Effect of Hypoglycemia on type III Neuregulin-1 Gene Expression and Lactate Dehydrogenase in Streptozotocin Induced Diabetic Rats.

A dissertation project
Submitted to
Nirma University
In Partial fulfilment of
Requirement for the Degree of

Master of Science
In
Biotechnology

Submitted by
Drashti Patel (13MBT019)
Aneri Panchal (13MBT017)

Under the guidance of
Dr. Amee K. Nair

Institute of Science, Nirma University
Ahmedabad-382481, Gujarat, India.

May 2015


Acknowledgment

Acknowledgement

First and foremost we dedicate our sincere thanks to the almighty for providing us a pure heart and a divine intervention. We shall seek his blessing in successfully going through the most challenging days of our graduate life.

In the first, we would like to express our sincerest gratitude towards ***Dr. Amee Nair, Assistant professor,*** *Institute of Science for her supervision, guidance and positive criticism throughout the duration of the dissertation project. It would have been very tough without her positive and straight forward advice. Her experience and dedication truly inspires us to be energetic and enthusiastic in every walk of life. Whatever we achieve in life shall be indebted to her with utmost happiness .We sincerely acknowledge* ***Prof. Sarat Dalai, Director****, Institute of Science, Nirma University for his advice, supervision, and passion , which made him a backbone of this institute.*

We are thankful to all the faculty members ***Dr. Nasreen Munshi, Dr. Mili Das, Dr. Sonal Bakshi, Dr. Shalini Rajkumar, Dr. Sriram Seshadri and Dr. Vijay Kothari, Dr Heena Dave*** *for their constant review and encouragement.*

We thank, ***Mr. Sachin Prajapati, Miss Sweta Patel and Mr. Rajendra Patel*** *for providing us with all kinds of possible help. We enjoyed the opportunity of working with our colleagues* ***Ankita Mishra, Manjusha Pal, Vinay Patel, Nikita Patel, Aditi Patel*** *who had been a great support in completion of this project. We would also like to thank our PhD scholar,* ***Madhavi Joshi*** *for her support and help throughout our dissertation work.*

We would also like to thank our friends for extending their helping hand always and for making this dissertation project memorable. Most important we would like to thank our parents who have been a source of encouragement and inspiration.

Finally, we would like to thank everyone who was important in the successful completion of this thesis. We express our sincere apologies to those whose names we could not mention individually.

Drashti Patel (13MBT019)
Aneri Panchal (13MBT017)

CONTENTS

List of Figures

List of Tables

IMPORTANT

ABBREVIATIONS

ABBREVIATIONS

ARIA: Acetylcholine receptor inducing activity

ANOVA: Analysis of Variance

ATP: Adenosine triphosphate

BLAST: Basic local alignment search tool

C: Control

C+IIH: Insulin Induced Hypoglycemia in Control rats

cDNA: Complementary DNA

CNS: Central nervous system

CRD: Cystein rich domain

D: Diabetic

D+IIH: Insulin Induced Hypoglycemia in diabetic rats

DNA: Deoxy ribonucleic acid

EGF: Epidermal growth factor

EGTA: Ethylene glycol tetraacetic acid

EGP: Endogenous glucose production

EPM: Elevated plus maze

GABA: Gamma- Aminobutyric acid

GAPDH: Glyceraldehyde 3-phosphate Dehydrogenase

GGF: Glial growth factor

HAAF: Hypoglycemia Associated Autonomic Failure

HEPES: (4- (2- hydroxylethyl)-1- piperlzineethenesulfonic acid)

HPA: Hypothalamus pituitary adrenal

IDT: Integrated DNA Technologies

KCL: Potassium chloride

LDH: Lactate Dehydrogenase

MDA: Malondialdehyde

NAD: Nicotinamide Adenine Dinucleotide

NADH: Nicotinamide adenine Dinucleotide

NCBI: National Centre for Biotechnology information

NDF: Neu differentiation factor

NO: Nitrogen oxide

NRG: Neuregulin

PCR: Polymerase Chain Reaction

PNS: Peripheral nervous system

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

SMDF: Sensory and motor neuron derived factor

STZ: Streptozotocin

TBA: Thio barbituric acid

TBARS: Thio Barbituric acid reactive substance assay

UV: Ultra violet

ABSTRACT

Brain is dependent on a continual supply of glucose diffusing from the blood. Hence low amount of blood glucose affect the brain first. Subtle reduction of cognitive efficiency is one of common side effect of hypoglycaemia can be observed when the glucose falls below 65 mg/dl. So Glucose homeostasis in humans is a critical factor for the functioning of nervous system. Hypoglycemia, diabetic hypoglycemia and hyperglycemia are found to be associated with central and peripheral nerve system dysfunction. Hyperglycemia and hypoglycaemia induces oxidative stress in neurons and results in activation of multiple biochemical pathways. These activated pathways are a major source of damage and are potential therapeutic targets in neuropathy. NRG-1 found both as in membrane-anchored regions and also as soluble form work as a cytokine that binds to their erbB2 receptor and activate to appropriate downstream signaling cascade and made a protein product that regulates developmental neuronal survival synaptogenesis, astrocytic differentiation, and microglial activation. Neuronal over expression of NRG-1 induces hypermyelination and demonstrates that NRG-1 type III is the responsible isoform. We carried out experiments to investigate the role of diabetes and insulin induced hypoglycemia by performing behaviour analysis test for the assessment of motor activity (Grid Walking test), cognition (Modified Karl Lashley's Maze) and anxiety (Elevated Plus maze). Lactate dehydrogenase (LDH) was used as a marker enzyme to study the gluconeogenic pathway. LDH is also involved in gluconeogenesis, the synthesis of glucose from smaller molecules and also catalyzes the conversion of lactate to pyruvate utilizing the NAD/NADH coenzyme system. Cellular oxidative damage (i.e. Lipid peroxidation) by Peroxidative damage to lipids was determined by measuring Malondialdehyde concentration in Liver by performing Thio Barbituric Acid Reactive Substance enzyme assay spectrophotometrically at 532 nm. Lipid peroxidation was significantly enhanced in Diabetic + insulin induced hypoglycaemic (D+IIH), Control+ insulin induced Hypoglycemia (C+IIH), and Diabetes (D) groups When compared to Control. The increase of reactive oxygen species levels can be because of neurochemical alterations during seizures in hypoglycemia groups.


Introduction

Introduction

1.1Hypoglycemia:

Hypoglycemic brain injury is a common and serious complication of insulin therapy associated with diabetes. Repeated episodes of hypoglycemia in diabetics result in neuronal loss because of impaired glucose supply and utilization. Recurrent hypoglycemia reduces the supply of glucose to the brain which will have deleterious effect to the functioning of brain cells (Sherin et al., 2010). Hypoglycemia is a true medical emergency which requires prompt recognition and treatment to prevent organ and brain damage. The symptoms depends on duration and severity of hypoglycemia and varied from autonomic activation to behavioural changes to altered cognitive function to seizures or coma. Severe untreated hypoglycemia can cause a significant economic and personal burden, therefore identification and prevention of hypoglycemia can reduce diabetes burden by prevention of hypoglycemia complications (Shafiee et al., 2012).

1.2 Effect of Hypoglycemia on Brain damage:

Hypoglycemia depresses brain metabolism. It is evident that insulin coma, deliberately induced as a therapy for mental disorders (a practice laudably abjured), markedly reduces cerebral oxygen consumption and disproportionately decreases brain glucose metabolism. Moderate hypoglycemia produces a partial energy failure of the CNS through brain fuel starvation or neuroglycopenia. The energy failure is due to synaptic release of glutamate (or aspartate) and a concomitant failure of energy-dependent reuptake of excitatory neurotransmitters (Mccall, 1992).

High concentration of insulin in brain extracts and expression of insulin receptors in CNS tissues have attention over the sources, and functions of insulin in the brain (Ghasemi et al., 2013). For insulin-stimulated glucose metabolism which occurs in the brain, insulin receptors, and insulin –sensitive glucose transporters are required (Hopkins and Williams, 1997). In late 1950s, it was showed that insulin elevates glucose uptake by the spinal cord

tissue, and that insulin can stimulate the resting nerve respiration and glucose utilization (Chowers, 1961).

Glucose homeostasis is maintained through a classical sensory motor integrative pathway. Fluctuations in glucose are monitored by specialized glucose-sensing cells located in the periphery (hepatic portal/mesenteric vein) and a number of discrete regions of the brain (particularly in the hindbrain andhypothalamus) (Banks et al., 1997). Glucose signal allows to incorporated with and influenced by inputs from other brain regions (*e.g.* circadian rhythms) is generated via one of a number of effector mechanisms (*e.g.* epinephrine or glucagon release). During hypoglycaemia, glucose-sensing neurons release neurotransmitters or neuropeptides rather than insulin (McCrimmon, 2012).

Insulin is a hormone which is secreted by the pancreas. The body can use glycogen for energy between meals. Fat can also be used for energy. Hypoglycaemia is the result of the interplay of relative or absolute insulin excess and compromised physiological defences against falling plasma glucose concentrations (Starlanyl et al., 2001).

1.3 Enzyme Assay:

1.3.1 Lactate Dehydrogenase Assay (LDH):

Lactate Dehydrogenase (LDH) is an important enzyme for the energy metabolism pathways. It is known that glucose is the major fuel for the brain which gets metabolized for different functions (Dienel, 2012). Most of the times, the cells breakdown this glucose completely, but this requires a lot of oxygen, and if this oxygen is not available, these pathways end-up at glycolysis and further energy generation and other physiological functions does not become possible. LDH is the connecting link between the energy generating pathways and the basic substrate glucose, which gets activated when there is a lack of oxygen (Beltran et al., 2012).

LDH catalyses a reversible redox reaction inter-converting pyruvate to lactate, coupled to NADH and NAD+ and vice-versa. LDH permits organisms to generate temporary oxygen debt energy in the form of accumulated lactate which later gets discharged by re-oxidation of lactate to pyruvate when oxygen becomes available (Markert, 1984). LDH overexpression shows decrease in the glucose oxidation rate at glucose concentrations little more than the control cells, whereas this condition remains unchanged at lower glucose concentrations.

Lactate release gets increased in parallel with a decrease in the glucose oxidation rate (Alcazar et al., 2000).

1.3.2 Thio Barbituric Acid reactive substance Assay (TBARS):

Oxidative stress is mostly assessed in plasma and increased concentrations of thiobarbituric acid-reactive species (TBARS) which is by product of lipid peroxidation. Lipid peroxidation is the consequence of oxygen free radicals, the role of which is well established in the pathogenesis of a wide range of diseases. Some metabolic diseases are also associated with an enhanced level of lipoperoxidation (Patockova et al., 2002).

The central nervous system (CNS) is extremely sensitive to free radical damage due to its poor antioxidant capacity. Furthermore, the central nervous system is extremely sensitive to hypoglycemic damage, because of the properties of the hematoencephalic barrier and because of the lack of other substrates in the CNS (Altomare et al., 1992).

Malondialdehyde (MDA) is a three-carbon compound formed from peroxidized polyunsaturated fatty acids, mainly arachidonic acid. It is one of the endproducts of membrane lipid peroxidation. Since MDA levels are increased in various diseases with excess of oxygen free radicals, many relationships with free radical damage were observed (Ohkawa et al. 1979; Guichardant et al. 1994).

1.4Behavioural Studies:

1.4.1 Elevated Plus Maze:

The Elevated plus Maze is a widely used behavioural assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents, and to define brain regions and mechanisms underlying anxiety-related behaviour. The Elevated plus maze assay essentially determines a preference between a comparatively safe and comfortable environment (the closed arms) and a risky environment (elevated open spaces) (Chathu et al., 2008).

1.4.2 Grid Walking Test:

The Grid Walking Test assesses the motor function ability in rats to accurately place their limbs during tasks. The use of a grid walking test in the evaluation of spinal cord injured animals has been endorsed as a sensitive technique for behavioural testing and for

providing an objective evaluation of neurological impairments. Animals are required to walk across a runway made of a wire grid. As the animals walk across the grid, the number of errors made and the amount of time they are actually walking will be video-recorded and measured to assess performance (Z'Graggen et al., 1998).

1.4.3 Modified Karl lashley's Maze:

Modified Karl Lashley's Maze is behavioural test to check the memory and cognition of the rats. Modified Karl Lashley's test is generally performed in experimental rats to check the memory and cognition power in control and other experimental groups. Primarly rats of all experimental groups were trained in Karl Lashley's without doors. During testing period to create complexity to the maze doors were added in model and then cognition and memory of rats were measures by observing the parameters (Wijk et al., 2008).

1.5 Role of Nrg 1 in Myelin Formation:

Myelin is a lipid-rich membrane produced by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Myelin is an outgrowth of a type of glial cell. The composition of myelin is ~70% lipid and 30% protein, which is in stark contrast to other membranes, which are typically 30–50% lipid. The low capacitance, high-resistance membrane envelops. The role of insulator is to provide support to the axon and maintains integrity. Thus myelin facilitates tri-directional communication between the neuron, the myelinating cell, and the environment (Nave et al., 2008; Tao et al., 2009).

The production of the myelin sheath is called myelination. Myelin thickness is depends upon the axon fiber size. Neuregulin protein (NRG-1) regulates developmental neuronal survival, synaptogenesis, astrocytic differentiation. NRG-1 binds to the ERBb receptor and activates appropriate downstream signaling cascade that regulates developmental neuronal survival (Chaudhury et al., 2003, G. Fischbach, 2007).

The Neuregulin-1 (NRG1) family comprises more than 15 membrane-associated and secreted proteins. All NRG1 isoforms share an epidermal growth factor (EGF)-like signaling domain that is necessary and sufficient for activation of their receptors (Nave et al., 2006).

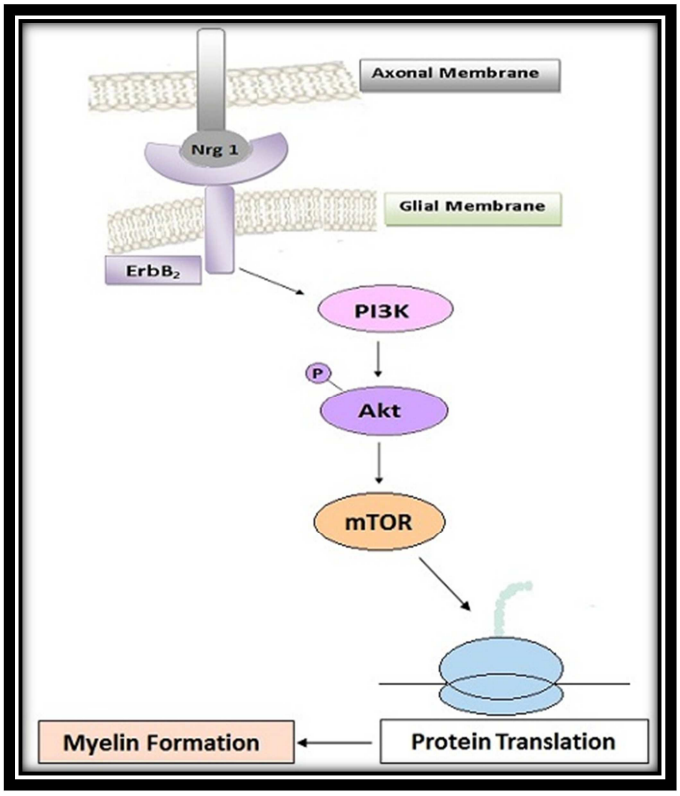


Fig-1 Role of NRG 1 in Myelin Formation

NRG1 type I (also known as Heregulin, neu differentiation factor, or acetylcholine receptor-inducing activity [ARIA]) and NRG1 type II (also known as glial growth factor [GGF]) have N-terminal immunoglobulin-like domains. NRG1 type III is defined by its cysteine-rich domain (CRD), which functions as a second transmembrane domain (Holmes et al., 1992).

Table- 1: Types of NRG 1

NRG 1	Name
NRG 1 type –I	Heregulin, or acetylcholine receptor inducing activity (ARIA)
NRG 1 type –II	Glial Growth Factor-2 (GGF2)
NRG 1 type –III	Sensory and motor neuron-derived factor (SMDF)

All isoforms, type I, II and III, contain an epidermal growth factor (EGF)-like signaling domain in the extracellular domain that is necessary and sufficient for activating erbB receptors on the Schwann cell surface. The type I and II isoforms contain an Ig-like domain whereas type III isoform contains a cysteine-rich domain, which functions as a second transmembrane domain (Neeraja, 2012; Calaora, 2001).

Neuronal over expression of NRG 1 induces hypermyelination and demonstrates that NRG 1 type III is the responsible isoform (Bare et al., 2011). So for the adequate myelination required activation of Schwann cell (responsible for myelination and neuronal degeneration in PNS) and Oligodendriocytes (responsible for myelination in CNS) that is ultimately dependent upon NRG 1 growth factor molecules (Michailov et al., 2004).

1.6 Hypothesis:

NRG 1 may be altered in Streptozotocin induced Diabetic and Insulin induced Hypoglycaemic rats.

1.7 Objectives:

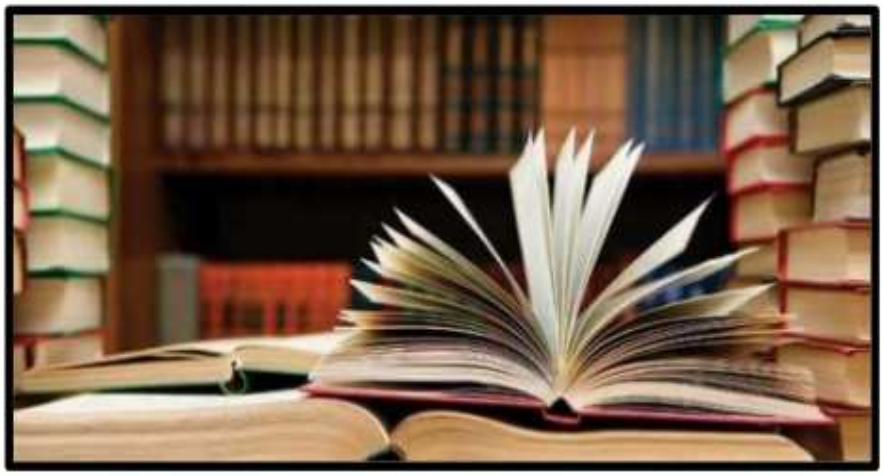
1. To study behavioral alteration in memory, anxiety and motor activity via neurobehavioral analysis in control & experimental group of rats (C, D, C+ IIH, D+ IIH).
2. To study Nrg 1 type III expression in cerebral cortex of brain in control & experimental group of rats (C, D, C+IIH, D+IIIH).
3. To assess the alteration in Lactate dehydrogenase, Malondialdehyde levels in control & experimental group of rats (C, D, C+IIH, D+IIH)

1.8 Aims to Attain Objectives:

1. To induce diabetes by Streptozotocin and hypoglycaemia by insulin in male adult Wistar rat.
2. To check food water consumption change in body weight and blood glucose level in control and experimental group of rats (C, D, C+IIH, D+IIH).
3. To evaluate Working memory (Modified Karl lashley's Maze), motor function (grid walking test), anxiety (elevated plus maze) in control and experimental group of rats (C, D, C+IIH, D+IIH).

4. To study expression of Nrg 1 (type- III) gene in control and experimental group of rats (C, D, C+IIH, D+IIH).
5. To assess enzyme activity of Lactate Dehydrogenase and Malondialdehyde levels in control and experimental group of rats (C, D, C+IIH, D+IIH).

Literature Review



2. Review of Literature

2.1 Hypoglycemia:

Hypoglycaemic coma and brain injury remains potential complications of insulin therapy (Robinson et al., 2009). Hypoglycaemic brain injury is a common and serious insequence of insulin therapy in diabetic individuals (Pramming et al., 1991). Hypoglycemia is a collection of symptoms due to abnormally low plasma glucose (less than 70 mg/dL) level than the normal glucose level. The brain and peripheral body cells require glucose for their proper functioning. Studies suggest that acute or chronic hypoglycemia leads to neurological dysfunction and injury (Robinson et al; 2009). Carbohydrates are the main dietary source of glucose which is the chief energy provider to the body. Insulin is a hormone secreted from the beta cells of Islet of Langerhans in the pancreas. Insulin helps the cells to metabolise the glucose for energy (Daniel, 2011).

2.1.1 General signs of Hypoglycaemia

Physical morbidity of an episode of hypoglycaemia ranges from unpleasant symptoms such as anxiety, palpitations, tremor and hunger. However prolonged hypoglycaemia can lead to permanent brain damage. Signs that result from activation of the sympathoadrenal system include pallor and diaphoresis which are often prominent along with a increased heart rate and systolic pressure (Stratton, 2000).

The glycemic threshold for decreased insulin secretion is approximately 4.5mmol/l (81mg/dl). Increments in pancreatic β cell glucagon and adrenomedullary epinephrine secretion normally occur as glucose levels fall just below the physiological range 68 mg/dl. If these defences fail to abort the hypoglycemic episode, lower glucose levels trigger a more intense sympathoadrenal response that causes autonomic symptoms; neuroglycopenic symptoms which occur at about the same glucose level 54 mg/dl (Boyle et al. 1994).

The lower glucose levels cause overt functional brain failure that can progress from measurable cognitive impairments 50mg/dl to aberrant behaviours, seizure, and coma. Coma

can occur at glucose levels in the range of 41–49 mg/dlas well as at lower glucose levels (Ami et al., 1999).

2.1.2 Symptoms of Hypoglycaemia:

The study conducted by David et al., in 1998 reported the most frequently autonomic symptoms during hypoglycaemia were sweating, trembling, and warmness, and the most frequently reported neuroglycopenic symptoms were inability to concentrate, weakness, and drowsiness. Neuroglycopenic symptoms were reported more commonly at the onset of hypoglycaemia. Other factor identified that included dizziness, confusion, tiredness, difficulty in speaking, shivering, drowsiness, and inability to concentrate, which was labelled a neuroglycopenic factor**.** The symptoms such as nausea, vomiting, and headache were induced non-specific symptoms (David et al., 1998).

2.1.3 Classification of Hypoglycemia in diabetes:

1. **Severe hypoglycaemia**

Severe hypoglycemia is an event requiring assistance of another person to actively administer carbohydrates, glucagon, or take other corrective actions. Plasma glucose concentrations may not be available during an event, but neurological recovery following the return of plasma glucose to normal glycemic is considered sufficient evidence that the event was induced by a low plasma glucose concentration (John, 2005).

2. **Documented symptomatic hypoglycaemia**

Documented symptomatic hypoglycemia is an event during which typical symptoms of hypoglycemia are accompanied by a measured plasma glucose concentration ≤70 mg/dL (John, 2005).

3. **Asymptomatic hypoglycaemia**

Asymptomatic hypoglycemia is an event not accompanied by typical symptoms of hypoglycemia but with a measured plasma glucose concentration ≤70mg/dL (John, 2005).

4. **Probable symptomatic hypoglycaemia**

Probable symptomatic hypoglycemia is an event during which symptoms typical of hypoglycemia are not accompanied by a plasma glucose determination but that was presumably caused by a plasma glucose concentration ≤70mg/dL (John, 2005).

5. Pseudo-hypoglycemia

Pseudo-hypoglycemia is an event during which the person with diabetes reports any of the typical symptoms of hypoglycemia with a measured plasma glucose concentration >70mg/dL but approaching that level (Elizabeth, 2013).

2.2 Effect of Diabetic Neuropathy in CNS:

Diabetic neuropathy is a chronic and often disabling condition that affects a significant number of individuals with diabetes. Long considered a disease of the peripheral nervous system, there is now increasing evidence of central nervous system involvement (Tesfayeet et al., 2011).

Diabetes may be at all levels of nervous system because brain micro/macro vascular disease in diabetes appears to be associated with cognitive decline and brain atrophy (Ian et al., 2011).

2.3 Model Induction by Streptozotocin:

The objective of this study is to induce experimental diabetes by Streptozotocin in control male adult Wistar rats (Burstow, 2003).

For induction of experimental diabetes in male adult rats weighted 200-250 grams (8-10 weeks), 55mg/kg of body weight was injected intravenously. Three days after induction diabetes was observed in all animals. The control and experimental group of rats were kept in the cages separately and their body weight, consumption of food and water, the levels of serum glucose in all animals were measured and then these quantities were compared. Induction of diabetes with streptozotocin decreases Nicotinamide-adenine dinucleotide (NAD) in pancreatic beta cells and causes damage through ROS and in beta cells which probably intermediates induction of diabetes (Rad, 2007).

In three days, streptozotocin reduces swelling in pancreas and ultimately causing degeneration in β cell which induces experimental diabetes. Consumption of water and food, increase in urination and serum glucose observed in diabetic animals in comparison with control rats (Farhangi, 2007).

Hypoglycemia was induced by administrating 1.5 IU/kg in control rats and 10-12 IU/kg diabetic rats. The key components of this counter-regulatory mechanism are:

1. Suppression of insulin secretion from the pancreatic β-cells. This happens even when plasma glucose is within the normal range, if there is a tendency for plasma glucose to decline (below 80 mg/dl.
2. Increase in glucagon secretion from the pancreatic α-cells, when plasma glucose is in the range of 65-70 mg/dl.
3. Increase in adrenomedullary epinephrine secretion when plasma glucose falls below the normal range (65-70 mg/dl.
4. Increased growth hormone and adrenocorticotropin secretion from the anterior pituitary (Liatis, 2011).

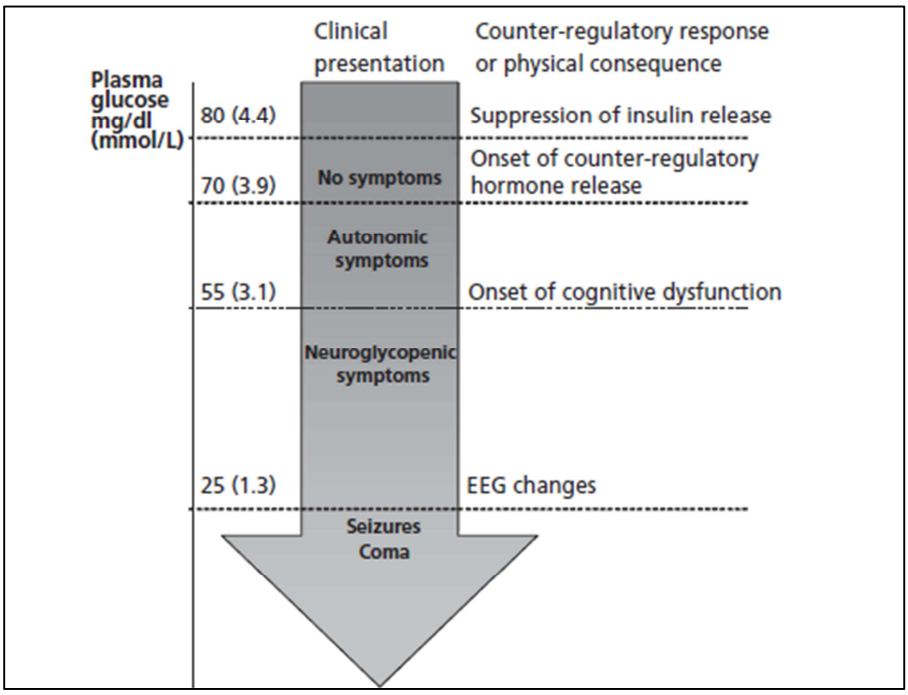


Fig-2 Normal glycaemic condition to hypoglycemic condition

2.4 Role of Insulin in the CNS:

Insulin plays important role in growth, development and metabolism as a neuromodulator. Insulin also considered being a link between adaptive feeding and changing energy requirements through its involvement with hypothalamic regulation of feeding behaviour. Hence, it might also be responsible for feeding habits which affects weight and energy regulation (Schulingkampet al., 2000). Suppression of cell death in the CNS is one of the most important protective effects of insulin. It also has protective effects against oxidative stress (Ghasemiet al., 2013).

Insulin controls the levels of sugar in the blood by inhibiting glucose release from the liver and by stimulating glucose utilization in muscle and adipose tissues. These metabolic effects are mediated by interaction of the hormone with specific cell surface receptors. In addition, insulin influences circulating glucose levels by modulating glucose production and its metabolism indirectly, through central nervous system (CNS) (Shafiee et al., 2012). Although insulin receptors exhibiting binding characteristics resembling those described for receptors in muscle and adipose tissues have been demonstrated in the CNS, it is presently unknown whether the central effect of insulin on plasma glucose is mediated by specific CNS insulin receptors similar to those found in the periphery or whether other molecular mechanisms, such as those mediating the growth-promoting functions of the hormone are involved (Ghasemiet al., 2013).

The presence of insulin receptors in CNS regions involved in regulation of energy balance and feeding (i.e. hypothalamus) and the ability of insulin to produce multiple receptor-mediated cellular effects in CNS tissue preparations in vitro, suggested that the hormone is likely to produce its central effect on circulating glucose by interacting with specific insulin receptors (Amir et al., 1987).

Hypoglycemia may cause alterations both in the central (CNS) and the peripheral (PNS) nervous system. Effects of hypoglycemia on the CNS include various symptoms such as irritability and lack of concentration, convulsions and unconsciousness. Reports show that a loss of neurons is found, in the cerebral cortex and the hippocampus (Hoffman, 2010).

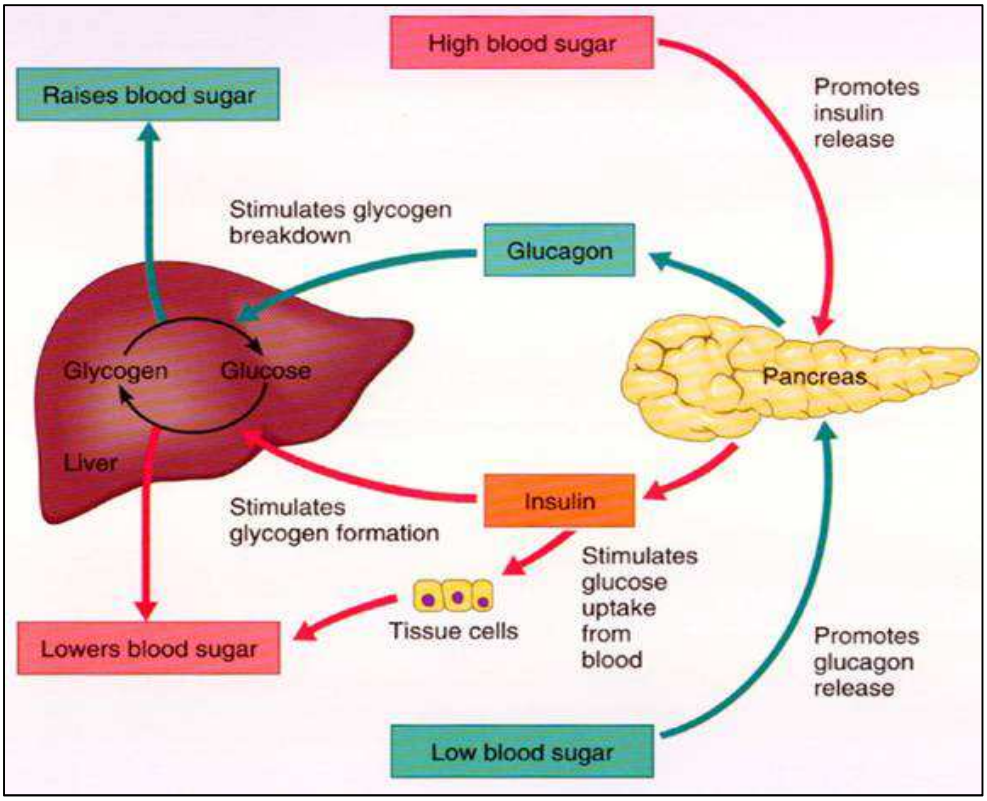


Fig: - 3 Pathophysiology of Hypoglycemia

Physiological responses to extremely low blood sugar levels in the central nervous system (CNS), which cause mainly depends on glucose which served as energy, lead to brain injury and death. Thus, during a hypoglycemic episode, the CNS can initiate a series of counter regulatory hormone responses that help to maintain the normal function of the brain. In the normal physiological condition, as blood glucose levels fall, secretion of insulin is stopped. As insulin levels falls the glucagon levels rise and the glycogen is converted into glucose in the liver, raising blood glucose levels (Zhou et al., 2014).

However, currently, the precise mechanism of glucagon release is not clear; it may rely on a combination of several mechanisms, such as local intra-islet response to hypoglycemia, or via classic hypothalamus-pituitary-adrenal (HPA) axis, or a CNS-dependent response of pancreatic tissue. The increase in glucagon secretion and complementary reduction in insulin

secretion provides a powerful mechanism to correct falling blood glucose, and rapidly restore normal blood glucose levels (Bakris et al., 2008).

In response to fluctuation glucose levels in the blood and brain, the CNS plays an important role in maintaining several of homeostatic functions, such as central control of endogenous glucose production, insulin secretion, and the appetite/feeding pathway. Sensitivity to hypoglycaemia may thus represent a functional response that depends on coordination of sensory information from neurons and perhaps also peripheral glucose sensors in order to control the fuel supply to the CNS. The CNS plays an important role in controlling defenses against hypoglycemia, thus stabilizing its fuel supply (Zhou et al., 2007).

Pathophysiology includes impairment of all three key defences against falling plasma glucose levels in the endogenous insulin deficient state:

1. Insulin levels do not decrease,
2. Glucagon levels do not increase,
3. The increase in epinephrine levels is typically attenuated.

2.5 Myelination:

The myelin sheath is a greatly extended and modified plasma membrane wrapped around the nerve axon in a spiral fashion. The myelin membranes originate from and are a part of the Schwann cells in the peripheral nervous system (PNS) and the oligodendroglial cells in the central nervous system (CNS). Each myelin-generating cell furnishes myelin for only one segment of any given axon. The periodic interruptions where short portions of the axon are left uncovered by myelin are the Nodes of Ranvier and they are critical to the functioning of myelin. Myelin is an electrical insulator, although its function of facilitating conduction in axons has no exact analogy in electrical circuitry (Waxman et al., 2004).

Myelin thickness depends on axon fibre size. Neuregulin protein (NRG-1) regulates developmentalneuronal survival, synaptogenesis, astrocytic differentiation and microglial activation. NRG-1 binds to erbB2 receptor and activates to appropriate downstream signalling cascade and made a protein product that regulates developmental neuronal survival synaptogenesis, astrocyticdifferentiation, and microglial activation (Chaudhury et al., 2003).

Between areas of myelin are non-myelinated areas called the nodes of Ranvier. Because fat acts as an insulator, membrane coated with myelin will not conduct an impulse. So, myelinated neuron, action potentials only occur along the nodes and,therefore, impulses 'jump' over the areas of myelin - going from node to node in a process called salutatory conduction (with the word salutatory meaning 'jumping'): Neurons with myelin conduct impulses much faster than those without myelin (French, 2004).

2.5.1 Demyelination:

Demyelination is the loss of the myelin sheath insulating the nerves, and is the hallmark of neurodegenerative diseases. Demyelination exposes these fibres and appears to cause problems in nerve impulse conduction that may affect many physical systems. This impairs the conduction of signals in the affected nerves, causing impairment in sensation,movement, cognition, or other functions depending on which nerves are involved (Filbin, 2004).

There is no cure for demyelinating diseases. In some cases, new myelin growth can occur in areas of damage. However, the new myelin is thinner and not as effective (George, 2014).

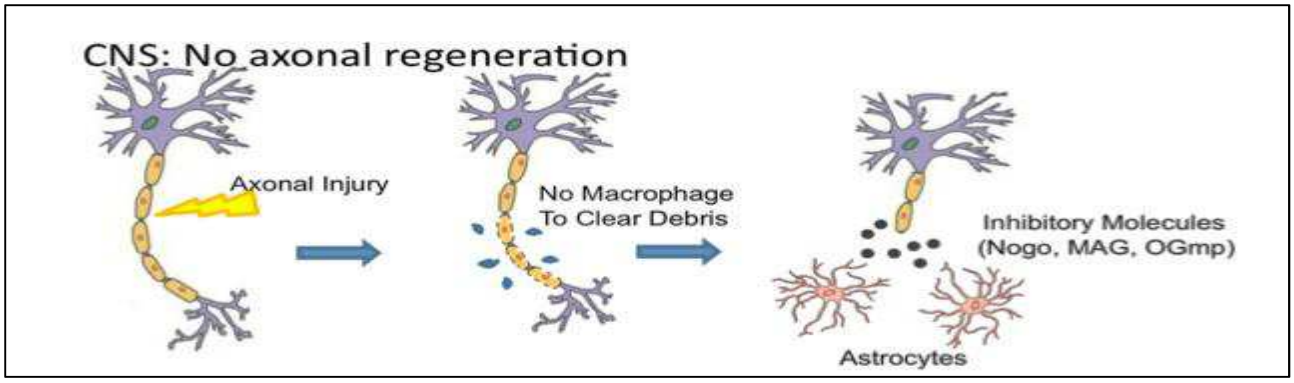


Fig:-4 Myeline degeneration

2.6 Neuregulin:

The neuregulins comprise a subfamily of at least four epidermal growth factor (EGF)-like growth factors that influence a variety of cellular events, including proliferation, differentiation, migration, survival, and fate. The most thoroughly examined neuregulin, neu differentiation factor NDF (AKA: neuregulin-1; NRG1; also heregulin, glial growth factor, or

acetylcholine receptor-inducing activity and), has been shown to play essential roles in the development of cardiac and neural tissues. (Diamonti, 2002).

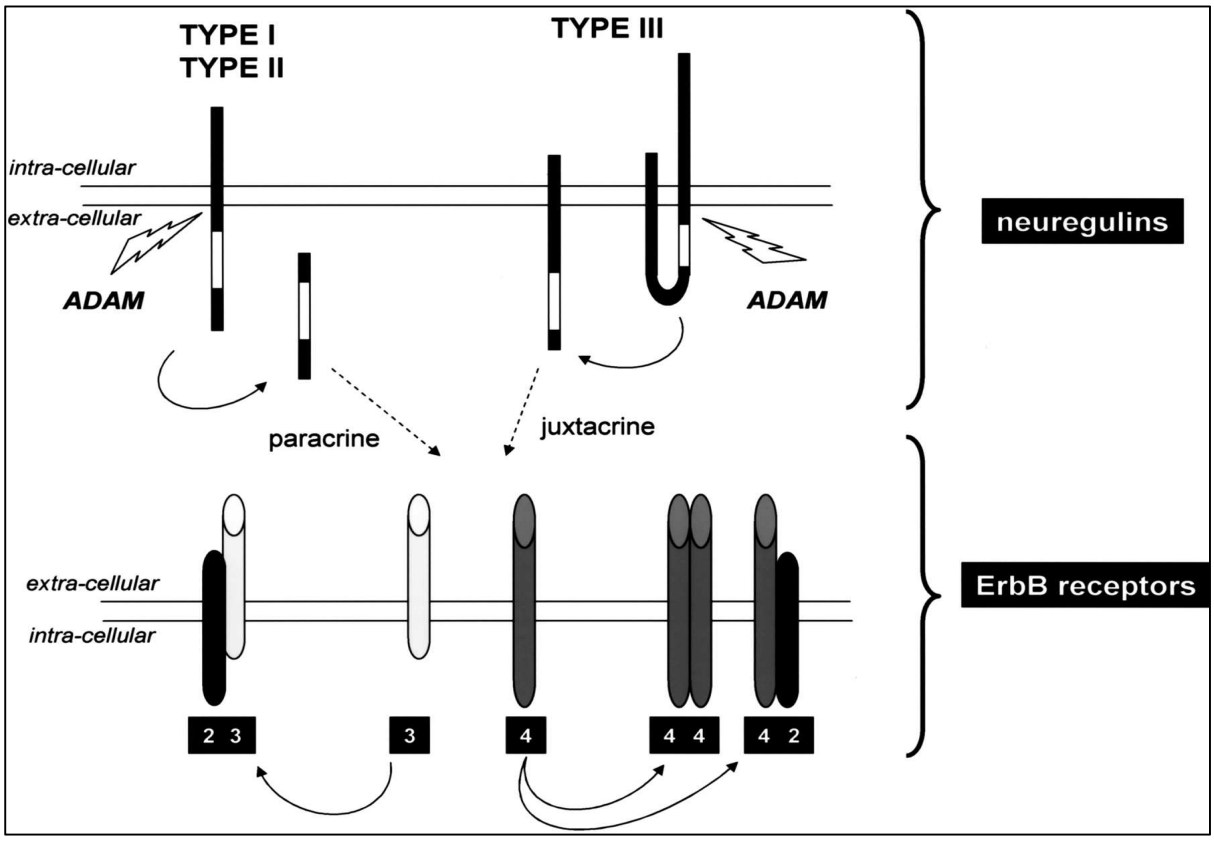


Fig-5 Neuregulin Regeneration

Type III NRGs have a cysteine-rich domain and are two transmembrane proteins. Cleavage of type III isoforms generates a transmembrane N-terminal fragment that includes the EGF-like receptor binding domain (symbolized as white fragments in the black NRG-1 bars). Therefore, type I and II isoforms are specialized for paracrine signaling, whereas types III NRGs serve as juxtacrine signals. NRG-1 can bind to 2 receptors: ErbB3 and ErbB4. Ligand binding induces formation of homodimers and heterodimers. ErbB3 homodimers are functionally defective because ErbB3 has impaired catalytic activity. ErbB2 cannot directly bind NRG-1, but it is the favoured partner for heterodimerization. On dimerization, the intrinsic kinase domain is activated, resulting in phosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptor and subsequent downstream signalling (Gilles, 2007).

2.6 Neuro-behavioural Analysis:

Behavioural and Neuroscientific Methods were invented by Shay M. Anderson and are used to get a better understanding of how our brain influences the way we think, feel, and act. There are many different methods which help us to analyze the brain and as well to give us an overview of the relationship between brain and behaviour. Analyses Techniques are for modulating brain activity, analyzing behaviour or for modelling brain-behaviour. In the these method, male adult wistar rats with brain damage are examined to determine which brain structures were damaged and to that extent this influences the rat behaviour comparing with the control rat without inducing Diabetes or Hypoglycemia (Marie, 2006)

2.6.1Elevated plus Maze

The Elevated Plus-Maze is a widely used animal model of anxiety that is based on two conflicting tendencies; the rodent's drive to explore a novel environment and it's aversion to heights and open spaces (Chathu et al., 2008). This assay essentially determines a preference between a comparatively safe and comfortable environment (the closed arms) and a risky environment (open arms). This is often discussed in terms of avoidance or fear, but this is not strictly accurate. The general principle is that the more "anxious" the subjects are, the less likely they will be to explore an uncomfortable, risky, or threatening environment. Thus, previous stress, presence of a predator odour, previous handling, manipulation of stress hormones and peptides all effect behaviour in the EPM (Garcia, 2005).

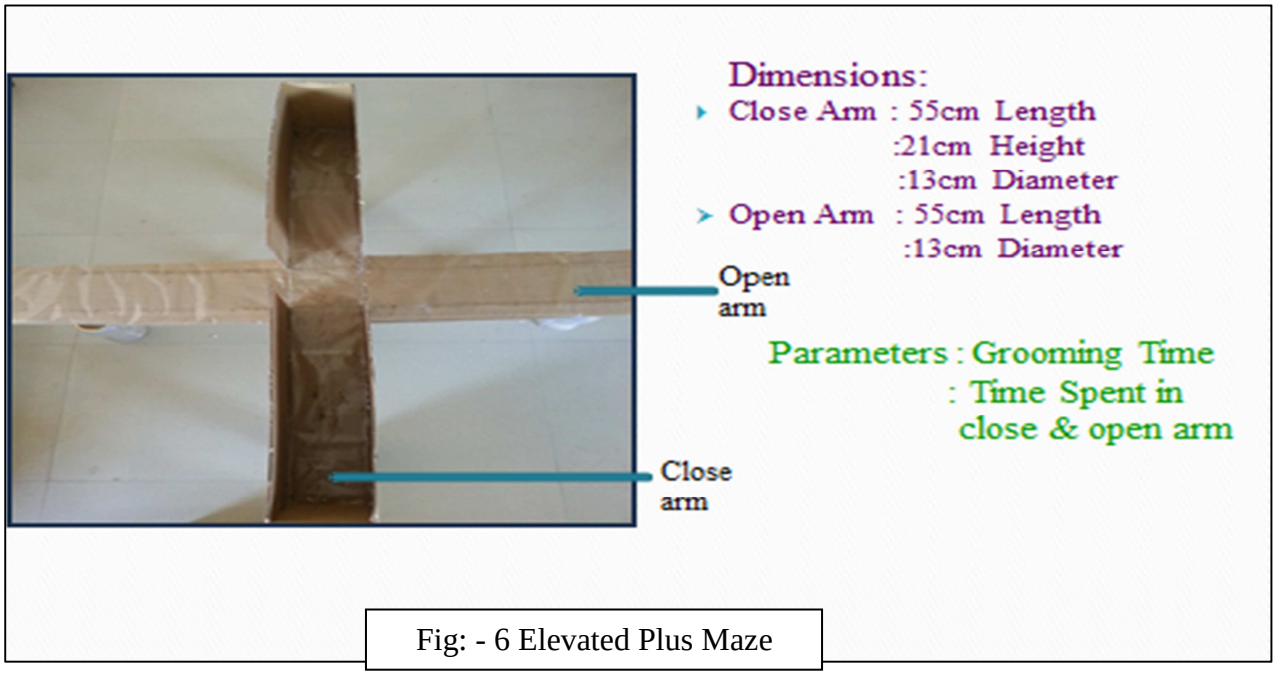


Fig: - 6 Elevated Plus Maze

2.6.2 Grid Walking Test

The Grid Walk Test is aimed to assess deficits in descending motor control. The Grid Walking test is a test of motor coordination. The apparatus consists of a grid floor. The grid is suspended or raised above a surface. Each rat is placed at one end of the grid and monitored or videotaped from the side as they walk across the grid. The number of forelimb and hind limb placement errors as the rat traverses the grid is scored. An error is counted whenever a limb misses a bar and extends downward through the plane of the bars.Padding is positioned under the grid in the event that the rat falls from the grid. Adult rat are placed on a wire or metal grid and are required to navigate over the grid. Monitoring foot placement assesses performance. One foot-miss is counted when the hind limb paw protrudes through the grid. This test is an indicator of forelimb–hind limb coordination: It assesses skilled walking and is assumed to rely on the additional contribution of pathways such as the corticospinal and rubrospinaltracts (Whishaw, 2002).

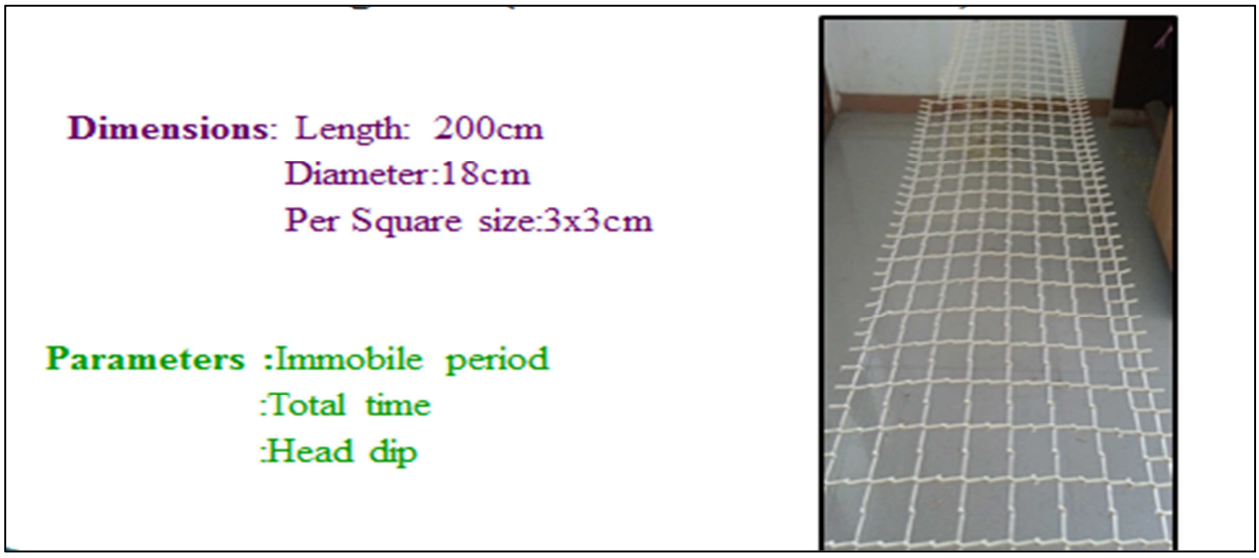


Fig: - 7 Grid Walking Test

2.6.3 Modified Karl Lashley's maze:

Karl Lashley, one of the world's foremost brain researchers, tried to locate the area in the brain where memory traces were stored. He removed sections of rat brains after teaching the rats to run mazes. He tried to find a specific location that stored a memory (Dale et al., 2008).

In 1950, Karl Lashley had distilled his research into two theories:

The principle of "**mass action**" stated that the cerebral cortex acts as one—as a whole—in many types of learning. Different areas of the brain participate in learning, and the memory traces are dispersed over the entire cortex. The principle of "**equipotentiality**" stated that if certain parts of the brain are damaged, other parts of the brain may take on the role of the damaged portion (Lashely 1950).

Maze running involves many parts of the brain. It involves vision (remembering the sight of correct pathways), spatial sense (remembering the direction to turn). If one type of clue is eliminated, there are many others remaining, allowing the rat to guide itself to the end of the maze. The maze was modified by adding doors and compartments (Baird et al., 1994).

In hypoglycaemia dysfunction of brain is seen so change in behaviour which is measured by performing behaviour study like Karl Lashley's modified model. Modified Karl Lashley's maze is test to check the memory and cognition of rats. The Memory and cognition were check by estimating parameters are : Immobilized time spent in the maze, Total time spent in the Maze, Number of false entry during total time (Baird et al., 1994).

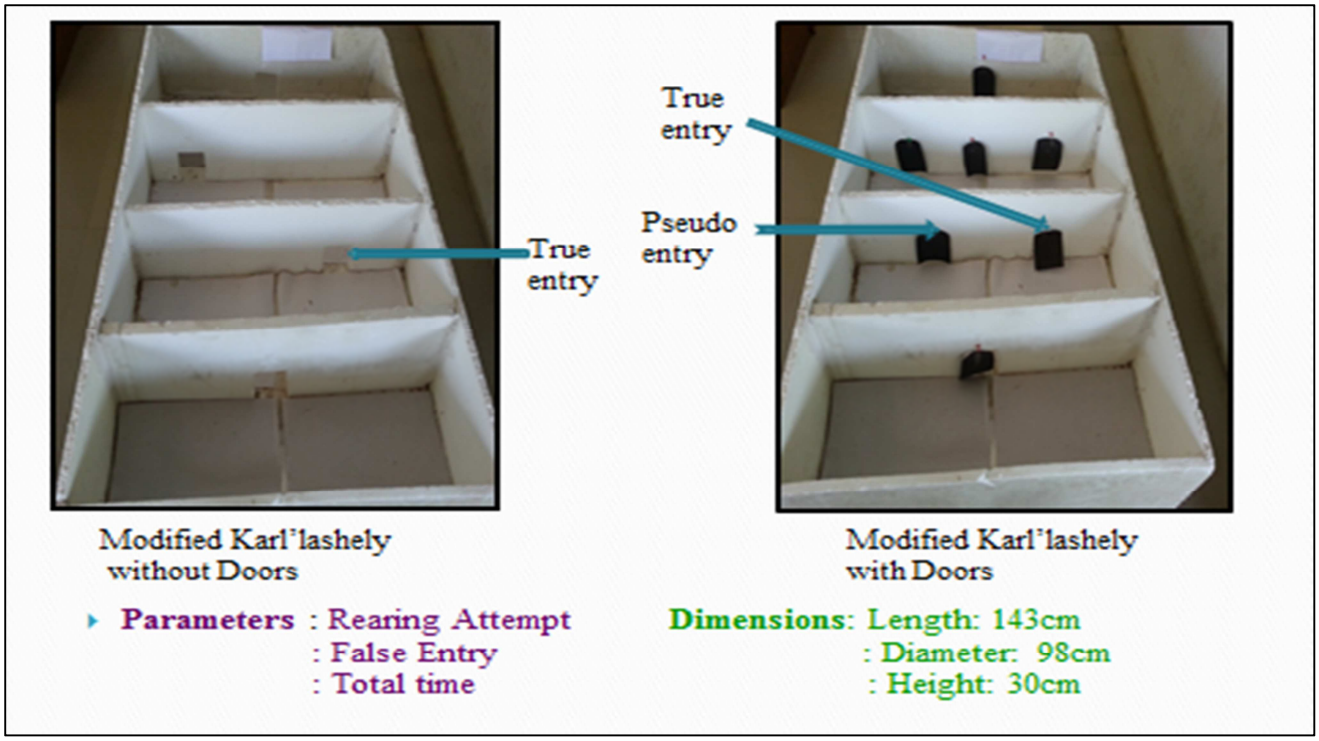


Fig: - 8 Modified Karl Lashley's Maze

2.7Enzyme assay

2.7.1 Lactate Dehydrogenase Assay (LDH):

LDH (EC no. – 1.1.1.27) catalyses reversible reaction from pyruvate to lactate. Under the influence of enzyme LDH, accumulations of lactate in brain are continued and the reversible conversation of NAD^+/NADH coupled redox reaction occurs. The extracellular lactate is transported to the cell and is oxidized to pyruvate and lactate (Beltran et al., 2012).

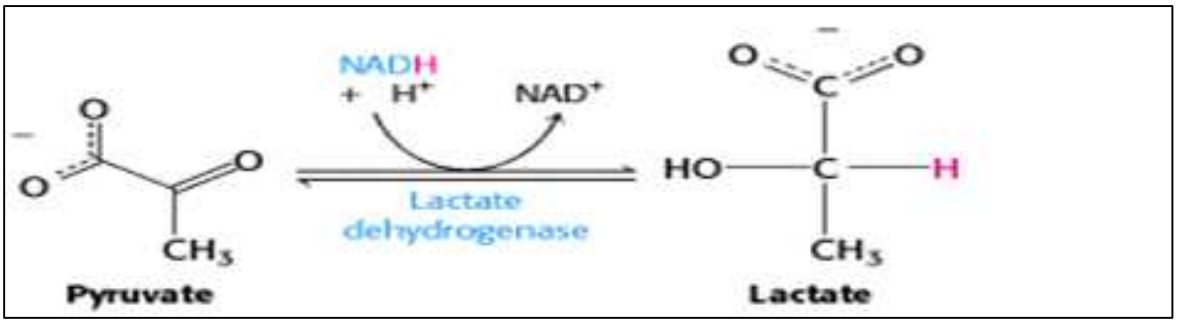


Fig: - 9 Enzymatic Reaction of Lactate Dehydrogenase (LDH)

The level of LDH expression in insulin secreting cells is an important determinant of the physiological insulin secretary capacity, which determines how pyruvate and lactate affect,

insulin secretion. Thus low level of LDH helps glucose to undergo anaerobic metabolism (Alcazar et al., 2000).

LDH allows to generating temporary oxygen debt energy in the form of accumulated lactate which later gets discharged by re-oxidation of lactate to pyruvate when oxygen becomes a valuable (Markert, 1984).

Lactate is a major product of exercising muscle cell and red blood cell metabolism. Pyruvate is converted into lactate primarily under anaerobic conditio. It is released into blood stream, and is eventually take up by the liver. The liver converts it back into glucose, which is released back into blood stream, where it is taken up by muscles recovering red blood cell and other tissues (Weatherby et al., 2002).

Cori Cycle:

Lactate in the muscle can go by way of the blood to the liver (where NAD^+ is not depleted) and be converted back to pyruvate and eventually back to glucose through a pathway called gluconeogenisis. The liver can export the glucose into the blood from where it can be taken up by the muscle for ATP production. This cycle is called the Cori cycle (Wantanbeet al., 2008).

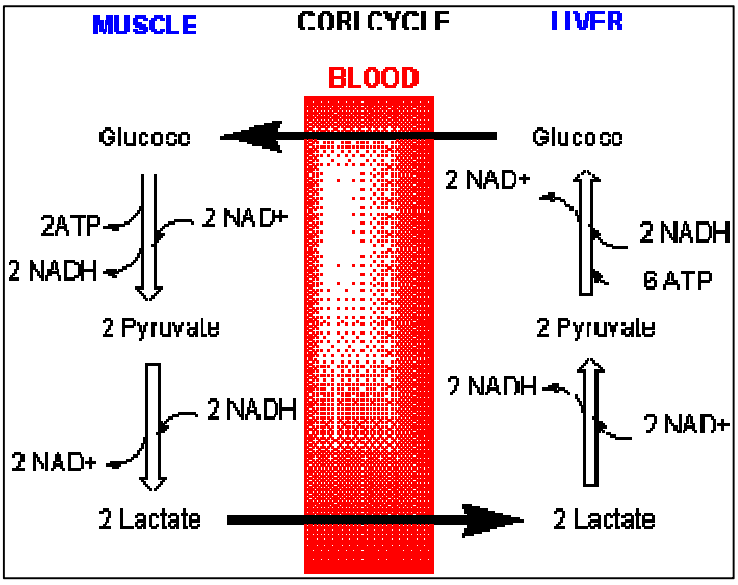


Fig: - 10 Cori Cycle

High levels of LDH indicate some form of tissue damage. High levels of more than one isoenzyme may indicate more than one cause of tissue damage. LDH catalyses the reversible reduction of pyruvate to lactate. Under the influence of enzyme LDH, accumulation of lactate

in brain is continued and the reversible conversion of NAD+/NADH coupled redox reaction occurs. The extracellular lactate is transported to the cell and is oxidized to pyruvate and lactate. Lactate synthesized within astrocyte and released into the interstitial space in the brain may serve as energy fuel for neurons (Beltran et al., 2012).

The level of LDH expression in insulin secreting cells is an important determinant of the physiological insulin secretary capacity, which determines how pyruvate and lactate affect, insulin secretion. Thus low level of LDH helps glucose to undergo aerobic metabolism (Alcazar et al., 2000).

2.7.2 ThioBarbituric acid reactive substance assay (TBARS):

Oxidative stress mostly assessed in plasma which showed increased concentration of thiobarbituric acid reactive species (TBARS) which is by product of lipid peroxidation (Colas et al., 2010).

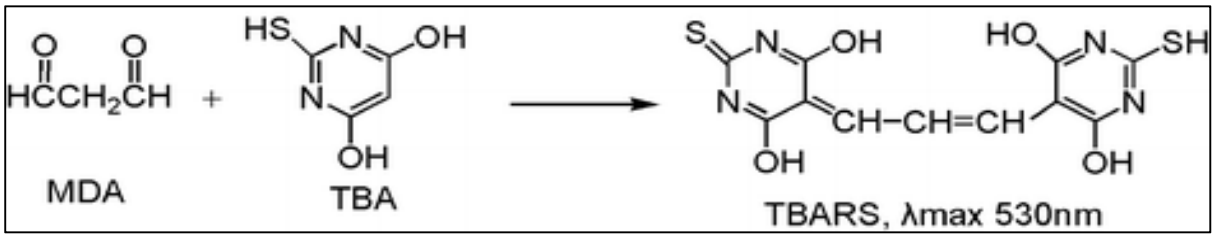


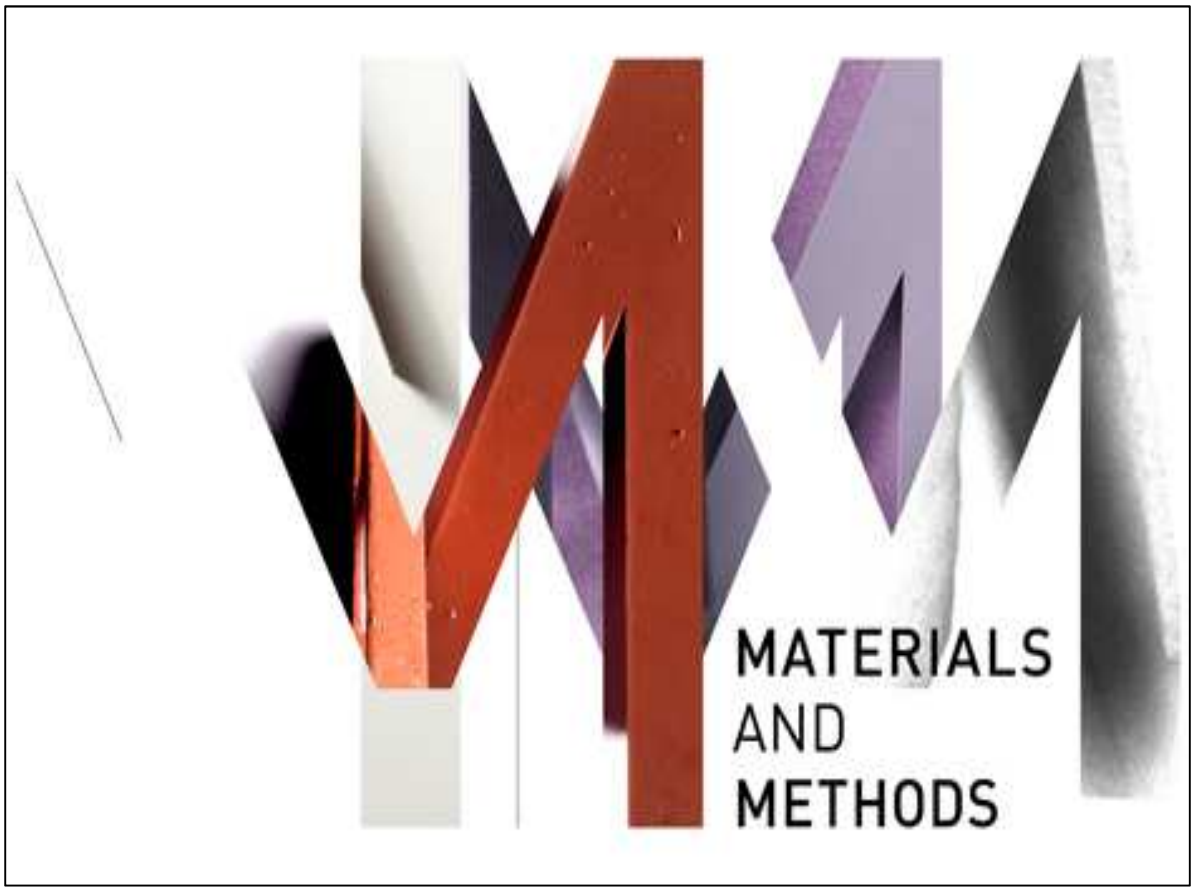
Fig: - 11 Enzymatic Reaction of Thio Barbituric acid reactive substance assay (TBARS)

At low PH and elevated temperature, MDA readily participated in nucleophilic addition reaction with 2-thiobarbituric acid. MDA itself participates in reaction with molecules other than TBA and is a catabolic substrate. Oxygen-free radicals, especially superoxide anion radical (O2**.-**), hydroxyl radical (OH**.-**) and alkylperoxyl radical (**.**OOCR), are potent initiators of lipid peroxidation. A free radical overload damages many cellular components: cellular proteins, DNA and membrane phospholipids (Andel, 2001).

The central nervous system (CNS) is extremely sensitive to free radical damage because of a relatively small total antioxidant capacity. Increased fatty acid metabolism in hypoglycemia may lead to acidosis, which enhances free radical aggressivity (Bhardwajet al., 1998).

Malondialdehyde (MDA) is a three-carbon compound formed from peroxidized polyunsaturated fatty acids, mainly arachidonic acid. It is one of the end products of membrane lipid peroxidation. Since MDA levels are increased in various diseases with excess of oxygen free radicals, many relationships with free radical damage were observed (Guichardantet al., 1994).

In severe hypoglycemia, the brain tissue suffers from substrate hunger, which decreases anabolic processes including enzyme constitution. It is evident that MDA is a sufficiently precise marker of oxidative stress in the brain following acute hypoglycaemia (Yatinet al., 1998).


MATERIALS
AND
METHODS

3. Materials and Methods:

3.1 Chemicals:

Streptozotocin (Sigma Aldrich), EGTA (Merck Specia;istsPvt. Ltd),Glucose Estimation Kit (Accu-check$_{Active}$), Actrapid Human Insulin (Novo Norisk India Pvt Ltd ,Bangalore), Sodium Hydroxide (NaOH, SiscoResearch Lab, Mumbai), Sodium Carbonate (Na_2CO_3, Sisco Research Lab, Mumbai); Bovine Serum Albumin (Central Drug House; Mumbai), Sodium Potassium tartrate ($NaKC_4H_4O_4$; Qualigens fine chemicals, Mumbai), Copper Sulphate ($CuSo_4$;RFCL New Delhi), Folin Ciocalteu Reagent (Sisco Research Lab,Mumbai), Sucrose (Sisco Research lab , Mumbai), HEPES (SDFCL, industrial estate,248 wroli road Mumbai-30), Chloroform, ethyl Alcohol, Tris HCL (Spectrochem Pvt Ltd,Mumbai) , Ascorbic Acid, Ferrus Sulphate, ThioBarbituric Acid (Central Drug House pvt ltd, New delhi), Hydrochloric Acid(HCL), Sodium Pyruvate (S. D. Fine – Chem Ltd., Mumbai), NADH (Sisco Research Laboratories Pvt. Ltd., Mumbai), di- Sodium hydrogen phosphate anhydrous (Merck specialities pvt ltd, Mumbai), Sodium Phosphate monobasic anhydrous (NaH2po4) (Sisco Research Laboratories pvt ltd, Mumbai), Citric Acid monohydrate ($C_6H_8O_7.H_20$),Tri-Sodium citrate di-hydrate($C_6H_5O_7$ $Na_3.2H_2O$), Acetic Acid, 1-Butenol, Pyridine (Merck specialities Pvt Ltd, Mumbai), Sodium lauryl sulphate (SDS) (HiMedia Laboratories Pvt Ltd., Mumbai), Tri reagent (Sigma Aldrich), DEPC water (Himedia Laboratories Pvt. Ltd.), cDNA synthesis kit (Fermentas, USA), PCR Master mix (Fermentas, USA), primers (IDT), Agarose (Sisco Research Lab, Mumbai), Ethidium bromide (Central Drug House, Mumbai), DNA ladder (Fermentas, USA), Primers (Sigma), PCR kit (Thermo scientific), Chloroform, Isopropanol, Ethanol etc . All chemicals used in the present study were of analytical grade.

3.2 Animals:

Adult male Wistar rats of 200-250 g body weight were purchased from Bharat Serum and Vaccine Ltd. Mumbai and used for all experiment. They were housed separate cage under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water. Animal care and procedures were done according to the Institutional and National Institute of Health Guide Lines.

3.3 Model Induction by Streptozotocin:

Chemical used for diabetes model induction was Streptozotocin (Sigma Aldrich, India). The mechanism through which streptozotocin produces its cytotoxic effects is still not clear. The STZ reduces the NAD+ content in several tissues and its effect is particularly harmful and necrotizing on pancreatic beta cells. It acts mainly by producing alkylation of DNA. The beta cell destruction is probably the consequence of low NAD+ levels caused by nuclear poly (ADPribose) synthetase during DNA reparation. The low levels of NAD+ also produce a decrease in intracellular ATP levels. On the other side, the participation of nitric oxide (NO) and reactive oxygen species (ROS) in cytotoxic effects of streptozotocin was proposed. As a conclusion, Okamoto proposes a common mechanism of action of Alloxan and streptozotocin toxicity suggesting that beta cells, trying to repair the damaged DNA, produce a suicidal response. The initial injury is produced by different causes: Alloxan acts mainly by production of reactive oxygen species and Streptozotocin by DNA alkylation (Rigalli et al., 2009).

3.3.1 Model Induction:

The experiment was completed in three sets and each set of animals were divided into the Following groups:

(i) Control (C)

(ii) Diabetic (D)

(iii) Insulin-induced hypoglycemia in control rats (Control + IIH)

(iv) Insulin-induced hypoglycemia in diabetic rats (Diabetic + IIH)

Each group consisted of 4 animals. Diabetic group was induced by a single intrafemoral dose (45 mg/kg body weight) of Streptozotocin (STZ) prepared in citrate buffer, pH 4.5 (Arison et al., 1967; Hohenegger et al., 1971; Robinson et al., 2009)**.** Blood glucose was estimated by glucose estimation kit (Lab Care Diagnostics Pvt. Ltd, Gujarat) using glucose oxidaseperoxidase method. The D+IIH group received daily 2 doses (10-12 Unit/Kg body weight) and C+IIH group received daily 2 doses (1.5-2.5 Unit/Kg body weight) of regular human insulin (Actrapid). D+IIH and C+IIH group had daily two episodes of insulin induced hypoglycemia for 10 days after confirmation of diabetes in experimental rats (Robinson et al., 2009).

Table (2): Model induction in Experimental groups rats

Group no.	Experimental group	Treatment
1	Control (C)	Saline treatment
2	Diabetic (D)	Streptozotocin (i.v) 55 mg/kg body weight
3	Control + Insulin-induced hypoglycemia (C+IIH)	Insulin (Subcutaneous) 1.5 IU/kg body weight, two doses daily
4	Diabetic + Insulin-induced hypoglycemia (D+IIH)	Streptozotocin 55 mg/kg body weight and Insulin (Subcutaneous)10 IU/kg body weight, two doses daily

3.4 Animal Group distribution for model induction:

The experiment completed in three sets and each set of animals were divided into the Following groups:

1) Control (C)
2) Diabetic (D)
3) Insulin-induced hypoglycemia in control rats (Control + IIH)
4) Insulin-induced hypoglycemia in diabetic rats (Diabetic + IIH)

Each group consisted of 4 animals consisted of animals. Diabetes was induced by a single intrafemoral dose (55 mg/kg body weight) of Streptozotocin (STZ) prepared in citrate buffer, pH 4.5 (Arison et al., 1967; Hohenegger et al., 1971; Robinson et al., 2009). The D+IIH group received daily 2 doses (10 Unit/Kg body weight) and C+IIH group received daily 2 doses (1.5 Unit/Kg body weight) of regular human insulin (Actrapid) (Flanagan et al., 2003; Robinson et al., 2009). D+IIH and C+IIH group had daily two episodes of insulin induced hypoglycemia for 10 days after confirmation of diabetes in experimental rats.

3.5 Water, Food consumption and Body Weight measurement:

Water, Food consumption and body weight was recorded throughout the experiments. Water intake and food consumption checked out during the active phase (12 hr) and inactive Phase (12 hr) through the experiment.

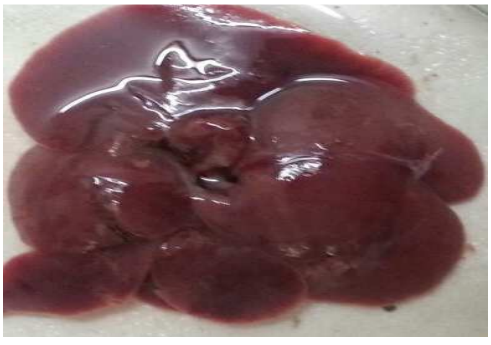
3.6 Glucose Estimation:

Glucometer:

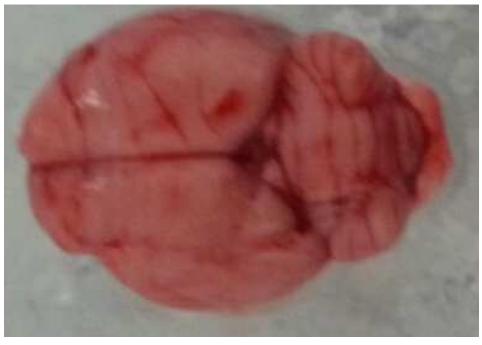
Glucose estimation was also carried out by Onetouch Glucometer. (Johnsons & Johnsons, USA). Blood is placed onto the Test strip. It reacts with a chemical called Glucose Oxidase producing gluconic acid from the glucose in the blood. At the other end of the test strip the meter transfers a current to the test strip. The test strip has electric terminal which allow the meter to measure the current betweens the terminals. The current between the terminals changes depending upon the level of gluconic acid that has been produced the blood glucose meter then uses an algorithm to work out the blood glucose level based upon the difference in current (Diabetes.cu.uk).

3.7 Dissection and Tissue preparation:

Rats were sacrificed on the 21st day of model induction by cervical dislocation. The cerebral cortex and body parts including liver, heart, kidney, muscle and pancreas were quickly dissect out and frozen in ice according to the procedure of (Iversen & Glowinski, 1966). The tissues were stored at -80^{o}C until assay.



Liver



Brain

Muscle

Spinal Cord

3.8 Enzyme Assay:

3.8.1 Lactate Dehydrogenase Assay:

LDH enzyme assay was done from skeletal muscle, Liver using a spectrophotometric method.

3.8.1.1 Preparation of Homogenate:

5% homogenate of the required tissues (skeletal muscle, Liver) was prepared using a homogenizer with an isolation medium containing 250 mM Sucrose, 10mM HEPES Buffer and 1 mM EGTA (pH – 7.2), according to the protocol of Ling et al., 2012.

3.8.1.2 Mitochondrial Isolation:

The prepared homogenate was the centrifuged at 1000 rpm for 8 min at 4ºC. The supernatant obtained, was collected and centrifuged at 10,000 rpm for 10 min at 4ºC. The pellet obtained here is the mitochondrial pellet, which is collected and washed twice with the washing medium containing 250 mM Sucrose, 10 mM HEPES and 0.1 mM EGTA. After this, the final pellets of mitochondria were suspended in 2 ml of isolation medium without EGTA i.e. the suspension medium (Ling et al., 2012).

3.8.1.3 Assay Mixture:

The assay was performed according to the protocol of Plummer, 2005. The assay was performed in a 1 ml reaction mixture. In which enzyme was used in varying concentrations ranges from 100µM to 600µM and 15µM sodium pyruvate was used as a substrate in constant concentrations, 0.1 mM NADH, 100µl of enzyme and 0.1 M sodium phosphate buffer, pH 7.4 to make up the volume to 1.0 ml. Here NADH acts as the reducing agent, which reduces the substrate pyruvate to produce lactate. The utilization of enzyme in making up the enzyme

– substrate complex, and thus the product is noted by taking the change in absorbance at 340 nm, by using Agilent Technologies Ltd. UV – Visible spectrophotometer.

3.8.1.4 Protein estimation:

Protein was measured by method of (Lowry et al., 1951) using bovine serum albumin as standard. The intensity of purple blue colour was proportional to the amount of protein, which was read in a U.V spectrophotometer (Agilent technologies) at 660 nm.

3.8.2 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.

3.8.2.1 Preparation of Homogenate:

10% homogenate of the required tissues (Liver) was prepared using a homogenizer with a medium containing 1.15% KCL (Beuge and Aust, 1978).

3.8.2.2 Estimation of MDA:

0.2 ml of the tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% TBA, and 0.6 ml distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95 1C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of butanol/ pyridine mixture (15:1 v/v) were added and vortexed. After centrifugation, absorbance of the organic phase was determined at 532 nm. The results were expressed as TBA reactive substances (nmol/g wet wt) (Wlostowski et al., 2008).

Calculations:

The Concentration of MDA was calculated using extinction coefficient of MDA-TBA complex which is 1.56×10^{5} M^{-1} cm^{-1} and the results were expressed as nmoles MDA/mg protein.

Formula: - $\frac{O.D\times Sample\ volume}{1.56\times10^{5}\times total\ volume\times mg\ protein/ml.}$

3.8.2.3 Protein estimation:

Protein was measured by method of (Lowry et al., 1951) using bovine serum albumin as standard. The intensity of purple blue colour was proportional to the amount of protein, which was read in a U.V spectrophotometer (Agilent technologies) at 660 nm.

3.9 Gene Expression

3.9.1 Total RNA Isolation

Total RNA was isolated using Tri reagent from the cerebral cortex of the control and experimental group of rats' brain. RNA purity was checked at 260/280 nm in Agilent Technologies Ltd. UV – Visible spectrophotometer. Isolated RNA was observed on an agarose gel using agarose gel electrophoresis.

3.9.2 cDNA synthesis

cDNA synthesis was performed from the total isolated RNA by using Thermo Scientific's cDNA synthesis kit.

3.9.3 PCR Amplification of Nrg 1

PCR amplification was performed with gene specific primers. Gene specific primers were designed using IDT (Integrated DNA Technologies) and NCBI's BLAST softwares. Amplified PCR product was observed on1.5% agarose gel using agarose gel electrophoresis.

Table (3): Nrg 1 gene specific Primer

Primers	Forward	Reverse	Product Length
Nrg 1 (Type III)	ATTTGAATACGACTCTCC	CACCAGTAAACTCATTTG	512 BP

Table (4): PCR Thermo Profile

Steps	Temperature	Time
Initial Denaturation	94^{O}	3 min
Denaturation	94^{O}	30 sec.
Annealing	52.9^{O}	45 sec.
Amplification	72^{O}	45 sec.
Final Extension	72^{O}	10 min.

3.10 Statistical Analysis

Statistical Evaluations were done by ANOVA by using Ms Excel (2010) software.

4 Results and Discussion:

4.1 Blood Glucose level in Experimental groups of Rats:

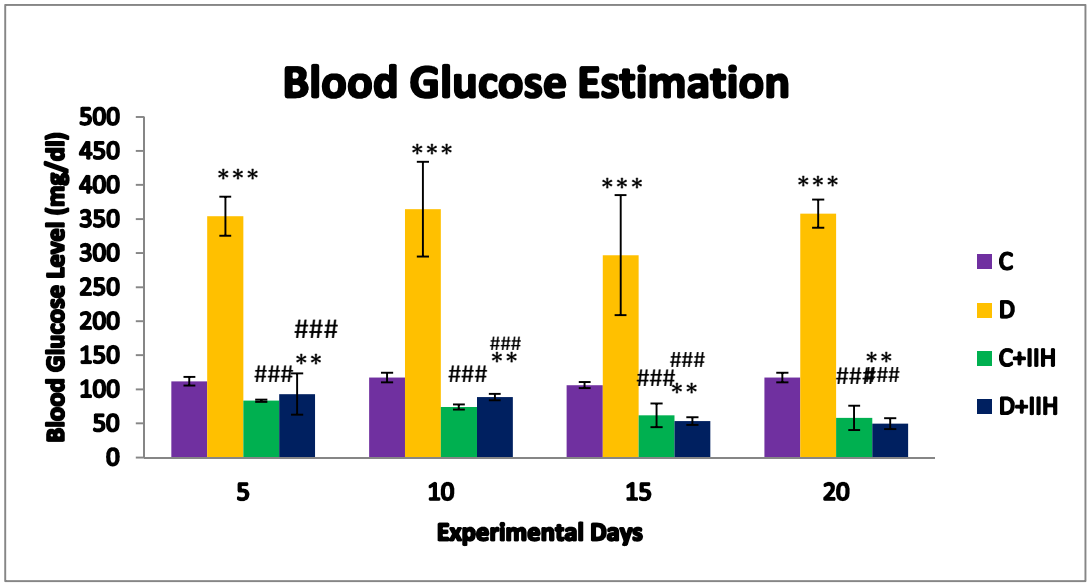


Fig: - 12 Blood Glucose Estimation

Table (5): Blood Glucose level in Control and Experimental group of Rats

Experimental Groups	Experimental Days			
	5th Day (mg/dl)	10th Day (mg/dl)	15th Day (mg/dl)	20th Day (mg/dl)
C	111.7±6.3	117.2±7.2	106.2±4.3	117.2±7.2
D	354±28.7	364.5±69.6	297±88.1	358±20.7
C+IIH	83.2±1.5	74.2±3.8	61.7±17.3	58±17.9
D+IIH	93±30.2	88.7±4.8	53.5±5.7	49.7±7.9

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.

*p<0.05 When compare to C ***p<0.001 When compare to C

**p<0.01 When compare to C ###p<0.001 When compare to D

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

A significant increase ($p<0.001$) was observed in the blood glucose level of Diabetic rats when compared to Control from 5th day of experiment. In C+IIH ($p<0.001$) and D+IIH ($p<0.001$) group the blood glucose level was significantly decreased as compare to C group. Deterioration in glucose homeostasis results in hypoglycemia that leads to neuronal injuries and cognitive function impairment. A significant increase in blood glucose level of D and D+IIH group was observed from 5th day of model induction with streptozotocin when compared to C. This elevated level in D group continued till 20th day of experiment. Blood glucose level of D group was 250mg/dL-350mg/dL. This is due to β cell destruction there is low level of Insulin, so muscle and fat cells reduces glucose uptake and also local storage of glucose as glycogen and triglycerides. Where as in liver cells results in reduced glycogen synthesis and storage and a failure to suppress glucose production and release into the blood (Mahmoud, 2009).

4.2 Change in Body weight:

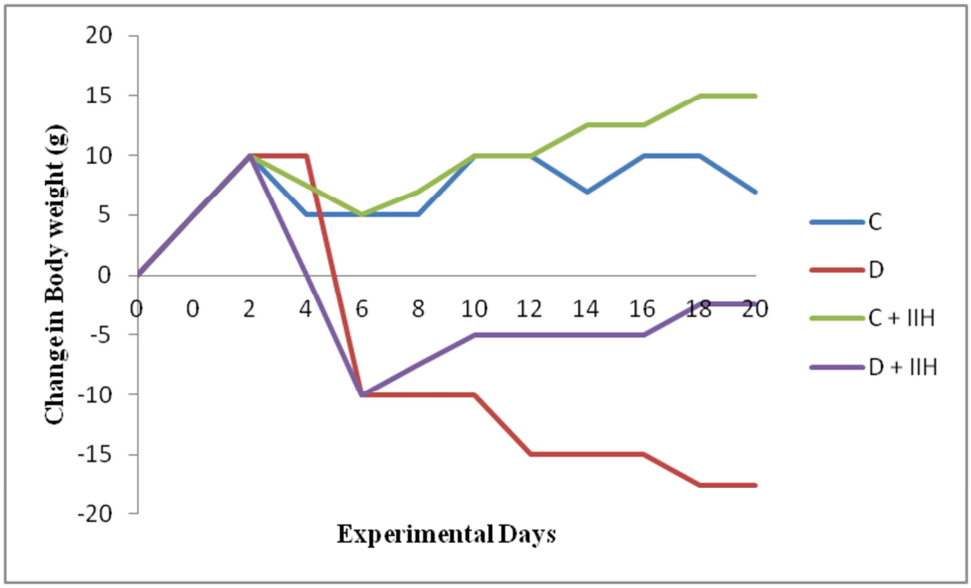


Fig: - 13 Body weight of Control and Experimental groups of Rats

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

A significant decrease in body weight of D was observed compared to C from 4th day of model induction corresponding to high blood sugar resulting in polyuria causes dehydration and loss of body fluids and electrolytes. During diabetes, lack of insulin prevents transporters mediated glucose entry into the cell. Hence initiating alternative mechanism for glucose production in cell via glycogenolysis and gluconeogenesis. Glycogenolysis leads to depletion of glycogen reserves signalling for lipolysis causing this unhealthy weight loss in D group. In C+IIH body weight increased and after it decreased compare to C. Insulin prevents excessive fat metabolism and thus an increase in body weight. In D+IIH body weight decreased but less compare to D (Jiang, 2003)

4.3 Food Consumption in Experimental groups of Rats:

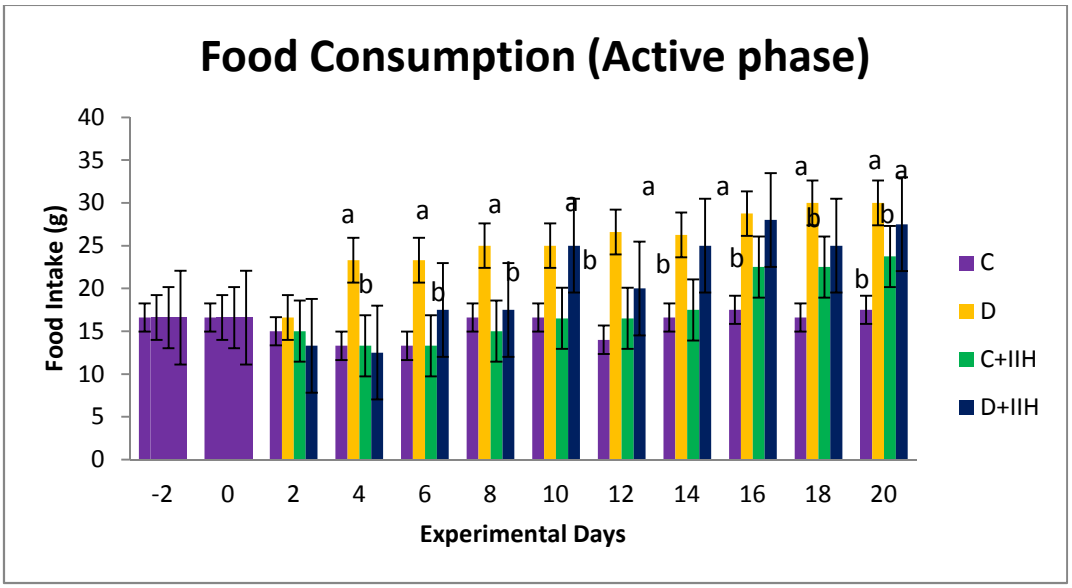


Fig: - 14 Food Consumption in Active Phase

a= ***p<0.001 When compare to C b=***p<0.001 When compare to D

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

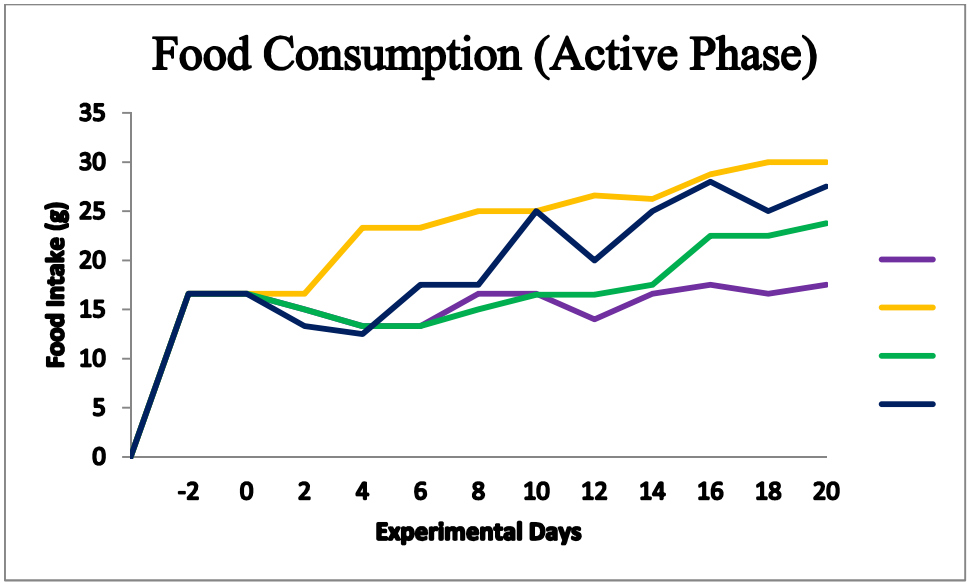


Fig: - 15 Food Consumption during Control and Experimental groups of Rats

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

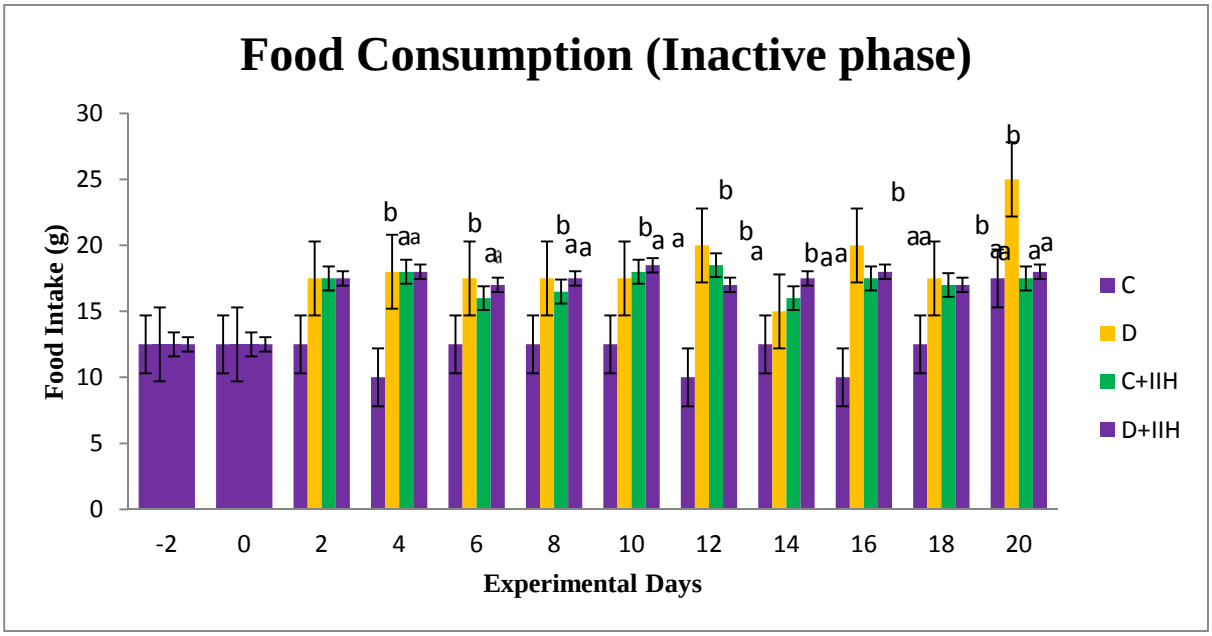


Fig: - 16 Food Consumption during Inactive Phase

a= ***p<0.001 When Compare to control b= ***p<0.001 When compare to D
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

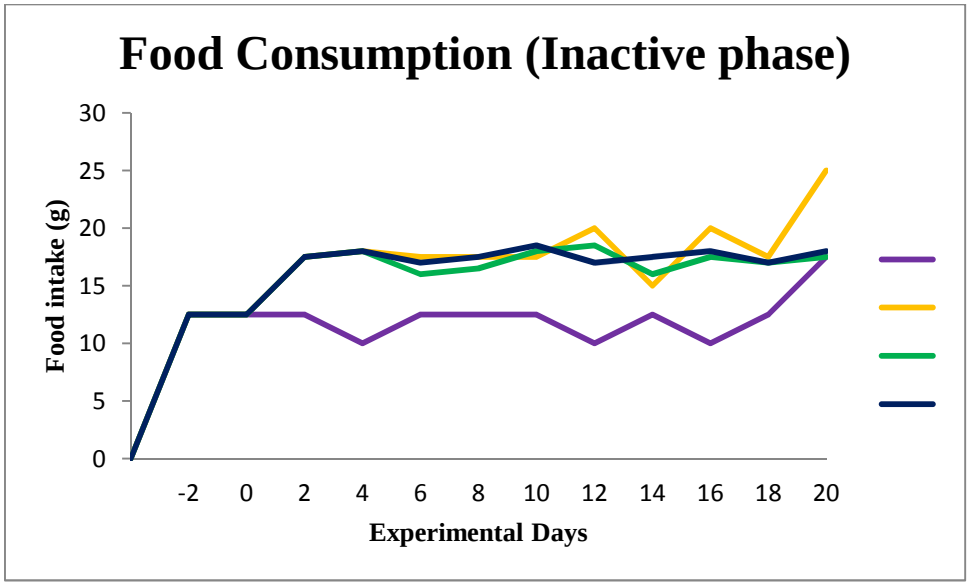


Fig: -17 Food Consumption during in Control and Experimental groups of Rats

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

In control and experimental rats a significant difference of food intake were observed in during experimental days. A significant increase in inactive period food consumption of D ($p<0.001$) was observed when compared to C. C+IIH group showed an increase in inactive period food consumption compared to C ($p<0.05$). D+IIH group showed an increase in food consumption compared to C ($p<0.001$) on experiment. From day 3rd onwards a significant increase in inactive period food consumption of D ($p<0.001$) was observed compared to C, till 6th day of experiment. In C+IIH ($p<0.001$) and D+IIH ($p<0.001$), there was a significant decrease in inactive period food consumption compared with D (Akbarzadeh et al., 2007).

4.4 Water Intake in Control and Experimental groups of Rats:

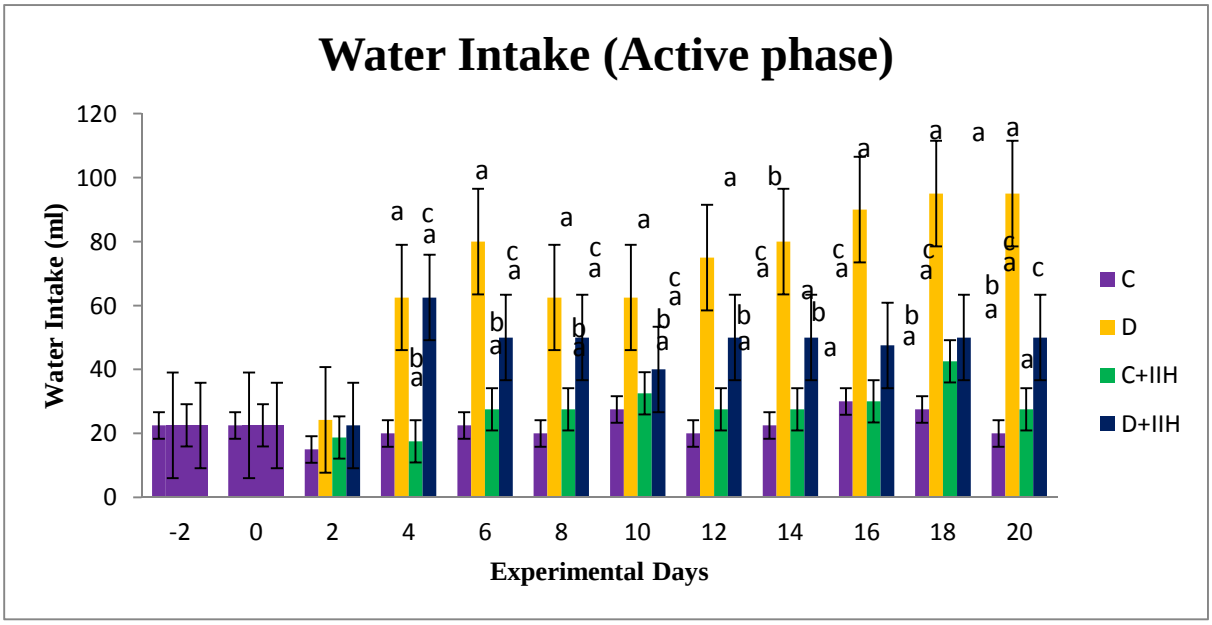


Fig: - 18 Water Intake during Active Phase

a = ***p<0.001 When compare to C a=*p<0.05 When compare to C
b=***p<0.001 When compare to D c=***p<0.001 When compare to C+IIH
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

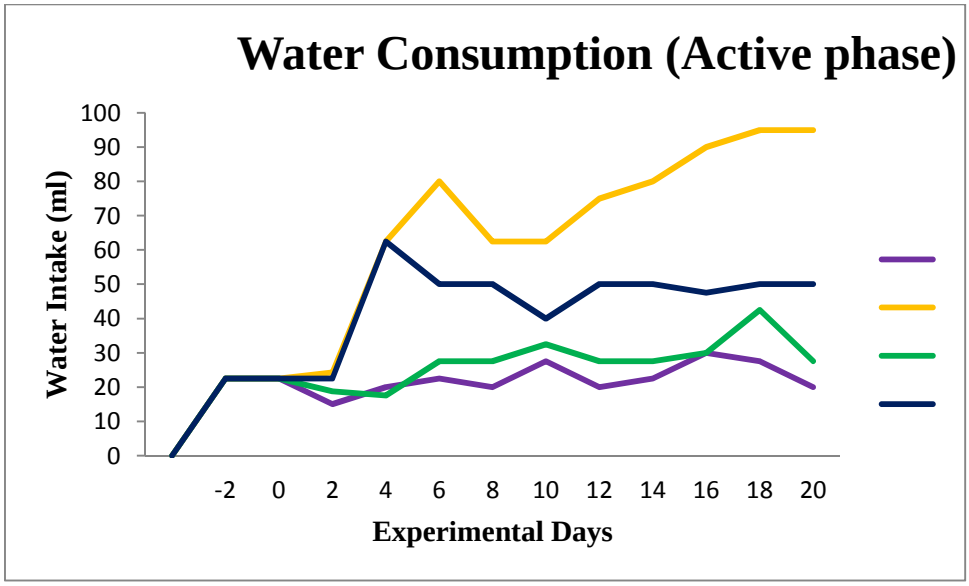


Fig: - 19 Water Intake in Control and Experimental groups of Rats

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

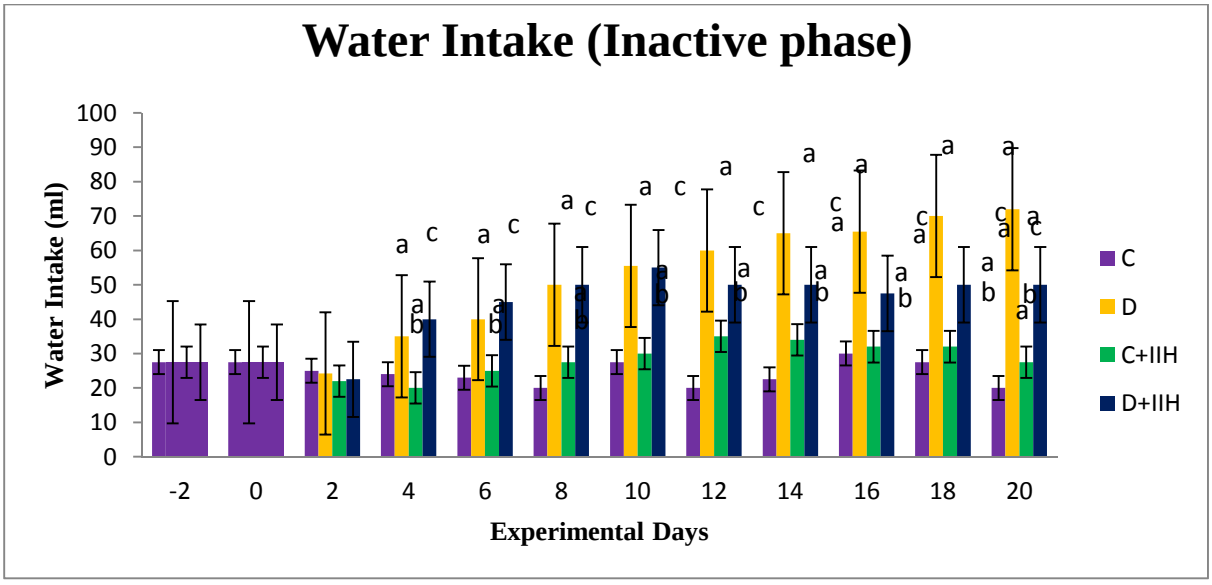


Fig: - 20 Water Intake during Inactive Phase

a= ***p<0.001 When Compare to C a=**<0.01 When compare to C
b= ***p<0.001 When compare to D c=***p<0.001 When compare to C+IIH
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

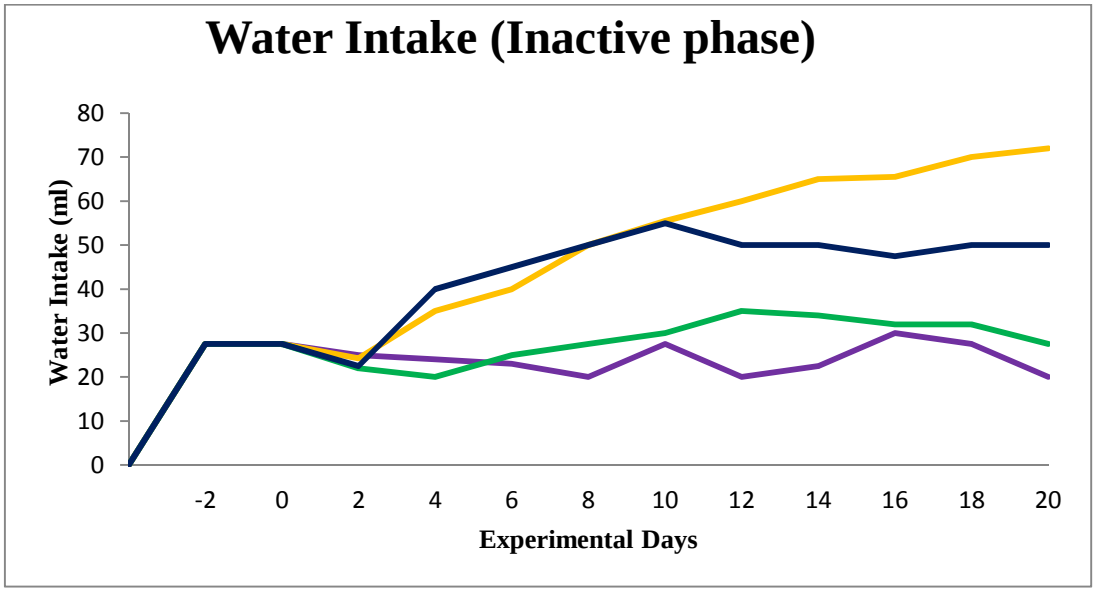


Fig: -21 Water Intakes in during Control and Experimental Groups of Rats

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

A significant increase in the water consumption was observed in D ($p<0.001$) and D+IIH ($p<0.001$) groups when compared to C, where as a little increase was observed in C+IIH when compared to the control rats. There was a significant decrease in water intake in C+IIH group during experiment. An increase in thirst (polydipsia) of diabetic rats is a known fact. As in case of diabetes when the blood glucose level exceeds about 160 – 180 mg/dl, the proximal tubule becomes overwhelmed and begins to excrete glucose in the urine. In renal diabetes, the threshold is abnormally low and glucose appears in the urine at a much lower concentration than normal. Thus to dilute the concentrated urine tubular secretion of water takes place and thus activating thirst center in brain to compensate for water loss. C+IIH showed significant increase in water consumption when compared to C. C+IIH and D+IIH showed significant decrease in water consumption when compared to D. Diabetic rats showed excess thirst due to polyurea condition. C+IIH and D+IIH groups showed lowered water intake as compared to D group after insulin administration. This may be due to the lowering of blood glucose level. Hence less water intake is observed in hypoglycaemic groups as compare to D groups but more as compared to C groups (Mahmoud et al., 2009).

4.5 Behavioural Studies:

4.5.1 Modified Karl Lashley's Maze Test:

To check the memory and cognitive ability in Experimental groups of rats they were put to solve the maze after training sessions. Three parameters were checked by the test:

1) Time Spent in 1st, 2nd, 3rd and 4th Compartment

2) Rearing Attempt

3) False entry

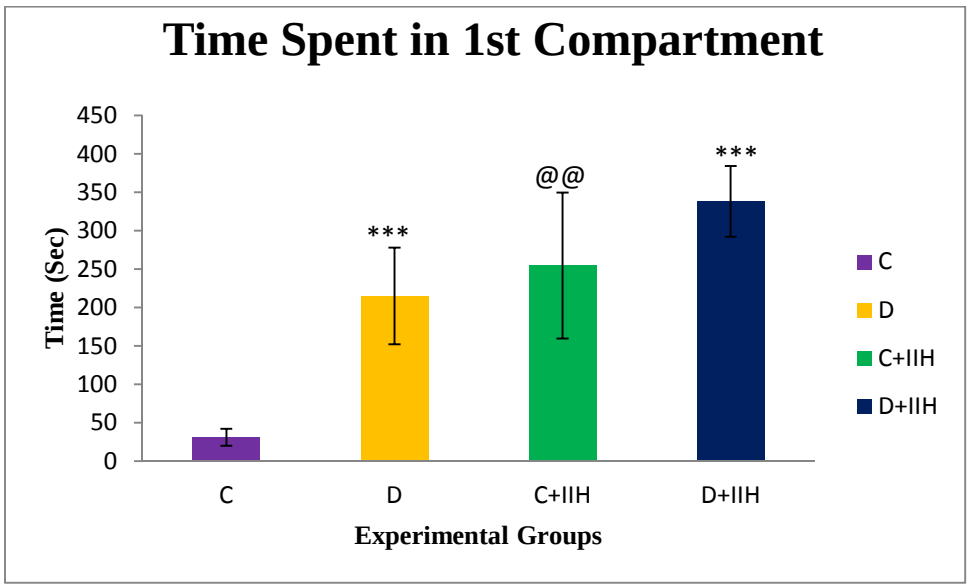


Fig: - 22 Times Spent in 1st Compartment by Experimental Groups to Solve the Maze

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.
***p<0.001 When compare to control
@@p<0.01 When compare to D
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

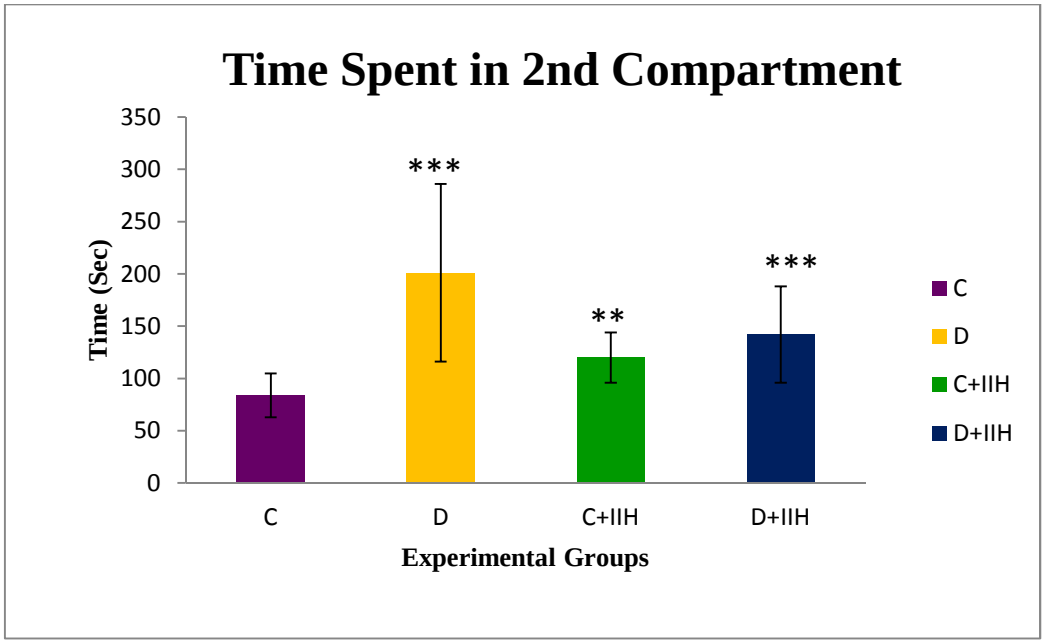


Fig: - 23 Times Spent in 2nd Compartment by Experimental Groups to Solve the Maze

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.
***p<0.001 When compare to control **p<0.01 When compare to control
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

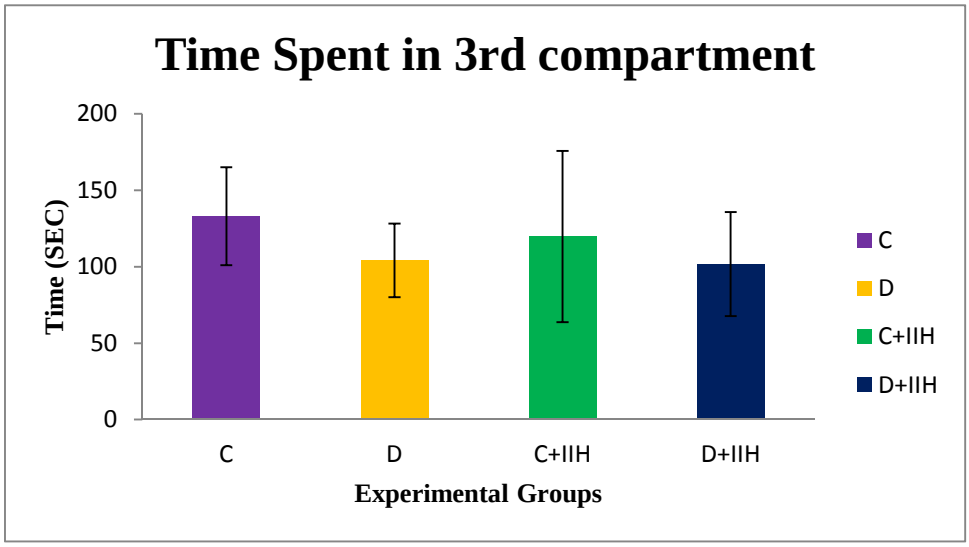


Fig: - 24 Times Spent in 3rd Compartment by Experimental Groups to Solve the Maze

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

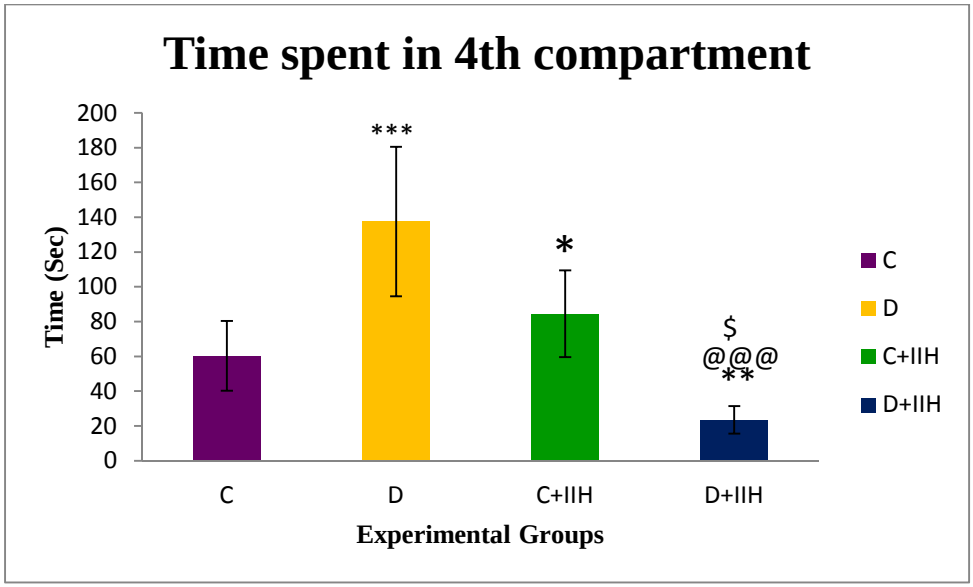


Fig: - 25 Times Spent in 4th Compartment by Experimental Groups to Solve the Maze

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.

***p<0.001 When compare to control *p<0.01 When compare to control

$p<0.05 When compare to C+IIH @@@p<0.001 When compare to D

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

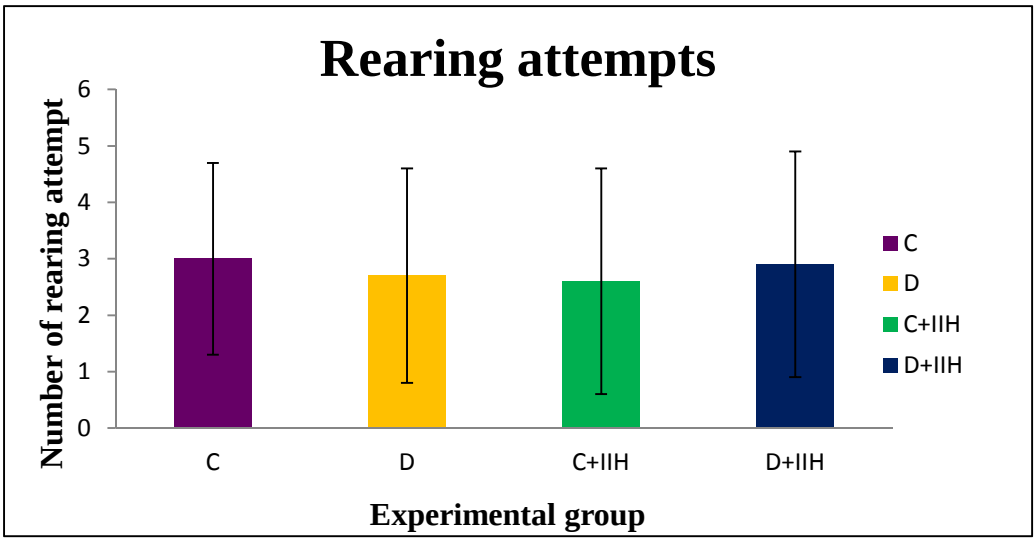


Fig: - 26 Total number of Rearing attempts performed by Control and Experimental groups of Rats

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

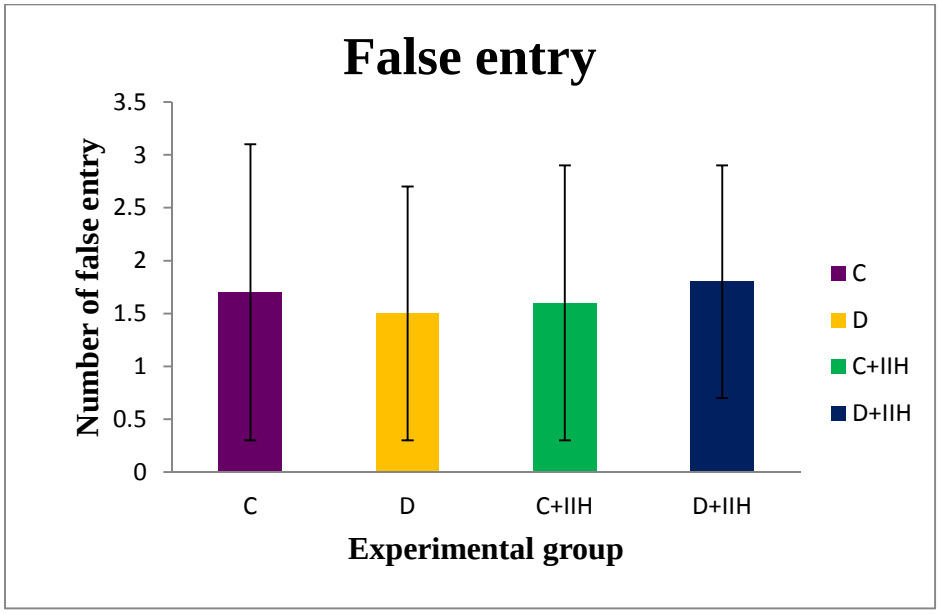


Fig: -27 Total numbers of False Hits performed by the Experimental groups of Rats

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

Time in 1st and 2nd compartment by D+IIH was the highest as compare to C. Time spent by D, C+IIH and D+IIH in 1st and 2nd compartment was significantly more as compare to C. There is no significant difference was observed for time in 3rd compartment between experimental group of rats. D+IIH group of rat were not an able to rich in 4th compartment hence, time spent by D+IIH was significantly less as compare to C. This result indicates impairment in working memory of D+IIH, D, C+IIH respectively. There is no significance was observed among experimental groups of rats for Rearing Attempt and false hits (Dewsbury, 2009).

4.5.2 Grid Walking Test:

To check the motor ability in Experimental groups of rats they were put to solve the maze after training sessions. Three parameters were checked by the test:

1) Total Time taken to cross the Grid
2) Immobile Period
3) Head dip

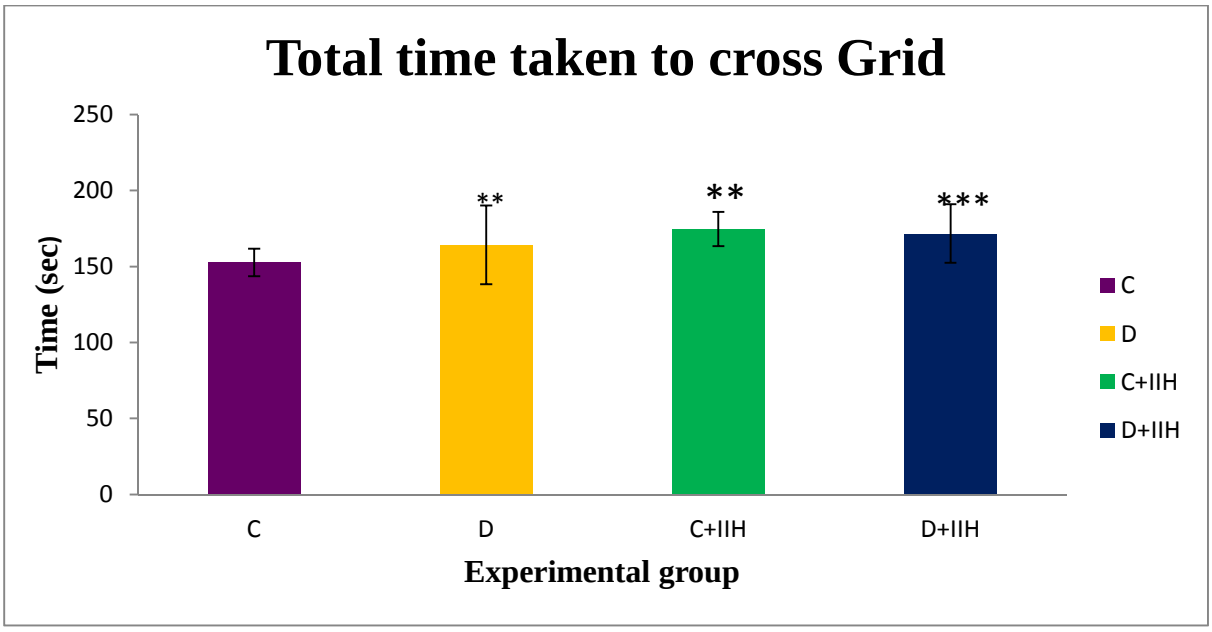


Fig: -28 Total time taken to Cross Grid by Experimental Groups to Solve the Maze

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.

***p<0.001 When compare to control **p<0.01 When compare to control

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

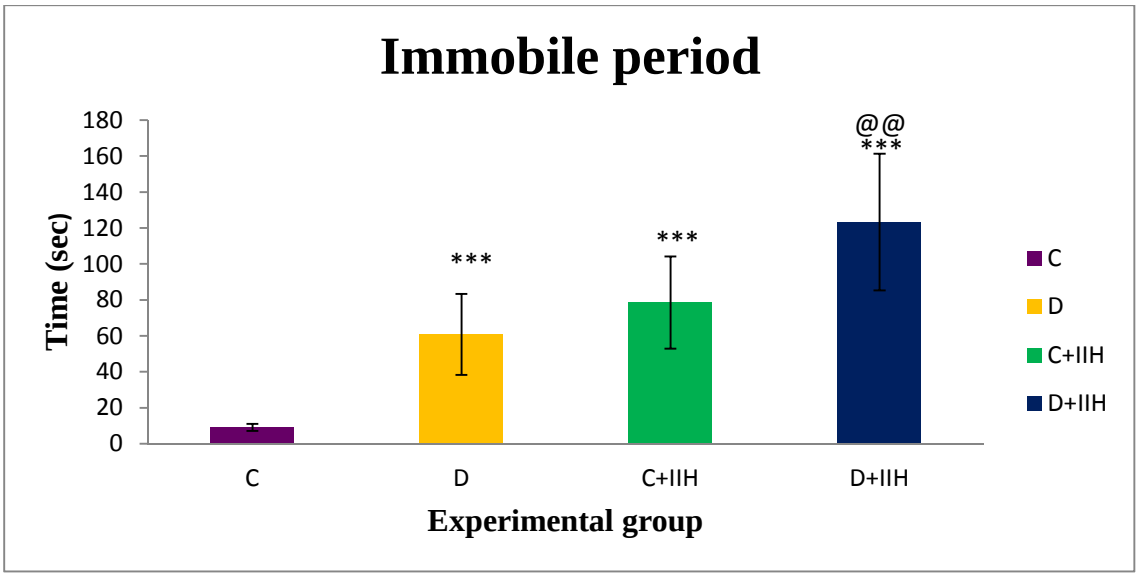


Fig: -29 Immobile time taken by experimental groups while solving the maze

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.

***p<0.001 When compare to Control @@p<0.01 When compare to Diabetic

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

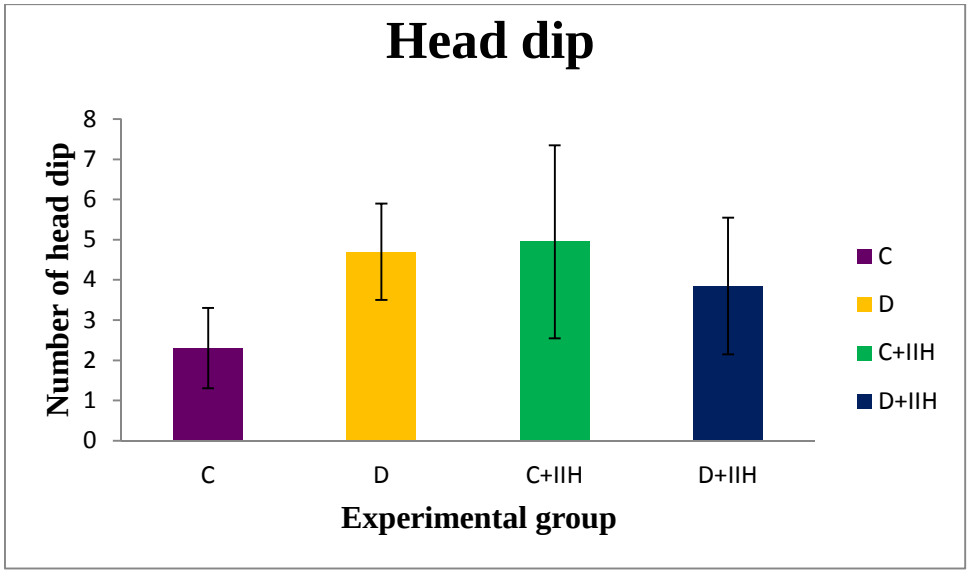


Fig: -30 Head dip performed by experimental groups while solving the maze

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

As per Result we get that, as compare to control (C) the Diabetic (D), Diabetes + Insulin Induced Hypoglycemia(D+IIH) and Control + Insulin Induced Hypoglycemia (C+IIH) took more time to Cross the grid. Which show that, motor function in Diabetes and Hypoglycemia is an indicative of altered neuron pathways linking Cerebellum with Frontal lobe of cerebral cortex.. In Immobile period- As compare to control (C) in Diabetic (D), Diabetes + Insulin Induced Hypoglycemia(D+IIH) and Control + Insulin Induced Hypoglycemia (C+IIH) is high. This shows impairment in motor function._As per result we get from this test, i.e there is no significance observed during control and Experimental groups of Rats while performing head dip (Methew et al., 2010).

4.5.3 Elevated Plus Maze:

To check the Anxiety in Experimental groups of rats they were put to solve the maze after training sessions. Three parameters were checked by the test:

1) Time Spent in Close arm
2) Time Spent in Open aem
3) Grooming Attempt

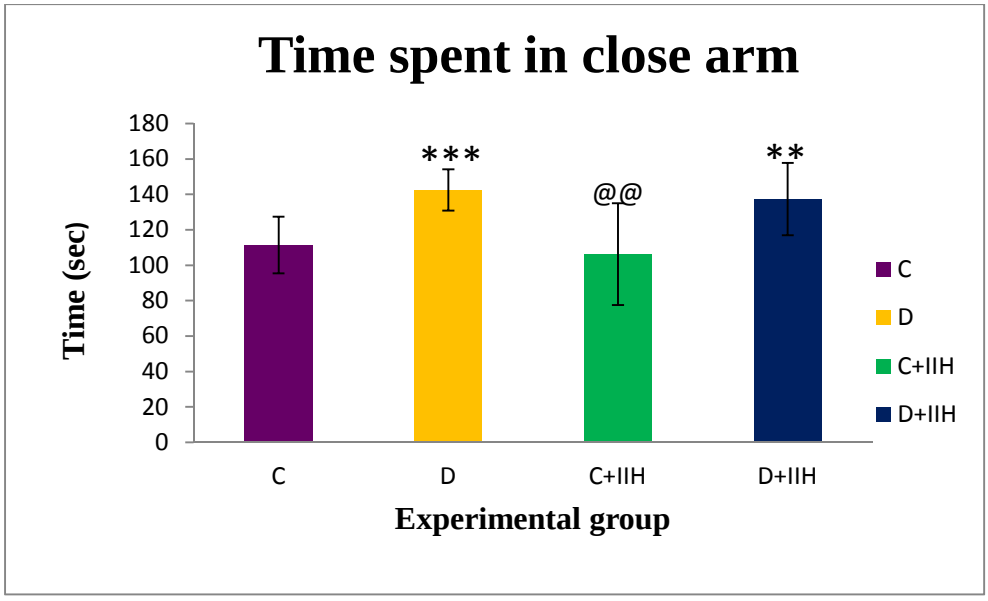


Fig: - 31 Time Spent in Close arm by Experimental Groups

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student‘s-Newman-Keul‘s Test.
***p<0.001 When compare to Control @@p<0.01 When compare to Diabetic
**p<0.01 When compare to Control
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

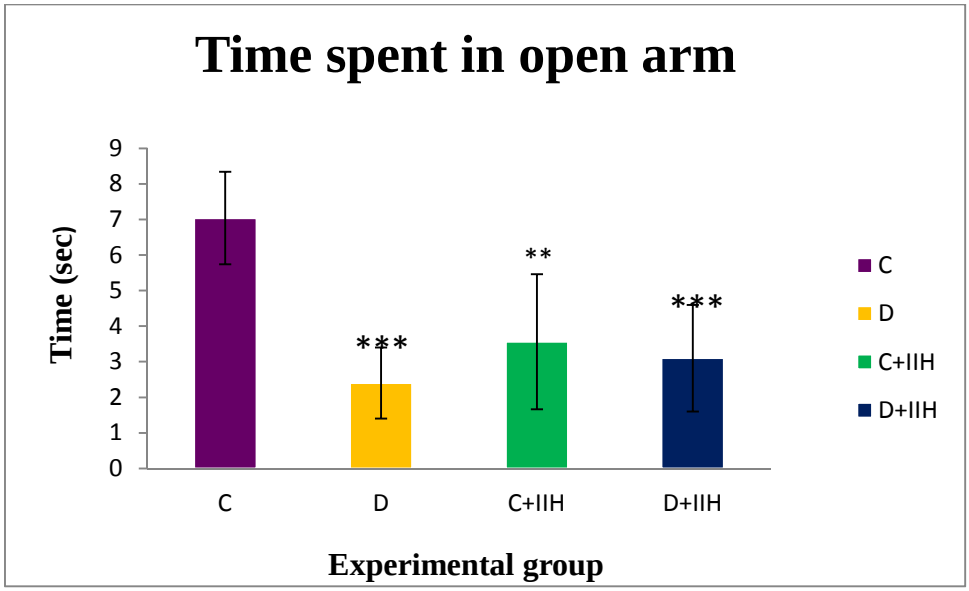


Fig: - 32 Time Spent in Open arm by Experimental Groups

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.
***p<0.001 When compare to Control **p<0.01 When compare to Control
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

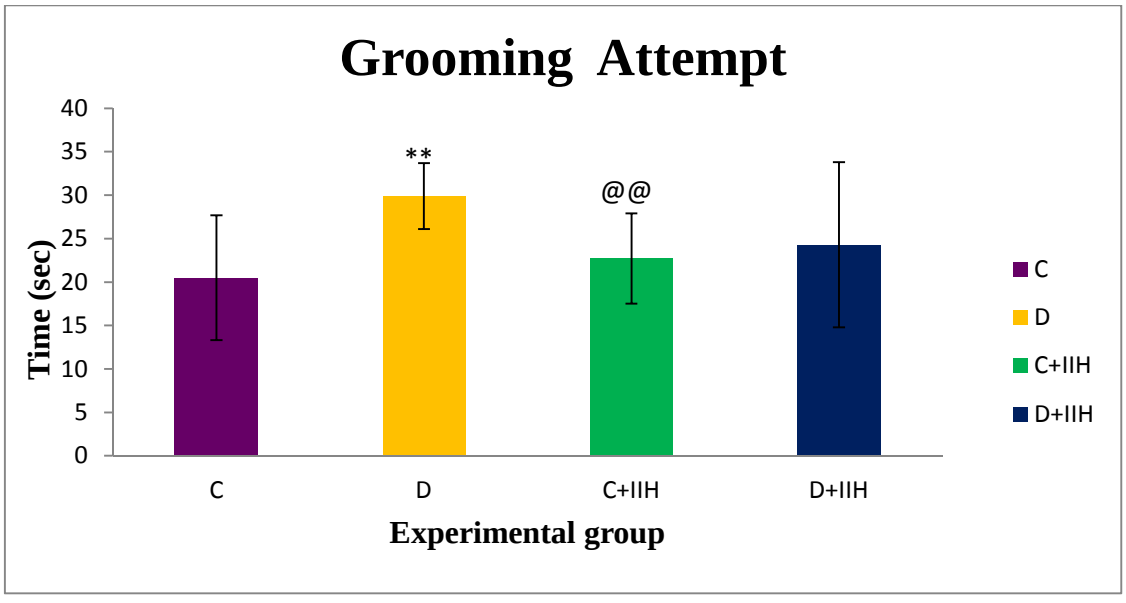


Fig: - 33 Grooming Attempt Performed by Experimental groups of Rats during task

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student‘s-Newman-Keul‘s Test.
**p<0.001 When compare to Control @@p<0.01 When compare to Diabetic
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats, D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

As compare to control (C) the Diabetic (D), Diabetes + Insulin Induced Hypoglycemia (D+IIH) and Control + Insulin Induced Hypoglycemia (C+IIH) spent more time in Close arm. This shows that the anxiety level is more in Diabetic and Hypoglycemia group as compare to Control.As per Result we get that, as compare to control (C) the Diabetic (D), Diabetes + Insulin Induced Hypoglycemia (D+IIH) and Control + Insulin Induced Hypoglycemia (C+IIH) spent less time in Open arm. This shows that the anxiety level is higher in Diabetic and Hypoglycemia group as compare to Control. The third parameter is Grooming Attempt during the session. As per Result we get that, as compare to control (C) the Diabetic (D), Control + Insulin Induced Hypoglycemia (C+IIH) and Diabetes + Insulin Induced Hypoglycemia (D+IIH) spent more time in grooming attempt. This shows that the anxiety level is higher in Diabetic and Hypoglycemia group as compare to Control (Tang et al., 2015).

4. 6 Enzyme Assay:

4.6.1 Lactate Dehydrogenase Assay (LDH):

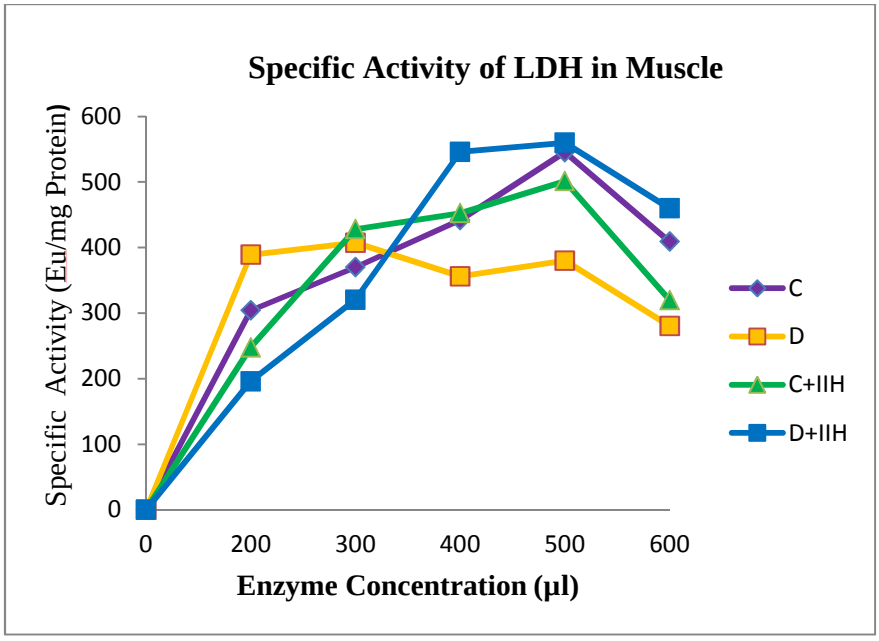


Fig: -34 Specific Activity of Lactate Dehydrogenase Assay in Muscle

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,

D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Table (6): Lactate Dehydrogenase Assay in muscle of Control and Experimental groups

Experimental Groups	Vmax	Km
C	670.7±115.6	321.2±89.9
D	120.5±69.7***	60.2±45.1
C+IIH	528.7±26.8 @@@ **	132.3±10.4
D+IIH	635.3±59@@@	147.3±64.1

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student‘s-Newman-Keul‘s Test.

***p<0.001 When compare to C @@@P<0.001 When compare to D

**p<0.01 When compare to C

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

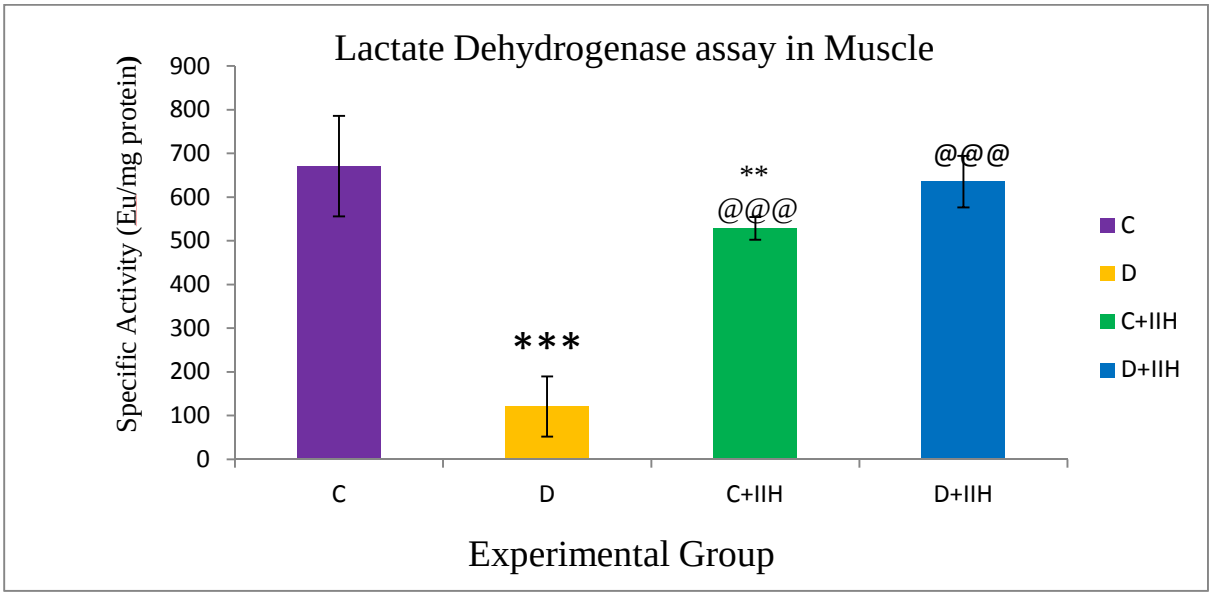


Fig: -35 Lactate Dehydrogenase assay in Muscle

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.
***p<0.001 When compare to C @@@P<0.001 When compare to D
**p<0.01 When compare to C

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

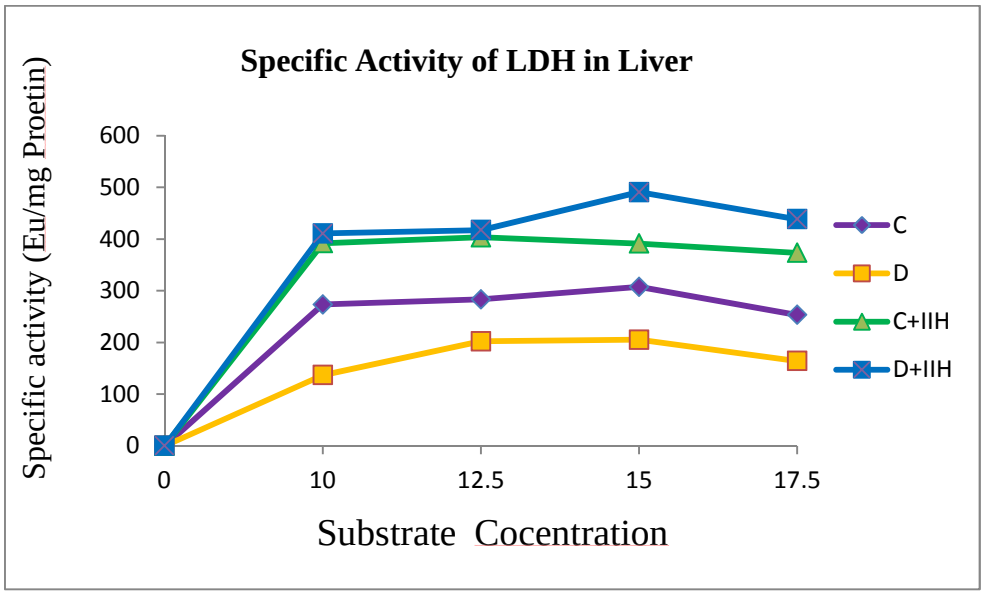


Fig: -36 Specific Activity of Lactate Dehydrogenase Assay in Liver

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Table (6): Lactate Dehydrogenase Assay in muscle of Control and Experimental groups

Experimental Group	Vmax	Km
C	354±109.4	74.4±33.2
D	221±86.5	78.5± 35.7
C+IIH	425.5±24.8@@	83.7±45.9
D+IIH	515.5±79.7@@	87.2±48.3

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). *ANOVA* followed by student's-Newman-Keul's Test.
@@p<0.01 When compare to D

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

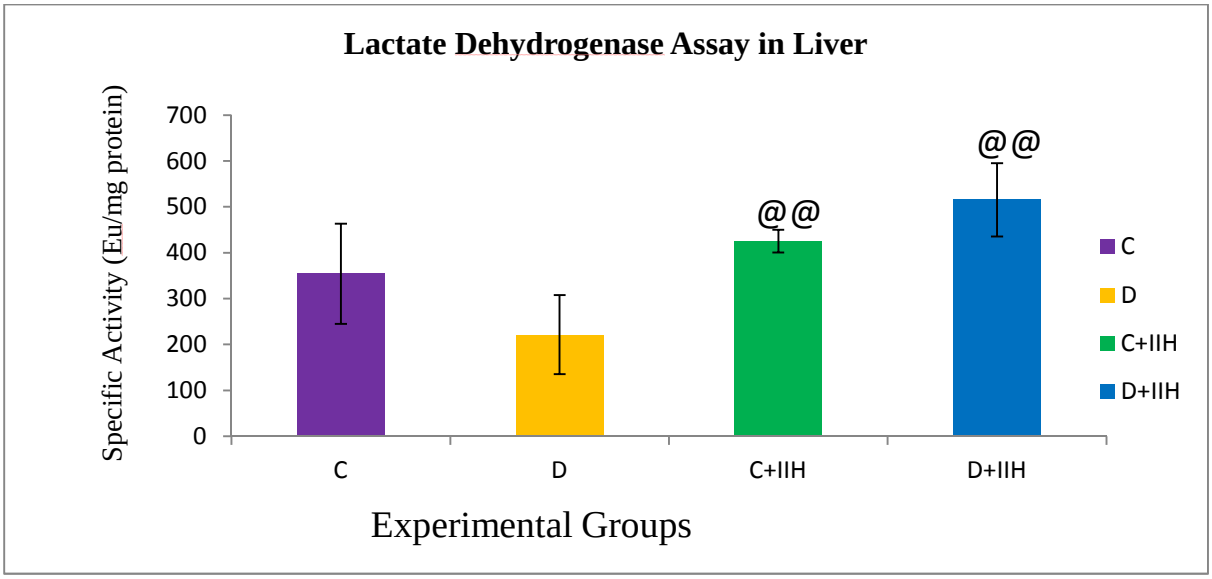


Fig: - 37 Lactate Dehydrogenase Assay in Liver

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). ANOVA followed by student's-Newman-Keul's Test.
@@p<0.01 When compare to D
C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

In muscle, LDH activity significantly decreases in D ac compare to C, where as LDH activity significantly increases in C+IIH and D+IIH as compare to C. In liver LDH activity significantly increases as compare to D. High concentration of LDH in tissue indicates decreased oxygen availability and anaerobic condition in muscles and liver (Gregory, 2013). Brooks (1986) postulated that for oxygen deficit (anaerobiosis) to be the primary cause of lactate accumulation, muscle anoxia must exist, since this was thought to be the stimulus for lactate production (Philip et al., 2005).

4.6.2 Thio Barbituric Acid Reactive substance assay (TBARS):

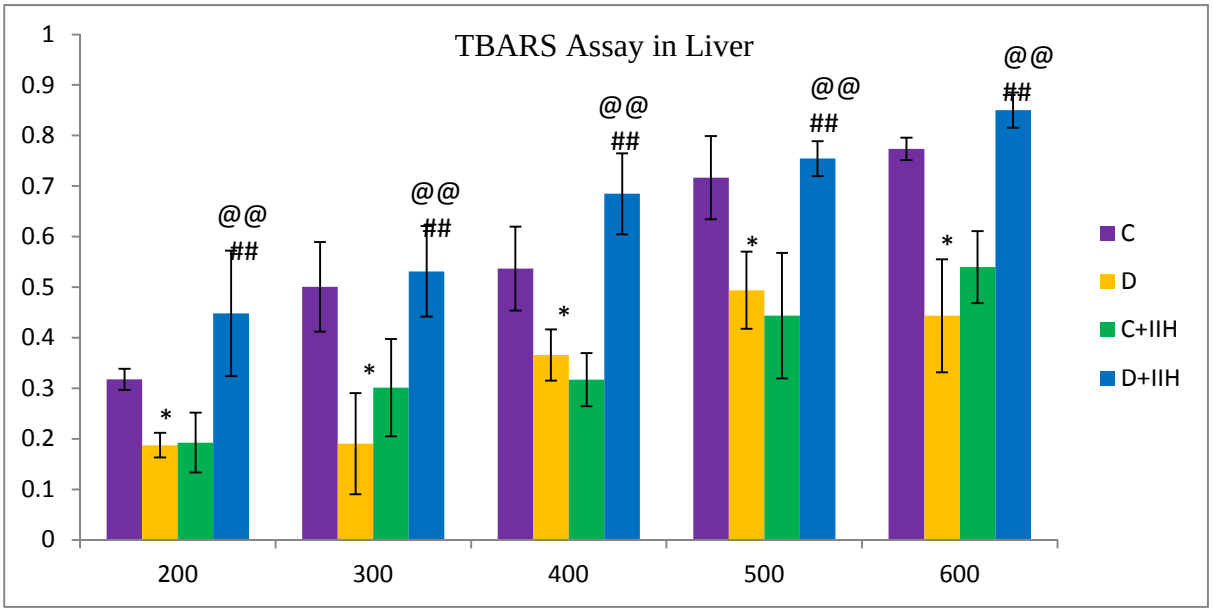


Fig: - 38 Thio Barbituric Acid reactive substance assay in Liver

Values are Mean ± SD of 4 separate experiments (n=4 rats per group). ANOVA followed by student's-Newman-Keul's Test.
@@p<0.01 When compare to D *p<0.05 When compare to C
##p<0.01 When compare to C+IIH

C=control, D=Diabetes, C+IIH=Insulin induced Control Hypoglycemic Rats,
D+IIH=Insulin induced Diabetic Hypoglycemic Rats.

Discussion:

MDA concentration was significantly decreased in D as compare to C. Whereas, In D+IIH group of rats MDA concentration was significantly increased which is suggestive of increased lipid peroxidation during hypoglycaemic condition (Lawrence, 1998). Lipid

peroxidation is a complex process which involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers (Dianzani & Barrera, 2008). Oxidative damage in liver is associated with hepatic lipid metabolism, and may be affecting the absorption and transport mechanisms. Hence, it can be said that increased lipid peroxidation causes more oxidative stress and tissue damage during hypoglycaemic condition (Repetto et al., 2010).

4.7 Nrg 1 Gene expression:

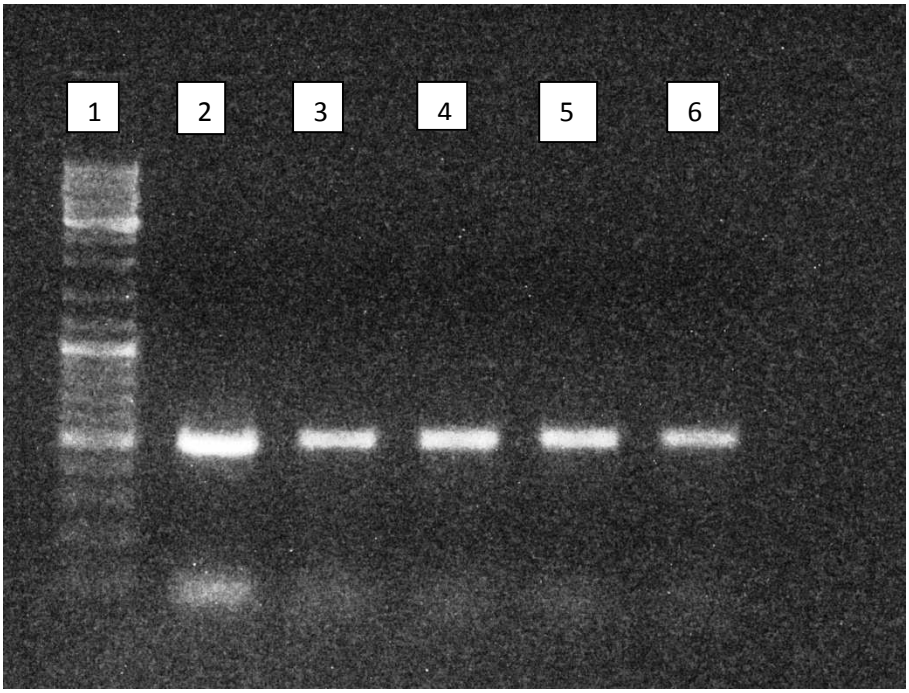


Fig: - 39 Expression of Nrg 1 Type III Gene

Product Length: 512bp

Lane 1: 100bp Leader

Lane 2: GAPDH

Lane 3: C

Lane 4: D

Lane 5: C+IIH

Lane 6: D+IIH

Discussion:

Good quality RNA was isolated from the cerebral cortex of experimental group of rats and cDNA was prepared. In D+IIH group gene specific amplicone seems to be down regulated. There is no significant difference was observed in D, C+IIH, and C. This is the result of only one time experiment hence, this study need to be repeated

5. Summary

Hypoglycemia is defined as the state of glucose emergency in which the blood plasma glucose falls critically low. A condition called Hypoglycemia-associated Autonomic Failure (HAAF) was detected in type-1 diabetes, where hypoglycemic condition provokes failure of the centrally mediated adrenal response so causing counter regulatory deficiency and impaired cognitive function during hypoglycemia. In our study we focus on effects of hypoglycaemia through the Nrg 1 Signalling pathway. NRG-1 type III regulates almost all steps of oligodendrocyte cell's differentiation, including control of myelin-sheath thickness, which is a function of axon size, it is thought that signalling between NRG-1-III and epidermal growth factor receptor family of proteins occurs at the glia-axon interface and activates several second-messenger cascades and made a product that is regulates developmental neuronal survival and synaptogenesis, astrocytic differentiation and microglial activation.

Neuro behavioural analysis was carried out to investigate the effect insulin induced hypoglycemia by performing behaviour analysis test for the assessment of motor activity (Grid Walking test), cognition (Modified Karl Lashley's Maze) and anxiety (Elevated Plus maze). In muscle, LDH activity was significantly enhanced in D+IIH froup of rats as compare to C. Where as in liver LDH activity was significantly decreased in D as compare to C. Lipid peroxidation was significantly enhanced in Diabetic + insulin induced hypoglycaemic (D+IIH), as compared to Control indicates increased lipid peroxidation and oxidative stress during hypoglycemic condition. The increase of reactive oxygen species levels can be because of neurochemical alterations during seizures in hypoglycemia groups. Cellular oxidative damage (i.e. Lipid peroxidation) by Peroxidative damage to lipids was determined by measuring Malondialdehyde concentration.

6. References:

- Allen I. Arieff , Tom Doerner, Harry Zelig, and Shaul G. Massry. Mechanisms of Seizures and Coma in Hypoglycemia,The Journal of Clinical Investigation Volume 54 *654-663 September 1974.
- Andreas Wiederkehr & Claes B. Wollheim, Impact of mitochondrial calcium on the coupling of metabolism to insulin secretion in the pancreatic –cell, Cell Calcium, 44, 64—76, (2008).
- Aneta Salińska, Tadeusz Włostowski, and Ewa Oleńska Differential Susceptibility to Cadmium-Induced Liver and Kidney Injury in Wild and Laboratory-Bred Bank Voles Myodes glareolus, Arch Environ Contam Toxicol. 65(2): 324–331(2013).
- Akbarzadeh A, Norouzian D, Mehrabi MR, Jamshidi SH, Farhangi A and Verdi A, Induction of diabetes by streptozotocin in rats. Indian Journal of Clinical Biochemistry.**22**:60-64 (2003).
- Banks WA, Jaspan JB, Huang W, Kastin AJ, Transport of Insulin across the blood brain barrier: saturability at euglycemic dose of insulin, Peptides, 18(9):1423-9, 1997.
- Bingham EM, Hopkins D, Smith D, Pernet A, Hallett W, Reed L, Marsden PK and Amiel SA. The role of Insulin in Human Brain Glucose Metabolism: An Fluoro-Deoxyglucose Positron Emission Tomography Study, Diabetes, 51 (2002).
- Cryer PE, Davis SN, Shamoon H: Hypoglycemia in diabetes. Diabetes Care, December 2012.
- Donald A. Dewsbury, Annals of Neurosciences, 10.5214/0972.7531, 2009.
- Emma M. Bingham, David Hopkins, Diarmuid Smith, Andrew Pernet, William Hallett, Laurence Reed, Paul K. Marsden, and Stephanie A. Amiel, vol. 51, december 2002.
- Ghasemi R, Dargahi L, Haeri A, Moosavi M, Brain insulin dysregulation: implication for neurological and neuropsychiatric disorder, Mol Neurobiology, 47(3):1045-65, 2013.
- Henderson JN, Allen KV, Deary IJ, et al: Hypoglycaemia in insulin-treated Type 2diabetes: frequency, symptoms and impaired awareness. Diabet Med, 20:1016–1021(2003).

- Jiang G, Zhang B.B, Glucogon and regulation of glucose metabolism, Am J Physiol Endocrinol Metabolism, 284(4):671-8, 2003.
- Joshua P Klein & Stephen G Waxman, The brain in diabetes: molecular changes in neurons and their implications for end-organ damage, Lancet Neurology,2: 548–554, (2003).
- Kae-JiunChang, KeiichiroSusuki, MariaT.Dours-Zimmermann, DieterR.-Zimmer mann, andMatthewN.Rasband, Oligodendrocyte Myelin Glycoprotein Does Not Influence Node of Ranvier Structure orAssembly, TheJournalofNeuroscience, 30(43):14476–14481, (2010).
- Kristjan R. Jessen & Rhona Mirsky, The origin and development of glial cells in peripheral nerves, Nature Reviews Neuroscience 6, 671-682, (2005).
- Leonard TR, Gregory W, Michael L, Gerald C, Raphael BO and David BO. Lipid Peroxidation and the Thio Barbituric Acid Assay: Standardization of the Assay When Using Saturated and Unsaturated Fatty Acids. The Journal of biochemistry and molecular bilogy 37(6): 749-752 (2004).
- Ling B, Peng F, Alcorn J, Lohmann K, Bandy B and Zello G. D-Lactate altered mitochondrial energy production in rat brain and heart but not liver, Nutrition and Metabolism, 9:6 (2012).
- Mahmoud A, Zuvair B.I, Khaled R.A, Sami A and Mohamed K, Induction of Diabetes mellitus in rat using intraperitonneal streptozotocin, European journal of Scientific research, 32:398-402, 2009.
- Mathew J, Antony S, Anju T.R, and Paulose C.S, Hypoglycemia induced changes in cholinergic receptor expression in the cerebellum of diabetic rats, J Biomed Sci, 5; 17:7, 2010.
- M N Hodgkin, C E Hills1 and P E Squires, The calcium-sensing receptor and insulin secretion: a role outside systemic control 15 years on, Journal of Endocrinology,199, 1–4, (2008).
- Mark W.J. Strachan, Ian J. Deary, fiona m.e. ewing, & brian m. frier, Recovery of Cognitive Function and Mood After Severe Hypoglycemia in Adults With Insulin-Treated Diabetes, Diabetes Care, 23(3),(2000).
- Masaaki Seki, Takayuki Tanaka, Hiroyuki Nawa, Tomoaki Usui, Takeo Fukuchi, Kazuhito Ikeda, Haruki Abe, and Nobuyuki Takei, Diabetes 53:2412–2419, 2004.

- Mauro Sola-Penna, Metabolic Regulation by Lactate, IUBMB Life, 60(9): 605–608, (2008).
- Marisa Repetto, Jimena Semprine and Alberto Boveris, Lipid perodixation: chemical mechanism, biological implication and analytical determination, Intech open science, 2012.
- McCall AL, The impact of Diabetes on CNS, 41(5):557-70, 1992.
- McCrimmon R, Ashford M, Beall C, Hypoglycemia: exercise for the brain, Journal neuroendocrinol, 24(10):1365-6, 2012.
- Nancy H. Sternberger, Yasuto Itoyama, Marian W. Kiest, T. and Henry , myelin basic protein demonstrated immunocytochemically inoligodendroglia prior to myelin sheath formation 1978.
- Philip E. Cryer, M.D. Hypoglycemia During Therapy of Diabetes, August 2011.
- Ranjna Chawla, Practical in Clinical Biochemistry- Method and Interpretations, chapter no. 38- Serum Calcium, Jaypee Brothers Medical Publishers (P) Ltd., third edition: 2003, reprint: (2006).
- Sandra Harper and David W. Speicher, Purification of proteins fused to glutathione S-tranferase, Methods Mol Biol , 681: 259–280, (2011).
- Sanjay Kalra, Jagat Jyoti Mukherjee,Subramanium Venkataraman, Ganapathi Bantwal, Shehla Shaikh, Banshi Saboo, Ashok Kumar Das, and Ambady Ramachandran, 17(5): 819–834 (2013).
- Shafiee Gita, Mohammadreza Mohajari-Tehrani, Mohammad Pajouchi and Bagher Larijani, The importance of Hypoglycemia in Diabetic Patients, Journal of Diabetes and metabolic disorder 2012.
- Sherin, K. T. Peeyush, S. Jayanarayanan, K. K. Amee & C. S. Paulose, Decreased Cholinergic Receptor Expression in the Striatum: Motor Function Deficit in Hypoglycemic and Diabetic Rats, Cell Mol Neurobiol, 26(1), (2011).
- Stephen A. Brunton, Nocturnal Hypoglycemia: Answering the Challenge With Long-acting Insulin Analogs, MedGenMed, 17;9 (2):38, (2007).
- Stephen Strittmatter, Spinal Cord Regeneration: Ready, Set, Nogo, Eukaryon, Lake Forest College, 6, 55-60, (2010).
- Tang ZJ, Zou W, Yuan J, Zhang P, and Tian Y, Anti depressant- like and anxiolytic- like effects of hydrogen sulphide in streptozotocin induced diabetic rats through inhibition of hippocampus oxidative stress, Behav Pharmacol, 2015.

- Vanessa Rodrigues Vilela ,Anyde Castro Ruiz Marques, Christiano Rodrigues Scham -ber, and Roberto Barbosa Bazotte, Hypoglycemia Induced by Insulin as a Triggering Factor of Cognitive Deficit in Diabetic Children, The Scientific World Journal, Article ID 616534, 1-9, (2014).