EFFECT OF INSULIN TREATMENT ON HEREGULIN MEDIATED REMYELINATION IN DIABETIC RATS

A Dissertation Project Submitted to Nirma University in Partial Fulfilment of Requirement for the Degree of

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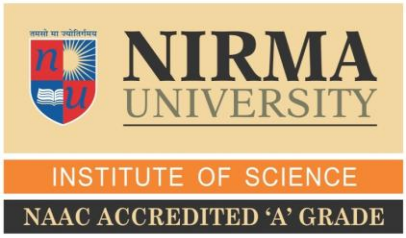
In

Biochemistry

&

Biotechnology



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Sruthi Sreenivasan

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ABRREVIATIONS USED IN THE TEXT

C	Control
D	Diabetic
D+I	Insulin Treated Diabetes
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phodphate
CNS	Central Nerve System
PNS	Peripheral Nerve System
ROS	Reactive Oxygen Species
STZ	Streptozotocin
NIDDK	National Institute of Diabetes & Digestive & Kidney Diseses
AGE	Advanced Glycation End Product
AGH	Alpha Glucosidase
GSH	Glucocorticide Suppressible Hyperaldeosteronism
SDH	Sorbitol Dehydrogenase
ECM	Extra Cellular Matrix
PKC	Protein Kinase C
GFAT	Glutamine Fructose 6 Phosphate Aminotransferase
UDP	Uridine Diphosphate N Acetyl Glucosamine
OPC	Oligodendrocytes Precursor Cell
EGF	Epidermal Growth Factor
CRD	Cysteine Rich Domain
G-6-P	Glucose 6 phosphate
G-6-Pase	Glucose 6 phosphatase
DNA	Deoxyribo Nucleic Acid

RNA	Ribonucleic Acid
ADP	Adenosine diphosphate
ATP	Adenosine tri phosphate
NO	Nitric Oxide
NRG 1	Neuregulin 1
HRG	Heregulin
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
PCR	Polymerase Chain Reaction
i.v.	Intravenous
sc.	Subcutaneous

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ABSTRACT

Glucose homeostasis has a significant role in the overall functioning of the nervous system. During hyperglycaemia the oxidative stress induced in the cell triggers various metabolic pathways capable of inducing damage to cell. Diabetic neuropathy encompasses a group of nervous system disorders and demyelination is one of its important complications. Remyelination in central nervous system is a difficult task as compared to Peripheral nervous system. In CNS, the NRG 1 -ErbB signalling pathway induces remyelination by promoting the survival and differentiation of myelin producing Oligodendrocytes. Heregulin (type I, NRG 1) and insulin both enhances the survival and differentiation of oligodendrocytes by inducing PI3/Akt pathway. In this study we hypothesised that the low insulin level could alter the Heregulin gene expression and subsequently remyelination of defected neurons.

A significant increase in food, water consumption and blood glucose level was observed in diabetic rats when compared with control group of rats. Glucose deficiency within the cell and glycosuria resulted in this increase and the decrease in body weight of diabetic rats could be an indication of excessive fat metabolism. The insulin treatment given to D+I group, brought down the readings near to control group. Blood glucose levels were also found to be higher in diabetic rats due to the lack of insulin. Motor impairment in diabetic rats were assessed by modified ladder rung test on the basis of foot fault scoring and calculation of total time and immobile time. Diabetic rats were having highest foot fault and immobile time suggesting their impaired motor function.

Specific activity of Glucose-6-Phosphatase was assayed in Liver, Muscle and Cerebral Cortex. The activity of this enzyme was found elevated in both diabetic and D+I group when compared with control rats. Also from the PCR results ,it was clear that the Heregulin gene expression gets down regulated during diabetes and the insulin treatment given to D+I group resulted in an up regulation of this gene suggesting that insulin plays a crucial role in the expression of Heregulin gene.

1. INTRODUCTION

1.1 Diabetes and diabetic neuropathy

Diabetes has become a common disorder in world population, gaining the status of an epidemic. Latest surveys show that there are up to 422 million diabetes cases worldwide. It is a chronic metabolic disorder which is characterized by high blood glucose level either due to inadequate or no production of insulin by the beta cells of pancreatic islets of langerhans (type 1) or insulin resistance of body cells (type 2) (Russell et al., 2014).

Diabetic neuropathy refers to the group of nervous system disorders caused by diabetes (NIDDK). About 60-70 % of people with diabetes have at least one form of neuropathy. The lifetime incidence of neuropathy is approximately 45% for patients with type 2 diabetes and 54% for patients with type1 diabetes. Various symptoms of diabetic neuropathy include numbness or tingling in legs and hands, indigestion, vomiting, dizziness etc. (Russell et al., 2014)

Four types of diabetic neuropathy are the following,

A. Peripheral neuropathy:

Most common type which affects mainly hands and legs

B. Autonomic neuropathy:

It causes significant alteration in digestion, bowel and bladder function. Also affects nerve which gives innervations to heart, lungs and eyes.

C. Proximal neuropathy:

It causes pain in hips, thighs, buttocks and leads to weakness in thigh.

D. Focal neuropathy:

It causes sudden weakness of one or a group of nerves causing muscle weakness/pain.

1.2 CNS Diabetic Neuropathy

The various diabetic complications of nervous system include axonal atrophy, demyelination, loss of nerve fibres and blunted regeneration of nerve fibres' (Yasuda et al., 2003). Diabetes increases the risk of stroke and other damages. Treatment with excess insulin or other oral agents can permanently damage the brain and diabetes may also increase the prevalence of seizure disorders. On long term basis diabetes changes brain transport, blood flow and metabolism which in turn results in a chronic encephalopathy. Also loss of cortical neurons and the subsequent decrease in brain volume are also complications associated with prolonged diabetes which ultimately leads to cognitive decline (Mc Call., 1992).

1.3 Patho-physiology of Diabetic Neuropathy

Diabetic neuropathy results from the oxidative stress generated in the cell and the subsequent activation of various metabolic pathways which are capable of inducing tissue damage. Some of the major metabolic pathways in diabetes contributing to diabetic neuropathy include Polyol pathway activity, formation of advanced glycation end products and various pro-inflammatory changes such as elevated NFkB and p38 MAPK signalling which results in reactive oxygen production (ROS) in the cells. Neurodegenerative changes associated with diabetes mainly occur due to the compromised vascular supply (Singh et al., 2013).

1.4 How does diabetic neuropathy cause damage to myelin sheath?

Myelin sheath is an extension of plasma membrane of Schwann cells in peripheral nervous system (PNS) and oligodendrocytes in central nervous system (CNS), which are wrapped around the nerve axon in a spiral fashion. The periodic interruptions where short portions of axons are not ensheathed by myelin sheath are the nodes of Ranvier which are critical for the functioning of the axon and the myelin. In myelinated axon, the axonal membrane is only exposed to the extra cellular space at the nodes of Ranvier which is the location of Na^+ channels. Thus, this node facilitates the saltatory conduction. Myelin sheath facilitates the nerve impulse conduction

by acting as an insulator. Myelin sheath is composed of mainly lipids (70%-85%) and a low proportion of protein (15%-30%). Also CNS myelin has some unique proteins like myelin basic protein and proteolipid protein (Siegel et al., 2007)

1.5 The major pathways through which hyperglycemia causes tissue damage are:

A. Increased flux of glucose and other sugars through the polyol pathway (Dam et al., 2013).

B. High rate of intracellular formation of advanced glycation end products (AGE'S) and activation of receptors for AGE'S (AGER).

C. Over activation of protein kinase C.

D. Increased activity of hexosamine pathway (Edwards et al., 2008).

Polyol pathway

This pathway is controlled by family of Aldo-keto reductase enzyme that acts up on a variety of carbonyl compounds like ketones which is then reduced by NADPH to their respective sugar alcohol (polyols).

During hyperglycaemic condition, the excess glucose is converted in to sorbitol by the action of the enzyme aldose reductase and then subsequently oxidized to fructose by the enzyme sorbitol dehydrogenase (SDH) with NAD as a cofactor. The increased consumption of NADPH causes an increase in redox stress. Therefore NADPH is a cofactor which is needed to generate glutathione (GSH) which is a scavenger of ROS (Edwards et al., 2008).

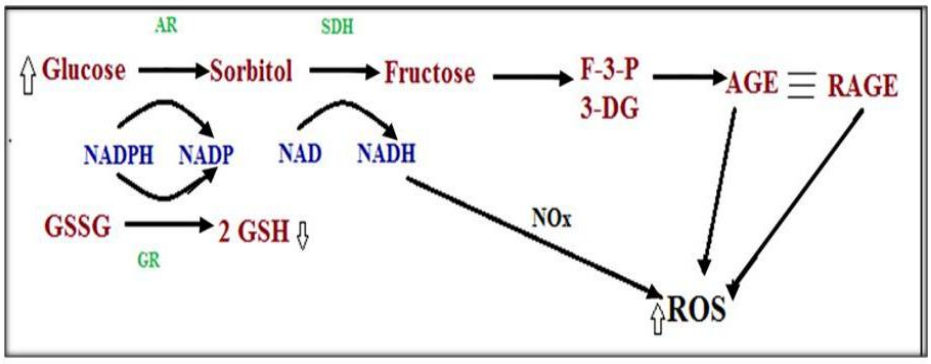

Figure 1: Schematic representation of polyol pathway

The diagram depicts the formation of ROS in the cell via the oxidation of fructose by sorbitol dehydrogenase using NAD^+ as a cofactor (Stephen et al., 2003)

Specific tissues or cells like mescengial cells in the glomerulus of renal system, neurons and Schwann cells in peripheral nerves system are more vulnerable to glucose toxicity due to the inefficient glucose uptake mechanism during hyperglycemia (Dam et al., 2013).

Three mechanisms by which intracellular production of AGE precursor cause tissue damage:

1. Alteration of intracellular proteins especially those involved in the regulation of gene transcription.
2. Diffusion of the AGE precursors in to the extracellular matrix will modify ECM molecules which then changes signalling between the cell matrix and the cell resulting in cellular function abnormalities.
3. Diffusion of the AGE precursors in to the circulation will modify circulating proteins. These modified proteins can bind to AGE receptors and subsequently activating them. This will further cause the production of inflammatory cytokines and growth factors. (Gessei et al., 2015)

PKC Activation

Hyperglycemia induces increased intracellular production of diacyl glycerol, which is a vital activating cofactor for the classic isoforms of protein kinase c, b, d, and a. once PKC is activated; it has a range of effects on gene expression (Edwards et al., 2008).

Increased hexosamine pathway activity

The excess glucose inside the cell is metabolized through glycolysis which involves several steps. The process begins with the conversion of glucose in to fructose and the subsequent steps of glycolytic pathway. However some of the fructose-6-phosphate enters to a different signalling pathway in which an enzyme called GFAT (Glutamine: fructose-6-phosphate aminotransferase) converts fructose-6-phosphate to glucosamine -6-phosphate and finally in to UDP (Uridine Diphosphate N-acetyl glucosamine). This then gets put on to serine and threonine residues of transcription factors just like the more familiar process of phosphorylation and over modification by this glucosamine often results in pathologic changes in gene expression (Dam et al., 2013).

1.6 Limitations of CNS Remyelination

There are mainly two types of factors affecting remyelination process. Disease specific like in case of multiple sclerosis and non-disease related factors such as age, sex, genetic back ground etc. One of the reasons for the failure of remyelination is the insufficient CNS pool of oligodendrocyte precursor cell (OPC's). The other prominent reason is the malfunction of OPC-recruitment which includes proliferation, migration and restoration of areas of demyelination (Franklin et al., 2008). Also myelin debris can restrict re-growth by causing oxidative stress and activating inhibitory signals (Yia et al., 2006).

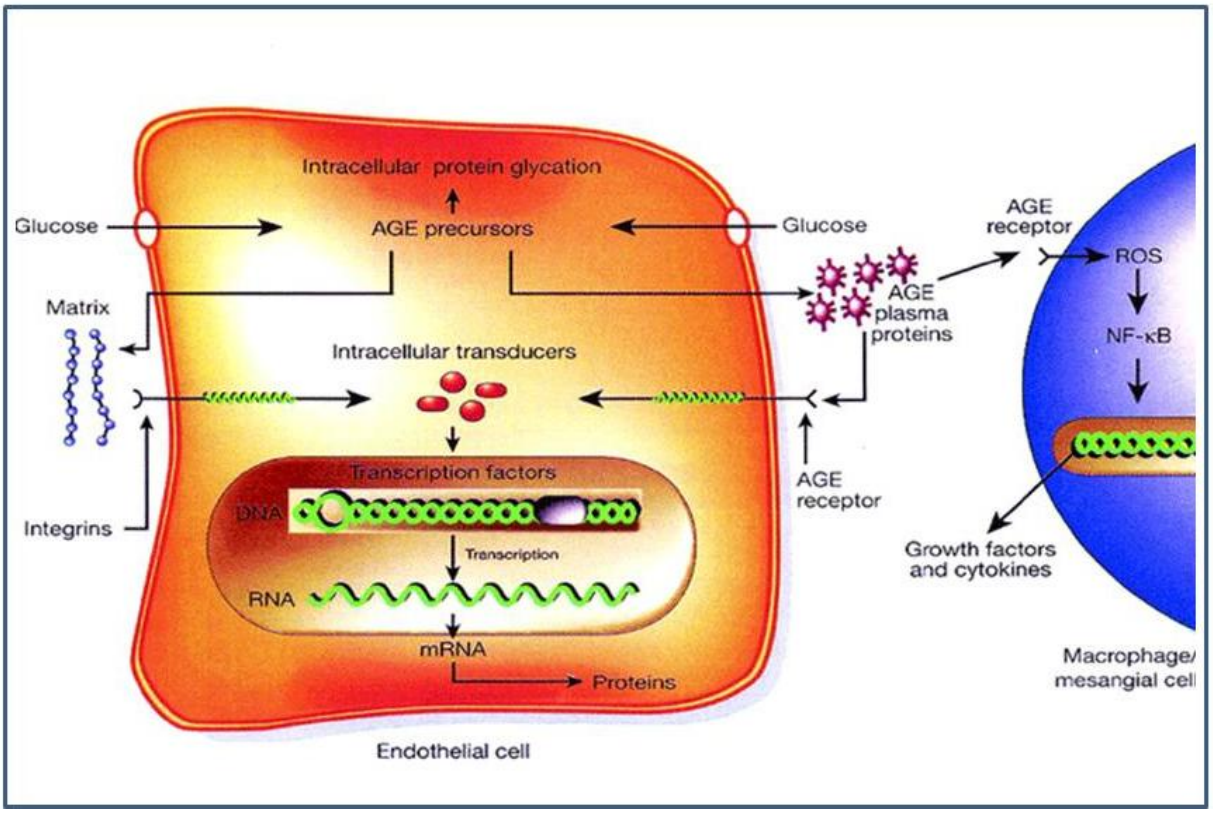

Figure 2: Schematic representation of Hexosamnie pathway

The diagram depicts the conversion of some of fructose 6 phosphate from glycolytic pathway to Uridine diphosphate N-acetyl glucosamine and subsequent changes in gene expression by phosphorylation and modification of transcription factors (Diabetes@2005 American diabetic association)

1.7 Remyelination

It is the process by which the myelin sheath is restored around the demyelinated axons and thereby restoring saltatory conduction and other functional deficits of neurons. remyelination process starts in response to demyelination which can be of two types.

A. *Primary demyelination:* due to a direct insult targeted at the myelin producing oligodendrocytes.

B. *Secondary demyelination or (Wallerian degeneration***):** myelin degenerate as a result of primary axonal loss.

There are two major reasons for primary demyelination, Genetic abnormality that affect glial cell and Inflammatory damage to myelin and oligodendrocytes. Remyelination occurs via the action of newly formed oligodendrocytes which are derived from the already existing adult oligodendrocyte precursor or stem cells distributed throughout CNS (Corfas et al., 1972)

1.8 Signalling cascades involved in CNS remyelination

Notch pathway

It is one of the fundamental signalling pathways for glial cell development and myelination in both CNS and PNS. Notch receptors comprise four members that are type 1 membrane proteins. Upon ligand binding, notch receptors are cleaved intracellularly by secretases. The resulting gamma secretase complex generates an cellular fragment iside, the notch intracellular domain which translocates to the nucleus to activate gene transcription. The type of the ligand determines whether the canonical or non-canonical signalling pathway is activated (Ables et al., 2011)

In the CNS, canonical notch 1 ligands which comprise members of delta or jagged family are expressed by neurons at early developmental stages. Notch 1 is only expressed by oligodendrocytes. Various studies have shown that binding of jagged-1 to notch inhibits OPC (oligodendrocyte precursor cells) differentiation and myelination. Notch promotes myelination

during embryonic development whereas NRG1-ErbB signals for myelination during developmental and adult stage (Taveggia et al., 2010).

Notch functions as a receptor and mammals have mainly four notch receptors (notch 1, 2, 3, 4) and many ligands including JAG1 and JAG2 and delta like proteins. Notch and its ligands are single pass trans-membrane heterodimers (Ables et al., 2011).

WNT signalling pathway

WNT's are cysteine rich glycoproteins which act as short range ligands to locally activate receptor mediated signalling pathways (Fancy et al., 2009). The hallmark of this pathway is that it activates the transcriptional activity of the multifunctional armadillo repeat-containing protein beta catenin which is the key mediator of WNT signalling (Fancy et al., 2009).

WNT pathway regulates nervous system patterning and the regulation of neural plasticity (Kahn., 2014).

Canonical WNT activity mediated through beta catenin activation inhibit oligodendrocyte maturation, independently of precursor proliferation and cell death (Feigenson et al., 2009).

Alterations in WNT-b catenin signalling in Oligodendrocyte precursor cells results in significant delay of both developmental myelination and remyelination (Fancy et al., 2009).

1.9 Neuregulin 1/ ErbB Signalling

Neuregulin 1 (NRG 1) gene produces various isoforms (NRG type 1, 2, 3) by alternative splicing. NRG 1 acts as a mitogen in CNS and reverses differentiation of myelin forming oligodendrocytes. Thereby it enables neurons to enhance the pool of pre-myelinating glial cells and only after NRG-ErbB signalling has been attenuated can myelination occur (Burden et al., 1997).

NRG1-ErbB signalling pathway regulates myelination in CNS. NRG1-ErbB Independent mechanism of myelination controlled by NRG-1 sub type share an epidermal growth factor (EGF) like signalling domain and can be divided into subgroup through their varied amino-termini. NRG-1 isoforms type and type have N-terminal IG like domain and Proteolytic cleavage. It can be released as soluble proteins from the surface of neurons.

NRG1 type defined by a cysteine rich domain (CRD) has two trans-membrane domain and is tightly associated with axonal membrane.

In the CNS, NRG1-ErbB signalling has been found to be involved in several functions like neuronal migration axonal path finding and synaptic function.

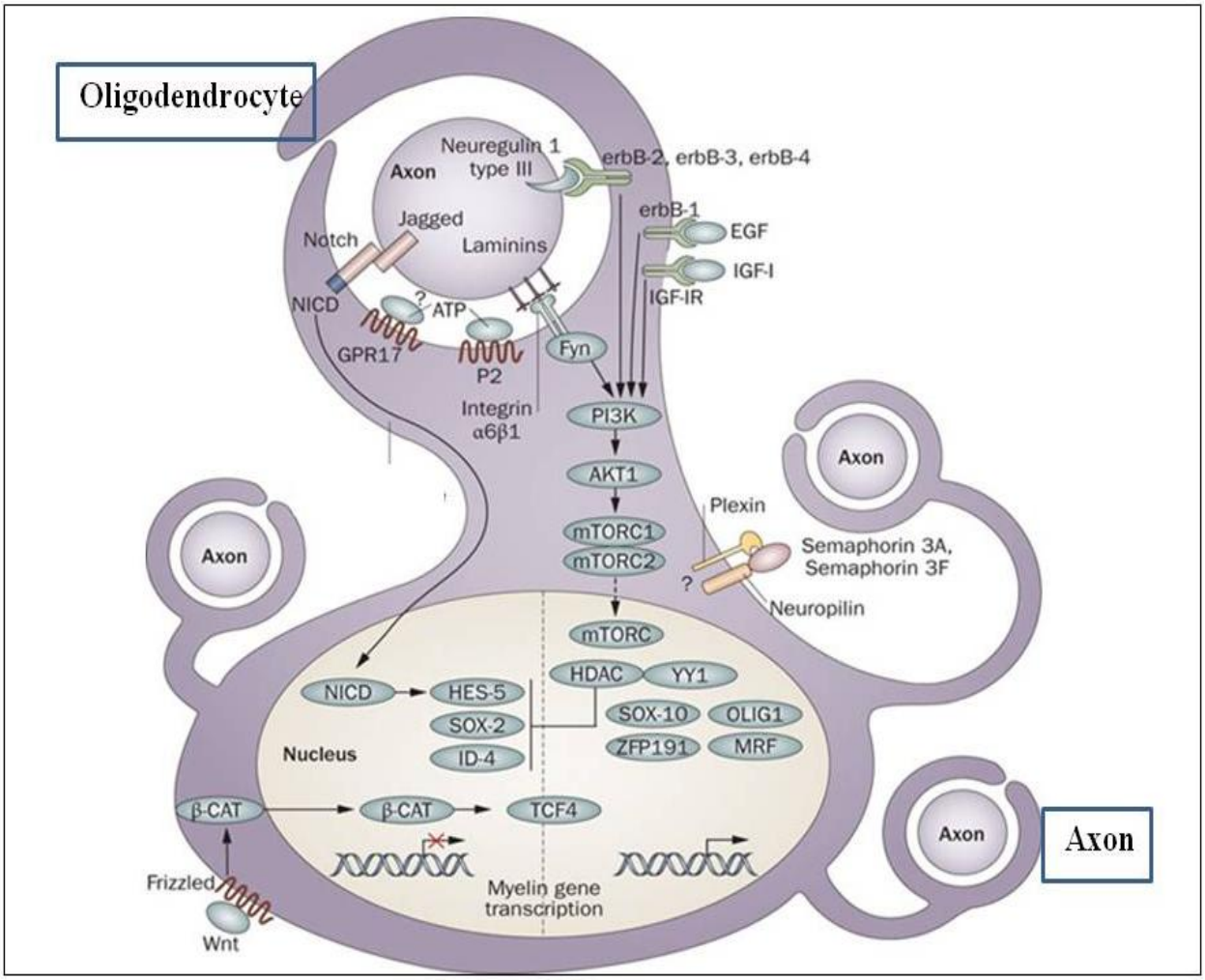

Figure 3: Schematic representation of NRG 1 - erbB pathway

This diagram depicts the Remyelination promoted by NRG1-erbB signalling pathway by inducing PI3/Akt pathway and mTOR pathway and subsequent changes in gene transcription factors (Taveggiaet et al., 2010).

NRG1 signalling also effects oligodendrocytes specification, differentiation, myelination and survival in vitro. Heregulin is one of the isoform of NRG1 which have a crucial role in the

differentiation of oligodendrocytes; critical for the myelination of neurons. Heregulin enhances the differentiation of preoligodendrocytes in to mature oligodendrocytes by inducing both mTOR mediated PI3 pathway and MAPK pathway (Canoll et al., 1996, Fernandez et al., 2000).

1.10 Glucose-6-Phosphatase

Glucose-6-phosphatase is an enzyme which catalyses the last biochemical reaction of glucose metabolism. i.e hydrolysis of glucose-6-phosphate in to glucose and phosphate.

Glucose-6-Phosphate + water $\xrightarrow{\text{G-6-Pase}}$ Glucose + Pi

This enzyme is found mainly in the liver tissue (the major Gluconeogenic and Glycogenolytic organ) and its presence is at least ten times less in other tissues of the body. Various studies have shown that there is an alteration in the gene expression of this enzyme depending on the glucose demand in the body (Liu et al., 1994).

1.11 Chemical agents for Diabetes Induction

Streptozocin

Streptozotocin is synthesized by *streptomycetes achromogenes* and is used widely to induce both insulin dependent and independent diabetes mellitus. Steptozotocin action in B cells is characterised by variation in blood insulin and glucose concentration. Two hours after injection ,increased blood glucose is observed with a significant drop in blood insulin. Pre-treatment of β cells with this inhibitor obstructs the STZ induced decrease of insulin secretion (Szkudelski., 2001).

Structure of Streptozotocin:

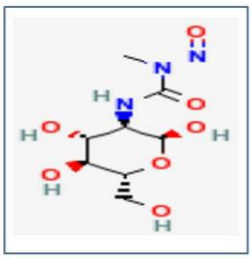

Figure 4: Structure of Streptozotocin (Rigalli et al., 2009)

Mode of Action of Streptozotocin

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 (ADP-ribose) synthatase 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 (Rigalli *et. al*., 2009).

1.12 Hypothesis

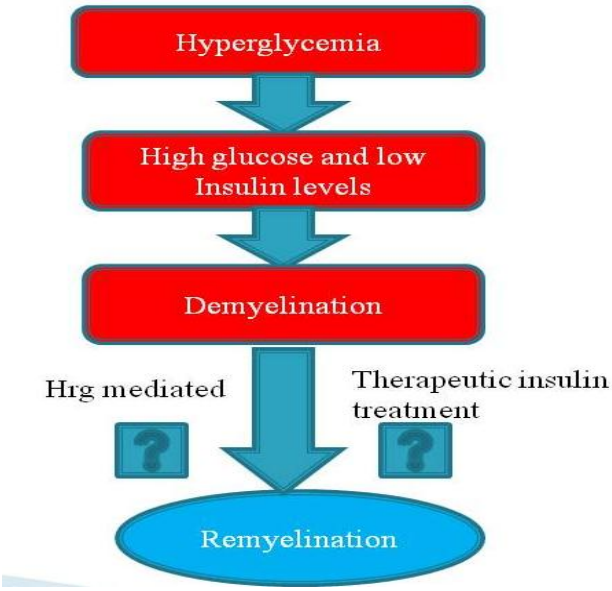

Figure 5: Schematic representation of Hypothesis

We hypothesized that the expression of NRG 1 (HRG) may reduced in diabetic condition and at Insulin treatment the expression may enhanced and which will lead remyelination in CNS.

Objectives

- To evaluate Food-water intake, change in body weight and blood glucose level in control and experimental group of rats (C, D, D+I).
- To assess Motor Function in control and experimental group of rats (C, D, D+I)
- To check the glucose-6-Phosphatase enzyme activity in Liver, Muscle and Cerebral Cortex.
- To study expression of Hrg in control and experimental group of rats (C, D, D+I).

2 MATERIALS & METHODS

2.1 Chemicals

Streptozotocin (MP Biochemicals), EDTA (Merck specialities private limited), Actrapid human insulin (Novo Nordisk India Pvt. Ltd), Sodium Hydroxide (HIMEDIA laboratories pvt. Ltd), Bovine serum albumin (Central Drug House), Sodium Carbonate, Folin Ciocalteu Reagent (Sisco Research Lab), Tris-HCl (HIMEDIA laboratories pvt. Ltd.), Sucrose (S.D. Fines), Tri reagent (Sigma Aldrich), cDNA synthesis kit (Thermo scientific), PCR Master Mix (Thermo scientific), Primers (Thermo scientific), Bromophenol blue (Thermo scientific), Agarose (Himedia laboratories pvt. Ltd.), Ethidium bromide (Genetix bitech asia pvt. Ltd.), DNA ladder (Thermo scientific). Nuclear free water (Genetix biotech asia pvt. Ltd.) Trichloro acetic acid (Merck specialities pvt. Ltd.) Sucrose (Merck specialities pvt. Ltd.), L-Ascortic acid (Himedia laboratories pvt. Ltd.), Ammonium heptamolybdate tetrahydrate (Merck limited), Hydrazine sulphate (Central Drug House pvt. Ltd.) Dithiothreoitol (Himedia laboratories pvt. Ltd.). All Chemicals used in the present study were of analytical grade.

2.2 Animals

Adult male Wistar rats of 200-250 g body weight were bought from Bharat serums and vaccine ltd Mumbai and were used for all experiments. They were kept in separate cages under 12-h light and 12-h dark periods and they were maintained at 25±3 °C. A definite amount of food and water were given to the rats on daily basis. Animal care and procedures were carried out according to the Institutional and National Institute of Health Guide lines.

2.3 Diabetes Induction

Animals were divided into following experimental groups as:

1.) Control [C]

2.) Diabetic [D]

3.) Diabetic +Insulin [D + I]

Each group consisted of 6 animals. Diabetes was induced by a single intrafemoral dose (i.v.) (50 mg/kg body weight) of Streptozotocin prepared in citrate buffer, pH 4.5 (Arison et al., 1967; Hohenegger et al., 1971; Robinson et al., 2009). D+I group received daily 2 doses (sc.) (1 IU/Kg body weight) of regular Human insulin (Actrapid) (Flanagan et al., 2003).

2.4 Water and Food consumption

Water and Food consumption were recorded throughout the experiments days. Water intake and food consumption checked out during the active phase (12 hour) and inactive Phase (12 hour) throughout the experiment.

2.5 Body Weight and Blood Glucose measurement

Body Weight Were recorded throughout the experiments days. Blood glucose was estimated by Glucometer Using Care-Sens N strips.

2.6 Arched ladder rung test

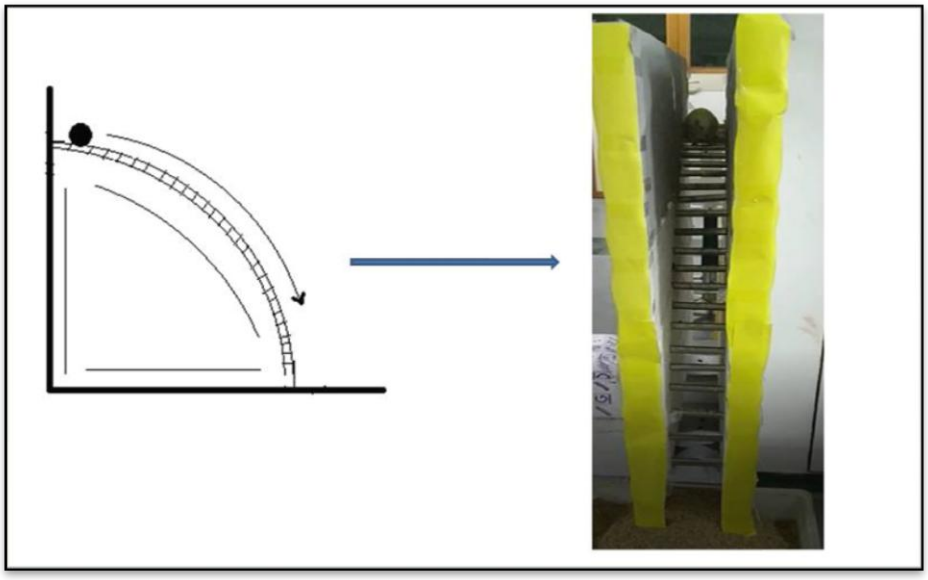

Figure 6: Diagrammatic representation of Arched ladder rung
Rung length – 10 cm, Rung diameter – 2.5 cm, Distance between two rungs – 5 cm

In this modified apparatus, ordinary ladder was modified in to the shape of an arc in order to give more complicated motor task for the experimental group of rats. Total distance across the ladder was 115 cm, dimension of rung 2.5 cm, width total rung length 10 cm and distance between two rung 5 cm. Home cage was the motivation stimulation to cross the ladder the rats.

2.7 Behavioural test and test analysis

All rats were trained in the model and videos were recorded for 5 min. most of the rats crossed the ladder from top to bottom.

2.8 Foot fault scoring

Table 1: Parameters for Foot Fault scoring (Metz and Whishaw, 2002)

Foot fault parameters	**Characteristics**
Total miss	Deep fall after limb missed the rung completely
Deep slip	Deep fall after limb slipped off the rung
Slight slip	Slight fall after limb slipped off the rung
Replacement	Limb replaced from one rung to another rung
Correction	Limb aimed for one rung but was placed on another rung Or: Limb position on same rung was corrected
Partial placement	Limb placed on rung with either digits/toes or wrist/heel

2.9 Tissue preparation

Rats ware sacrificed on 6 week of model induction by cervical dislocation and then brain parts (cerebral cortex) and body parts (liver, muscle) were dissected out. The tissues were stored at -20°C.

2.10 Specific Activity of Glucose-6-Phosphatase

- Sucrose (0.25 M)
- Triethanolamine (20Mm)
- Dithiothreitol (0.1Mm)
- Glucose-6-phosphate
- Tris-chloride buffer (135Mm)
- EDTA (5Mm)
- Trichloroacetic acid (10%)
- Ammonium molybdate (2.5%)
- Potassium dihydrogen phosphate
- Reducing agent (Ascorbic acid and hydrazine sulphate)

Isolation of Glucose 6 phosphatase

Glucose 6 phosphatase activity in liver, muscle and cerebal cortex was done using spectrophotometric method. Homogenate (liver 25%, muscle 10% & cerebal cortex 10%) was prepared in isolation buffer (0.25 M sucrose buffered with 20 mM triethanolamine pH 7.4 and 0.1 mM dithiothreitol) and was then centrifuged at 1000xg for 10 min at 4º C. supernatant was collected and used as a enzyme source.

Estimation of Glucose 6 phosphatase activity

Then final volume 1 ml assay mixture was prepared (135 mM trischloride buffer, 5 mM EDTA and substrate Glucose 6 phosphate (1.25 mM to 15 mM)). Enzyme extract was added and then put it in incubation at room temperature for 15 min. Ice cold 10% Trichloroacetic acid was added to stop the reaction and centrifuged at 8000xg for 10 min and supernatant was collected for phosphate estimation (Baginski et al., 1974).

In phosphate estimation, supernatant was collected and amoniummolybdate (2.5% in 3 N H_2SO_4) was added and kept for 10 min incubation at room temperature. Reducing agent (2% L ascorbic

acid and hydrazine sulphate in 0.1 N H_2SO_4) was added. Incubated for 40 min at room temperature and read at 660 nm. Activity of enzyme was expressed as specific activity measured in μmoles/min/mg protein.

Protein Estimation: Protein was estimated by the method of Lowry et al.,1951 using Bovine serum albumin as standard.

2.11 PCR analysis

Total RNA was isolated from the cerebral cortex using Tri reagent. Total cDNA synthesis was performed by using cDNA synthesis kit. PCR analyses were conducted with gene-specific primers.

Table 2: Primer sequence of Hrg gene

Gene	**Sequence**	**Length**	**Product Size**	**Tm (C)**	**GC Content (%)**
Heregulin	F:CCTCTTATTTGTCTGAAC R:CCTTCCACATTACATTAC	18 18	102	52 52	48.9% 48.9%

3 RESULTS AND DISCUSSION

3.1 Food consumption

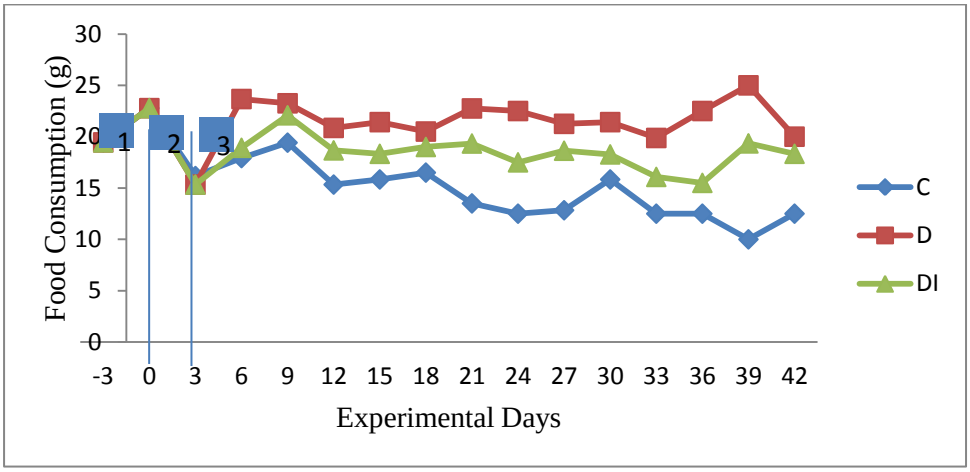

Figure 7: Representative Diagram of food Consumption during active phase in control and experimental group.

1= Pre diabetic condition, 2= Post diabetic condition, 3=Insulin treatment

C= Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

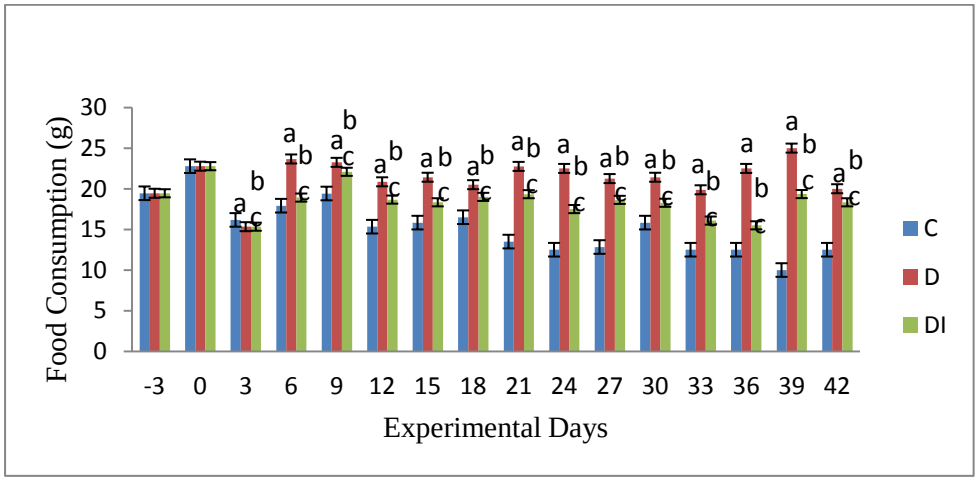

Figure 8: Food Consumption during active phase in control and experimental groups

C= Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

Values are Mean ± SD of 3 separate experiments (n=5 to 6 rats per group).

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

a-$p<0.001$ when compared to C, b-$p<0.05$ when compared to C, c-$p<0.05$ when compared to D

During active phase, a significant increase (P<0.01) in food consumption of D was observed compared to C. D+I have decrease (P<0.05) in food consumption as compared to D.

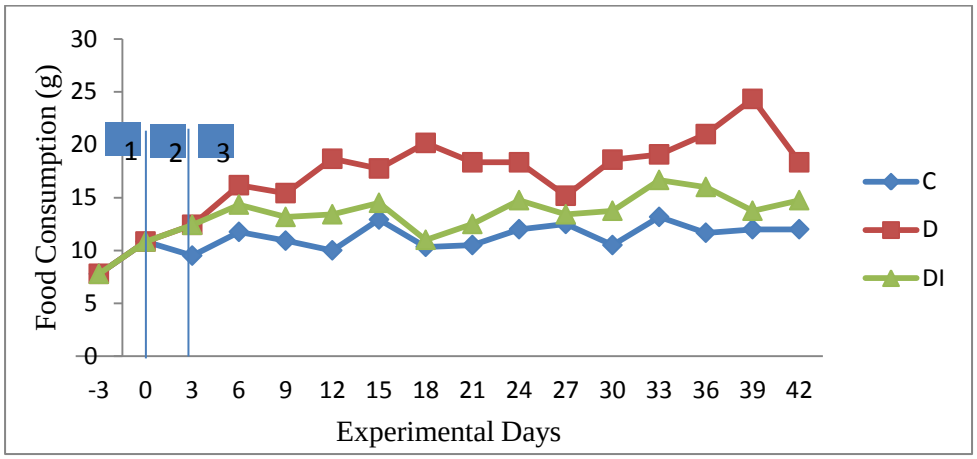

Figure 9: Representative diagram of food Consumption during inactive phase in control and experimental groups

1= Pre diabetic condition, 2= Post diabetic condition, 3=Insulin treatment
C= Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

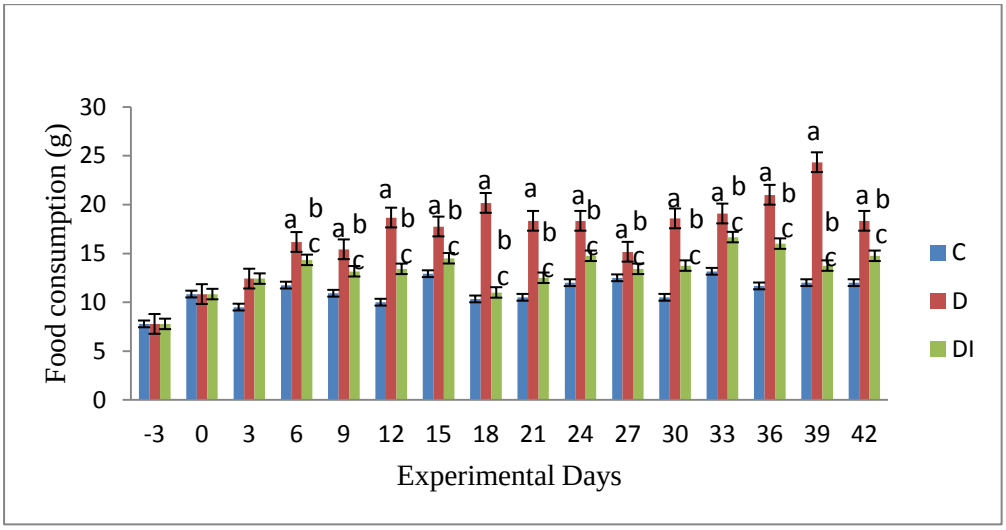

Figure 10: Food Consumption during inactive phase in control and experimental groups

C= Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
Values are Mean ± SD of 3 separate experiments (n=5 to 6 rats per group).
ANOVA followed by Student's-Newman-Keul's Test.
a-p<0.001 when compared to C, b-p<0.01 when compared to D, c-p<0.05 when compared to C

During inactive phase, a significant increase (P<0.01) in food consumption of D was observed compared to C. D+I have decrease (P<0.05) in food consumption as compared to D.

In D group, increase in food consumption that is because of lack of Insulin that prevents the entry of glucose in the cell which lead low energy production or no energy to perform cellular activities. This sends the continuous hunger signal from the appetite centre in the brain causing the body to crave more food thus increasing food consumption. Food consumption in D+I group gradually decreases as compared to D (Akbarzadeh et al., 2003).

3.2 Water consumption

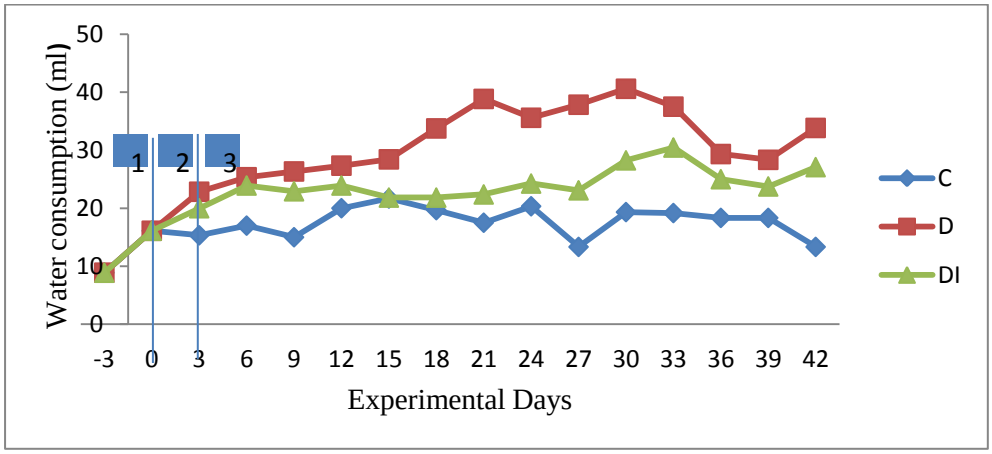

Figure 11: Representative diagram of Water Consumption during active phase in control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
1= Pre diabetic condition, 2= Post diabetic condition, 3=Insulin treatment

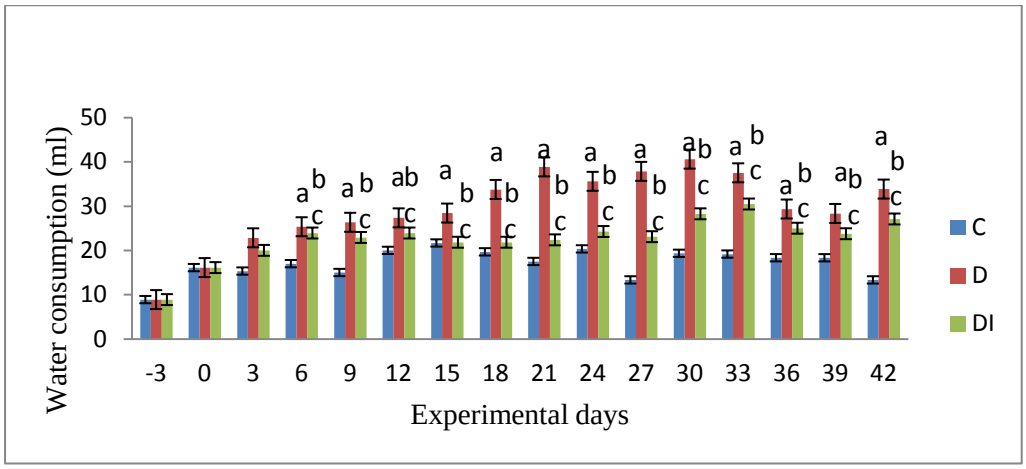

Figure 12: Water Consumption during active phase in control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
Values are Mean ± SD of 3 separate experiments (n=5 to 6 rats per group).
ANOVA followed by Student's-Newman-Keul's Test.
a-$p<0.05$ when compared to C, b-$p<0.05$ when compared to C, c-$p<0.001$ when compared to D

During active phase, a significant increase ($p<0.01$) in water consumption of D was observed compared to C and in D+I there was significant decrease ($p<0.05$) in water consumption compared to D.

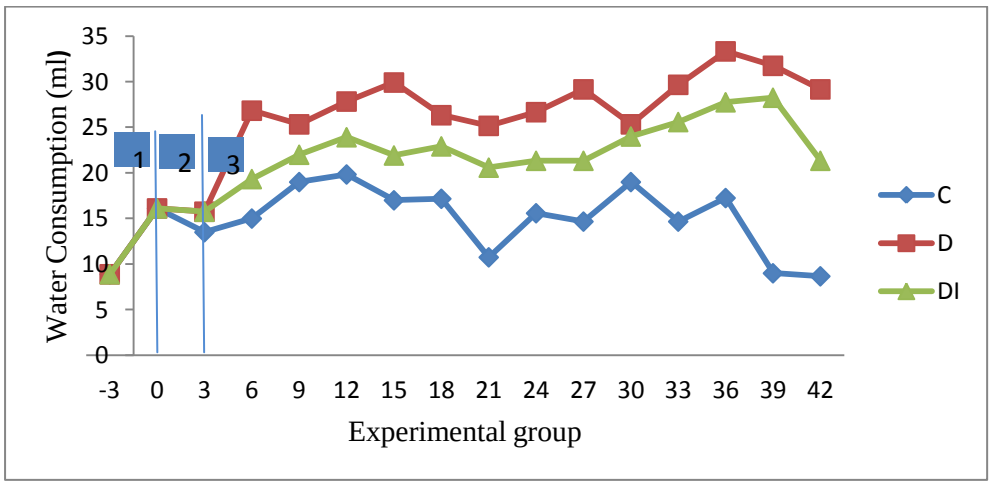

Figure 13: Representative diagram of Water Consumption during inactive phase in control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
1= Pre diabetic condition, 2= Post diabetic condition, 3=Insulin treatment

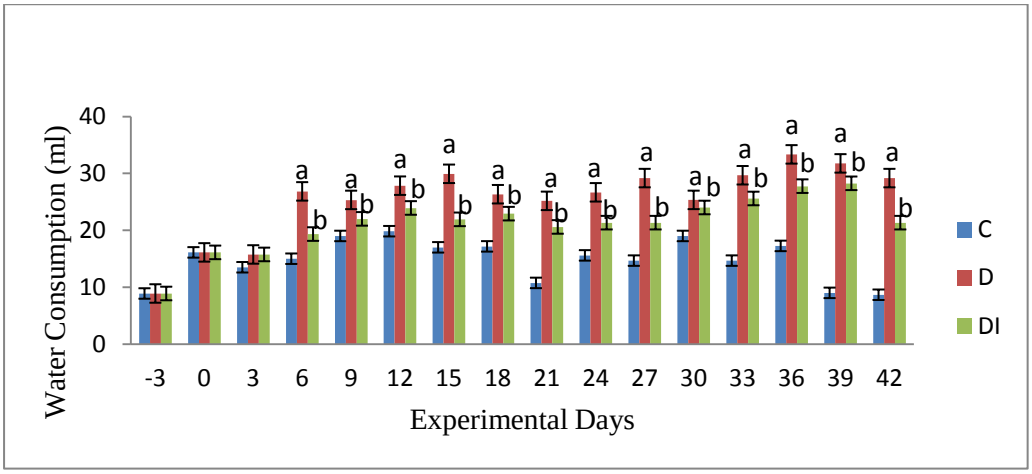

Figure 14: Water Consumption during inactive phase in control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
Values are Mean ± SD of 3 separate experiments (n=5 to 6 rats per group).
ANOVA followed by Student's-Newman-Keul's Test.
a-$p<0.001$ when compared to C, b-$p<0.01$ when compared to C

During inactive phase, a significant increase ($p<0.01$) in water consumption of D was observed compared to C and in D+I there was significant increase ($p<0.01$) in water consumption compared to C.

Increase in blood glucose alters the glomerular filtration rate of the kidney. Because of excess blood glucose (glycosuria), leads presence of high glucose in urine. Glycosuria leads decrease in blood glucose levels by excreting excessive glucose in urine. Dilution of this concentrated urine leads more secretion of water from tubular region of nephrons which activates thirst centre in brain to compensate for water loss thus increasing water consumption (Akbarzadeh et al., 2003).

3.3 Change in Body Weight

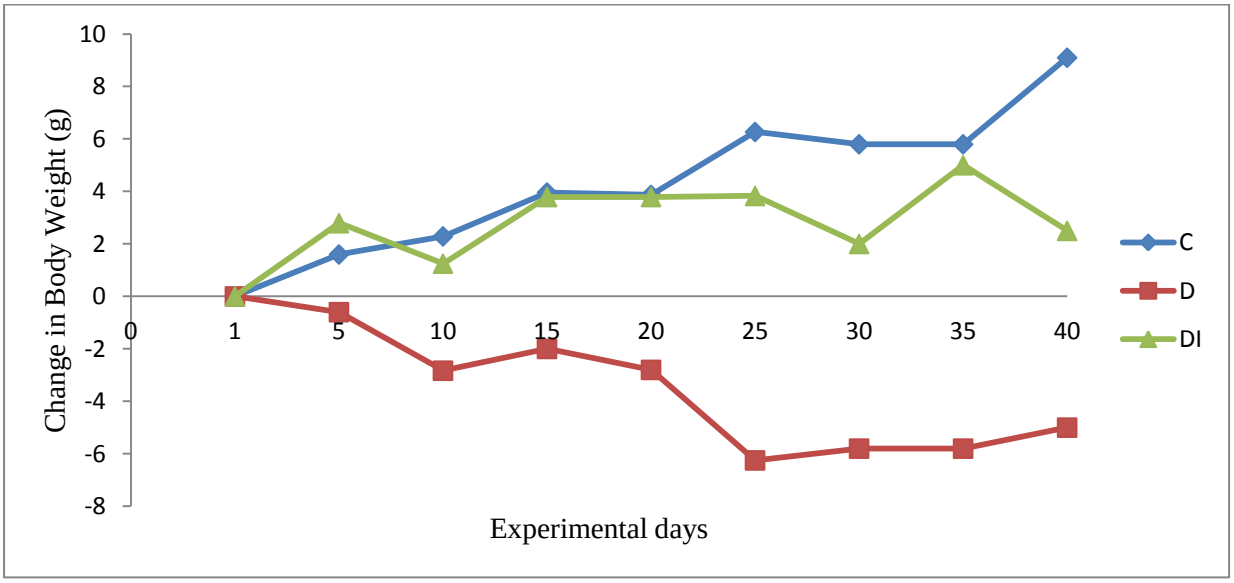

Figure 15: Representative diagram of Change in Body Weight in control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

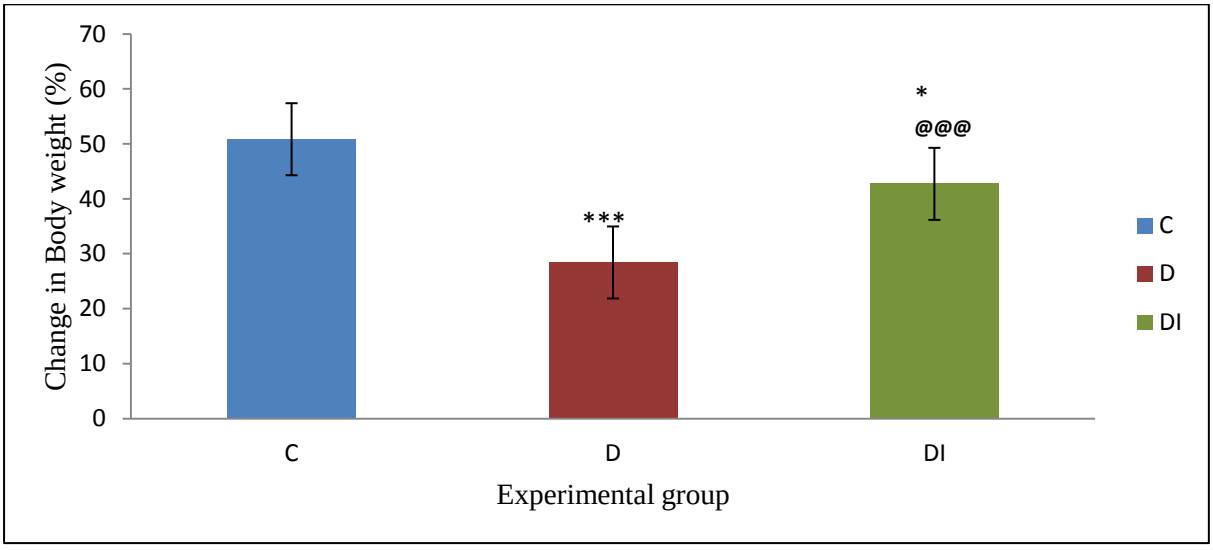

Figure 16: %Change in Body Weight in control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

Values are Mean ± SD of 3 separate experiments (n=5 to 6 rats per group).

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

***-p<0.001 when compared to C, *-p<0.05 when compared to C, ###-p<0.001 when compared to D

When compared with the control group of rats,there was a significant decrease in the body weight of diabetic group of rats. In diabetic condition, the excess glucose level in blood prevents the insulin mediated entry of glucose in to the body cells as a result of which the cell triggers alternative pathways for glucose production like glycogenolysis and glucogenolysis. Glycogenolysis would cause the depletion of glycogen reservoirs and will result in lipolysis. Also polyuria during diabetes would cause the loss of body fluids and electrolytes from the body (Wood et al.,2004). All these factors lead to the decrease in body weight of diabetic group of rats when compared to control rats. On the other hand ,a significant increase in the bodyweight of D+I group of rats can be attributed to the insulin treatment given to this group which prevents excessive metabolism.

3.4 Blood Glucose

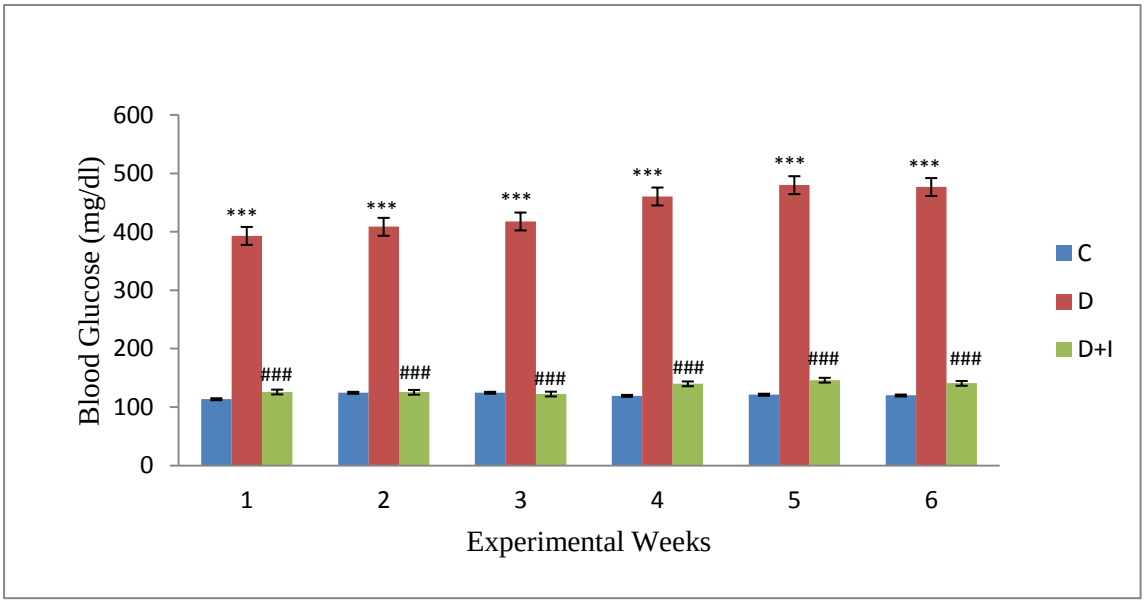

Figure 17: Blood Glucose in control and experimental group

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
Values are Mean ± SD of 3 separate experiments (n=5 to 6 rats per group).
ANOVA followed by Student's-Newman-Keul's Test.
***-p<0.001 when compared to C, ###-p<0.001 when compared to D

A significant increase (p<0.001) in blood glucose observed in D group compared to C. D+I group showed a decrease (p<0.001) in blood glucose compared to D.

In the adipose and muscles tissue, Insulin activates glucose transportation and also promotes glycogen and triglyceride's synthesis (Genuth et al, 2001). In diabetes blood glucose levels

elevates due to low Insulin production or Insulin resistance. Thus increase in blood glucose levels are observed in D as compared to C. In D+I, due to insulin administration, blood glucose levels lower down.

3.5 Arched Ladder Rung Test

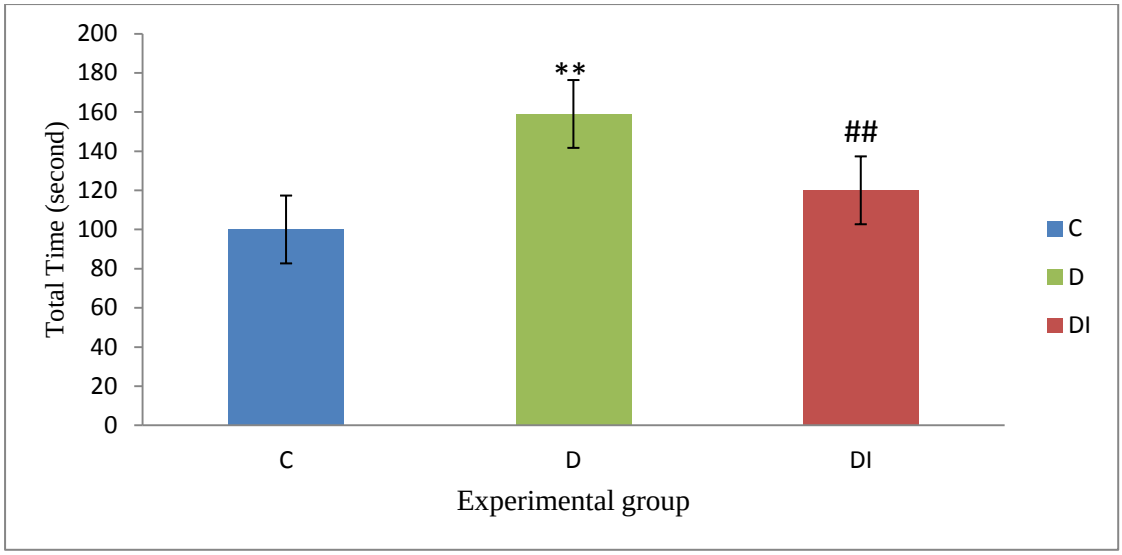

Figure 18: Total time taken to cross the ladder by control & experimental groups
Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
Values are Mean ± SD of 3 separate experiments (n=3 rats per group).
ANOVA followed by Student's-Newman-Keul's Test.
**-$p<0.01$ when compared to C, ##-$p<0.01$ when compared to D

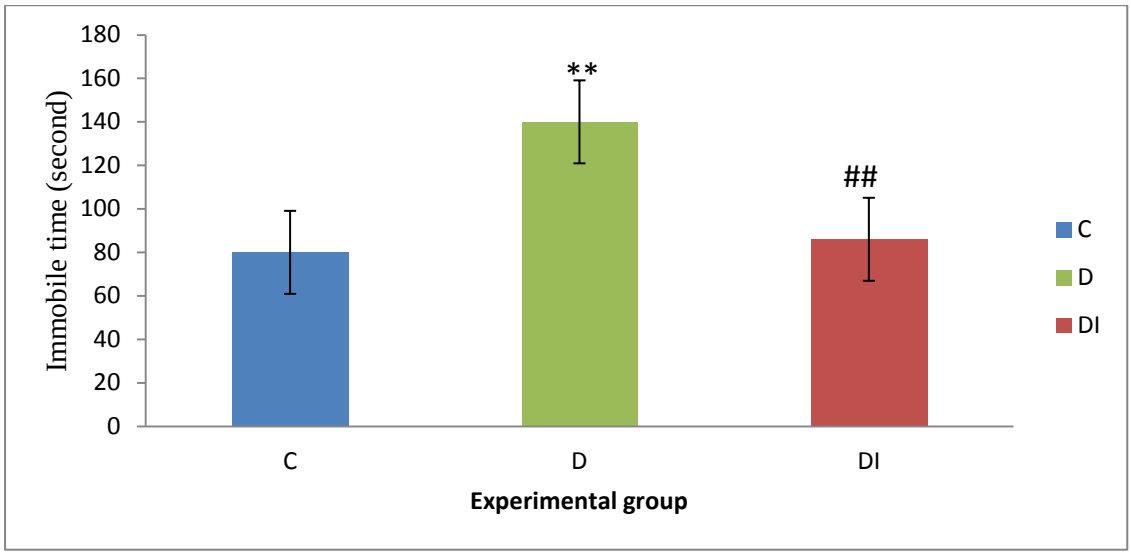

Figure 19: Immobile time of control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
Values are Mean ± SD of 3 separate experiments (n=3 rats per group).
ANOVA followed by Student's-Newman-Keul's Test.
**-p<0.01 when compared to C, ##-p<0.01 when compared to D

A significant increase in total time to cross the ladder and immobile time was observed for diabetic rats when compared with control rats and no significance was obtained for comparison among the three experimental groups.

3.6 Foot fault Scoring

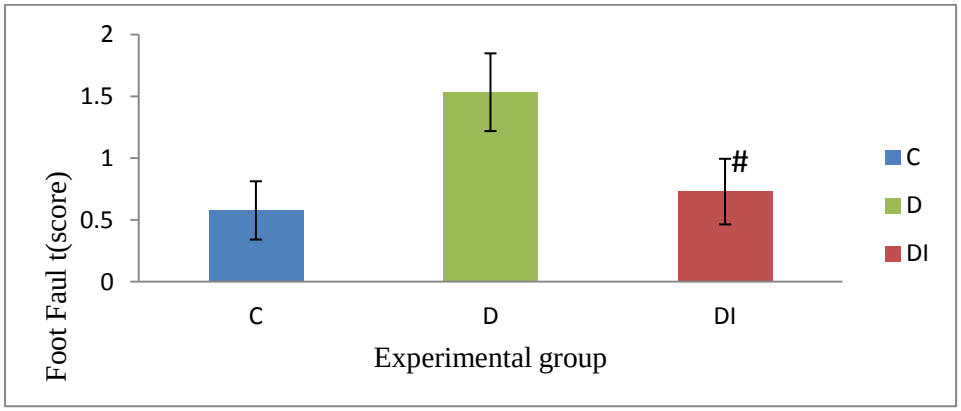

Figure 20: Foot Fault score of control and experimental groups

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats
Values are Mean ± SD of 3 separate experiments (n=3 rats per group).
ANOVA followed by Student's-Newman-Keul's Test.
#-p<0.05 when compared to D

A significant increase ($p<0.05$) in foot faults was observed for diabetic rats when compared to control rats and a decrease ($p<0.05$) for D+I group of rats when compared with diabetic rats

Modified ladder rung was used for assessing the motor function impairment in the experimental group of rats. The idea behind the modification of the ladder was to assign a complicated motor task for the experimental groups. Performance on the ladder rung were analysed on the basis of foot-fault scoring and by counting the mobile and immobile time period in every quadrant of the arc model. When compared with control and D+I group of rats, diabetic group of rats were having high foot faults indicating the impairment in their ability to coordinate their limb movements and to maintain balanced posture. Also mobile time period was highest for diabetic group of rats which can be related with their slow movements in taking steps. Control group of rats crossed the ladder within a short period of time and same with D+I group of rats when compared with diabetic group. Most of the rats remained in the first quadrant (top portion of the arc) initially and after a short time period, they moved down to home cage. The longer duration of time spend by the diabetic group of rats could be an indication of the anxiety developed in this group of rats. Insulin treatment given to the D+I group was evident from their walk performance on the ladder; comparable to control group. The decreased myelination of neurons during diabetes due to ROS production could result in the impairment in motor function

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3.7 Glucose 6 phosphatase enzyme activity

Glucose 6 phosphatase enzyme activity in Liver

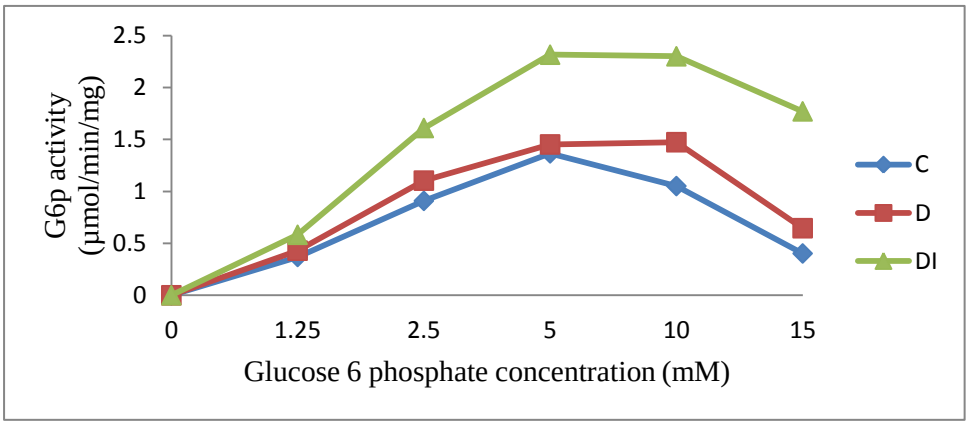

Figure 21: Glucose 6 phosphatase activity in Liver in control and experimental group
Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

Table 3: Glucose 6 phosphatase activity in liver in control and experimental group

Experimental groups	Vmax	Km
C	0.12	1.23
D	0.13	1.61
DI	0.23	1.87

Glucose 6 phosphatase enzyme activity in Muscle

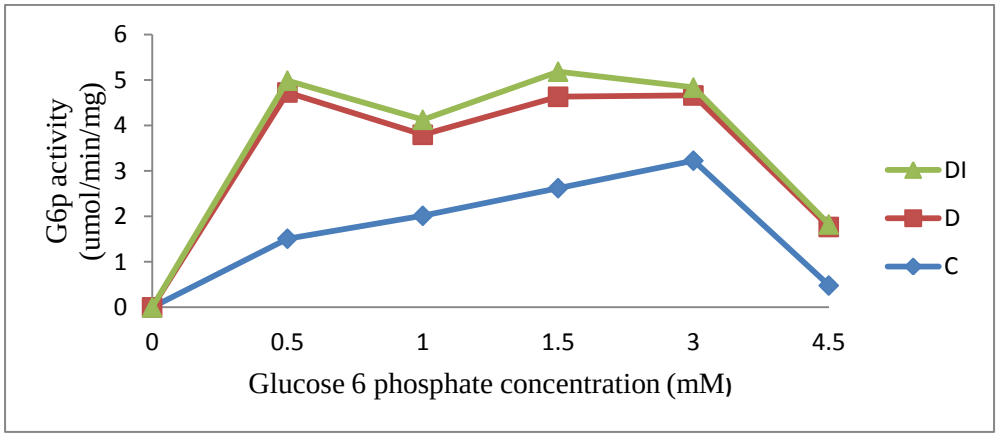

Figure 22:Glucose 6 phosphatase activity in Muscle in control and experimental group
C= Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

Table 4: Glucose 6 phosphatase activity in liver in control and experimental group

Experimental groups	Vmax	Km
C	0.1084	0.55
D	0.0934	0.25
DI	0.1446	0.55

Glucose 6 phosphatase enzyme activity in Cerebral Cortex

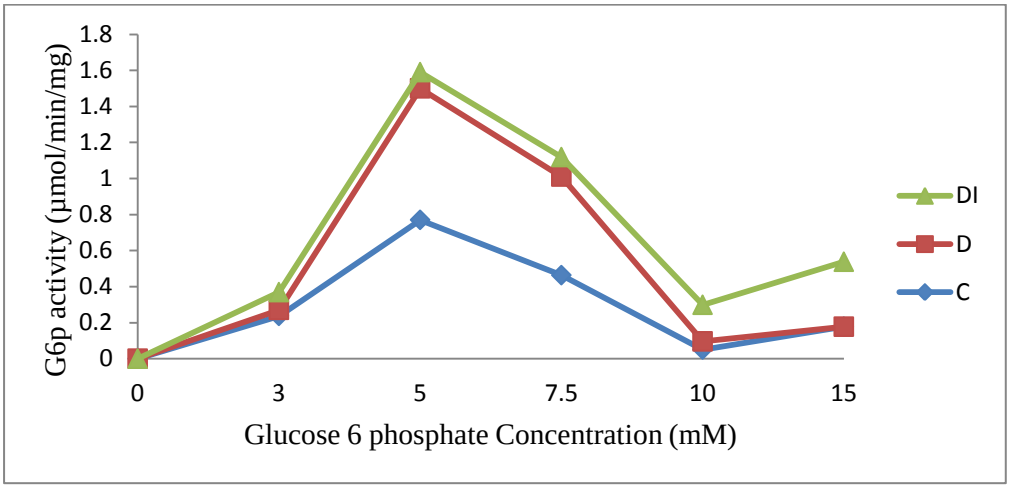

Figure 23: Glucose 6 phosphatase activity in Cerebral Cortex in control and experimental group

Control Rats, D= Diabetic Rats, D+I = Insulin treated Diabetic Rats

Table 5: Glucose 6 phosphatase activity in Cerebral Cortex in control and experimental group

Experimental group	Vmax	Km
C	0.13	1.63
D	0.06	0.75
DI	0.14	1.13

Glucose-6-Phosphatase enzyme is predominantly present in liver which is the major gluconeogenic tissue to meet the glucose requirements of the body. In diabetic condition, the glucose demand of the cell is fulfilled by both gluconeogenesis and glycogenolysis. The increase in G-6-Pase activity and resultant endogenous glucose production was associated with a reduction in insulin secretion. Ideally in case of D+I group, the insulin treatment should have bring down the plasma glucose to normal level. But in contrast D+I group shows highest G-6-Pase activity in all the three tissues (Liver, Muscle and Cerebral cortex).

Since we did not get positive results for the repeated experiments, results are represented without standard deviation and significance value.

3.8 Hrg gene expression

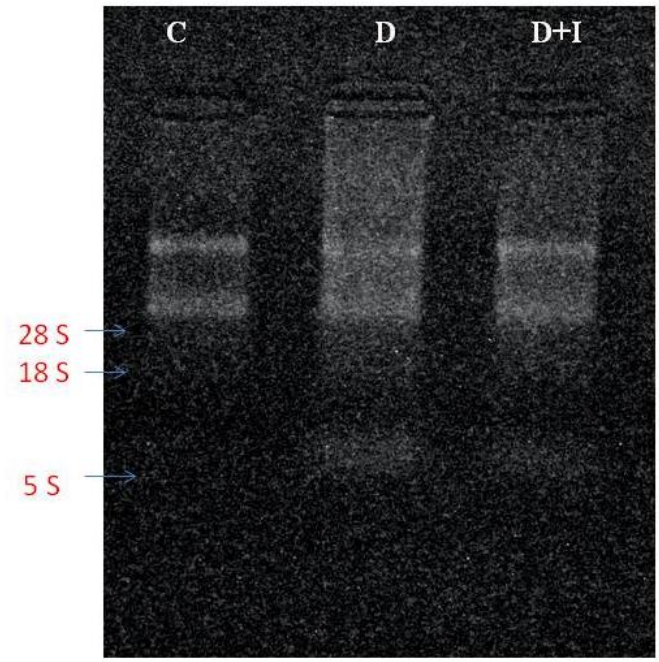

Figure 24: Total RNA bands in control and experimental group

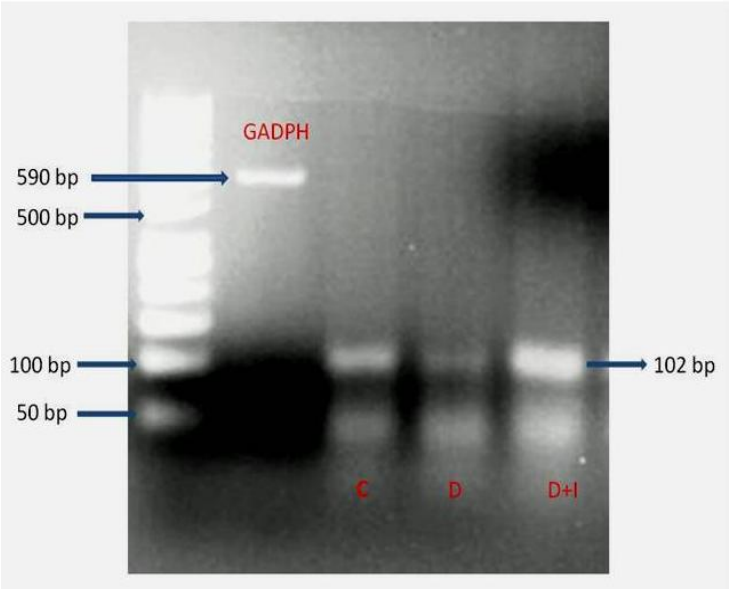

Figure 25: Hrg gene expression in control and experimental group

To check the expression of Hrg gene RNA was extracted from Cerebral Cortex tissue of control as well experimental group rats. Total RNA was converted to cDNA using reverse transcriptase assay. Routine PCR was done to amplify Hrg gene in control and experimental group by using converted cDNA as a templete. GAPDH was amplified from cDNA to check RNA extracted from each tissue was intact.

Agarose gel electrophoresis phoresis based qualitative analysis was done to check the expression of Hrg gene and GAPDH control. Gel electrophoresis of the same depicts that Hrg gene was more expressed in experimental group D+I in compared to control group, in contrast hrg gene in experimental group D was less expressed. But the experiment was performed only once hence, this study needs to be repeated (Mitchell et al., 2010)

SUMMARY

Glucose is the major source of energy for cellular activities especially for the brain function. Normally glucose transport in the cell by GLUT4 transporter is mediated by the insulin secreted by the pancreatic beta cell. Diabetes causes damage to nervous system and termed as diabetic neuropathy. Due to the lack of insulin, body cells do not get sufficient glucose for its activity which in turn triggers numerous alternative pathways like gluconeogenesis and glycogenolysis. Hyperglycemia induces various pathways in the cell resulting in oxidative stress. Demyelination is one of the important Complications of diabetes which affects the whole functioning of the nervous system. Demyelination itself can trigger remyelination process but when compared with Peripheral nervous system, it is an unlikely event in CNS

Neuregulins constitute a family of neuronal growth factors which signal through erbB family of receptors. This system enhances the myelin producing oligodendrocytes survival and differentiation. NRG1 has several isoforms and Hrg is type1 of neuregulin 1 and it promotes the differentiation of pre oligodendrocytes in to mature oligodendrocytes.

In order to validate the hypothesis we set four objectives which include blood glucose measurement, change in body weight and food- water consumption measurements of experimental rats during the 6 weeks of study, For assessment of motor function arched lader rung test, evaluation of gene expression of Heregulin and specific activity of Glucose-6-Phosphatase in Liver, Muscle and Cerebral Cortex of experimental rats were carried out. A significant increase in food and water consumption was observed in diabetic rats when compared with control and comparatively less consumption in D+I group of rats than diabetic group. Increased blood glucose level in diabetic rats indicates the lack of insulin whereas the insulin treatment reduced the blood glucose level in D+I rats. Performance on the ladder was analysed on the basis of foot-fault scoring and by noting mobile and immobile time. When compared with control and D+I group. Diabetic rats showed highest foot fault. When compared with control and D+I group, diabetic rats showed highest foot fault and immobile time indicating impairment in their ability to coordinate limb movements and to maintain balanced posture . The demyelination caused by diabetes results in their inability to integrate their sensory impulses and motor signals.

Specific activity of Glucose-6-Phosphatase enzyme was assayed in Liver, Muscle and Cerebral Cortex. Increased enzyme activity was observed in both diabetic and D+I group of rats , activity in D+I group found more than that of diabetic group. Since the experiment was carried out only once, we did not get significant result. Also we might go a step further in enzyme purification to ensure the purity and to get good result. The major focus of our study was the change in the expression of Heregulin gene during diabetes. From the PCR results we observed that there is a down regulation in the Heregulin gene expression during diabetes and the insulin treatment given to the D+I group resulted in the up-regulation of the gene. This suggests that insulin has an important role in the expression of Heregulin gene.

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