Incidence of Hypoglycemia in Diabetic population and the Effect of Hypoglycemia on the gene expression of NRG 1 and ErbB 2 receptor in Streptozotocin induced Diabetic rats

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Submitted by

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Under the guidance of

Dr. AMEE K. NAIR

Dedicated

TO

GOD...

&

My Family and My Teachers

&

Especially

To

My Parents

Dedicated to my beloved

Parents and lovely Niece

Acknowledgement

"It is good for the heart to be strengthened by grace"

First and foremost I dedicate my sincere thanks to God with pure heart and soul. He always leads me to a right path and gives me enough strength to reach all success. I wish to be strengthened by divine grace forever.

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Mukesh Kumar Jat

ABRREVIATIONS USED IN THE TEXT

1,3 BPG	1,3 bisphosphoglycerate
D+IIH	Diabetic + insulin induced hypoglycemia
AGE	Advanced glycation e nd
NADP	Nicotinamide adenine dinucle otide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National centre for biological information
NRG-1	Neuregulin-1
HRG	Heregulin
NO	Nitric oxide
D	Diabetic
AChR	Acetylcholine receptor
NDF	Neu differentiation factor
AR	Aldose reductase
ARI	Aldose reductase inhibitor
BLAST	Basic local alignment search tool
BP	Blood pressure
C	Control
CNS	Central nerve system
DEPC	Diethyl pyrocarbonate
EGF	Epidermal growth fac tor
EGFR	Epidermal growth fac tor rece ptor
PCR	Polymerase chain rea ction
PKC	Protein kinase C

PNS	Periphe ral nerve system
RNS	Reactive nitrogen species
ROS	Reactive oxygen spec ies
SDH	Sorbitol dehydrogenase
ErbB-2	Epidermal growth fac tor rece ptor B-2
C+IIH	Control + insulin induced hypoglyc emia
GGF	Glia l growth factor
GLUT-4	Glucose transporter 4
GOD-POD	Glucose oxidase peroxidase
Mt.	Mitochondria
NAD+	Nicotinamide adenine dinucle otide
NADH	Nicotinamide adenine dinucle otide, reduced form
NADPH	Nicotinamide adenine dinucle otide phosphate, reduced form
PARP	Poly (ADP-ribose) polymera se-1
GSSG	Oxidized glutathione
GSH	Glutathione
GR	Glutathione reductase
DNA	Deoxy ribonucleic ac id
Km	Michaelis constant
PKC	Protein kinase C
PLC	Phospolipase C
Vmax	Maximal velocity
D+B	Diabetes+blood pressure
D+N	Diabetes+Neuropathy
D+R	Diabetes+Retinopathy
D+H	Diabetes+Hypoglycemia

D+H+N Diabetes+Hypoglycemia+Neuropathy

D+B+G Diabetes+Blood pressure+gastric

D+E Diabetes+Eye

D+B+R Diabetes+Blood pressure+Ratinopathy

CONTENTS

ABSTRACT

ABSTRACT

Glucose homeostasis in humans is a critical factor for the functioning of nervous system. Hypoglycemia and hyperglycemia is found to be associated with central and peripheral nerve system dysfunction. In the present study, we surveyed the incidence of hypoglycemic events and complications associated with diabetes, occurring in diabetic patients in cross section of population in Ahmedabad. We also investigated the effects of insulin induced hypoglycemia and streptozotocin induced diabetes on the SDH, NRG 1 and ErbB 2 receptor. A single intrafemoral dose of streptozotocin was administered to induce diabetes. Hypoglycaemia was induced by appropriate doses of insulin subcutaneously in control and diabetic rats. The kinetic parameters Vmax and Km of SDH were studied spectrophotometrically at different substrate concentrations of fructose. Results showed enhanced SDH activity in D ($p<0.001$) and D+IIH ($p<0.001$) group than C group and decrease in D+IIH ($p<0.001$) and C+IIH ($p<0.001$) groups compare to D group. NRG 1 gene expression was not obtained due to some experimental condition but ErbB 2 receptor gene expression obtained and showed a downregulation in diabetic and hypoglycemic groups of rats compare to C group and also showed downregulated expression in both D+IIH and C+IIH group compare to D group in cerebral cortex. While in cerebellum and brain stem upregulation of ErbB 2 receptor gene expression were observed in D, C+IIH and D+IIH groups compared to C group and D+IIH group showed increase Erb 2 expression compared to D group. These studies demonstrated that a fluctuation in glucose leads to functional disturbance in the neuronal ErbB 2 in the cerebral cortex, cerebellum and brain stem during insulin induced hypoglycemia in diabetic rats. Incline beam test result conclude motor coordination impairment in Diabetic and Hypoglycemic groups than Control. Altered expression of ErbB 2 in cerebral cortex, cerebellum

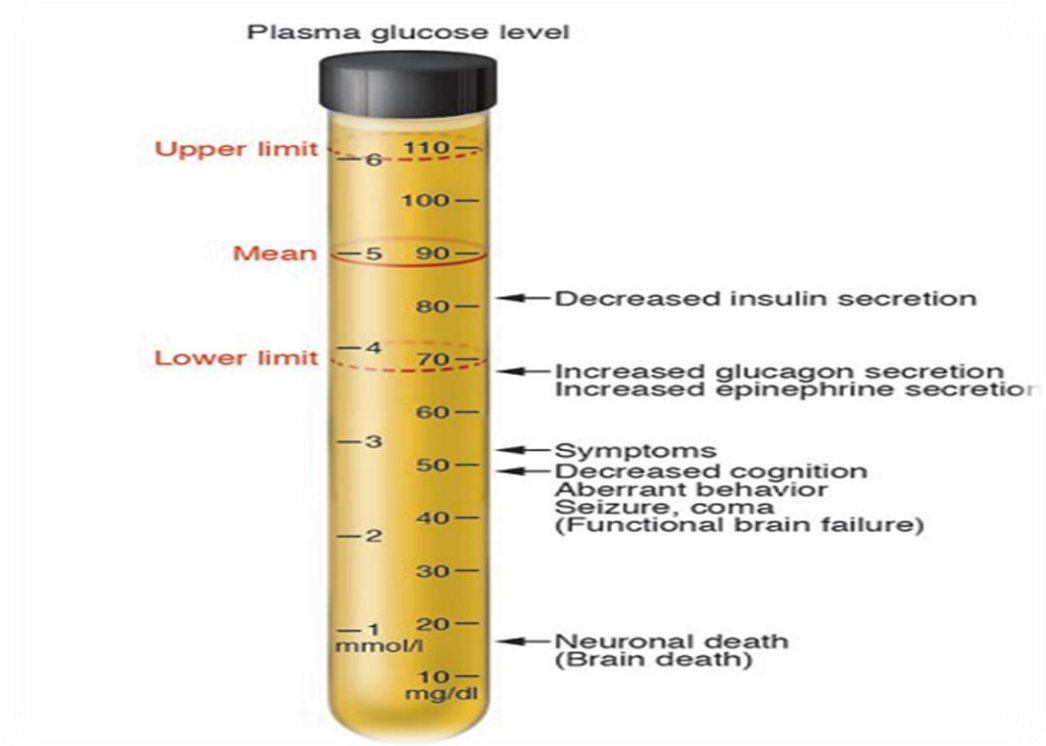
and brain stem of diabetic and hypoglycemic rats along with motor coordination impairment results suggested causing cognitive impairment and motor dysfunction. These results conclude that the expression of ErbB 2 is functionally compromised during hyperglycemia and hypoglycemia compared to hyperglycemia, hypoglycemia causes prominent imbalance in ErbB 2 activity which is suggested to be involved in demyelination, hypermyelination and dysfunction associated with hypoglycemia.

INTRODUCTION

1. INTRODUCTION:

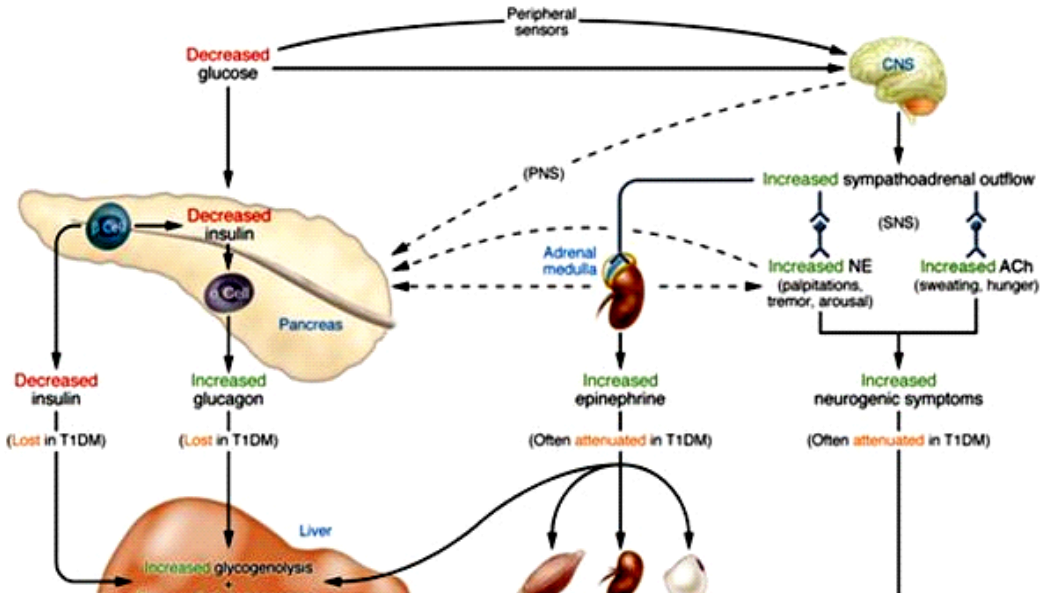
Hypoglycemia is a collection of symptoms due to abnormally low plasma glucose (less than 70 mg/dL) level then the normal glucose level. The brain and peripheral body cells require glucose for their proper functioning. Studies suggest that acute or chronic hypoglycemia leads to neurological dysfunction 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. Excess glucose gets converted into glycogen and is stored in the liver and muscles. Under hypoglycemic condition, glycogenolysis causes the conversion of glycogen to glucose to maintain normal blood glucose level.

Figure 1: Sequences of responses to falling arterial plasma glucose concentration



Hypoglycemia is a relatively common condition primarily affecting diabetic patients undergoing insulin treatment. It can also results from other medications, diseases, hormones, insulinomas and glucagons deficiency. Hypoglycemia can happen suddenly and it can be treated quickly by administration of glucose-rich food. If left untreated, hypoglycemia can lead to confusion, clumsiness and fainting. Severe hypoglycemia can triggers a cascade of events in vulnerable neurons that culminate in cell death even after glucose normalization (Joseph *et al.*, 2009) and can also leads to seizures, coma and even death.

Figure 2: Physiological and behavioural defences against hypoglycemia in humans.



In diabetic patients, hypoglycemic brain injury (Pramming *et al.*, 1991; Boyle *et al.*, 1994), peripheral nerve damage is very common and serious complications associated with insulin administration (Robinson *et al.*, 2009). With respect to the PNS, scattered clinical observations in humans and experimental studies in animals show that hypoglycemia causes a distal axonopathy including both degenerative and regenerative events. In this respect, motor axons seem to be more vulnerable than sensory axons (Mohseni, 2001).

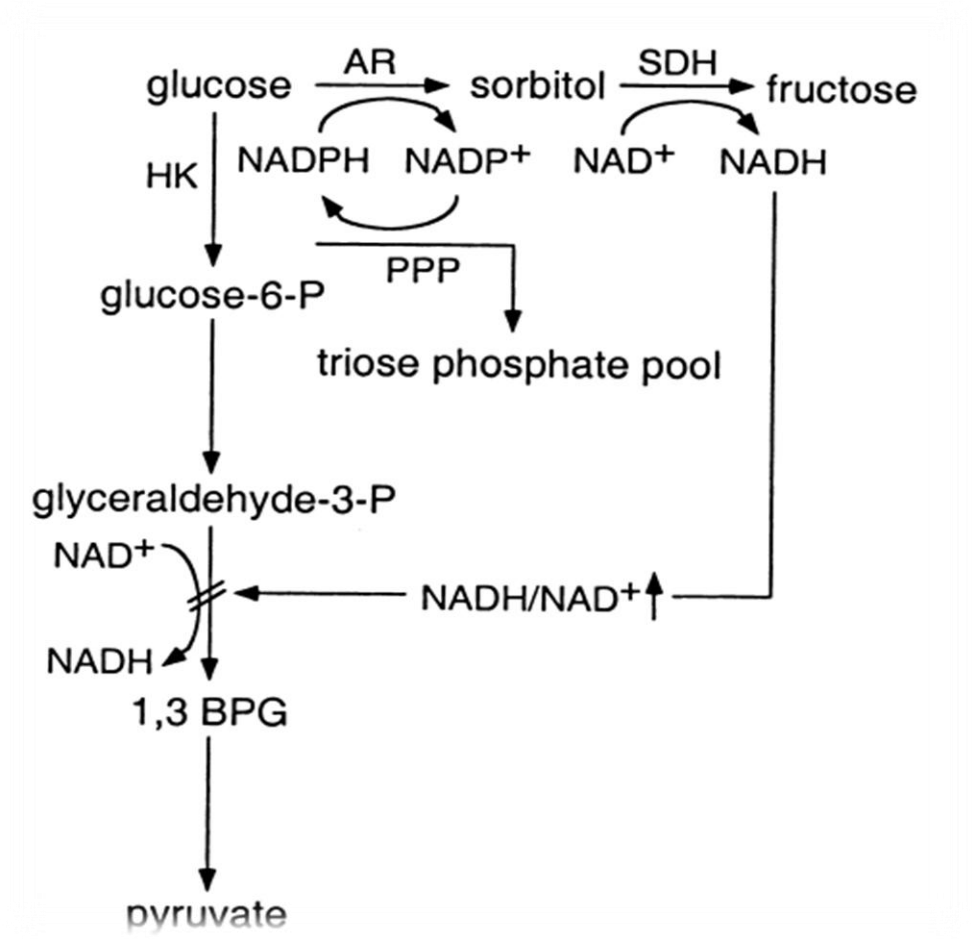
1.1 Polyol Pathway:

High level of blood glucose activates and maintains polyol pathway in diabetic patients which leads to the development of secondary diabetic complications (Srivastava *et al.*, 2005) including neuropathy (Sango *et al.*, 2008), cardiovascular diseases and retinopathy (King *et al.*, 2007). Among these proposed pathogenic mechanisms, the polyol pathway model has received the most attention and scrutiny. Aldose Reductase (AR) (EC 1.1.1.21) is the first enzyme in the polyol pathway, converting excess glucose to sorbitol, which is then further metabolized to fructose by Sorbitol Dehydrogenase (SDH) (Cheung *et al.*, 2005). In diabetes patient AR has been implicated in some clinical complications such as cataract formation, retinopathy, neuropathy and nephropathy (Chung *et al.*, 2003).

Obrosova and co-workers (2001) reported a high level of AR in diabetes and treatment with an adequate dose of Aldose Reductase Inhibitor (ARI) ameliorate diabetes-induced key metabolic abnormalities such vascular (Qing li *et al.*, 2008), mitochondrial and cytosolic NAD/NADH redox imbalances and energy deficiency in peripheral nerve animal models of diabetes (Fong *et al.*, 1998).

The polyol pathway has an important role in metabolism of glucose and on pyridine nucleotide flux. The metabolism of glucose to Sorbitol and fructose in the polyol pathway by AR and SDH, respectively, alters cytosolic pyridine nucleotides to provide an increased ratio of $NADP^+$/NADPH and NADH/NAD^+.

Figure 3: The effect of the polyol pathway on pyridine nucleotide flux and metabolism of glucose.

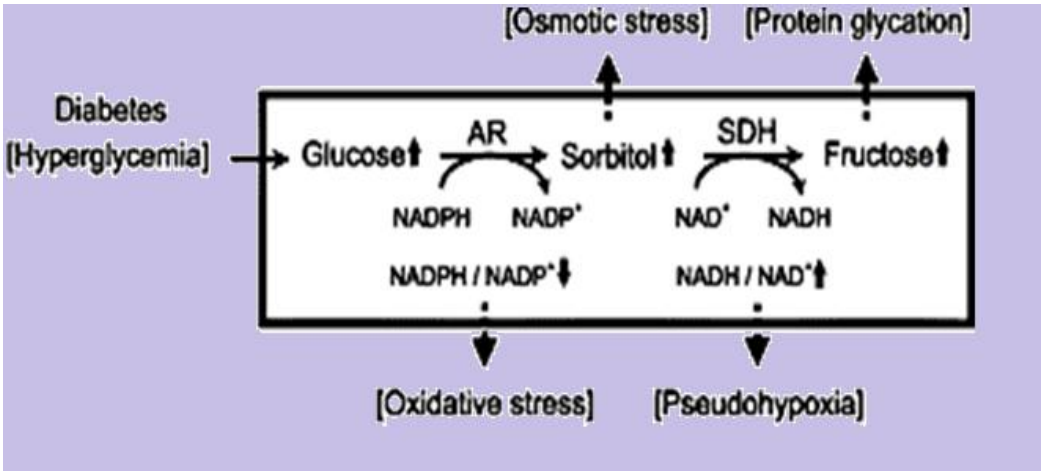


Utilization of NADPH provides conditions for sustained action of the pentose phosphate pathway (PPP) whereas use of NAD^+ may inhibit formation of 1, 3 bisphosphoglycerate (1,3 BPG) from glyceraldehyde-3-phosphate resulting in an increased triose phosphate pool (Dunlop, 2000). Formation of fructose promotes glycation as well as depletes NADPH, further augmenting redox imbalance. Activation of AR may also increase formation of diacylglycerol, which activates the deleterious PKC pathway (Dunlop, 2000; Yamagishi *et al.*, 2003; Uehara *et al.*, 2004; Edwards *et al.*, 2008).

1.2 Sorbitol Dehydrogenase:

The SDH (EC 1.1.1.14) is an important enzyme after AR in polyol pathway which mediates oxidation of sorbitol to fructose. Fructose, the second product of polyol pathway is increased several times in tissues with activated polyol pathway (Tilton *et al.*, 1995) and can contribute to non-enzymatic fructosylation of proteins and provide 3-deoxy glucosone, the precursor to advanced glycation end (AGE) products (Niwa, 1999). Reduction of glucose to sorbitol uses NADPH and oxidation of sorbitol increases NADH with a resultant rapid change in the cytoplasmic redox state. Decreased NADPH (altered cytosolic ratio of NADPH: $NADP^+$) may compromise reduction of glutathione in oxidatively stressed cells. Increased formation of NADH, following oxidation of sorbitol to fructose, favours a condition of hyperglycemia-induced pseudo hypoxia in diabetic tissue (Tilton *et al.*, 1992; Williamson *et al.*, 1993; Dunlop, 2000). SDH also contributes to oxidative stress due to depletion of its co-factor NAD^+ leading to more glucose being channelled through the polyol pathway (Lee *et al.*, 1999; Chung et al., 2003).

Figure 4: Role of polyol pathway enzymes Aldose reductase and Sorbitol Dehydrogenase in Diabetes.

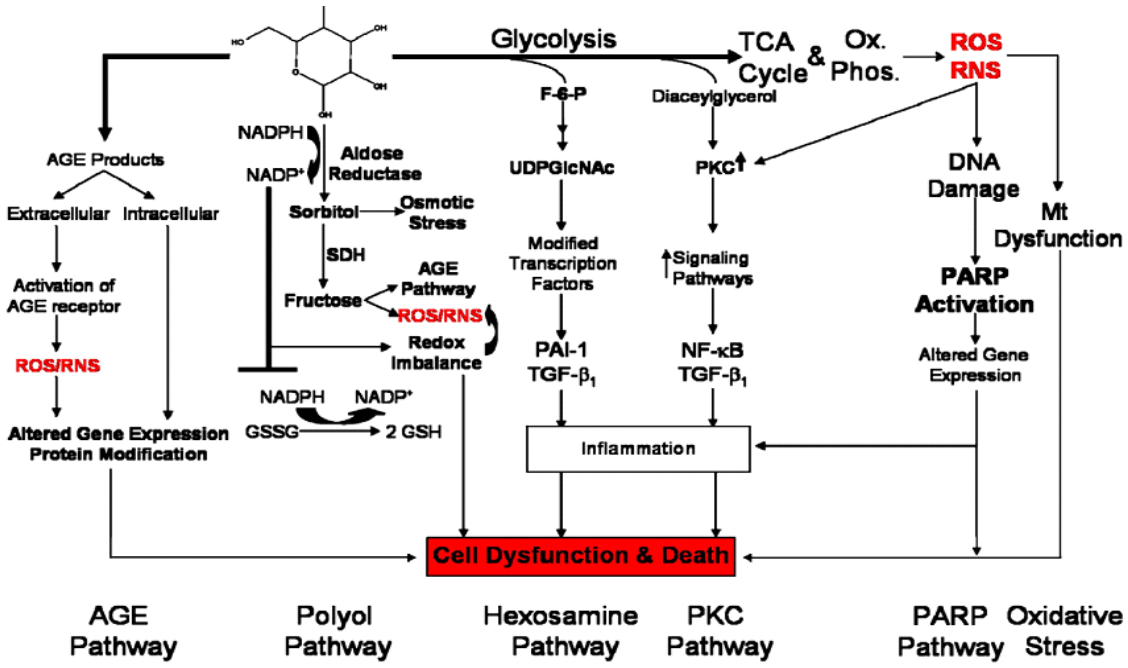


1.3 Diabetic Neuropathy:

Diabetic neuropathy is a descriptive term that encompasses a spectrum of clinical and subclinical syndromes with differing anatomical distributions, clinical courses and possibly differing underlying pathogenetic mechanisms. Each is characterized by diffuse or focal damage to peripheral somatic or autonomic nerve fibers resulting from diabetes, although indistinguishable syndromes may occur idiopathically or in association with other disorders in non diabetic individuals (Edwards *et al.*, 2008).

Hyperglycemia leads to increased mitochondrial activity, raising Reactive Oxygen Species (ROS) production in the mitochondrial peroxynitrite, the primary Reactive Nitrogen Species (RNS) is formed by the reaction of superoxide and nitric oxide (NO). RNS induces a number of cytotoxic effects including protein nitrosylation and activation of Poly ADP Ribose Polymerase (PARP) (Obrosova *et al.*, 2005; Edwards *et al.*, 2008).

Figure 5: Schematic representation of hyperglycaemic effects on biochemical pathways in diabetic neuropathy.



Clinical experience and some experimental studies reported that hypoglycemia can cause alterations both in the Central Nervous System

(CNS) and in the Peripheral Nervous System (PNS) (Mohseni, 2001). Hypoglycemic effects on the CNS include various symptoms such as irritability, lack of concentration, disruption of cognitive functions, convulsions and unconsciousness. Pathological changes have been reported in cerebral cortex and hippocampus. Loss of neurons has been observed in brainstem, cerebellum and spinal cord (Simons *et al.*, 2001). As for pathological aspect, a loss of neurons has been noted, being more obvious in the cerebral cortex (CC) and the hippocampus than in the brain stem, cerebellum and spinal cord. Myelin damage and glial changes have also been observed in the CNS. (Mohseni, 2001).

1.4 NEUREGULIN AND ITS RECEPTOR

Neuregulin

Neuregulins (NRG) are a multi-isoform family of epidermal growth factors that activate members of the ErbB family of receptor tyrosine kinases. The membrane-anchored isoforms contain the receptor-activating ligand in their extracellular domain, a single membrane-spanning region and a long cytoplasmic tail (Liu *et al.*, 1998). EGF results in cellular proliferation, differentiation and cell survival.

NRG 1 found both as in membrane-anchored regions and also as soluble form work as a cytokine that binds to their ErbB 2 receptor and activate to appropriate downstream signalling cascade and made a protein product that regulates developmental neuronal survival synaptogenesis, astrocytic differentiation and microglial activation (Chaudhury *et al.*,2003).

In the nervous system of vertebrates, myelination is essential for rapid and accurate impulse conduction. Myelin thickness depends on axon fiber size. Axonal Neuregulin 1 (NRG 1) signals information about

axon size to Schwann cells. Reduced NRG 1 expression causes hypomyelination and reduced nerve conduction velocity.

Neuronal over expression of NRG 1 induces hypermyelination and demonstrates that NRG 1 type III is the responsible isoform (Michailov *et al.*, 2004). 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.

Neuregulin growth factors are paracrine, autocrine and juxtacrine signaling peptides that belong to the epidermal growth factor (EGF) family belongs to a complex family of proteins that is structurally related to the epidermal growth factor (EGF). Four NRG 1 genes have been identified (Neuregulin 1 to 4). All the products encoded by NRG 1 genes have a characteristic EGF-like domain that distinguishes them from the rest of the EGF family members. The NRG 1 gene encodes more than 15 transmembrane and secreted protein isoforms, generated by alternative promoter usage and mRNA splicing (Liu *et al.*, 1998; Michailov *et al.*, 2004). Isoforms are expressed mainly by cells of endothelial, mesenchymal and neuronal origin and are thus critical for the proliferation, survival, migration and differentiation of several cell types, including epithelium, nerve, cardiac and skeletal muscle. The best studied and most characterized products are those encoded by neuregulin-1 gene. In the early 1990s, a number of groups isolated proteins encoded by this gene. These were named Neu differentiation factor (NDF), heregulin (HRG), glial growth factor (GGF), acetylcholine receptor (AChR)-inducing activity and sensory and motor nerve-derived factor. Later, it was proposed that all the members of this family be included in the term “neuregulin” (Guma *et al.*, 2009).

NRG 1 is subject to extensive alternative splicing, giving rise to a rich and diverse number of distinct peptides. NRG 1 produces at least 15 distinct proteins that are developmentally (Brown *et al.*, 2004).

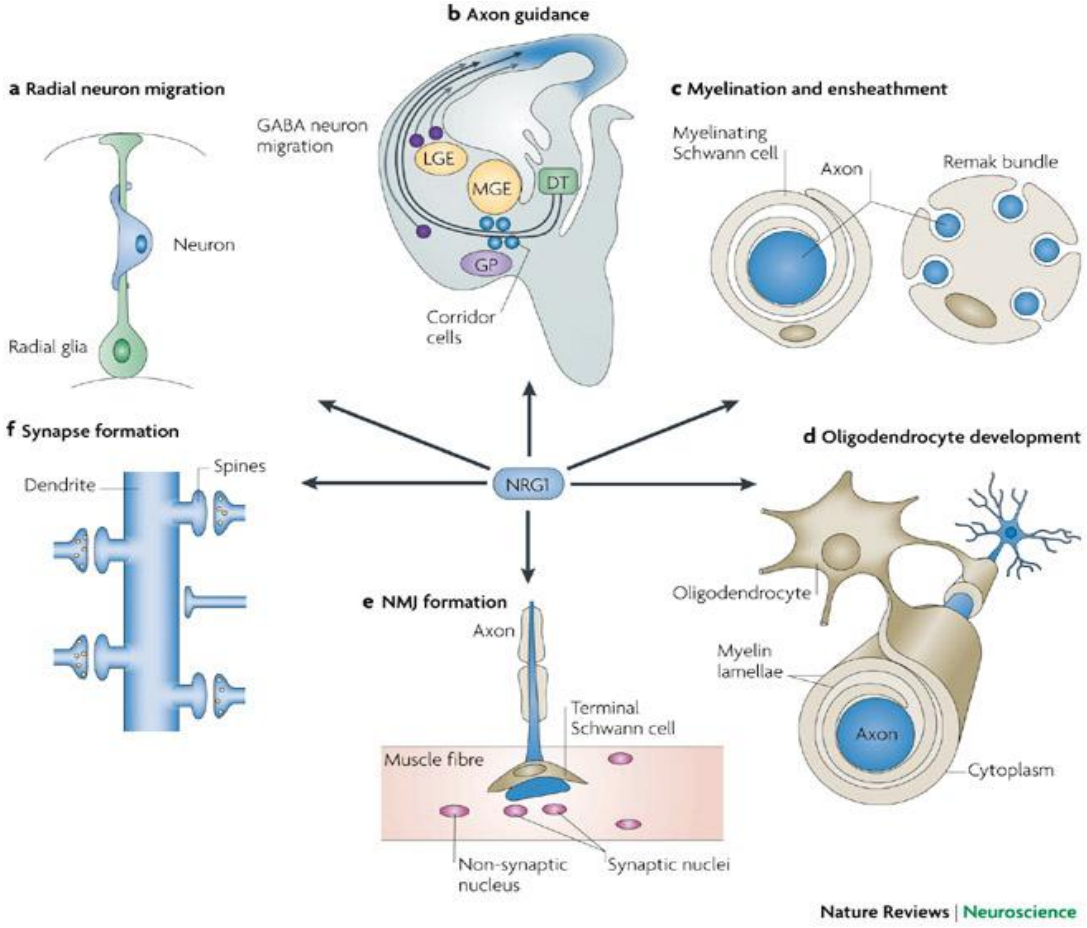
NRG 1 and ErbB 2 Receptor

ErbB is the cell-surface receptor for members of the epidermal growth factor family (EGF family) of extracellular protein ligands (Herbst, 2004). The epidermal growth factor receptor is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB 1), HER2/c-neu (ErbB 2), Her3 (ErbB 3) and Her 4 (ErbB 4). EGFR dimerization stimulates its intrinsic intracellular protein tyrosine kinase activity. As a result, autophosphorylation of several tyrosine (Y) residues in the C-terminal domain of EGFR occurs. These include Y992, Y1045, Y1068, Y1148 and Y1173 (Downward *et al.* 1984). This autophosphorylation elicits downstream activation and signalling by several other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains.

NRG 1 activate ErbB receptor protein tyrosine kinases and are involved in neural and heart morphogenesis as well as cell growth and differentiation. They are a diverse multi-isoformprotein family encoded by a single gene. NRG 1 growth factor binds with ErbB (2-4) tyrosine kinase receptor and regulates developmental neuronal survival, synaptogenesis, astrocytic differentiation and microglial activation (Chaudhury *et al.*, 2003). Myelination requires axonal signals myelin sheath thickness in peripheral nerve system is determined by Schwann cell development that is dependent on NRG 1 and ErbB signaling cascade which is one determinant of nerve conduction velocity, thus must be carefully controlled in development (Michailov *et al.*, 2004). NRG 1 is essential for the cell-cell interactions development and function of multiple organ systems including nervous system, heart, breast and other organ systems and its deregulation has been linked to long lasting diseases such as cancer and schizophrenia (Tan *et al.*, 2007). Given these NRG 1 actions, we hypothesized that there is some role of NRG 1 and its ErbB 2 receptor

in the demyelination, synaptic loss and neuronal death in clinical hypoglycemic individuals.

Figure 6: Multiple functions of NRG 1 growth factor.



NRG 1 and the ErbB 2 receptors have emerged as key mediators of axon–Schwann cell interactions and regulators of Schwann cell development. Some recent *In vitro* studies suggested that NRG 1 induces neural crest cells to adopt the Schwann cell fate which induces Schwann cell proliferation, promotes the survival of embryonic Schwann cell precursors and immature Schwann cells and induces Schwann cell migration.

Thus we required focusing on hypoglycemia because it has some strong scientific reasons including, severe hypoglycemia able to triggers a cascade of events in vulnerable neurons that culminate in cell death even

after glucose normalization (Sang *et al.*, 2003, 2004, 2005, 2007, Suh *et al.*, 2003, 2004, 2005; Joseph *et al.*, 2007; Robinson *et al.*, 2009).

Brain is the major glucose consuming system that consuming 25% of total body glucose and has very little glucose storage capacity so completely dependent on circulatory glucose level and unable to consume other than glucose except ketone bodies but higher level of ketone bodies also create further complication like decrease in blood pH. So finaly conclude that hypoglycemic condition directly affect the brain and ultimately leads to neuronal dysfunctioning.

OBJECTIVES

2. OBJECTIVES

1) To conduct survey studies for indentify the number of diabetic patients afflicted with neuropathy and hypoglycemia.

2) To induce diabetes and hypoglycemia in experimental groups of rats.
    a) Control [C]
    b) Diabetic [D]
    c) Insulin-induced hypoglycemia in control rats [Control + IIH]
    d) Insulin-induced hypoglycemia in diabetic rats [Diabetic + IIH]

3) To study and compare the gene expression of NRG 1 and ErbB 2 receptor in brain regions of experimental groups of rats.

4) To study the effect of hypoglycemia on motor co-ordination by conducting behavior study in control and experimental groups of rats.

MATERIALS AND METHODS

3.MATERIALS AND METHODS

3.1 Chemicals

All chemical used were of analytical grade. Streptozotocin (Qualigens fine chemicals, Mumbai), Glucose estimation kit (Lab Care Diagnostics Pvt. Ltd., Gujarat), Actrapid human insulin (Novo Nordisk, Denmark), copper sulphate ($CuSO_4$;RFCL New Delhi), Sodium Potassium Tartarate ($NaKC_4H_4O_6$; Qualigens fine chemicals, Mumbai), Sodium Carbonate (Na_2C0_3; Sisco research Lab, Mumbai), Sodium Hydroxide (NaOH; Sisco Research Lab, Mumbai), Bovine Serum Albumin (Central Drug House, Mumbai), Folin Ciocalteu Reagent (Sisco Research Lab, Mumbai), β NADH (Sisco Research Lab, Mumbai), D-fructose (Sisco Research Lab, Mumbai), Tris HCl (Spectrochem Pvt. Ltd, Mumbai), Sucrose (Sisco Research Lab, Mumbai), tri reagent (Sigma Aldrich), DEPC Water (Himedia laboratories pvt. Ltd.), chloroform ethylalcohol, cDNA synthesis kit- 100 reactions (Fermentas,USA), PCR Master mix- 200 reactions (Fermentas), Custom Oligonucleotide NRG 1, ErbB 2 (50 nmol; Fermentas USA), Bromophenol Blue, Agarose (Sisco Research Lab, Mumbai) Ethidium bromide (EtBr) and DNA Ladder (Fermentas,USA).

3.2 Laboratory Animals

Adult male Wistar rats of 150–250 g body weight were purchased from Haffkine institute, Parel, Mumbai and used for all experiments. They were housed in separate cages under 12-h light and 12-h 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 Survey Studies

A cross-sectional survey study in Ahmedabad was conducted to describe diabetes management, diabetes control and late complication status among diabetic patients managed in primary health care clinics and hospitals. All the information and data contained in the survey has been collected from Gayatri Mandir Trust Hospital (Shahibaug), Shri Kanti Manilal Trust Hospital (Shahibaug), Police welfare Hospital (Shahibaug), Rajasthan hospital under guidance of Dr. Kiran K. Shah, Dr. N. D Khimesra, Dr. K. D Tiberwala, Dr. Bharat Shah, Dr. Faruk Memon and Dr. Anil Chadha. The patients were interviewed and the medical records such as demographic data, management practice, glycemic control and complications were retrospectively reviewed for a period of one month. All data were entered in the case record forms and analyzed statistically.

3.4 Model Induction

The experiment complete in two 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 3 animals. Diabetes was induced by a single intrafemoral dose (50 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 (Fermetas, USA) using glucose oxidase-peroxidase method. 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) (Flana gan *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 Sorbitol Dehydrogenase Assay

SDH catalyzes the reversible oxidation-reduction reaction between sorbitol and fructose.

$$\text{D-Fructose + NADH} \xrightleftharpoons{\text{SDH}} \text{D-Sorbitol + NAD}$$

The rate of oxidation of NADH is directly proportional to the rate of conversion of D-Fructose to D-Sorbitol. The rate of decrease in absorbance of sorbitol at 340 nm allows measurement of SDH activity.

SDH activity was assayed according to the method described by Hayman and Kinoshita. The assay mixture in 1 ml contained 6 mM NADH, 4 mM D-Fructose, Tris HCL buffer, pH 6.6, 1% w/v Standard BSA and crude enzyme (from rat liver). Appropriate blanks were employed for corrections. The change in the absorbance at 340 nm due to NADH oxidation was followed in a Shimadzu spectrophotometer (Clive *et al.*, 1975).

Kinetic Parameters

K_m and V_{max} of Sorbitol dehydrogenase were determined with varying concentrations of fructose. K_m and V_{max} were estimated by Michaelis-Menten kinetics.

3.6 Glucose estimation

GOD-POD method was used for confirmation of model induction and for determination of blood glucose level in control and experimental rats. Glucose is oxidised to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Red quinoneimine dye is measured at 505 nm. The absorbance at 505 nm is proportional to concentration of glucose in the sample.

$$\text{Glucose} + O_2 + 2H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + 4\ \text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye} + H_2o$$

3.7 Tissue Preparations

Rats were sacrificed on the 15th day of model induction by cervical dislocation and then decapitated (Robinson *et al.*, 2009). The brain parts cerebellum, cerebral cortex, hypothalamus and brain stem 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 -20 C until assay.

3.8 NRG 1 and ErbB 2 receptor gene expression:

Ribonucleic acid (RNA) was isolated from the cerebellum, cerebral cortex and brain stem using Tri reagent. Total cDNA synthesis was performed by using fermentas cDNA synthesis kit. PCR analyses were conducted with gene-specific primers. Gene specific primers were designed by using BLAST software.

Sequence of gene specific primers:

	Forward (5'-->3')	**Reverse (5'-->3')**	**Produ-ct size**
NRG 1 primer	CGTCACCGTCACCCAACCGG	AGCTGGTTTCGCACCGGAGC	230 bp
ErbB 2 primer	CCTGCCTCCACTTCAATCAT	CAGGATCCCATTCCGTAGA	172 bp

3.9 Behaviour Studies

Inclined beam walk test

Fine motor coordination can be assessed by using a beam walking or beam balance test. This test essentially examines the ability of the animal to remain upright and to walk on an elevated and relatively narrow beam. Inclined beam walking test was employed to evaluate fore and hind limb motor coordination. Wooden beam with a dimensions-55 cm long and 1.5 cm wide was taken for conducting inclined beam walk. Rough surface is assigned to prevent excessive slipping of rats. Each animal was individually placed on a metallic bar 55 cm long inclined at increasing angles of 0º,30º,45º and 60º from the ground. Time (s) for which each animal stays on the beam before fall off was recorded. Time Scale ranging from 0 to 4 was used to grade motor performance. A grade

of 0 was assigned to animal that could readily traverse the beam, grade 1 was given to animal demonstrating mild impairment, grade 2 was assigned for moderate impairment, grade 3 was given for moderate impairment and grade 4 was assigned to animal completely unable to walk on the beam. This test is performed during active phase (Ahmad *et al.*, 2011; Feeney *et al.*, 1981).

Figure 7: Inclined beam walk test at 60° and 45° angle

RESULTS

4. RESULTS

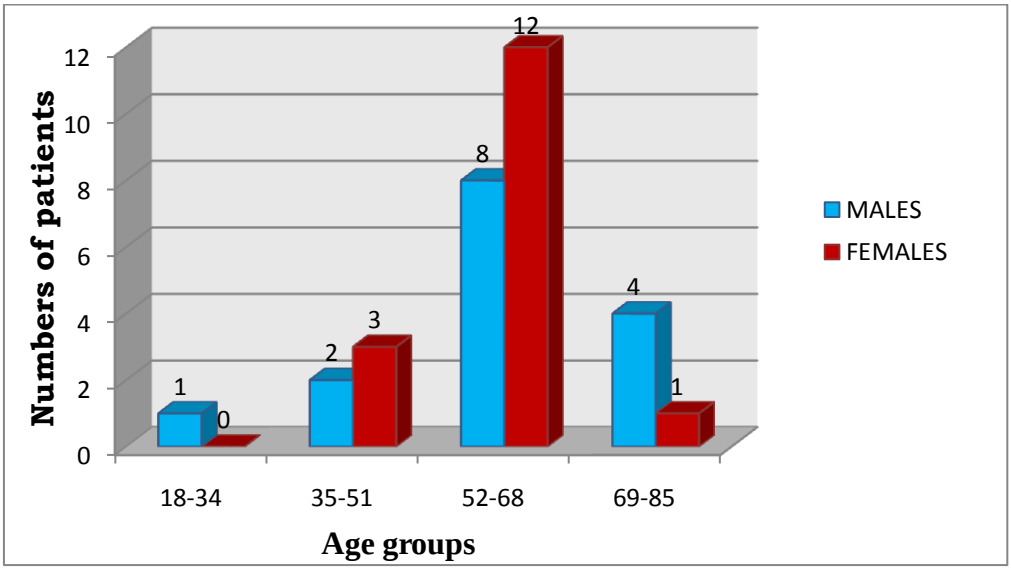
4.1 Survey studies

Survey studies were performed in the different age groups of diabetic patients. A total of 31 diabetic patients were studied. The highest numbers of diabetic patients were found in the age group between 52-68 years in which 8 males and 12 females were afflicted with diabetes. The lowest numbers of patients were found in the age group between 18-34 years in which only 1 male patient was found to be diabetic whereas no female diabetic patients were reported. Age group of 35-51 years were contained 2 males and 3 females and the age group of 69-85 years were contained 4 male and 1 female diabetic patients (Table 1 and Figure 8).

Table 1: Numbers of male and female diabetic patients in different age groups.

AGE GROUP (Years)	**MALES (No. of patients)**	**FEMALES (No. of patients)**
18-34	1	0
35-51	2	3
52-68	8	12
69-85	4	1
Total	**15**	**16**

Figure 8: Numbers of Male and female diabetic Patients in different age groups.

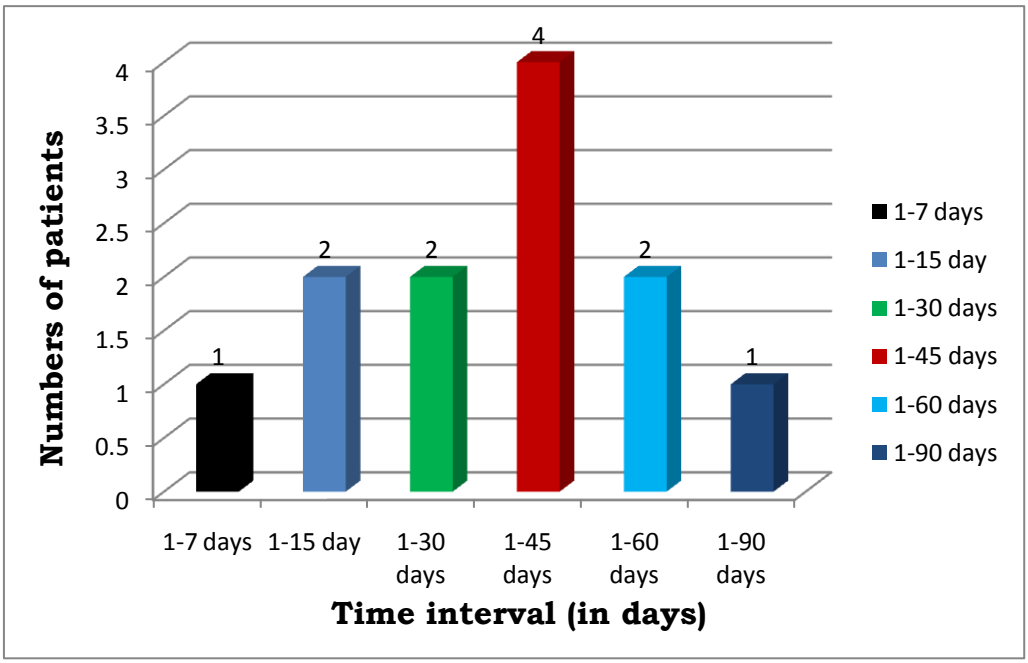


We obtained 12 patients suffered from hypoglycemic episodes from a total of 31 diabetic patients. In these, we found the 2 patients in each group who experience hypoglycemic episodes in 1-15 days, 1-30 days and 1-60 day. One patient was found to afflict with hypoglycemic episodes within 1-7 days and one patient within 1-90 days. Four diabetic patients were found to suffer from hypoglycemic event in every 1-45 days of time interval. (Table 2, Figure 9).

Table 2: Number of diabetic patients experience hypoglycemic episodes in a specific time of interval.

Time interval (in days)	No. of Patient
1- 7	1
1-15	2
1-30	2
1-45	4
1-60	2
1-90	1

Figure 9: Number of diabetic patients experience hypoglycemic episodes in a specific time of interval.

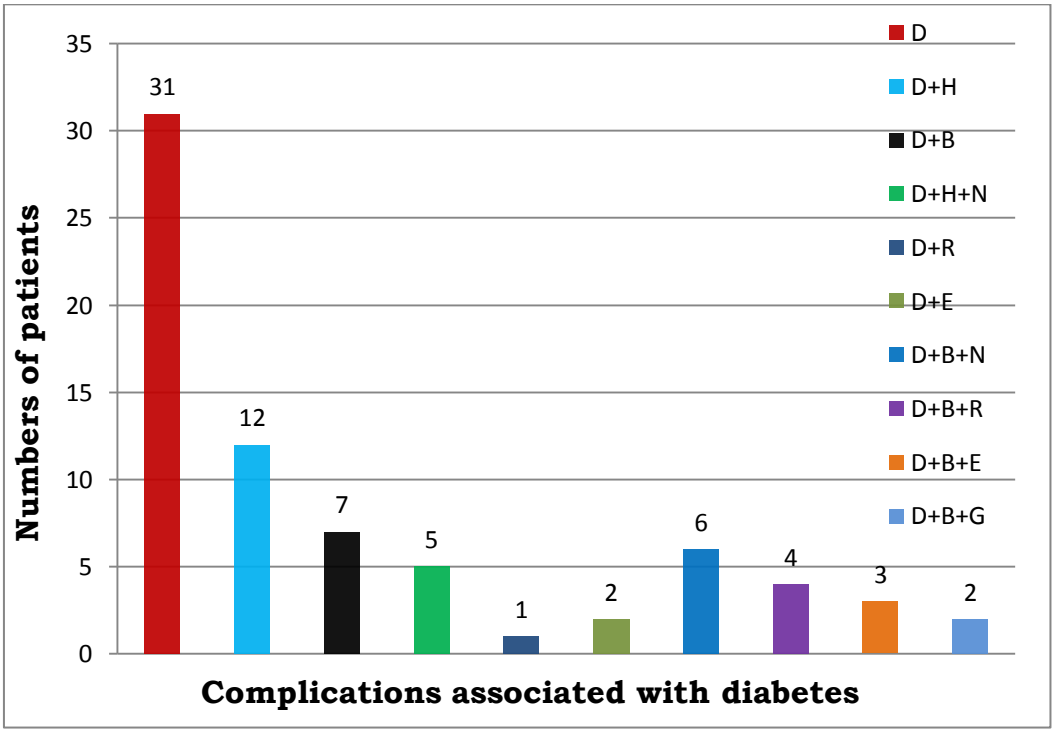


From a total 31 diabetic patients, 12 of diabetic patient were found to experience of hypoglycemic events during their treatment period. 5 patients out of these 12 hypoglycemic patients were also found to be suffering from neuropathy. 22 diabetic patients were found to suffer from high or low blood pressure (BP) problems. 6 in out of 22 patients suffer from both BP and neuropathy and another 4 patients were affected with BP and retinopathy. 3 diabetic patients were found to affect with eye related diseases along with BP and another 2 diabetic patient found to suffer along with only vision related diseases. And 2 diabetic patients were found to associate with BP and gastric complication (Table 3 and Figure 10).

Table 3: Complications reported with diabetes patients in our studies. (Abbreviations stands for, D- Diabetes, H- Hypoglycemia, B- Blood pressure, N- neuropathy, R- Retinopathy, E- Eye related diseases, G- Gastric problems)

Group	No. of Patients
D	31
D+H	12
D+B	7
D+H+N	5
D+R	1
D+E	2
D+B+N	6
D+B+R	4
D+B+E	3
D+B+G	2

Figure 10: complications reported with diabetes patients in our studies.



4.2 Blood glucose level in experimental groups of rats

A significant increase ($p<0.001$) was observed in the blood glucose level of Diabetic rats when compared to Control from 3rd day of experiment. In C+IIH and D+IIH group the blood glucose level showed a significantly decrease ($p<0.001$ & $p<0.001$) respectively when compared to control group. These levels were observed from 4th day to till 14th day of experiment (Table 4, Figure 11a, 11b and 11c).

Table 4: Blood glucose level (mg/dL) on different experimental days in four experimental groups of rats

Experimental groups	Blood glucose (mg/dL)		
	Day 1	Day 7	Day 14
Control	99.00±2.00	99.23±1.36	99.30±0.81
Diabetic	92.66±3.05	251.76±2.04***	261.26±2.13***
C+IIH	93.50±7.50	42.25±2.62***¶¶¶	34.45±3.87***¶¶¶
D+IIH	95.12±5.23	45.25±9.34***¶¶¶	38.97±4.01***¶¶¶

Value are mean ± SD of 2 separate experiments with 6 rats per group.

IIH - Insulin-Induced Hypoglycemia

*** P < 0.001 when compared to Control, ** P < 0.01 and * P < 0.05

¶¶¶ P < 0.001 when compared to Diabetic, ¶¶ P < 0.01 and ¶ P < 0.05

$$$ P < 0.001 when compared to Diabetic+ IIH, $$ P < 0.01 and $ P < 0.05

Figure 11a: Blood glucose (mg/dL) level in experimental groups of rats during experimental days.

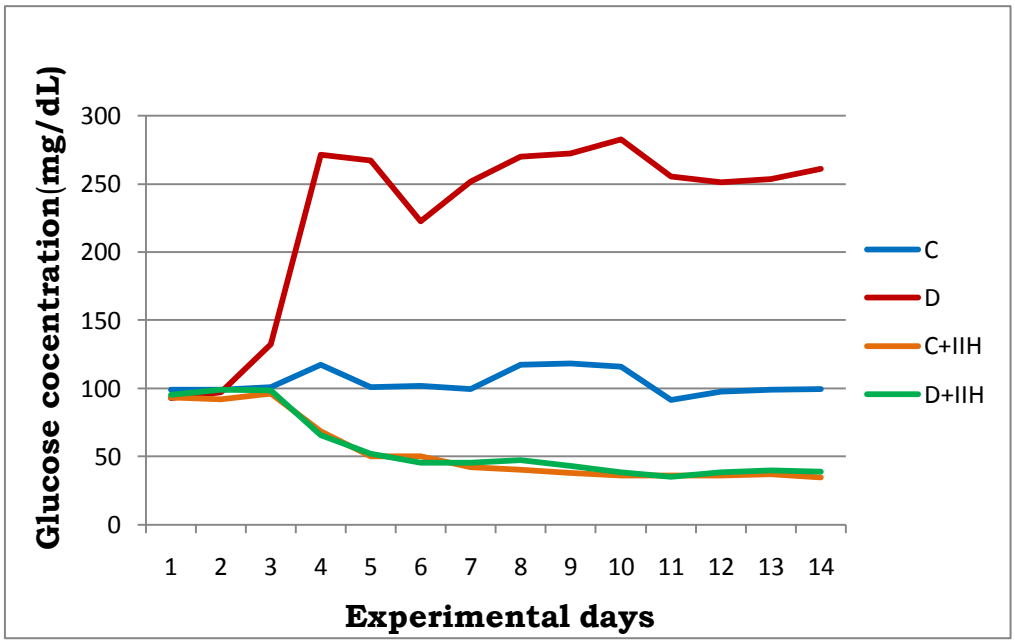


Figure 11b Blood glucose (mg/dL) level in experimental groups of rats in initial 1-7 experimental days.

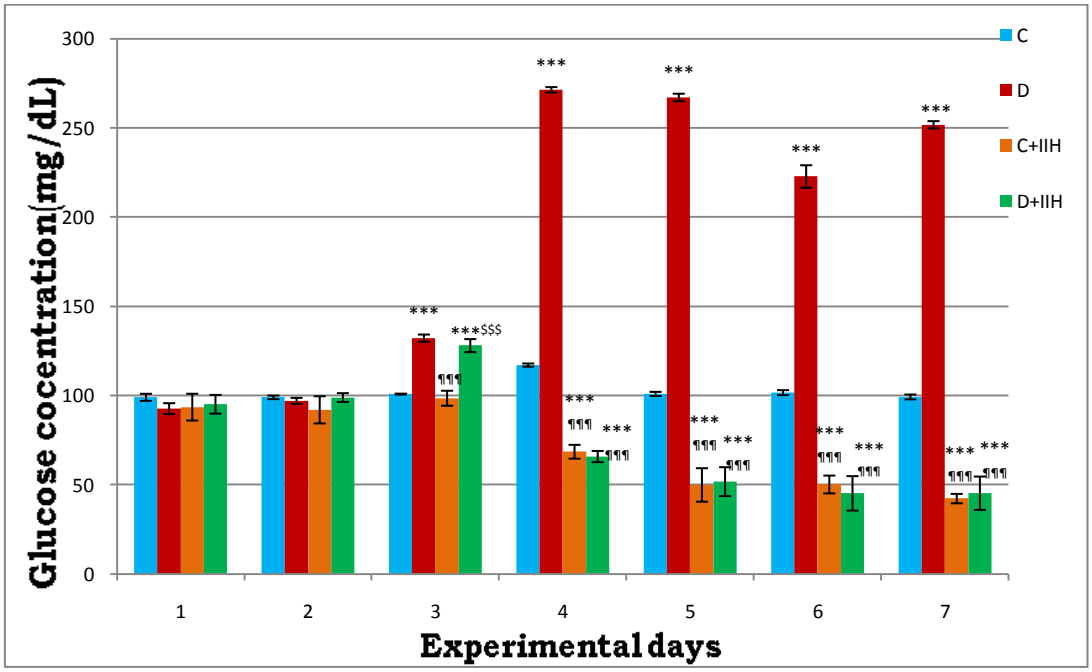
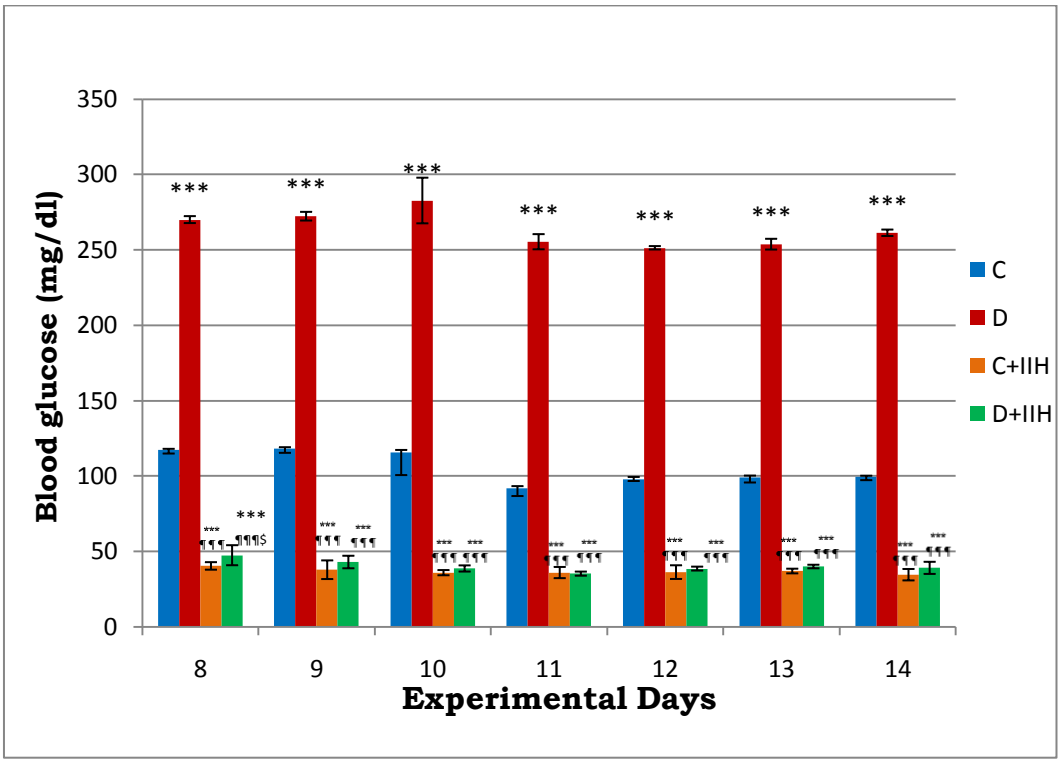


Figure 11c Blood glucose (mg/dL) level in experimental groups of rats in 8-14 experimental days.



4.3 Food and water intake in experimental groups of rats

In control and experimental rats a significant difference of food and water intake were observed in during experimental days. A significant increase in food intake were observed respectively in diabetic ($p<0.001$), D+IIH ($p<0.001$) and C+IIH ($p<0.001$) groups as compared to control group from 3rd day to 14th day. A significant decrease in food intake were observed in D+IIH($p<0.001$), and C+IIH groups ($p<0.001$) compared to D group from 3rd day to till end of 14th day respectively. In D+IIH group a significant increase ($p<0.001$) of food intake was also observed with compared to C+IIH group from 3rd day to till last experimental day (Table 5, Figure 12a, 12b and 12c).

Table 5: Food intake (g) in experimental groups of rats during experimental days.

Experimental Groups	Total Food consumption (g)		
	Day 1	**Day 7**	**Day 14**
Control	4.16 ± 0.15	8.06 ± 0.20	9.73 ± 0.64
Diabetic	4.23 ± 0.20	25.86 ± 0.80***	18.53 ± 0.68***
C+IIH	4.2 ± 0.21	16.96 ± 0.28***¶¶¶	14.13 ± 1.20***¶¶¶
D+IIH	4.21 ± 0.03	21.41 ± 0.42***¶¶¶$$$	16.33 ± 0.15***¶¶¶$$$

Value are mean ± SD of 2 separate experiments with 6 rats per group.

IIH - Insulin-Induced Hypoglycemia

*** P < 0.001 when compared to Control, ** P < 0.01 and * P < 0.05

¶¶¶ P < 0.001 when compared to Diabetic, ¶¶ P < 0.01 and ¶ P < 0.05

$$$ P < 0.001 when compared to Diabetic+ IIH, $$ P < 0.01 and $ P < 0.05

Figure 12a: Food intake (gm) in experimental groups of rats during experimental days.

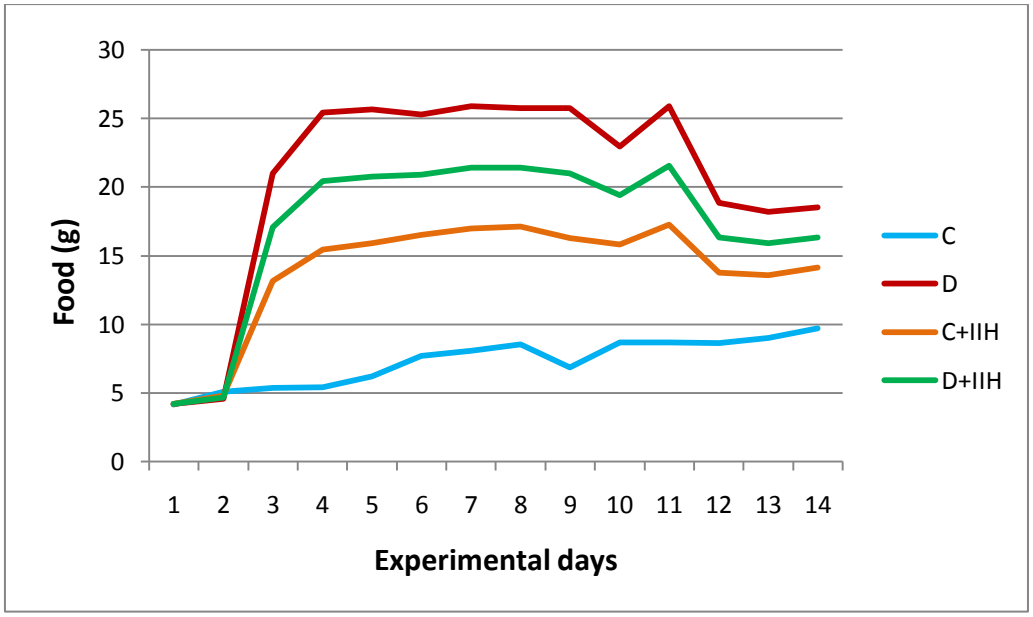


Figure 12b: Food intake (gm) in experimental groups of rats during initial 1-7 experimental days.

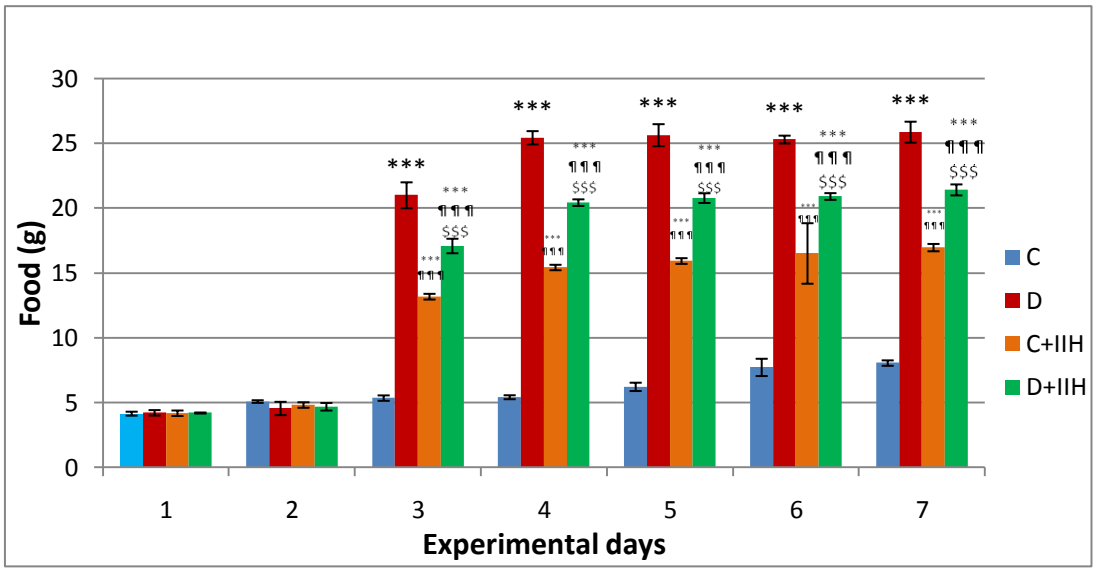
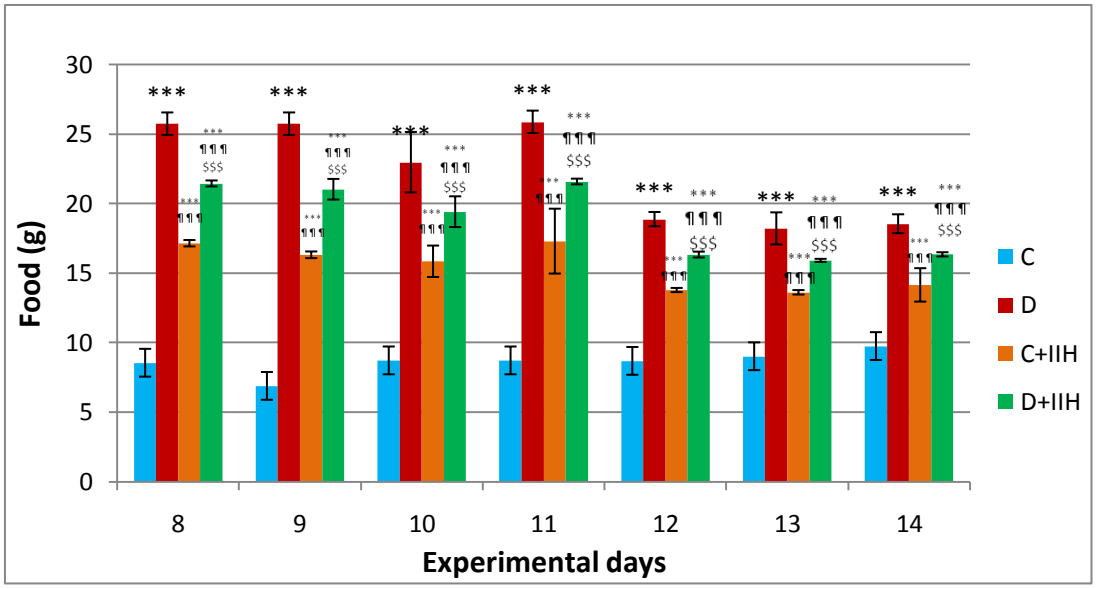


Figure 12c: Food intake (gm) in experimental groups of rats during 8-14 experimental days.



A significant increase in water consumption were observed respectively in D ($p<0.001$), C+IIH ($p<0.001$) and D+IIH ($p<0.001$) groups from 3rd day to 14th day. A significant decrease ($p<0.001$), ($p<0.001$) in water

consumption were also observed in D+IIH and C+IIH groups respectively with compared to D group from 3rd day to till 14th day (Table 6, Figure 13a, 13b and13c).

Table 6: Water intake (mL) in experimental groups of rats during experimental days.

Experimental Groups	Total water consumption (mL)		
	Day 1	**Day 7**	**Day 14**
C	8.3 ± 0.3	8.93 ± 0.30	10.23 ± 0.20
D	8.63 ± 0.35	51.2 ± 1.05***	56.06 ± 0.92***
C+IIH	8.3 ± 0.35	27.5 ± 3.53***¶¶¶	21 ± 2.82***¶¶¶
D+IIH	8.46 ± 0.14	30.06 ± 3.53***¶¶¶$$$	33.15 ± 2.33***$$$

Value are mean ± SD of 2 separate experiments with 6 rats per group.

IIH - Insulin-Induced Hypoglycemia

*** P < 0.001 when compared to Control, ** P < 0.01 and * P < 0.05

¶¶¶ P < 0.001 when compared to Diabetic, ¶¶ P < 0.01 and ¶ P < 0.05

$$$ P < 0.001 when compared to Diabetic+ IIH, $$ P < 0.01 and $ P < 0.05

Figure 13a: Water intake (mL) in experimental groups of rats during experimental days.

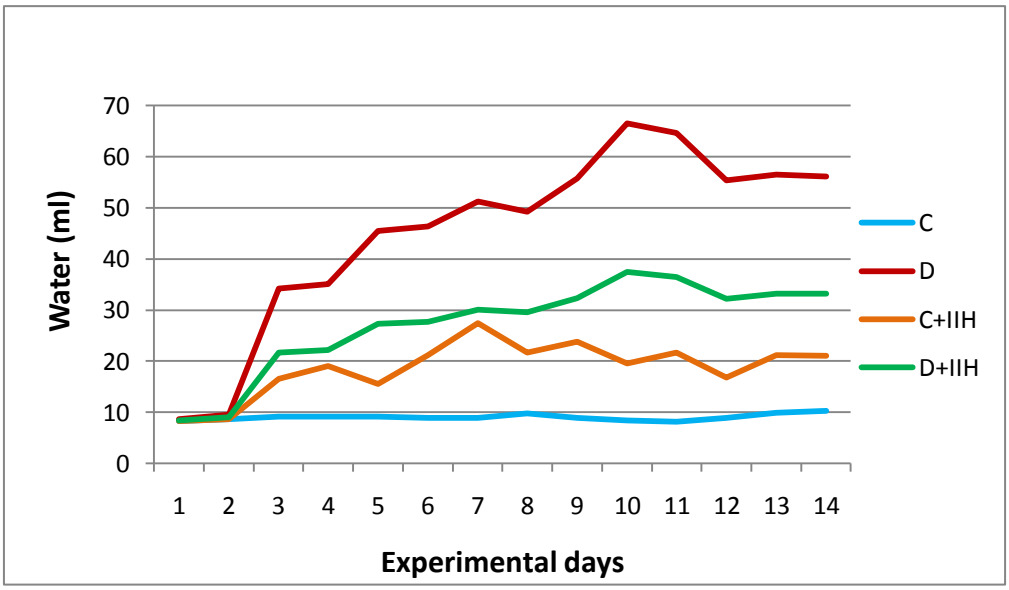


Figure 13b: Water intake (mL) in experimental groups of rats in initial 1-7 experimental days.

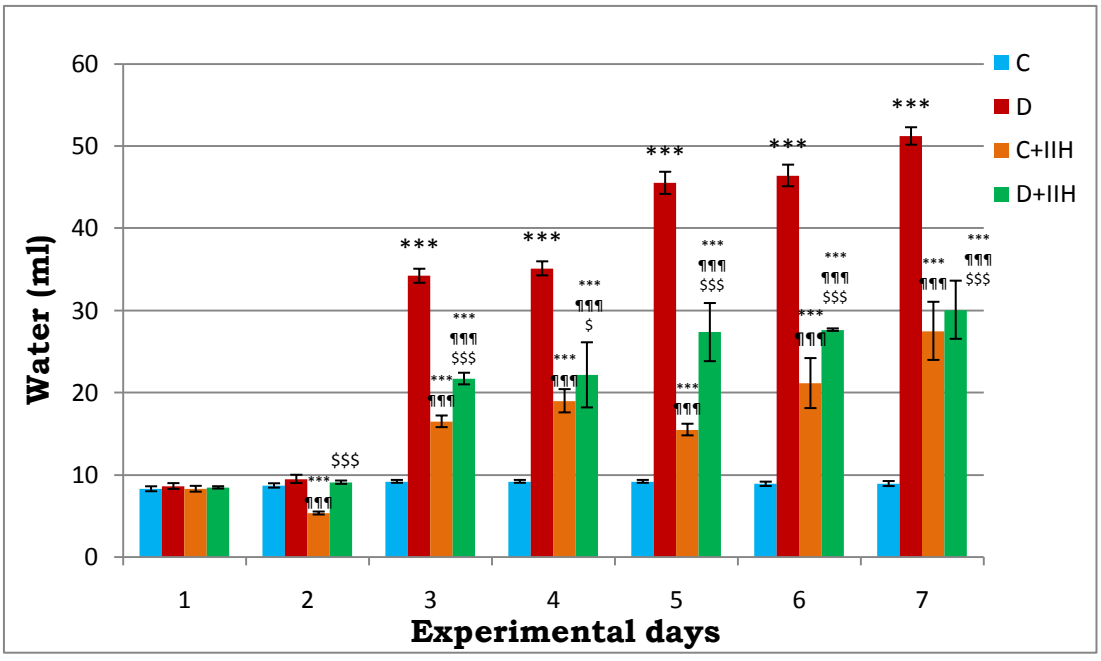
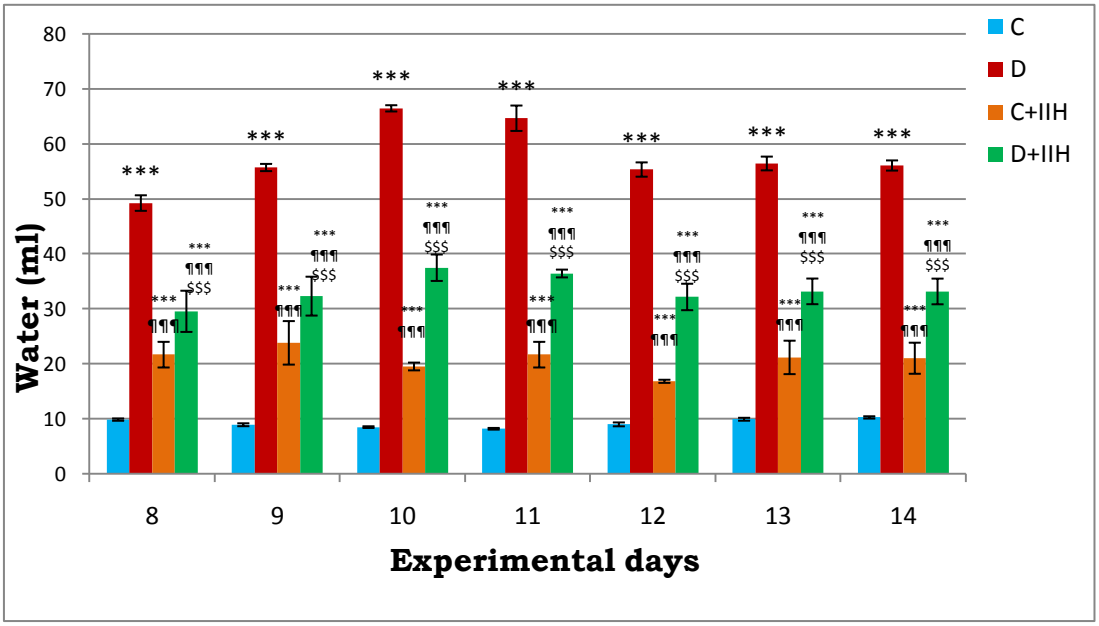


Figure 13c: Water intake (mL) in experimental groups of rats in 8-14 experimental days.



4.4 Weight Fluctuation in the experimental groups of rats

A significant fluctuation in weight was observed in experimental groups compare to C group of rats. A significant decrease in body weight were observed in D ($p<0.001$), D+IIH ($p<0.001$) and C+IIH ($p<0.001$) groups of rat compare to C group. A significant increase in body weight were observed in C ($p<0.001$) and C+IIH ($p<0.001$) groups compare to D group. A significant loss ($p<0.001$) in body weight was also observed in D+IIH group compare to C+IIH group of rat (Figure 14a, 14b and 14c).

Figure 14a: Change in body weight (g) in experimental groups of rats during experimental days.

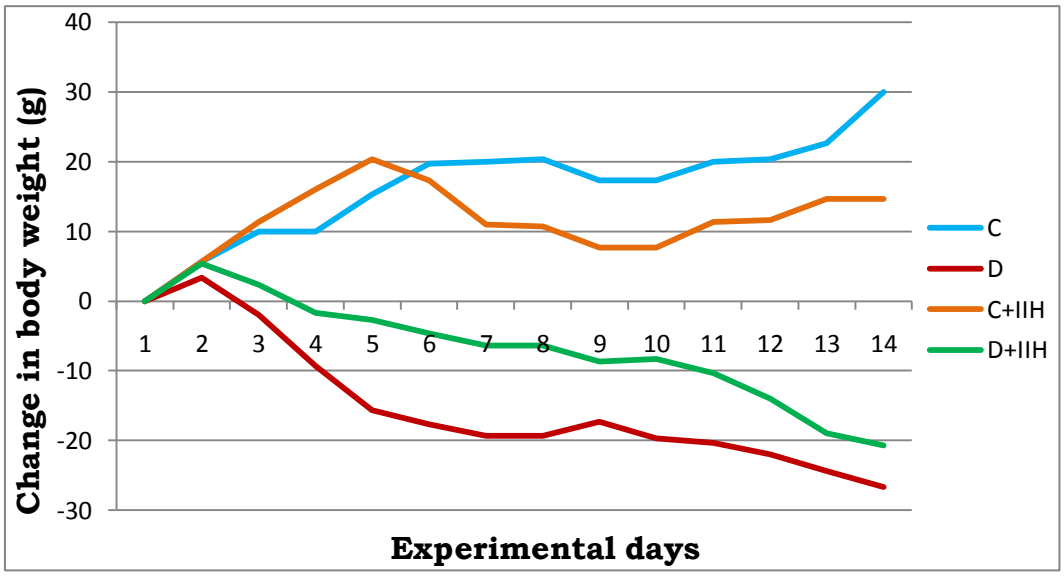


Figure 14b: Change in body weight (g) in experimental groups of rats during 1-7 experimental days.

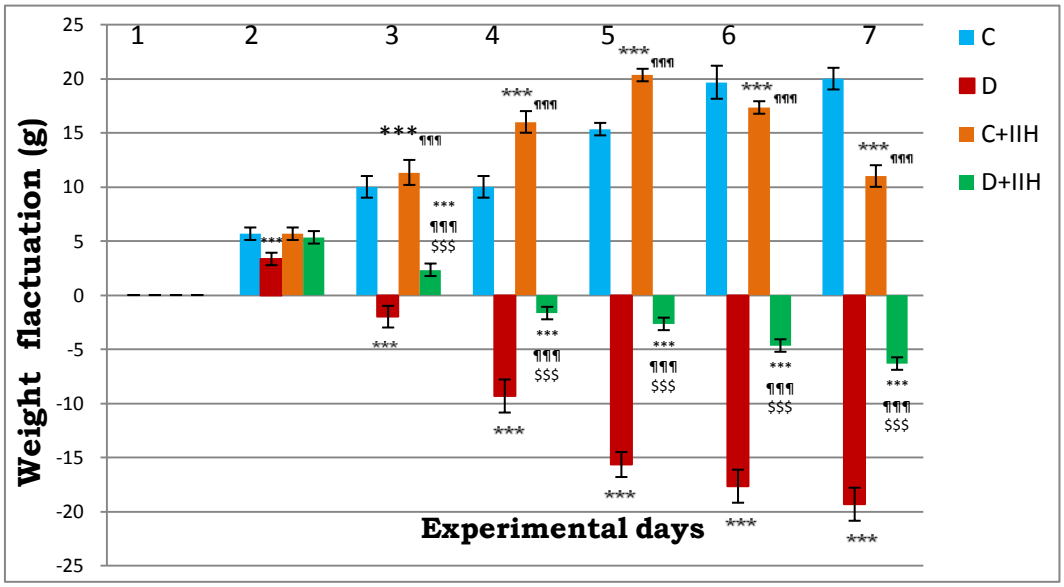
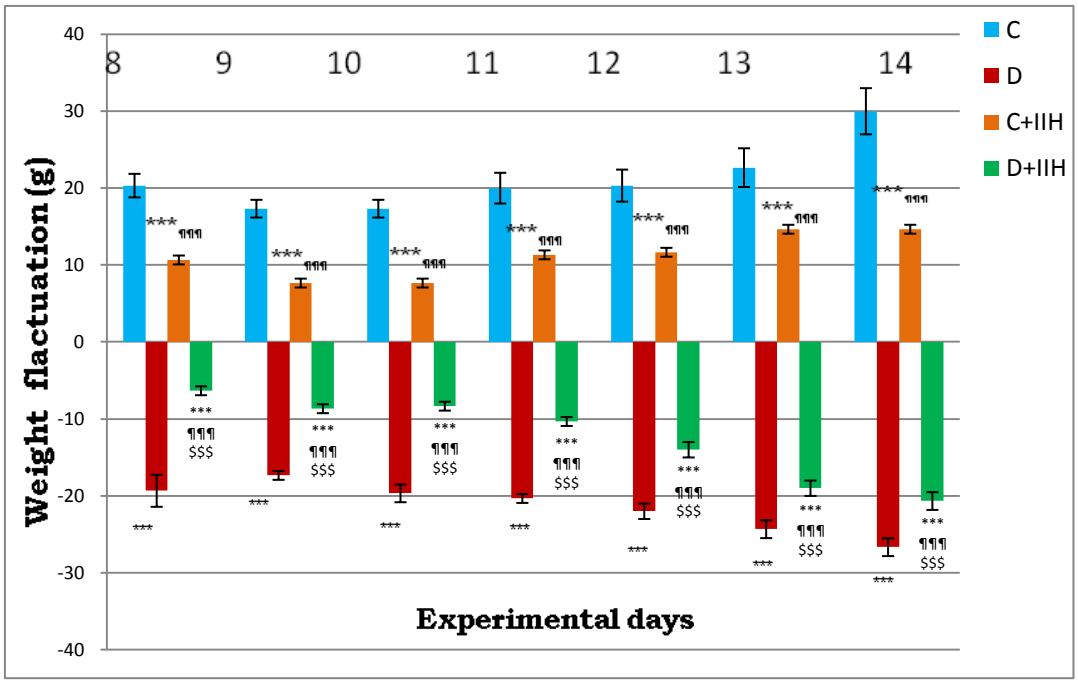


Figure 14c: Change in body weight (g) in experimental groups of rats during 8 - 14 experimental days.



4.5 Sorbitol Dehydrogenase assay

Liver SDH activity showed the activation and maintenance of polyol pathway. A significant increase in SDH activity were observed in D and D+IIH group of rats than compare to C group of rats. D and D+IIH groups were showed a significant increase ($p<0.001$) & ($p<0.01$) respectively in Vmax compared to C group. While C+IIH and D+IIH were showed significant decrease ($p<0.001$) & ($p<0.001$) respectively in Vmax compared to D group. D+IIH were showed increase ($p<0.05$) in Vmax compared to C+IIH group.

Km was observed significantly decreased in D group ($p<0.001$) and increased in C+IIH and D+IIH groups ($p<0.05$) & ($p<0.001$) respectively compared to C group. Km was observed significantly increase ($p<0.001$) &

(p<0.001) in C+IIH and D+IIH groups respectively compared to D group. D+IIH group was also observed significantly increased (p<0.001) in Km compared to C+IIH group (Table 7 and Figure 15).

Table 7: Liver SDH activity at different fructose concentration in four experimental groups.

EXPERIMENTAL GROUPS	**V_{max}** (U/mg protein)	**Km** (m mol/L)
Control	0.432 ± 0.015	0.060 ± 0.001
Diabetic	0.686 ± 0.005 ***	0.046 ± 0.005***
Control + IIH	0.450 ± 0.023¶¶¶	0.065 ± 0.001* ¶¶¶
Diabetic + IIH	0.480 ± 0.011 **¶¶¶ $	0.076 ± 0.002 *** ¶¶¶ $$$

Value are mean ± SD of 2 separate experiments with 6 rats per group.

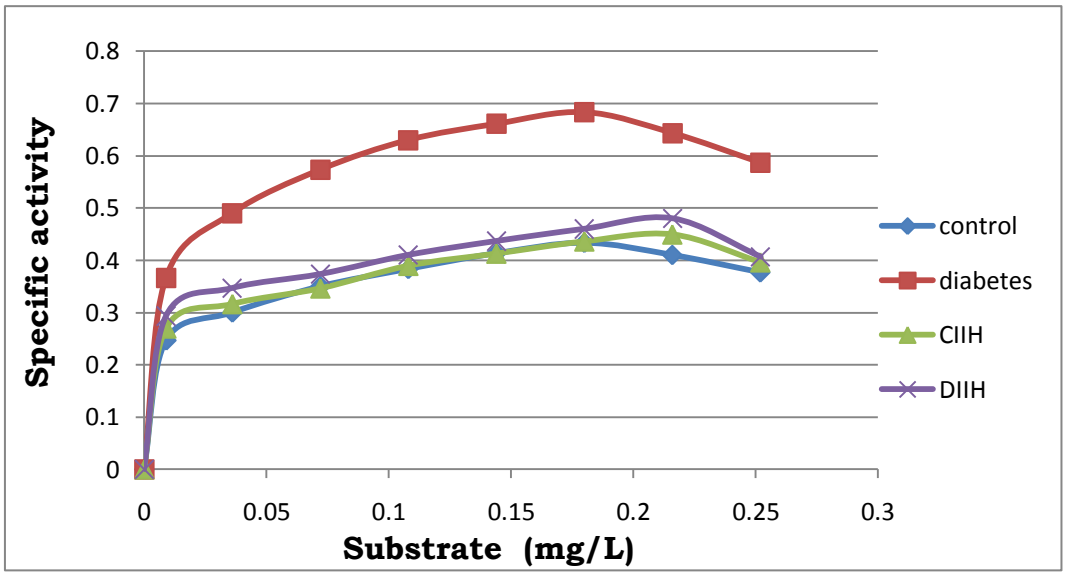
IIH - Insulin-Induced Hypoglycemia

*** P < 0.001 when compared to Control, ** P < 0.01 and * P < 0.05

¶¶¶ P < 0.001 when compared to Diabetic, ¶¶¶ P < 0.01 and ¶ P < 0.05

$$$ P < 0.001 when compared to Diabetic+ IIH, $$ P < 0.01 and $ P < 0.05

Figure 15: Effect of substrate (fructose) concentration on liver SDH activity.

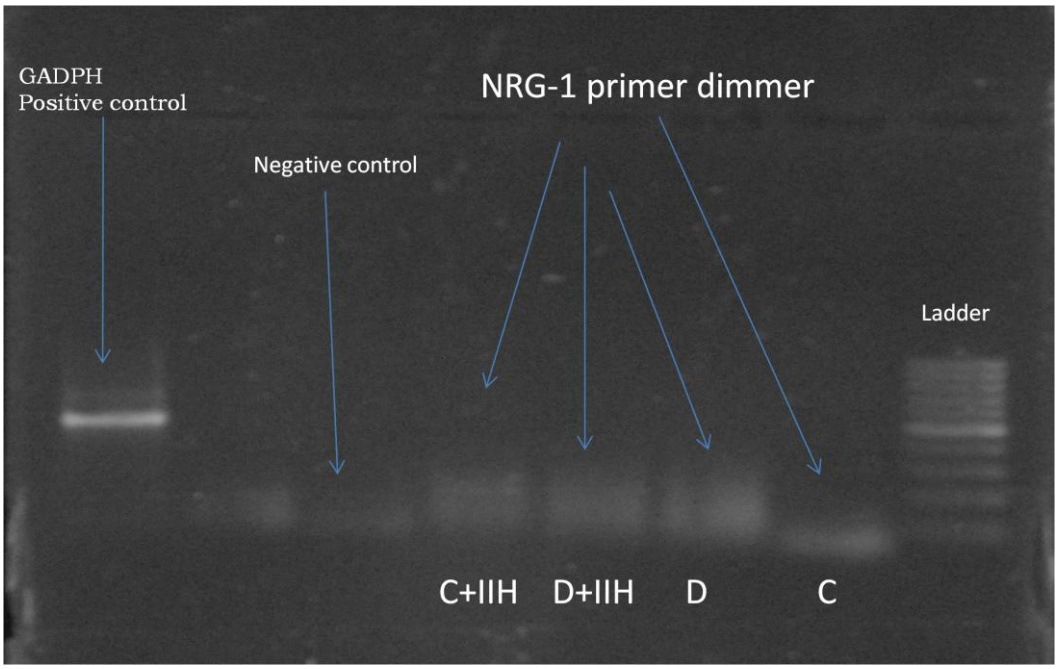


4.6 NRG 1 and ErbB 2 Receptor gene expression.

4.6.1 NRG 1 gene expression

Though we could standardize the thermo cycling profile for NRG 1 gene in control rat, we were unable to obtain a proper band corresponding to the product size of NRG 1 gene expression in experimental groups. This could possibly be due to high GC content in the NRG 1 gene primer designed by us. Forward primer contained 70% and reverse primer had 65% of GC content. GADPH gene specific primer was used as a positive control to ensure that prepared cDNA from total RNA is good enough to obtain appropriate product. We also used a negative control containing only NRG 1 Forward and Reverse primers without cDNA for confirmation of primer dimerization (Figure 16).

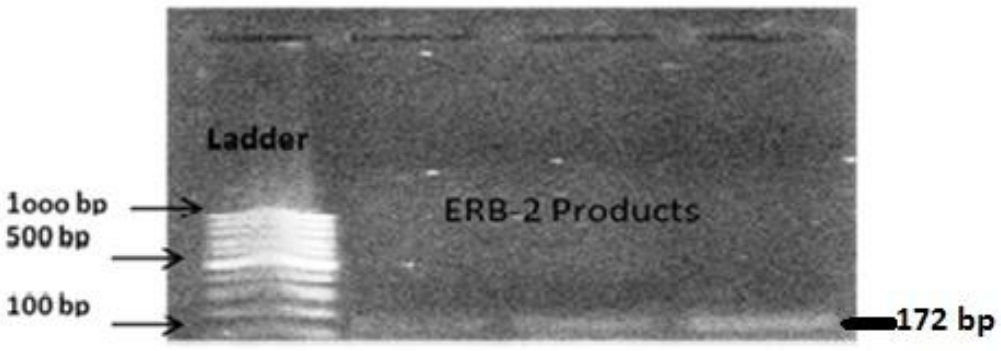
Figure 16: NRG 1 gene product in different experimental groups



4.6.2 ErbB 2 Receptor

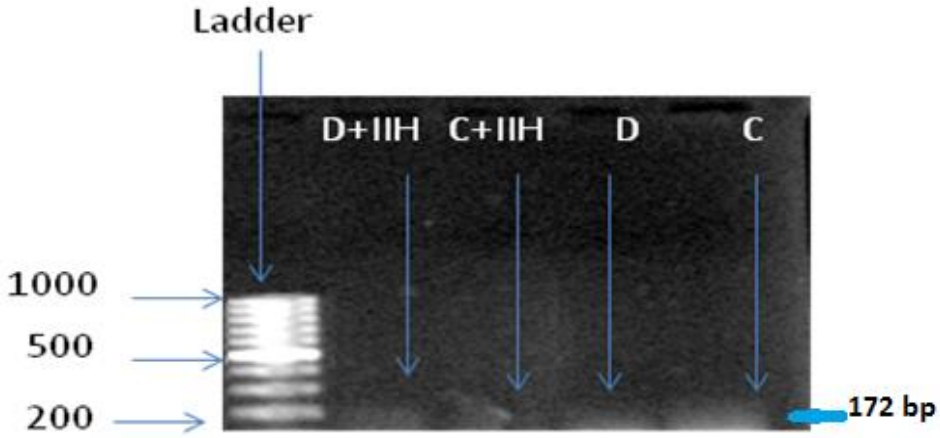
We obtained ErbB 2 receptor gene products in control and experimental groups. Figure 11 shows ErbB 2 receptor gene product in control rats at different concentration of cDNA in brain stem (BS) region (Figure 17).

Figure 17: ErbB 2 receptor gene product in control rat at different cDNA concentration



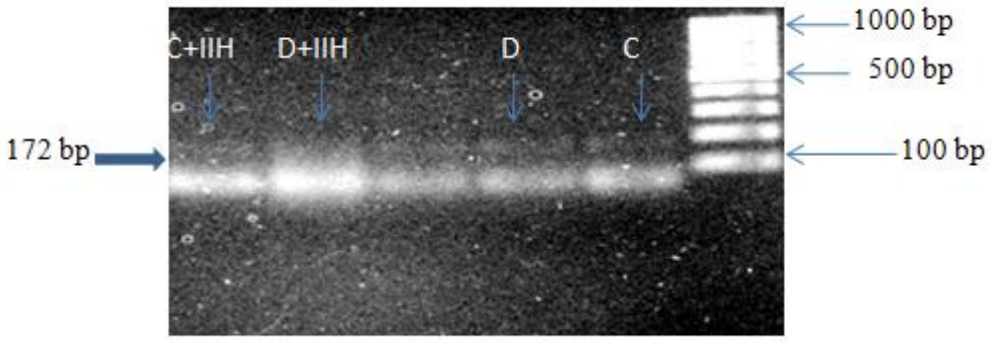
A decrease ErbB 2 expression were observed in D, C+IIH and D+IIH groups of rats compared to C group in cerebral cortex. Both D+IIH and C+IIH groups showed a small decrease in ErbB 2 as compared to D group (Figure 18).

Figure 18: ErbB 2 receptor gene expression in Cerebral cortex



In cerebellum, D+IIH group showed an increase ErbB 2 receptor gene expression compare to C, D and C+IIH groups. D group showed an increased ErbB 2 expression compare to C and C+IIH group but less then D+IIH group (Figure 19).

Figure 19: ErbB 2 receptor gene expression in cerebellum



An increase ErbB 2 expression was observed in all three experimental groups than compare to C group in brain stem. D+IIH group showed an increase ErbB 2 expression with D and C+IIH groups. Same pattern of ErbB 2 expression has been observed in D and C+IIH groups (Figure 20).

Figure 20: ErbB 2 receptor gene expression in brain stem

4.7 Behavioural studies

At 60˚ angle of beam, a significant decrease ($p<0.01$), ($p<0.01$) & ($p<0.01$) were observed in retention time on inclined beam in D, C+IIH and D+IIH groups respectively compared to C.

Table 8: The retention time (s) on beam at the different angles (30°, 45° and 60°) by the four experimental groups of rats**.**

EXPERIMENTAL GROUPS	Incline beam Angle		
	30°	**45°**	**60°**
CONTROL	30.00	8.33±0.57	5.00±1.00
DIABETIC	30.00	2.66±1.15*	1.00±0.00**
C+IIH	30.00	4.66±3.05*	1.66±1.15**
D+IIH	30.00	4.00±1.00*	1.33±0.57**

Values are mean ± SD of 2 separate experiments with 6 rats per group

IIH - Insulin-Induced Hypoglycaemia

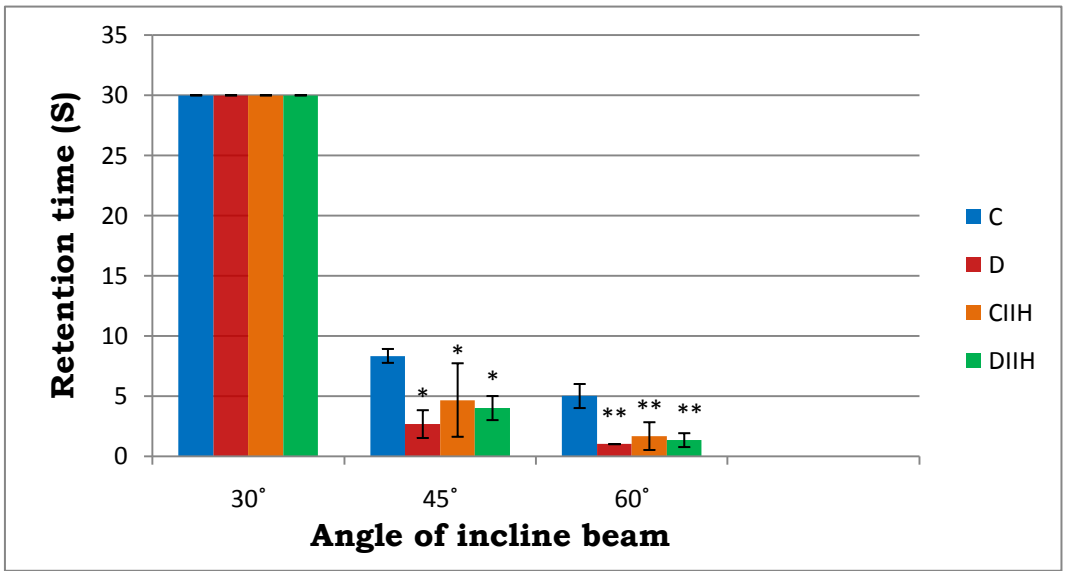
*** P < 0.001 when compared to Control, ** P < 0.01 and * P < 0.05

¶¶¶ P < 0.001 when compared to Diabetic, ¶¶ P < 0.01 and ¶ P < 0.05

$$$ P < 0.001 when compared to Diabetic+ IIH, $$ P < 0.01 and $ P < 0.05

Significant decrease ($p<0.05$), ($p<0.05$) & ($p<0.05$) were observed in D, C+IIH and D+IIH groups compared to C group at 45˚ angle. No differences were found in retention time on beam in all four experimental groups of rats at 30˚angle of beam. C+IIH and D+IIH group did not show any significant difference in retention time compared to D groups at any angles (Table 8 and Figure 21).

Figure 21: The retention time (s) on beam at the different angles (30°, 45° and 60°) by the four experimental groups of rats.



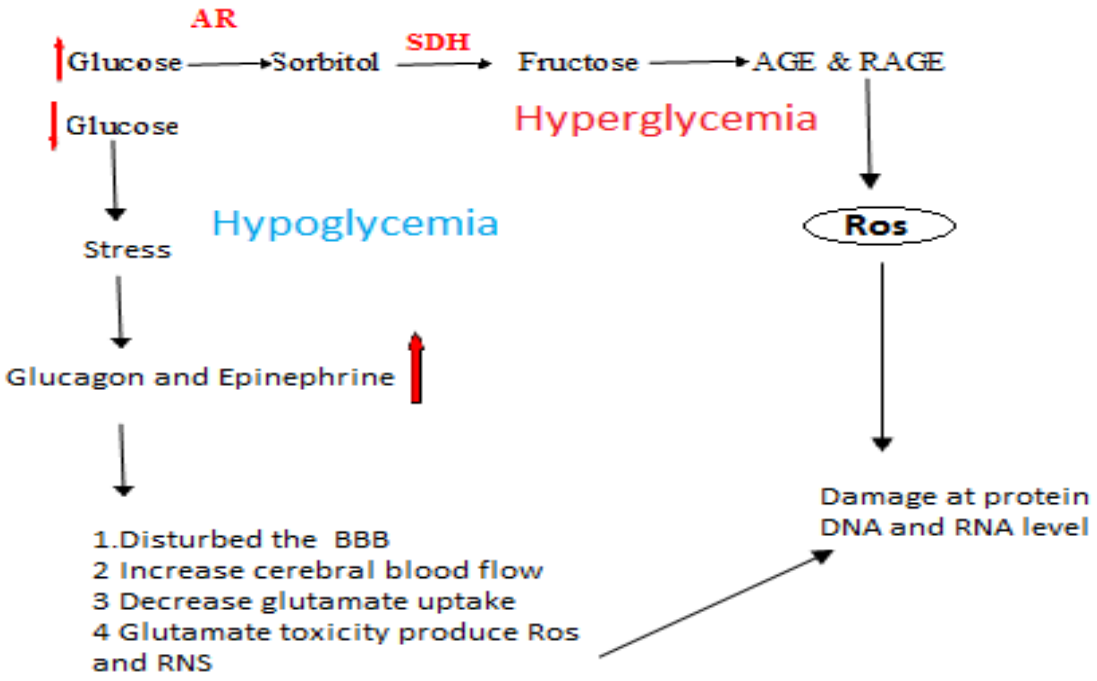
DISCUSSION

5. Discussion

Hypoglycemia is the major obstacle to optimal blood glucose control in diabetic patients. Clinically, hypoglycemic unawareness is a complication of diabetes, especially in aged patients with cognitive deficits (Widom *et al.*, 1994; McCall, 1992; Ott *et al.*, 1999; Joseph *et al.*, 2007). Hypoglycemia-induced brain injury is a significant obstacle to optimal blood glucose control in diabetic patients (Suh *et al.*, 2005). Chronic hyperglycemia leads to metabolic adaptations that render the diabetic brain more susceptible to mild hypoglycemia (Jacob *et al.* 1995). Deterioration in glucose homeostasis resulted to hyperglycemia or hypoglycemia that leads to neuronal injuries and cognitive function impairment.

We were performed a survey studies in diabetic population in Ahmedabad. Although our sample (diabetic population) size was small but available clinical data suggested that hyperglycemia leads to neuropathy (Bril, 2005; Sango *et al.*, 2008) via activation of polyol pathway (increase oxidative stress). Hypoglycemia actively contributes in the cognitive impairments, muscle weakness and loss of sensibility (desensitization), which is due to myelin damage or abnormal myelination in insulin administrated diabetic patients (Baker, 1939; Winkelman *et al.*, 1940; Mohseni., 2001). Here, a most valuable question arises weather hyperglycemia alone or intermittent during diabetes can be cause of neuropathy in insulin administered diabetic patients while they are opposite to each other. Studies by Westfall and co-workers (1983) suggested that fluctuations of the blood glucose level rather than maintained hyperglycemia underlie the development of diabetic neuropathy. Even a moderate hypoglycemia compatible with a grossly normal behaviour may leads neuropathy (Mohseni., 2001).

Figure 21: Diabetic Neuropathy due to combination of hypoglycemia and hyperglycemia.



In the present study the liver SDH enzyme activity in D group rats were significantly higher ($p<0.001$) than D+IIH and C+IIH and C groups indicating activation and maintenance of polyol pathway that leading to oxidative stress, formation of ROS resulted to neuropathy (Edwards *et al.*, 2008). Very high levels of ROS can alter signal transduction, enzyme activation, protein synthesis, DNA and RNA synthesis, cause base damage as well as strand breaks in DNA and directly influence the cell cycle, cell growth and development. Excessive oxidation damages nucleic acids, proteins and lipids, disrupting mitochondrial energy metabolism and causing increased calcium influx across the plasma membrane resulting in cell death (Sultana et al., 2006; Chathu et al., 2008).

In hypoglycemia an increased epinephrine release in the system in respond to stress condition. Yagiela (1985) reported to that epinephrine

altered the distribution of blood by its effects on the cardiovascular system. Sokrab and Johansson (1980) found that epinephrine induced hypertension resulted in increased cerebral blood flow in conscious rats. Epinephrine induces disturbance of the blood–brain barrier (Johansson and Martinsson, 1979; Chathu *et al.,* 2008) even functioning as excitatory neurotransmitter (Barchas, Ciaranello, and Steinman, 1969) decreasing the threshold for convulsions (Yokoyama, Hirakawa, and Goto, 1995) which augments an early onset of seizures during hypoglycemic state. Paulose and Co-workers (2007) reported that epinephrine decreases the uptake of glutamate in the brain causing persistent activation of glutamate receptors which is capable of causing glutamate toxicity. Glutamate activation of neuronal glutamate receptors leads to production of nitric oxide and superoxide, which combine to form peroxynitrite and related species with high reactivity towards DNA and other cell constituents (Aral *et al.*, 1998; Cavaliere *et al,.* 2001; Joseph *et al.,* 2007). Disturbance in blood-brain barrier changes the permeability of barrier to peripheral substances that might be also increase the brain toxicity.

In diabetic rats food intake and water consumption was more than that of control rats. Also the blood glucose was found to be higher than control and hypoglycemic group of rats. It is known fact that hyperglycemia causes extracellular glucose accumulation for the want of insulin activated transporters to enter into the cell. GLUT-4 is a insulin responsive glucose transporter on adipocytes, skeletal and cardiac muscle. Adipocytes have capicity to stored high amount of glucose in the form of glycogen. Skeletal and cardiac muscle show high glucose utilization. When the mass of adipose tissue increases, released leptin inhibits feeding by acting on receptors in the hypothalamus to curtail appetite and fat synthesis and stimulates oxidation of fatty acids. When the mass of adipose tissue decreases, a lowered leptin production favors a greater food intake and less fatty acid oxidation (Nelson and Cox, 2007). Hypoglycemia show low glucose at inside and outside of these cells. But the difference is low blood glucose able to initiate glycogenolysis and

gluconeogenesis pathway via release of glucagon (Jiang and Zhung, 2003) and adrenaline that increase leptin to some extent resulted hunger centre less activates then hyperglycemia.

Diabetic rats showed excess thirst due to polyurea condition. In diabetic condition high glucose leads electrolyte imbalance and exerts osmotic pressure by changing osmolariy. Osmolariy maintained by specific osmoreceptors found in the anterior hypothalamus, these modulate the release of ADH and affect thirst. A high Anti diuretic hormone (ADH) activates thirst centre supraoptic nucleus in hypothalamus.

Hypoglycemia can causes deleterious effect on both CNS (Mohseni, 2001; Pramming *et al.* 1991; Boyle *et al.*, 1994) and PNS (Fujioka *et al.*, 1997; Robinson *et al.*, 2009) Myelin damage and glial changes have also been observed in the CNS during hypoglycemia (Mohseni, 2001). NRG 1 growth factors play an essential role in myelination, neuronal survival and migration (Chaudhury *et al.*, 2003; Michailov *et al.*, 2004). NRG 1 binds with Erb2 receptor and activates downstream signalling pathway essential for glial cells activation and differentiation. Evidence proved that NRG 1–ErbB signaling is necessary for normal myelination and sensory function (Chen *et al.*, 2006). In diabetic neuropathy demyelination and axonal damage are more obviously observed. These data provide compelling support that altered expression of the NRG/ErbB ligand-receptor pair has a strong implication in demyelination. Hence, changes in the expression of proteins that affect ErbB 2 activity may have an important impact in promoting Schwann cell and oligodendriocytes degeneration.

Here we observed a significantly increased SDH activity of D group compared to C, C+IIH and D+IIH groups show activation of polyol pathway. Hypoglycemia induces stress release excess epinephrine. Chathu and Co-workes (2008) suggested that a high epinephrine increase cerebel blood flow, induces disturbance of the blood–brain barrier, act as

a excitatory neurotransmitter and also decreases the uptake of major excitatory glutamate neurotransmitter. These action of high epinephrine possibly affect the neuronal functioning and neuronal myelination in hypoglycemic groups of rats.

Our experimental data of ErbB 2 activity in diabetic and hypoglycemic groups compare to control supports the fact that low levels of ErbB 2 receptor expression in the brain might be implicated in demyelination occurring in CC during diabetes. A reduced Erb 2 receptor activity in the CC can lead to decreased oligodendriocytes development (Park *et al.*, 2001) which is chiefly responsible for myelination and ensheathment of neuron in the CNS. Also the decreased Erb 2 activity also has the potential to reduce radial neuron migration (Rio *et al.*, 1997; Anton *et al.*, 1997; Yau *et al.*, 2003) and synapse formation (Chaudhury *et al.*, 2003) ultimately leading to altered neural circuit formation and relay deficits. Neuronal circuit formation and activity remains the basis for proper sensory and motor function in the cortical region. Low activity of ErbB 2 associated with memory and cognitive function impairment in cerebral cortex. Both C+IIH and D+IIH groups shown a decrease in gene expression of Erb 2 compare to D group suggested more demyelination in CC of hypoglycemic groups.

Experimental evidence indicate the involvement of the cerebellum in variety of human mental activities including language, attention, cognitive affective syndromes (Imazhumi *et al.*, 2000) and motor relearning (Diener *et al.*, 1989, Antony *et al.*, 2010). Cerebellum participates in learning and coordination of anticipatory operations which are necessary for the effective and timely directing of cognitive and non-cognitive resources (Marchell *et al.*, 1976, Antony *et al.*, 2010). Our results shows an increased gene expression of ErbB 2 in cerebellum of diabetic and hypoglycemic groups compare with control group. D+IIH group shown increase ErbB 2 activity compared to diabetic group. A higher Erb 2 receptor activity leads to hypermyelination. Increased insulation of axons by hypermyelination restricts their access to

extracellular metabolic substrates (Nave, 2010). Cerebellar ErbB 2 impairment is maximal during insulin induced hypoglycemia leading to hypermyelination and neuronal dysfunction. These result showed that the cerebellum is not invariably resistant to hypoglycemia.

Recent studies have addressed the role of descending brain stem pathways in the development of dorsal horn hyper excitability and chronic or persistent pain (Ren *et al.*, 1999; Ren *et al.* 2000). We obtained an increased gene expression of ErbB 2 in brain stem of D group and the hypoglycemic groups compared to control group that demonstrate ErbB 2 impairment in brain stem.

Inclined beam walk test is a good indicator of impairment in motor function and coordination of limb movement (Ahmad et al., 2011) in diabetic and hypoglycemic rats. A significant decrease in the retention time on the inclined beam was observed in D compared to C suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture and at the same time adjust their limb movements on inclined beam. A decreased myelination in the limb (Feeney et al., 1981) as a result of excessive ROS formation due to activation of the Polyol pathway which is evident from increased Vmax of sorbitol enzyme activity is suggested to be indicative of neuropathy. C+IIH and D+IIH groups were also showed the significant decrease ($p<0.01$) in retention time on beam at 60° compared to C group that show the motor dysfunction possibly due to high epinephrine that might be lead to the demyelination of neurons and motor function impairment by changes in blood-brain barrier and by decreasing glutamate uptake. Proper motor functioning is controlled by cerebellum and upregulation of ErbB 2 in cerebellum suggest that the change in ErbB 2 activity directly related with demyelination and motor dysfunctioning.

CONCLUSION

6. Conclusion

A higher SDH activity, excess epinephrine, Motor impairment and altered ErbB 2 receptor expression along with clinical data suggested that diabetic neuropathy a combination effect of hyperglycemia and hypoglycemia in during diabetes and changes in NRG 1 and ErbB 2 receptors in diabetes have been implicated in the pathophysiology of major diseases of the central nervous system (CNS).

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