adduct with TBA. On boiling, it produces pink colored complex, which absorbs maximally at 532 nm.

Procedure:-

- 0.1ml homogenate, 0.1 ml Tris-HCl buffer, 0.1 ml FeSO4 and 0.1ml Ascorbic acid were added in a test tube then 0.6 ml DDW was added to make the volume 1.0 ml.

- It was incubated at 370C for 15 min. Then 1.0 ml TCA and 2 ml TBA were added to the reaction mix.
- Tubes were plugged and incubated for 15 min. at boiling water. Appropriate blanks were employed for corrections.
- Centrifugation was done at 3000 Rpm for 10 min.
- Readings were taken at 532 nm. The change in the absorbance at 532 nm due to formation of MDA was followed in a colorimeter.

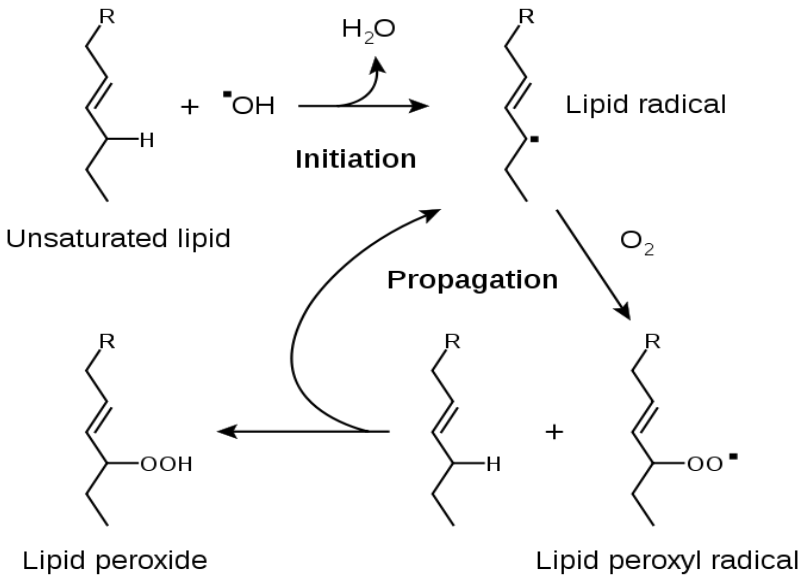


Fig. 11:- Formation of free radicals during lipid peroxidation.

Kinetic Parameters: - Concentration of MDA was calculated using formula

Concentration of MDA= O.D. × sample volume× / 1.56× 10^5Total volume × mg protein per ml.

Results expressed in nmoles MDA / mg protein.

2.9 PCR assay for PLCβ1: -

Primers for PLCβ1 receptor were designed using BLAST, IDT and sigma software.

Table 2:- Primer Sequence for PLCβ1

Gene	**Sense primer**	**Antisense Primer**	Product Size
PLCβ1	5'ATGTCAGTGGATGGATTC3' (18 bases)	5'GGCTTCTATGACTTCCTTG3' (19 bases)	334bp

2.9.1 Polymerase Chain Reaction:-

.

RNA was isolated from brain stem using Tri-reagent. Total RNA was reverse transcribed into first-strand cDNA using the Fermentas cDNA synthesis kit. cDNA (2µl) from the RT reaction was used as the template for quantitative RT PCR reaction with a final PCR reaction volume of 25 µl, with the 5‘ and 3‘ gene-specific PCR primer, concentrations at 50 nM each.

The thermo cycling profile conditions were as follows: 94°C for 4 mins—initial denaturation, 94°C for 53s - denaturation , 52°C for 60s-annealing, 72°C for 45 sec– extension, 34cycles, 72°C for 5 min- final extension.

2.9.2 Analysis of PCR products: -

The polymerase chain reaction product was loaded on a 2% Agarose gel with 2% Ethidium Bromide. Bromophenol blue dye was used as a indicator dye.100V current was used for all the runs. The image was captured using a gel documentation system. PLCβ1 receptor expression in the brain stem of C,C+SD, E ,E+SD were analyzed.

Statistics: -Statistical evaluations were done with ANOVA followed by the Student–Newman–Keuls test using the Instat (Version 2.04a) computer program.

RESULTS

AND

DISCUSSION

RESULTS

3. Results:

3.1 Model induction and behavioral observation:

Initially, pilocarpine induced hyper salivation or occasional foam at the mouth, chewing, licking, tearing, and was observed. Then face washing started and lasted without respite for a few minutes; in some animals, face washing reoccurred at 50–60 min. Creep walking began a few min after face washing, followed by head nodding .Ultimately, the animals alternated creep walking with long periods of sitting with head nodding and chewing. Approximately 70 min after pilocarpine administration, this abnormal behavior were gradually replaced by repeated episodes of exploration–searching activity and self-grooming. Nodding persisted longer; it disappeared approximately after 90 min.

Following are the behavior observations after model induction that model induction was successfully done and rats were induced for epilepsy.

1. Hunch Back:-

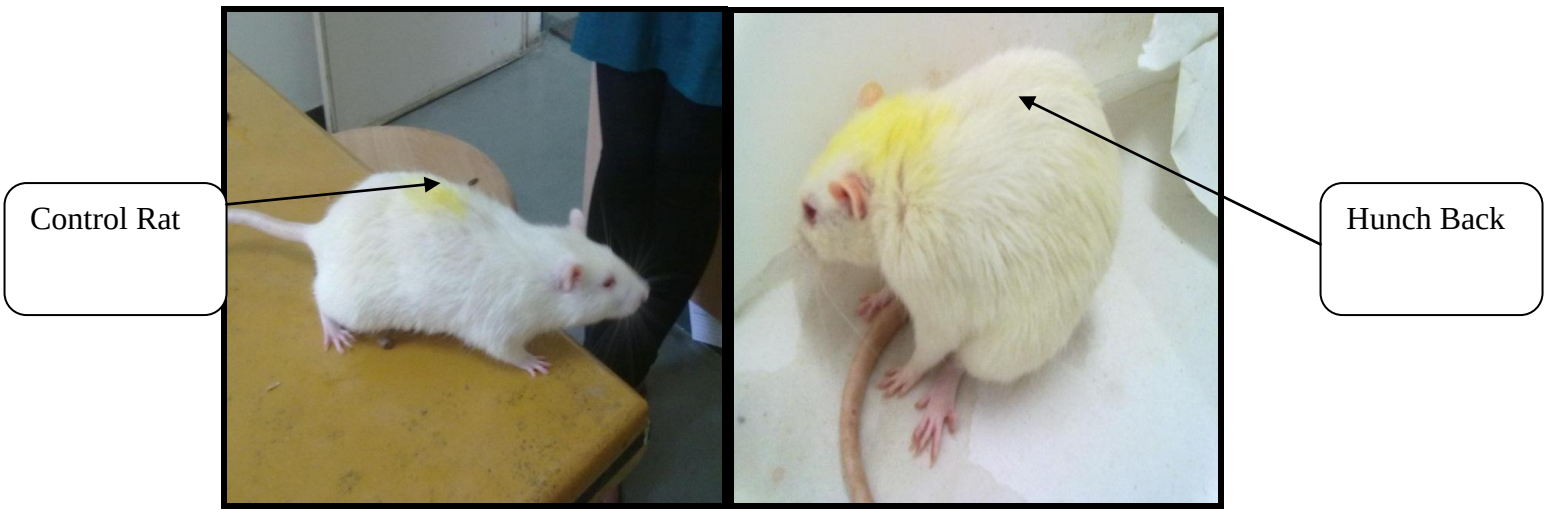
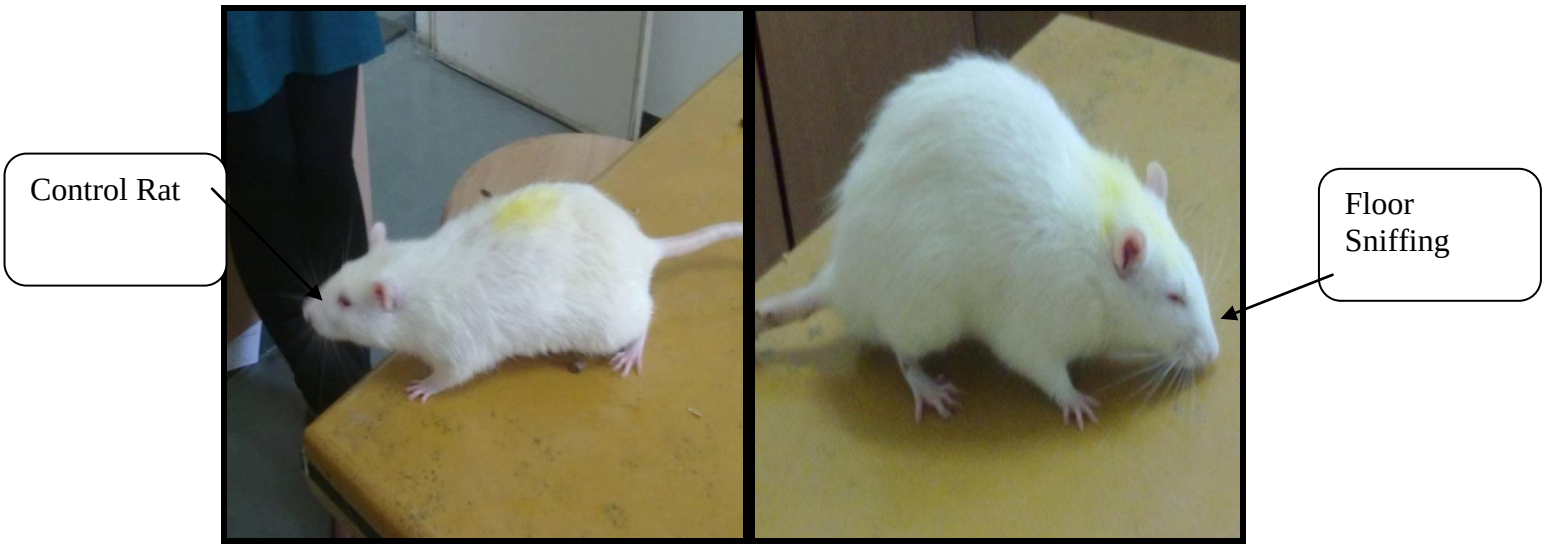


Fig. 13:- Hunch back formation in epileptic rats

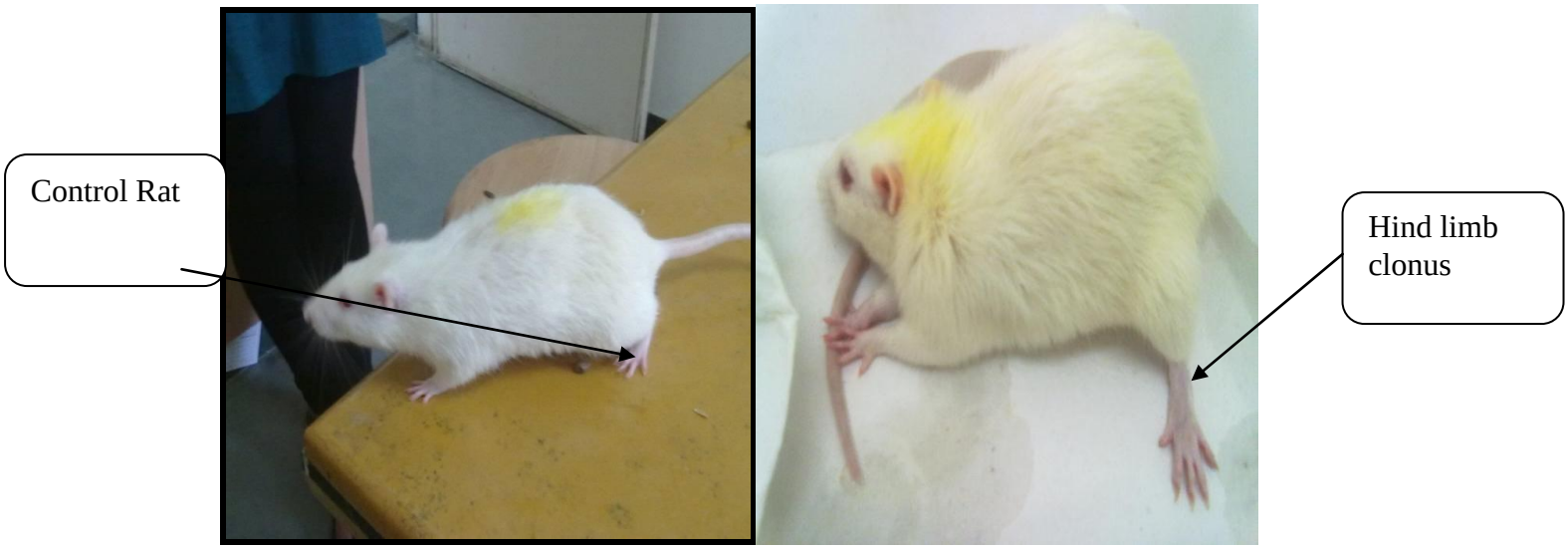
2. Floor sniffing:-



Control Rat Epileptic Rat

Fig. 14:- Floor sniffing in epileptic rat

3. Hind limb Clonus



Control Rat Epileptic Rat

Fig. 15:- Hind limb clonus in epileptic rat

4. Forelimb Clonus :-

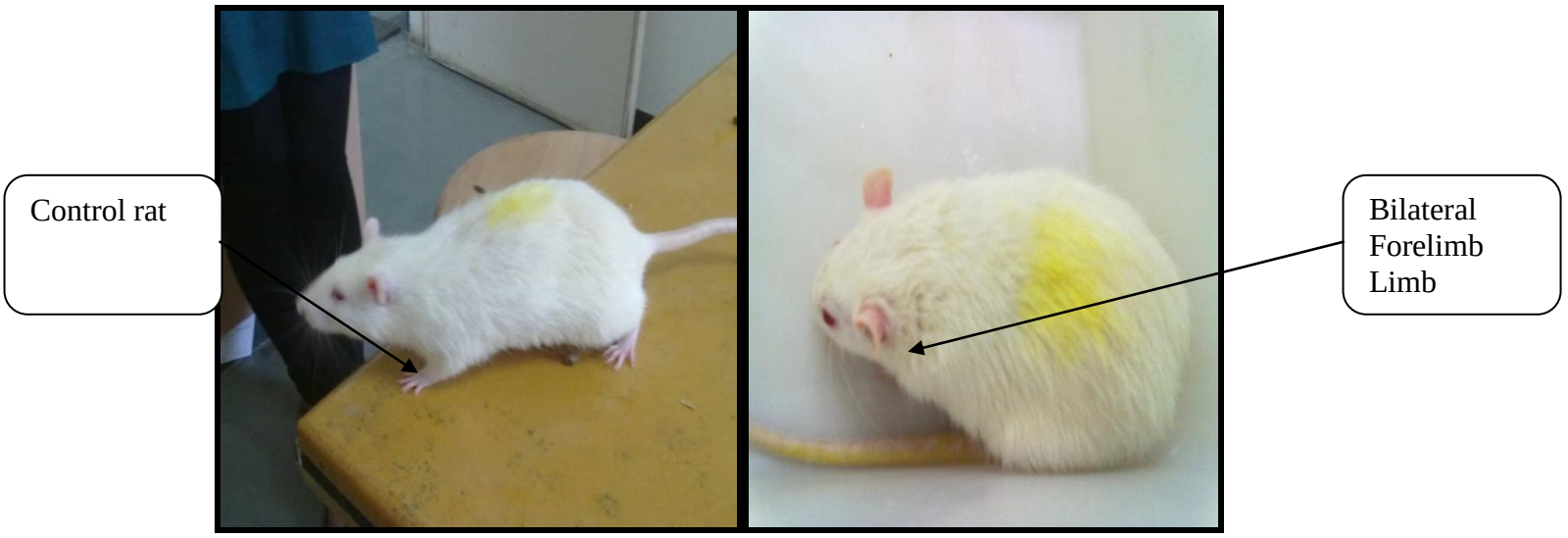


Fig.16: - Bilateral Forelimb clonus in epileptic rat

5. Excessive salivation and Urination ;-

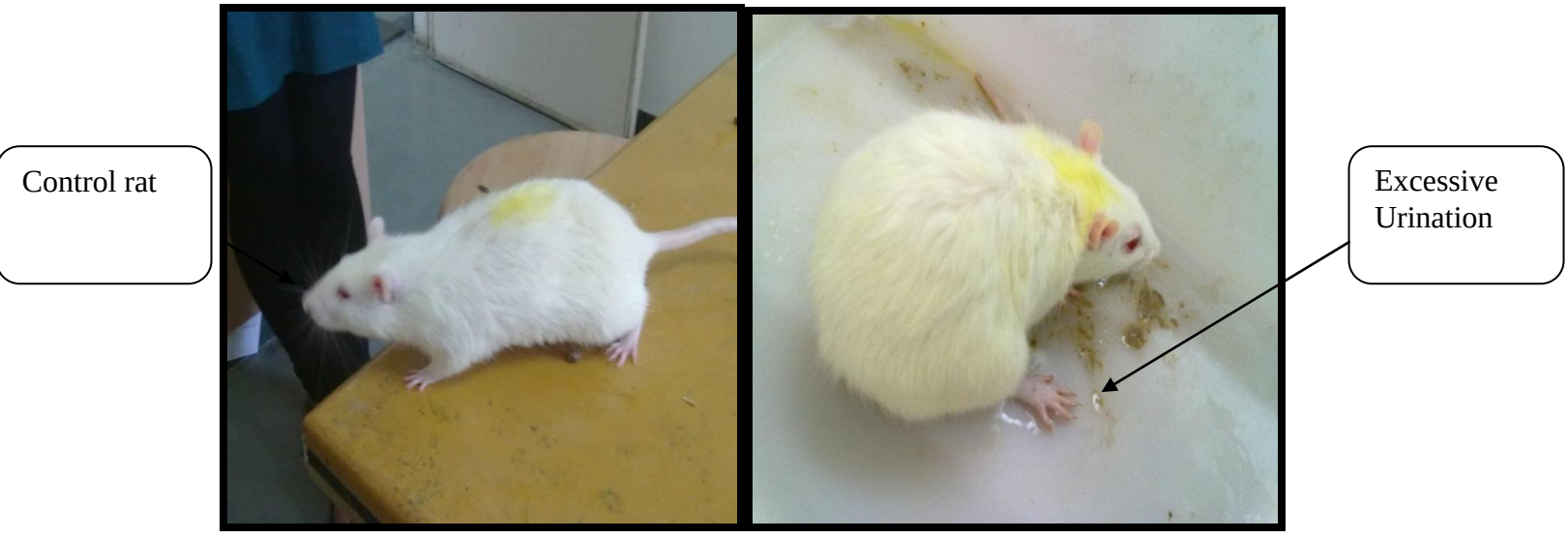
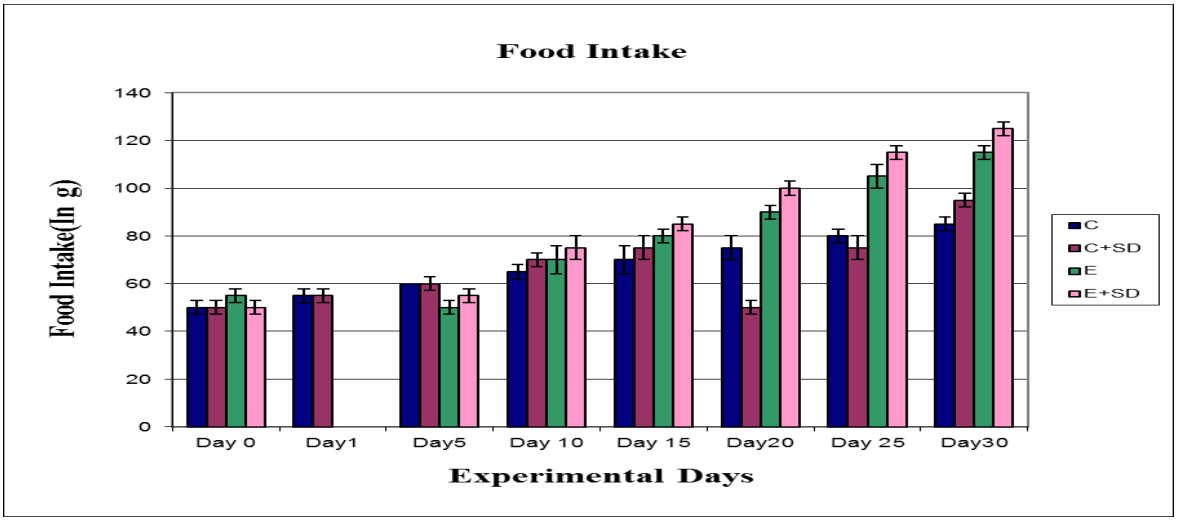


Fig.17:- Excessive salivation and Urination in Epileptic Rat.

3.2 Food Consumption

Fig.18 Total food intake in control and experimental group of rats

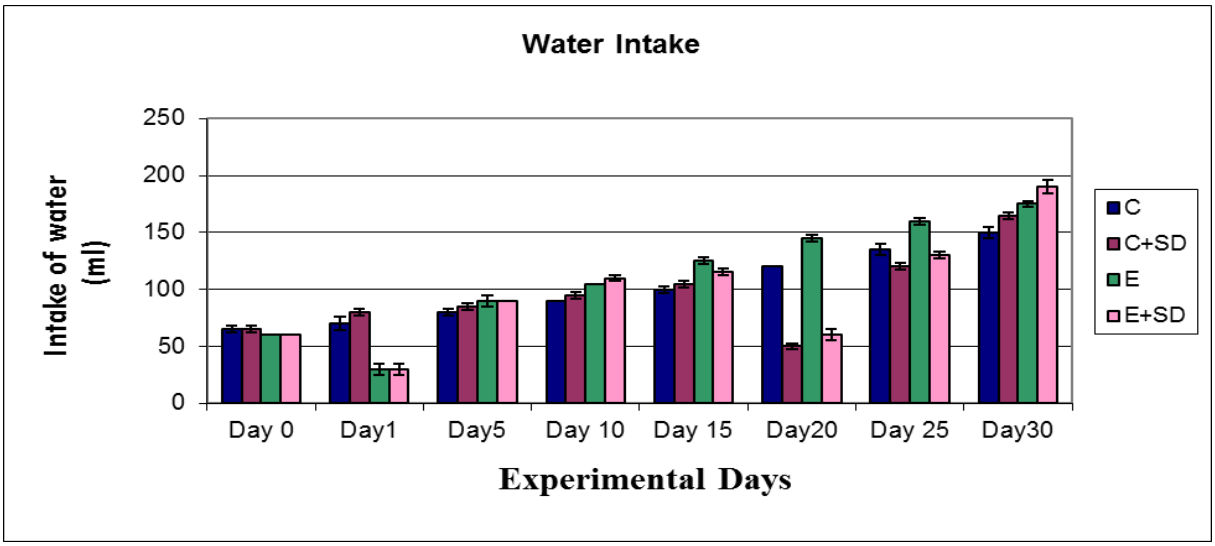


Values are Mean of 3 separate experiments (n=3 rats per group).

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

3.3 Water consumption

Fig.19:- Total water consumption in control and experimental group of rats

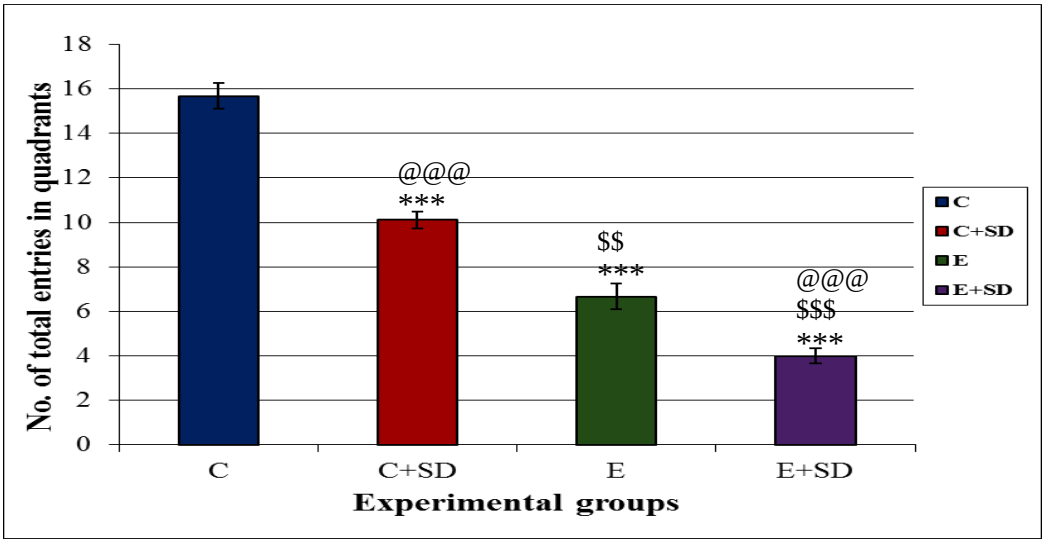


Values are Mean of 3 separate experiments (n=3 rats per group).

C-control, E-epileptic, C+SD-control sleep deprived, E+SD-epileptic sleep deprived.

3.4 Open Field Test

Fig .20:- Total Entries in quadrants in control and experimental groups.



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.001 compared to C+SD.

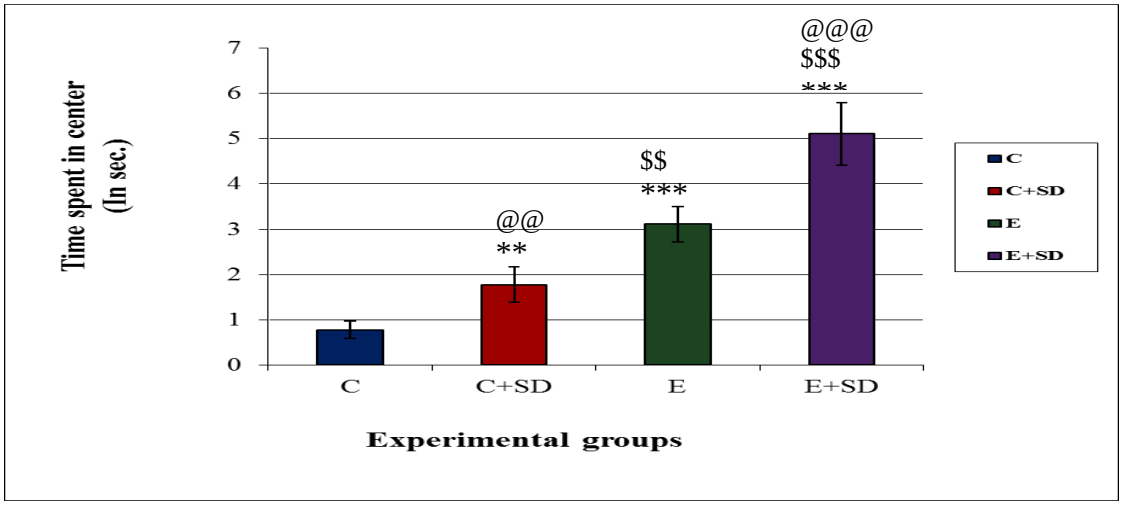
@ @@p<0.001 compared to E. $$ p<0.01 compared to C+SD.

C-control, E-epileptic, C+SD-control sleep deprived, E+SD-epileptic sleep deprived.

A significant decrease in Total Entries in Quadrants of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant decrease in Total Entries in Quadrants of E+SD (P<0.01) when compare to C+SD rat. Decrease in Total Entries in Quadrants of E+SD (P<0.001) as compare to E rat.

The Open Field Test provides simultaneous measures of locomotion, exploration and anxiety (Walsh & Cummins, 1976). The number of Total Entries in Quadrants is usually used as measures of locomotor activity, but it also measures exploration and anxiety. A high frequency of this behavior indicates increased locomotion and exploration and/or a lower level of anxiety. The number of central square entries measures anxiety. A high frequency/duration of this behavior indicates low anxiety levels.

Fig. 21:-Time spent in center in control and experimental group



Values are Mean±SD of 3 separate experiments (n=3 rats per group)

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.001 compared to C+SD

** p<0.01 compared to C. $$ p<0.01 compared to C+SD.

@@@ p<0.001 compared to E.

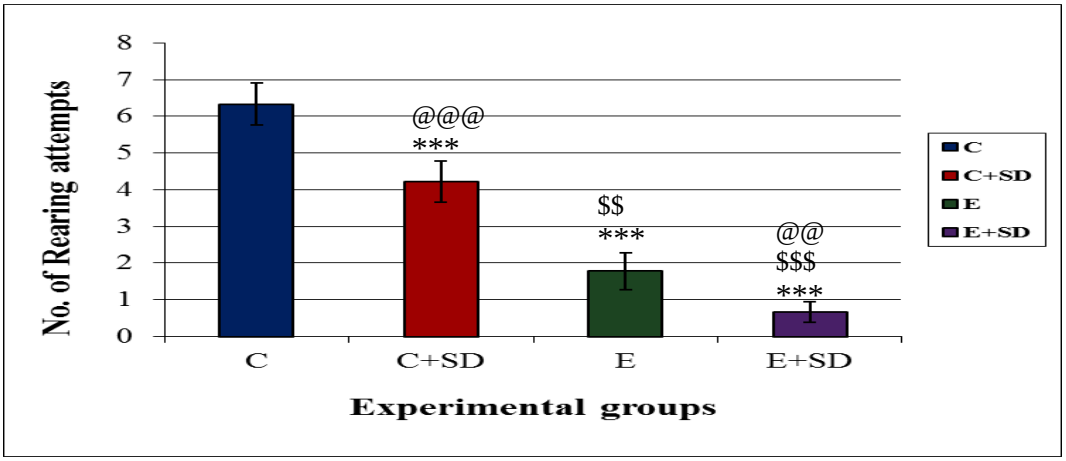
.@@p<0.01 compared to E.

.C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant increase in Total time spent in Centre of C+SD (P<0.01), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant increase in Total time spent in Centre of E+ when compare to C+SD (P<0.01) rat. Significant increase in Total time spent in Centre of E+SD as compared to C+SD (P<0.01) and E (P<0.001) rat.

The duration of time spent in the central square is a measure of exploratory behavior and anxiety. A high frequency/duration of these behaviors indicates high anxiety levels (Hall et al., 1983).

Fig. 22: - Rearing attempts in control and experimental groups.



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C . $$$ p<0.01 compared to C+. $$ p<0.01 compared to C+SD.

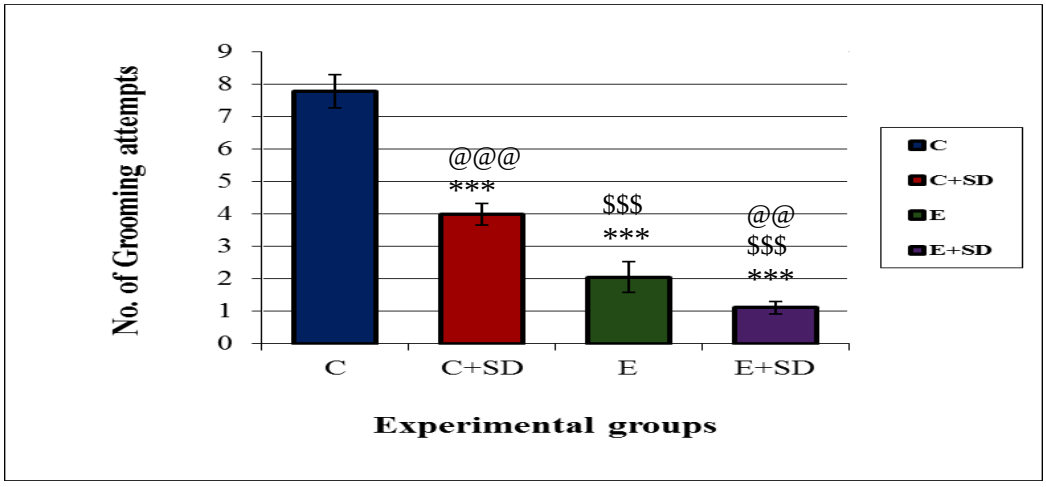
@@@ p<0.001 compared to E. @@ p<0.01 compared to E.

. C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant decrease in Number of rearings of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant decrease in Number of rearings of E when compare to C+SD (P<0.01) rat. A Significant decrease in Number of rearings of E+SD as compared to C+SD (P<0.01) and E (P<0.001) rat.

Rearing is usually used as a measure of locomotor activity. More number of rearing indicates high level of locomotor activity and less anxious nature.

Fig. 23: - Grooming attempts in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

A*NOVA* followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.001 when compared to C+SD.

@@@ p<0.001 compared to E.

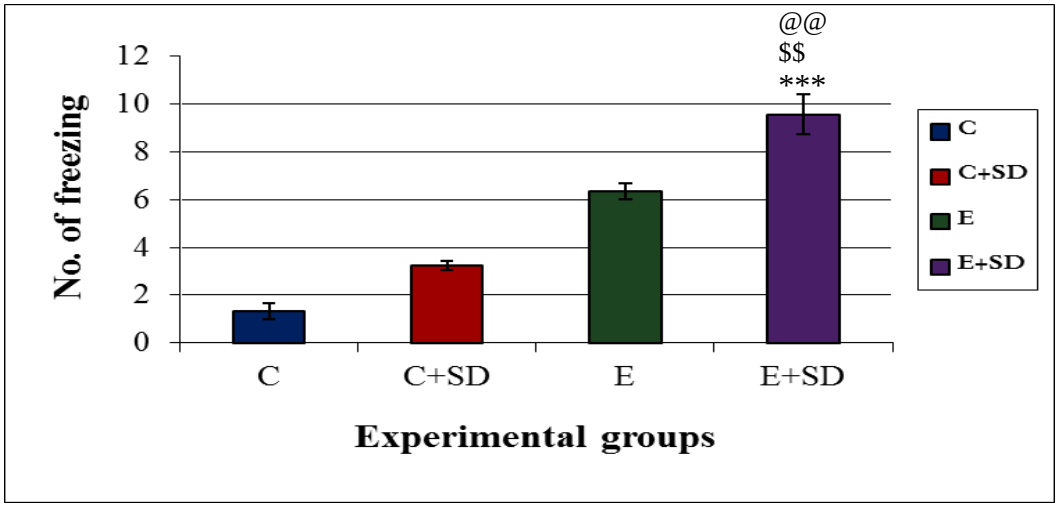
@@p<0.01 compared to E.

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant decrease in Number of grooming attempts of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant decrease in Number of grooming attempts of E when compare to C+SD (P<0.01) rat. Significant decrease in Number of grooming attempts of E+SD as compared to C+SD (P<0.01) and E (P<0.001) rat.

Grooming behavior is a displacement response and is expected to be displayed in a novel environment (Espejo, 1997). Rats groom to maintain their hygiene as well as to acclaimatize themselves into a novel environment.

.Fig. 24: -No. of freezing in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

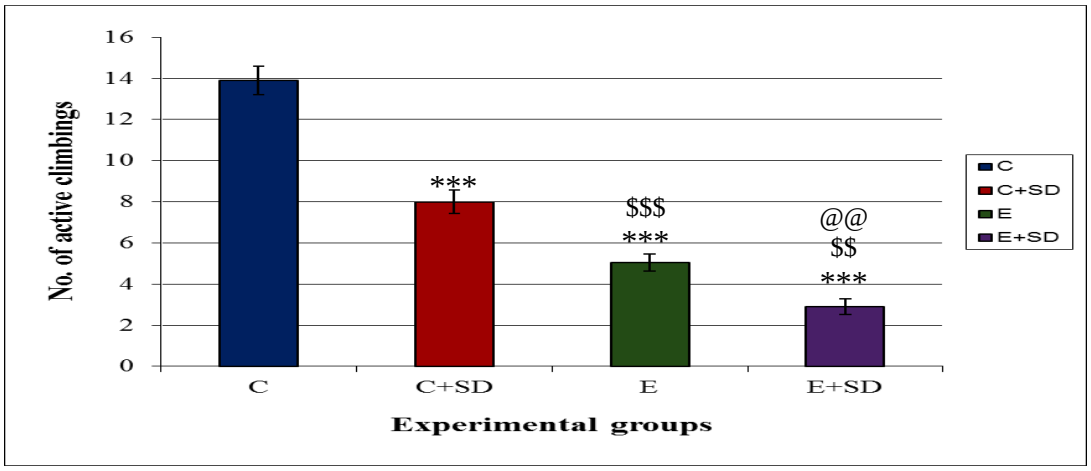
*** p<0.001 compared to C. $$ p<0.01 compared to C+SD.

@@p<0.01 compared to E.

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A Significant increase in Number of freezing of E+SD as compared to C(0.001), C+SD (P<0.01) and E (P<0.01) rat.

Fig. 25:- No. of active climbings in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.001 compared to C+SD.

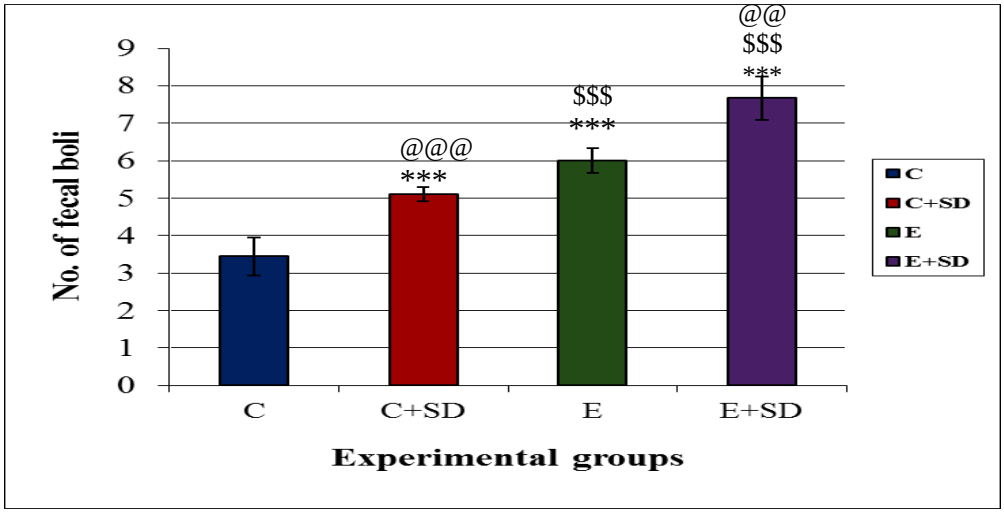
@@p<0.01 compared to E. $$ p<0.01 compared to C+SD.

C-control, E-epileptic, CSD-control sleep deprived.

A significant decrease in Number of active climbings of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant decrease in Number active climbings of E when compare to C+SD (P<0.001) rat. A Significant decrease in Number of active climbings of E+SD as compared to C+SD (P<0.01) and E (P<0.01) rat.

Active climbing is measure of escaping behavior in Open Field test (Walsh & Cummins, 1976). More number of climbing shows more active behavior to escape from the novel arena.

Fig. 26: - No. of fecal Boli in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.001 compared to C+SD.

@ @@p<0.001 compared to E. @ @p<0.01 compared to E.

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

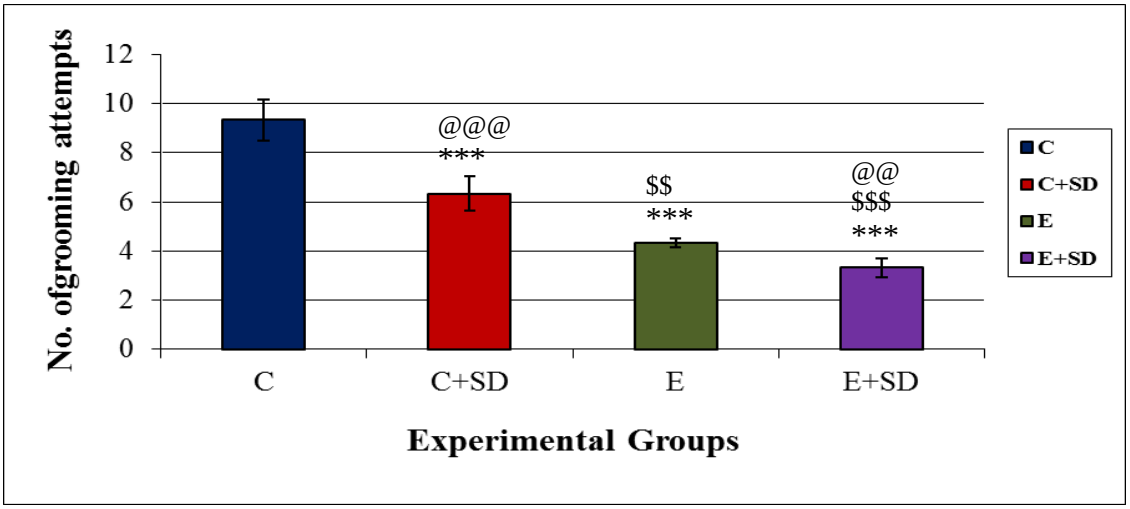
A significant increase in Number of Fecal Boli of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Increase in Number of Fecal Boli of E when compare to C+SD (P<0.001) rat. A Significant increase in Number of Fecal Boli of E+SD as compared to C+SD (P<0.01) and E (P<0.01) rat.

Defecation and urination as indices of anxiety in rodents (Hall, 1934).

Repeated exposure to the open field apparatus results in time dependent changes in behavior (Choleris et al., 2001). At first, when the apparatus is novel to the animals, more fear-related behaviors (such as stretch attends and activity in the corners and walls of the open field) are displayed. However, with repeated trials, more exploration and locomotor activity (such as rearing and line crosses) is observed.. Major nerve connections of the motor and sensory nerve system of the brain pass through the brain stem before entering the peripheral nervous system (Gergard et al., 2003). These results also suggest imbalance in excitatory and inhibitory neurotransmitter in the brain stem region which impairs the locomotor ability in experimental group of rats.

3.5 Splash Test

Fig.27: - No. of Grooming attempts in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

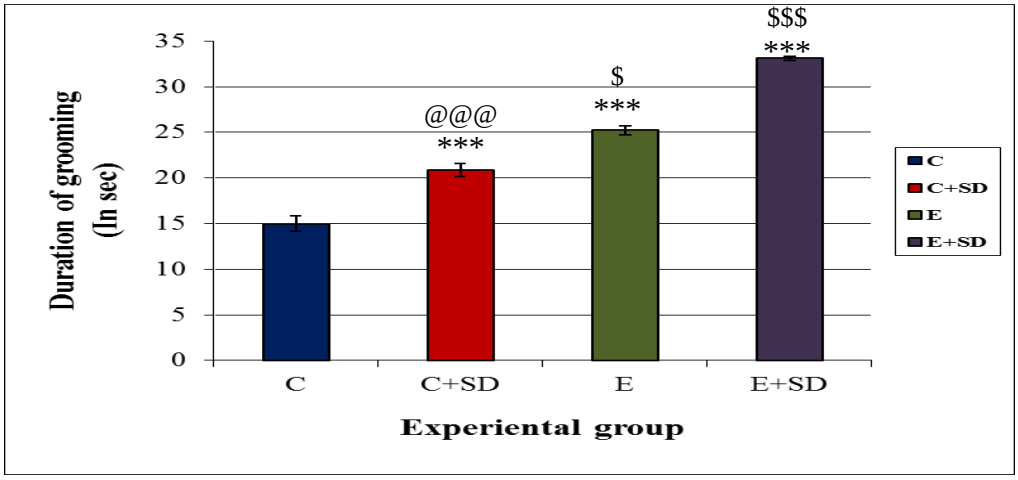
*** p<0.001 compared to C. $$$ p<0.001 compared to C+SD . $$ p<0.01 compared to C+SD.

@ @@p<0.001 compared to E. @ @p<0.01 compared to E.

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant decrease in Number of grooming attempts of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant decrease in Number of grooming attempts of E when compare to C+SD (P<0.01) rat. Significant decrease in Number of grooming attempts of E+SD as compared to C+SD (P<0.001) and E (P<0.01) rat.

Fig. 28: - Duration of grooming in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.01 compared to C+SD.

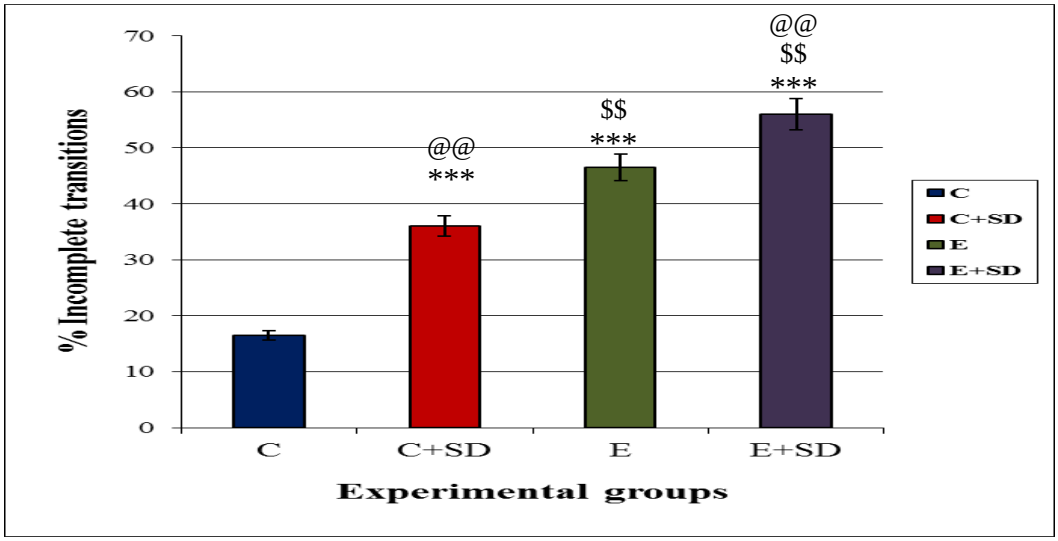
@ @@p<0.001 compared to E. $ p<0.05 compared to C+SD

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant increase in duration of grooming of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant increase in duration of grooming of E when compare to C+SD (P<0.05) rat. A Significant increase in duration of grooming of E+SD as compared to C+SD (P<0.001) rat.

A "splash test" is the indirect measure of body care efficiency. The test consists of splashing a 10%sucrose solution on the dorsal coat of an animal and then counting the animal's grooming bouts (Ducottet and Belzung, 2004). A decrease in the number of grooming bouts and increased time duration can be interpreted as a stress-induced attenuation of sucrose preference rather than an alteration in hygienic behavior (Ducottet and Belzung, 2004).

Fig.29: - % incomplete transitions in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

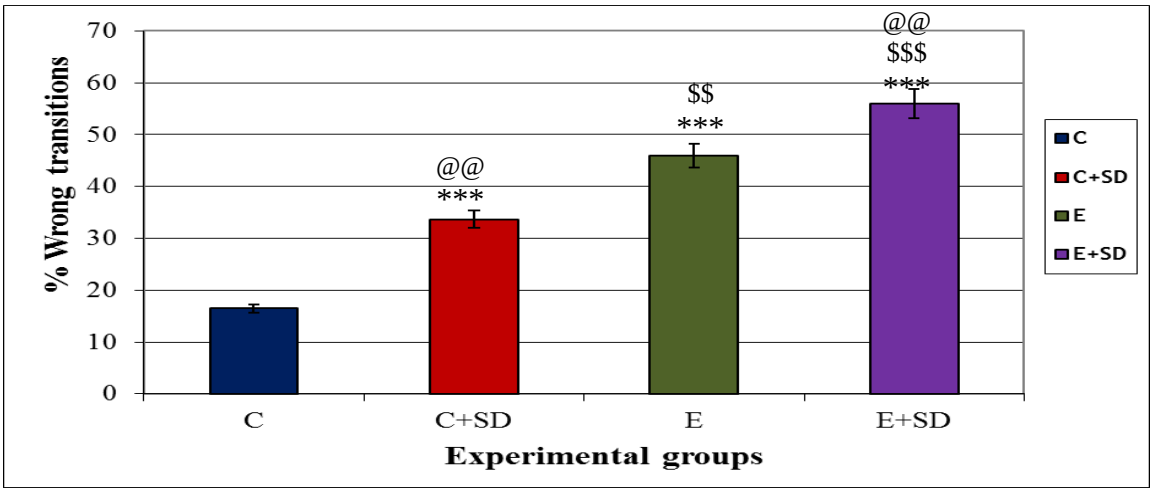
*** p<0.001 compared to C. $$p<0.01 compared to C+SD.

@ @p<0.01 compared to E.

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant increase in % incomplete transitions of grooming of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant increase in % incomplete transitions of grooming of E when compare to C+SD (P<0.01) rat. Significant increase in % incomplete transitions of grooming of E+SD as compared to C+SD (P<0.01) rat and E+SD(p<0.01).

Fig.30: - % Wrong transitions in control and experimental groups



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$ $P<0.001 compared to C+SD .

@@p<0.01 compared to E. $$ P<0.01 compared to C+SD.

. C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant increase in % incomplete transitions of grooming of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant increase in % incomplete transitions of grooming of E when compare to C+SD (P<0.01) rat. A Significant increase in % incomplete transitions of grooming of E+SD as compared to C+SD (P<0.001) rat and E+SD (P<0.01).

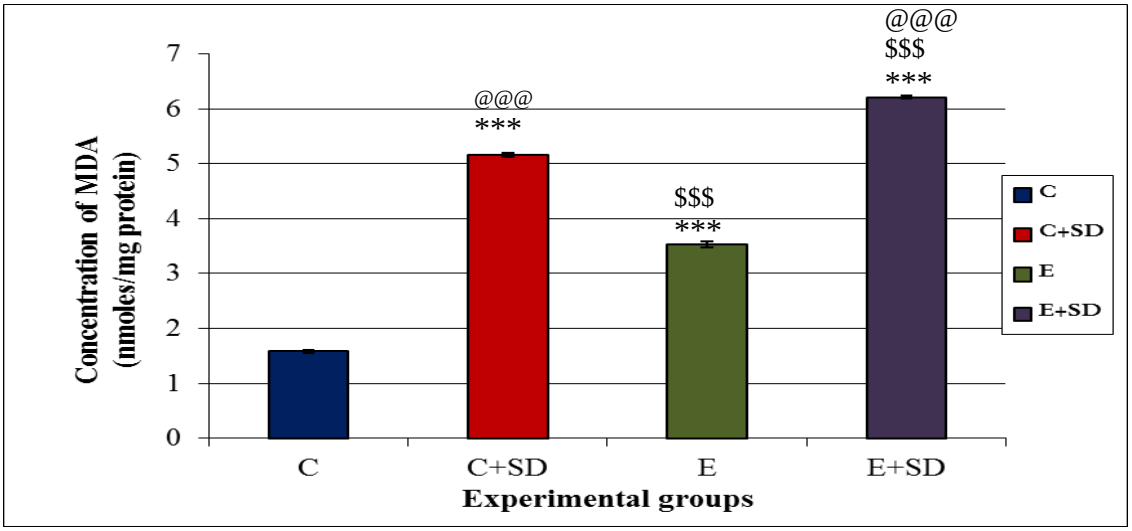
Body care (hygienic) behavior in most animal species is a natural adaptation aimed at removal of litter particles, pathogenic microbes, and parasites from fur and skin (Hart, 1990). It entails a sequence of movements (grooming) such as licking the fur and paws, washing the head, and cleaning the genital area (Berridge 1990; Hart and Pryor 2004). Hygienic behavior also serves as an indicator of animal health. A healthy rat spends about 30-50% of its waking time on body care and its fur is usually neat and tidy. On the contrary, the pelage of a sick animal is dirty

and oily from lack of grooming (Hart, 1988). Chronic stress is frequently used to produce sick and Depressive behavior in rodents.

The results showed altered pattern of grooming and more number of incorrect bouts in epileptic and sleep deprived rats which can be interpreted as a result of stress induction in the novel environment.

3.6 TBARS enzyme assay

Fig. 31:- Concentration of MDA in the liver of control and experimental group of rats



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.01 compared to C+SD.

@@@ P<0.001 compared to E.

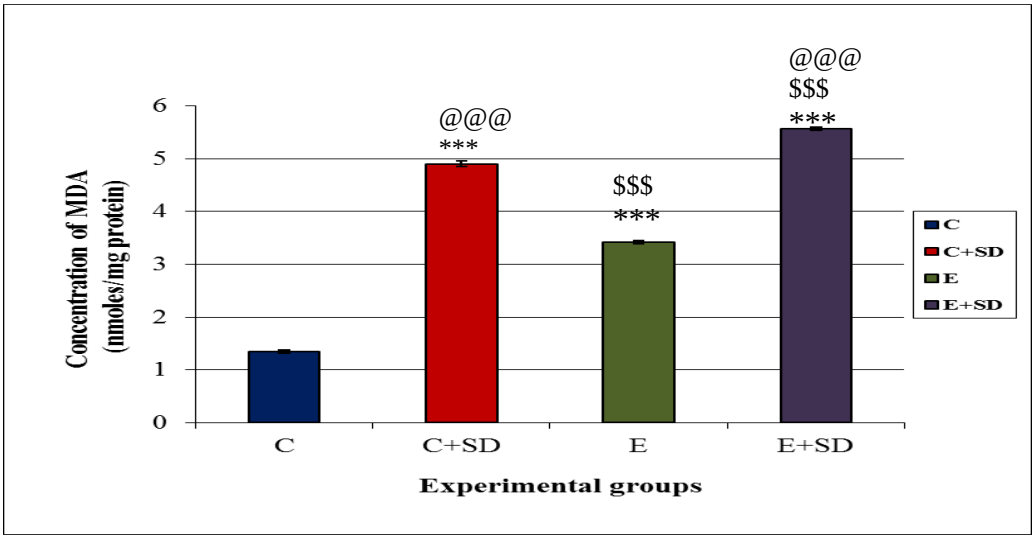
C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant increase in concentration of MDA of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant increase in concentration of MDA of E when compare to C+SD (P<0.001) rat. A Significant increase in concentration of MDA of E+SD as compared to C+SD (P<0.001) rat and E+SD (P<0.001).

Oxidative stress has been involved in the mechanisms of biologic aging, as well as in the pathogenesis of cancer, atherosclerosis, diabetes, and neurodegenerative disorders (Droge et al., 2002). Oxidative stress occur whenever there is an imbalance between oxidant production and antioxidant defenses, either because the former is increased, because the latter are decreased, or both. At the cellular level, such imbalance can result in structural damage due to oxidative modifications of proteins, lipids, and nucleic acids. Major cellular oxidants include reactive oxygen species (ROS, Eg, O2 and H2O2) (Droge et al., 2002). Numerous neurochemical studies using animals have revealed that oxidative stress-related seizures produce changes in antioxidant enzymatic activity and receptor binding (Xavier et al., 2007). ROS and lipid peroxides, which are

produced by free radical chain reaction, have been implicated in the pathogenesis of a variety of conditions (Yagi et al., 1999). Lipid peroxidation due to the reaction of free radicals with lipids is considered a hallmark of cellular oxidative damage (Yu et al., 1994). Once established, such damage can affect the membrane lipid bilayer and, specifically, the mitochondrial electron transport chain, thus becoming a major cause for a further increase in oxidant production. Our result showed significantly high concentration of MDA in liver of E group suggesting cellular damage due to Peroxidative damage in liver. The increased lipid peroxidation in sleep deprived groups indicates that inadequate sleep may activate pathways for ROS formation ultimately cause change in membrane permeability.

Fig.32: - Concentration of MDA in the kidney of control and experimental group of rats



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.001 compared to C+SD.

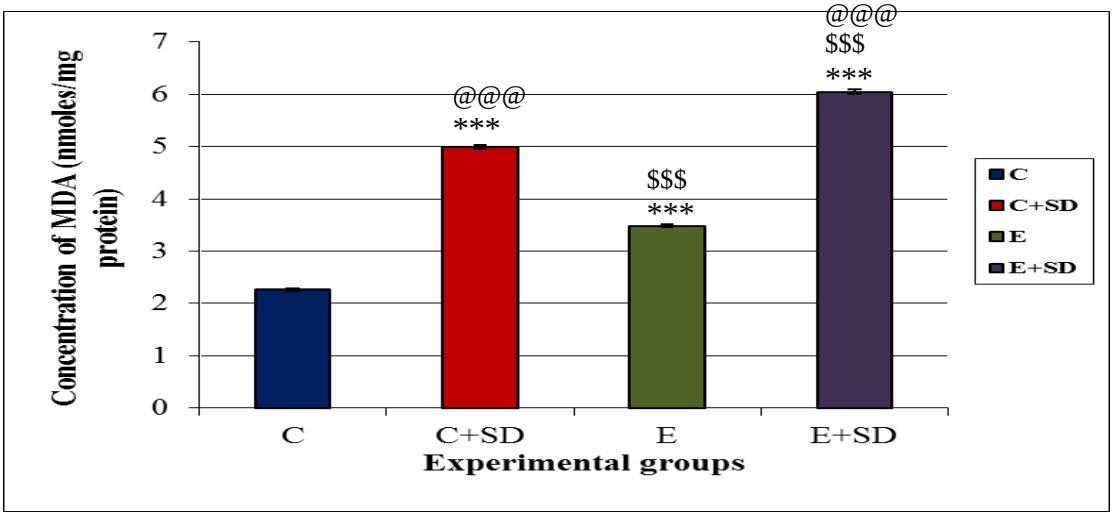
@@@ P<0.001 compared to E.

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant increase in concentration of MDA of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant increase in concentration of MDA of E when compare to C+SD (P<0.001) rat. A Significant increase in concentration of MDA of E+SD as compared to C+SD (P<0.001) rat and E+SD (P<0.001).

Kidney is another major organ where many of the metabolic pathways take place. Increased amount of stress may result into different diseases of kidney. There is also evidence that ROS play an important role in the pathogenesis of many diseases, particularly in kidney diseases due to the vulnerability of the kidneys to oxidative stress (Simsek et al., 2005). Epilepsy is one of the most common neurological disorders. There is emerging focus on the role of oxidative stress and mitochondrial dysfunction both a consequence and a cause of epileptic seizures (Patel et al., 1951). Increased MDA in the kidney of C+SD and E+SD Showed increased ROS formation in kidney.

Fig. 33: -Concentration of MDA in the brain stem of control and experimental group of rats.



Values are Mean±SD of 3 separate experiments (n=3 rats per group).

ANOVA followed by student's-Newman-Keul's Test.

*** p<0.001 compared to C. $$$ p<0.001 compared to C+SD.

@@@ p<0.001 compared to E.

C-control, E-epileptic, CSD-control sleep deprived, ESD-epileptic sleep deprived.

A significant increase in concentration of MDA of C+SD (P<0.001), E (P<0.001), E+SD (P<0.001) was observed as compared to C. Significant increase in concentration of MDA of E when

compare to C+SD ($P<0.001$) rat. A Significant increase in concentration of MDA of E+SD as compared to C+SD ($P<0.001$) rat and E+SD ($P<0.001$).

Brain stem controls sleep-wake cycle. Structurally continues with the spinal cord and help in reflexes. The brain may be particularly vulnerable to oxidative stress because of its high rate of oxygen consumption, high content of polyunsaturated fatty acids, and low levels of natural antioxidants (Sun et al., 1975). Since most of the effects of sleep deprivation in humans are on higher cognitive functions. Several hypotheses about the functions of sleep rest on the assumption that wakefulness represents an oxidative challenge for the brain. It has been proposed that, during sleep, uridine and glutathione may facilitate the oxidative detoxification of the brain by potentiating GABAergic transmission and inhibiting glutamatergic transmission, respectively. Populations of neurons responsible for the onset and maintenance of paradoxical sleep (PS) are restricted to the brainstem (Verret et al., 2005). In the brain stem, the phenomena of excitotoxicity has been related to an over production of free radicals by the neurons during seizures and SE induced by pilocarpine (Erakovic et al., 2000) and it has been suggested as a possible mechanism for the neurotoxic effects observed during epileptic activity. (Freitas et al., 2004). Increased Lipid peroxidation in E rat suggests that excitotoxicity in the brain stem may lead to more free radical formation which in turn increases the Peroxidative load on brain stem resulting in higher lipid peroxidation. Moreover facilitation of the oxidative detoxification of the brain by potentiating GABAergic transmission and inhibiting glutamatergic transmission during sleep was inhibited in C+SD and E+SD group, resulted into increased ROS formation and lipid peroxidation.

SUMMARY AND CONCLUSION

Summary:

Epilepsy is one of the most common disorders of the central nervous system characterized by recurrent seizures unprovoked by an acute systemic or neurologic insult. One of every ten people will have at least one epileptic seizure during a normal lifespan, and a third of these will develop epilepsy. Evidences show that epilepsy can disrupt sleep as well as sleep deprivation can increase susceptibility for seizures. Epilepsy was induced in adult Wistar male rat with a single intraperitonial dose of pilocarpine, which is known one of the best models for temporal lobe epilepsy. Rats were partially sleep deprived by modified float over water method. The rats were kept awake for 10 hours daily during their sleep cycle by stand over water method. I investigated the change in feed intake and water consumption in experimental group of rats and alterations in general behaviors in epileptic and sleep of epileptic rats due to sleep deprivation. The pineal gland secrets melatonin into the bloodstream where it modulates the brain stem circuit which plays a key role in influencing the sleep-wake cycle and activate MT1 receptors which then activates PLC-PKC signaling pathway. My last objective was to evaluate the effect of sleep deprivation and epilepsy on PLCß 1 gene expression. A good quality total RNA was isolated which was used for C-DNA preparation but I could not get the amplified product of PLCß 1 gene. The open field test is a good indicator of general exploratory activity which provides simultaneous measures of locomotion, exploration and anxiety. A significant decrease in the exploratory activity in the open field was observed in Control sleep deprived ($p<0.001$), Epileptic ($p<0.001$) and Epileptic sleep deprived ($p<0.001$) showed that epileptic sleep deprived rats show high level of anxiety and depression as compare to epileptic and control sleep deprived, while sleep deprivation in control rats can make their situation worse. This finding suggest imbalance in excitatory and inhibitory neurotransmitters in epileptic rats and higher level of anxiousness and stress in sleep deprived rats. “Splash Test” is the indirect measure of body care efficiency. A significant decrease in the number of grooming bouts and increase in duration of grooming was observed in Control sleep deprived ($p<0.001$), Epileptic ($p<0.001$) and Epileptic sleep deprived ($p<0.001$) which can be interpreted as a stress induced attenuation. Thus our findings in behavior studies suggest alterations in motor and cognitive functions in Epileptic and Sleep deprived rats. I also investigated an interesting fact that during sleep deprivation and “Splash test” epileptic rats showed difference in their sitting posture. Epileptic rats tend to sit on

the extreme corners of tripod which shows highly fearful conditions. It has been hypothesized that sleep deprivation represents an oxidative challenge for the brain and that sleep may have a protective role against oxidative damage. Cellular oxidative damage (i.e. Lipid peroxidation) by Peroxidative damage to lipids was determined by measuring Malondialdehyde concentration in Brain Stem, Liver and Kidney by performing Thio Barbituric Acid Reactive Substance enzyme assay spectrophotometrically at 532 nm. Lipid peroxidation was significantly enhanced in Control sleep deprived ($p<0.001$), Epileptic ($p<0.001$) and Epileptic sleep deprived ($p<0.001$) groups When compared to Control. The increase of reactive oxygen species levels can be because of neurochemical alterations during seizures in epileptic groups. The increased lipid peroxidation in sleep deprived groups indicates that inadequate sleep may activate pathway for reactive oxygen formation ultimately causing changes in membrane permeability.

CONCLUSION

Thus from our studies we can conclude that Epilepsy and sleep deprivation produces ROS in central as well as peripheral tissues which can be because of neurochemical alterations during seizures. We also concluded that epilepsy and sleep deprivation produces anxiety and decreased locomotor and exploratory activity.

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