Effect of Insulin on Wnt/β-catenin Pathway through Fzd3 Receptor Gene Expression in Cerebral Cortex of Streptozotocin Induced Diabetic Rats and Role of Occupation, Inheritance, Migration and Diet on Prevelance of Diabetes in Surat.

A Dissertation Project
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In
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Dedicated

To

Our

Family....

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Jignasha Mangukiya
Gargi Adhvaryu

INDEX	
	PAGE NO.

LIST OF TABLES

LIST OF FIGURES

ABRREVIATIONS USED IN THE TEXT

C: Control

D: Diabetes

D+I: Diabetes Insulin

ROS: Reactive Oxygen Species

SCs: Schwann Cells

STZ: Streptozotocin

ANOVA: Analysis of Variance

BS: Brain stem

CS: Corpus Striatum

CB: Cerebellum

CC: Cerebral Cortex

CRD: Cysteine-rich Domain

IDF: International Diabetes Federation

CNS: Central nervous system

DPN: Diabetic peripheral Neuropathy

EGF: Epidermal Growth Factor

IDDM: Insulin Dependent Diabetes Mellitus

NIDDM: Non Insulin Dependent Diabetes Mellitus

LADA: Latent autoimmune diabetes in adult

MS: Multiple Sclerosis

MPNSTs: Malignant Peripheral Nerve Sheath Tumors

NCBI: National Centre for Biotechnology information

NF-Kb: Nuclear Factor kappa B

NO: Nitric Oxide

Wnt Pathway: Wingless Pathway

DVL: disheveled

Fzd 3: Frizzled Receptor 3

Fz: frizzled

CK1α: casein kinase

Wnt: wingless signaling transduction

LRP5/6: lipoprotein receptor related protein

GSK3β: glycogen synthase kinase

APC: adenomatosus polyposis coli

PCR: Polymerase Chain Reaction

PKC: Protein Kinase C

PNS: Peripheral Nervous System

TCF7L2: Transcripation Factor 7 –like 2

WS: Williams Syndrome

Abstract

Glucose is the obligatory energy substrate for brain. Hyperglycemia is found to be associated with central and peripheral nervous system dysfunction. Hyperglycemia induces oxidative stress and results in activation of multiple biochemical pathways. These activated pathways are a major source of damage and are potential therapeutic targets in diabetic neuropathy. In our present study, we surveyed many complications associated with diabetes in a cross section of population in Surat. We also investigated the effect of diabetes and therapeutic role of insulin on Fzd3 gene expression in cerebral cortex and succinate dehydrogenase (SDH) enzyme assay of streptozotocin induced diabetic (D) rats. Behavioural study to check their motor impairment and memory level was conducted.

We observed a significant increase in the blood glucose, food intake and water consumption while a decrease in the body weight was observed in D group compared to C. Insulin treatment showed a reversal in the body weight, blood glucose, food intake and water consumption to C. The enzyme activity of succinate dehydrogenase was studied spectrophotometrically at different enzyme concentrations in liver, kidney and muscle. A significant decrease in enzyme activity was observed in liver of D group compared to control while in kidney and muscle, SDH activity showed a significant increase in D group compared to control. Insulin treatment to diabetic rats showed a partial reversal in the SDH activity in liver, kidney and muscle. The impairment in the liver SDH may be possibly as a result of mitochondrial impairment. In kidney, increase in enzyme activity could be attributed to the activation of RAS (renin-angiotensin) system which eventually results in accumulation of succinate enzyme in kidney. Loss in the body weight causes increased capillary to fibre ratio as a result of oxidative stress which increases the SDH activity in the muscle during diabetes. Insulin treatment in D+I group causes a partial reversal of the SDH activity to C level.

D group showed impairment in their memory and cognition on modified Karl Lashley's test while they showed motor impairment on Staircase Test. Therapeutic effect of insulin to D+I group was confirmed through both tests.

1. Introduction

Diabetes is a group of metabolic diseases in which the individual has high blood sugar, either because the body does not produce enough insulin, or because β cells do not respond to the insulin that is produced. Diabetes is a disorder where in human body does not produce or properly use insulin, a hormone that is required to convert sugar, starches, and other food into energy. Human body maintains the blood glucose level at a very narrow range, with the help of insulin.

Diabetes is a chronic disease characterized by relative or absolute deficiency of insulin, leading to glucose intolerance. The lack of insulin activity results in failure of transfer of glucose from the plasma into the cells. This situation so called "starvation in the midst of plenty."

1.1 Types of Diabetes

The most common forms of diabetes mellitus are Type 1 and Type 2 diabetes.

Type 1 diabetes mellitus is characterized by an absolute deficiency of insulin secretion resulting from autoimmune destruction of pancreatic beta cells. Type 2 diabetes mellitus accounts for 90%– 95% of all diabetes cases, and it develops when the production of insulin is insufficient to overcome the underlying abnormality of increased resistance to its action. Absolute deficiency in insulin, as in Insulin Dependent Diabetes Mellitus (IDDM) is due to autoimmune destruction of insulin producing pancreatic β-cells. Classical symptoms include polydipsia, polyphagia, polyuria and weight loss.

Non-insulin dependent diabetes mellitus (NIDDM) is caused by relative deficiency in insulin due to peripheral insulin resistance and β-cell secretary defect. Approximately 90% of diabetic patients have NIDDM. Genetics play a big role in the etiology of NIDDM and is often associated with obesity. Type 2 diabetes results from a genetic predisposition and from lifestyle factors, especially those of the so-called Western lifestyle, characterized by high calorie intake and little exercise. Also known as non-insulin-dependent or adult-onset diabetes. Until recently,

type 2 diabetes henceforth simply 'diabetes'- was viewed as a disease of overfed sedentary people of European ancestry (Diamond *et. al.*, 2011). But it is now exploding around the world owing to the spread of Western habits (Diamond *et. al.*, 2011). This form of the disease far more common than type 1 (insulin dependent or juvenile onset) diabetes (Diamond *et. al.*, 2011). Usually presentation is slow and often insidious with symptoms of fatigue, weight gain, and poor wound healing and recurrent infection.

Latent autoimmune diabetes in adults (LADA) is the most common term describing patients with a type 2 diabetic phenotype. LADA is a disorder in which, despite the presence of islet antibodies at diagnosis of diabetes, the progression of autoimmune β-cell failure is slow. LADA patients are therefore not insulin requiring, at least during the first 6 months after diagnosis of diabetes.

In gestational diabetes, women without previously diagnosed diabetes exhibit high blood glucose levels during third trimester of their pregnancy. Gestational diabetes which is reported to affect 3-10% of pregnancies is a treatable condition and women who have adequate control of glucose levels can effectively decrease these risks.

1.2 Epidemiology

India leads the world with largest number of diabetic subjects earning the dubious distinction of being termed the "diabetes capital of the world". According to the Diabetes Atlas 2011 published by the International Diabetes Federation (IDF), the number of people with diabetes in India is currently around 61 million –an increase from 50.8 million last year is expected to rise to 100 million by 2030 unless urgent preventive steps are taken. Our country is also the largest contributor to regional mortality with 983, 000 deaths caused due to diabetes the year 2011. The so called "Asian Indian Phenotype" refers to certain unique clinical and biochemical abnormalities in Indians which include increased insulin resistance, greater abdominal adiposity. A part of this is due to genetic factors. Even though the prevalence of micro vascular complications of diabetes like retinopathy and nephropathy are comparatively lower in Indians, the prevalence of premature coronary artery disease is much higher in Indians

compared to other ethnic groups. The most disturbing trend is the shift in age of onset of diabetes to a younger age in the recent years (Mohan *et. al.*, 2007).

According to the latest comparative report of "International Diabetes Federation", 61.3 million people in India had diabetes in 2011. That figure is projected to rise to 101.2 million by 2030. IDF data reveal that India has more diabetes than the United States. In fact, India is ranked second in the world in diabetes prevalence after China (Safia Fatima Mohiuddin Jan 21, 2012).

Insulin is the most abundant peptidergic hormone secreted by the pancreatic islets of langerhans and plays an important role in organic metabolism. In recent years, various functions for insulin receptor signaling in the brain have been suggested in normal neurophysiology, and a dysregulation of insulin secretion or insulin receptor signaling has been reported in serious mental illnesses.

Fig. 1 Insulin Function in Organs (www.news-medical.net)

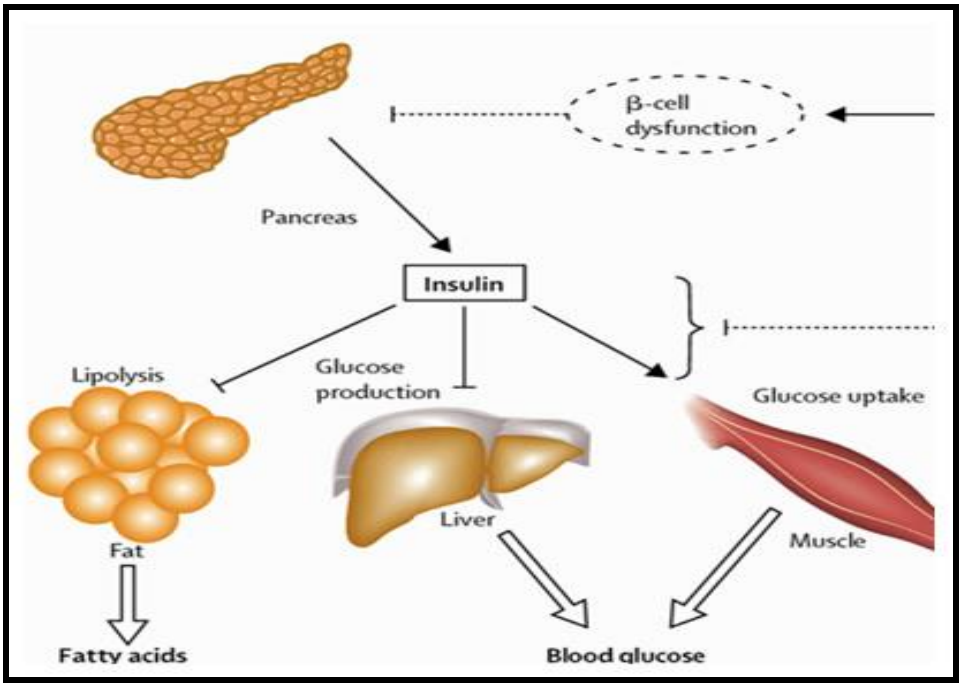
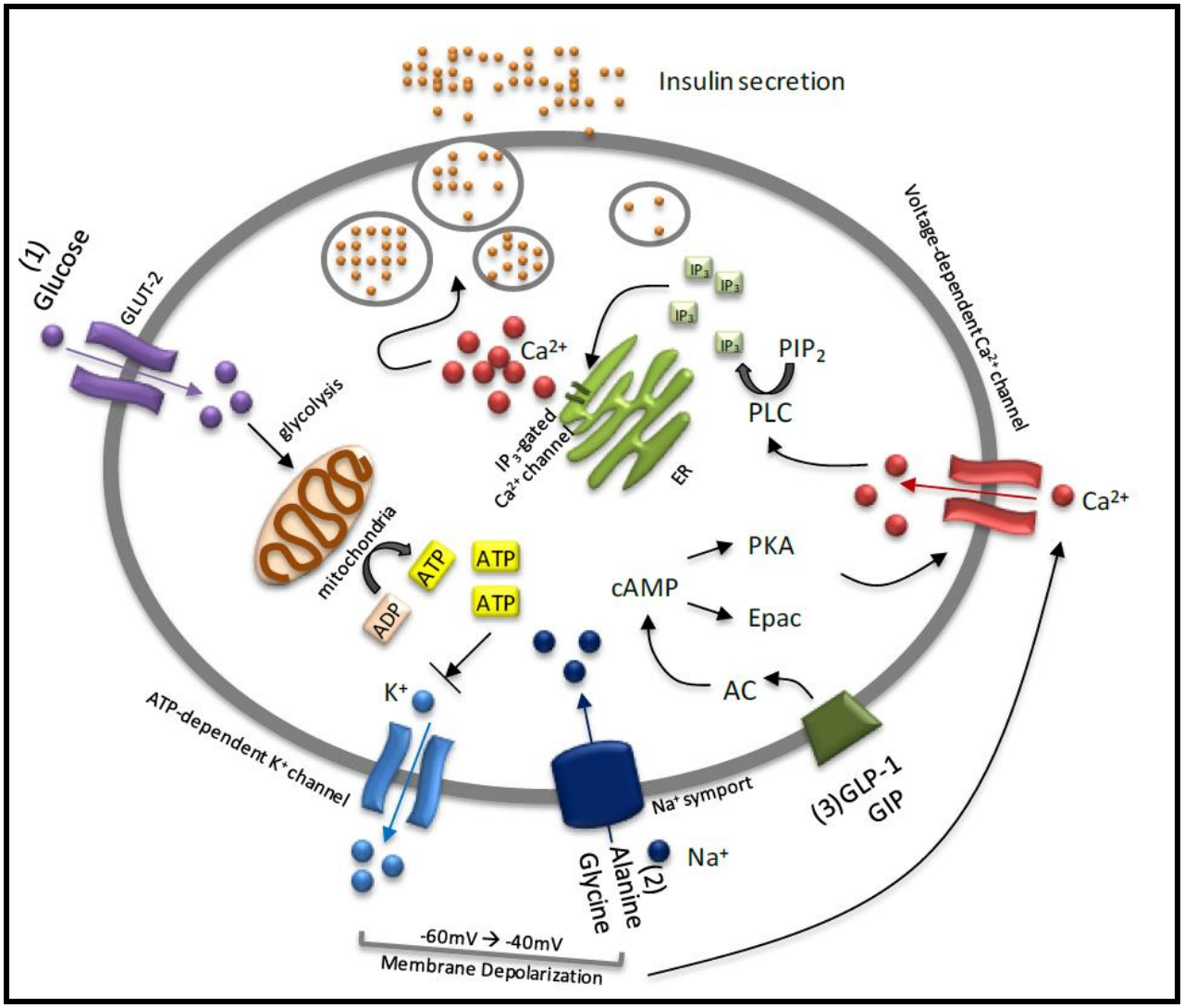


Fig. 2 Mechanism of Insulin Secretion (©Copyright by Jane Sun 2009)



Insulin, a 51-amino-acid peptide, is the key hormone responsible for maintaining glucose homeostasis. It is a hormone released from beta cells of islet of langerhans of pancreas which allows glucose to be transported into cells so that they can produce energy or store glucose. Diabetes results when body does not produce enough insulin to maintain normal blood sugar levels or when cells don't respond appropriately to in-vivo insulin. Glucose builds up in the blood, overflows into the urine and passes out of the body. Thus, the body loses its main source of energy even though the blood contains large amounts of glucose (Gung *et. al*., 2010).

Mechanism of insulin release in normal pancreatic beta cells - insulin production is more or less constant within the beta cells. Its release is triggered by absorbable glucose. Insulin is the principal hormone that regulates uptake of glucose from the blood into most cells (primarily muscle and fat cells, but not central nervous system cells). Therefore, deficiency of insulin or the insensitivity of its receptors plays a central role in all forms of diabetes mellitus. If the amount of insulin available is insufficient, if cells respond poorly to the effects of insulin (insulin insensitivity or resistance), or if the insulin itself is defective, then glucose will not have its usual effect, so it will not be absorbed properly by those body cells that require it, nor will it be stored appropriately in the liver and muscles. The net effect is persistent such as high levels of blood glucose, poor protein synthesis, and other metabolic derangements, such as acidosis.

The glucose absorbed during a meal is not metabolized at the normal rate and therefore accumulates in the blood (hyperglycemia) leading to glycosuria. Glucose in the urine causes osmotic diuresis, leading to increase urine production (polyuria). Stimulation of protein breakdown to provide amino acids for gluconeogenesis results in muscle wasting and weight loss.

The following symptoms may be associated with acute or chronic hyperglycemia, with the first three composing the classic hyperglycemic triad:

Polyphagia - frequent hunger, especially pronounced hunger

Polydipsia - frequent thirst, especially excessive thirst

Polyuria - frequent urination

Blurred vision

Fatigue (sleepiness).

Weight loss

Poor wound healing (cuts, scrapes, etc.)

Dry mouth

Dry or itchy skin

Tingling in feet or heels

Erectile dysfunction

Cardiac arrhythmia

For the individual who are suffering from diabetes, Hyperglycemia Symptoms is the alarming sign. Thus, an instant medicinal attention is necessary. This disease leads to damage of organs when level of glucose in blood rises to chronic level.

1.3 Objective

1. To check the therapeutic effect of insulin in STZ-induced experimental group of rats.
2. To conduct Behavioral study to assess motor impairment and anxiety levels.
3. To check the Effect of Frizzled 3 receptor gene expression of Wnt/β-catenin pathway in brain.
4. To carry out Succinate Dehydrogenase Assay by spectrophotometric methods.(Liver , Kidney, Muscle)
5. Demographic study in Surat to find incidence and factor contributing to the occurrence of disease.

2. Review of literature

The term diabetes describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes include long–term damage, dysfunction and failure of various organs. Diabetes may present with characteristic symptoms such as thirst, polyuria, blurring of vision and weight loss. In its most severe forms, ketoacidosis or a non–ketotic hyperosmolar state may develop and lead to stupor, coma and in absence of effective treatment, death. Often symptoms are not severe, or may be absent and consequently hyperglycemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long–term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (Report of a WHO Consultation, 1999).

2.1 Symptoms

Following are some of the common signs and symptoms of hyperglycemia:

Unusual increase of thirst and urination: One of the most common symptoms of diabetes is an urge of excessive thirst and frequent urination. This condition arises due to excessive secretion of fluids by the kidney that makes the body dehydrated, leading to reduction of the essential amount of fluid in the body (Muranyi *et. al.,* 2006).

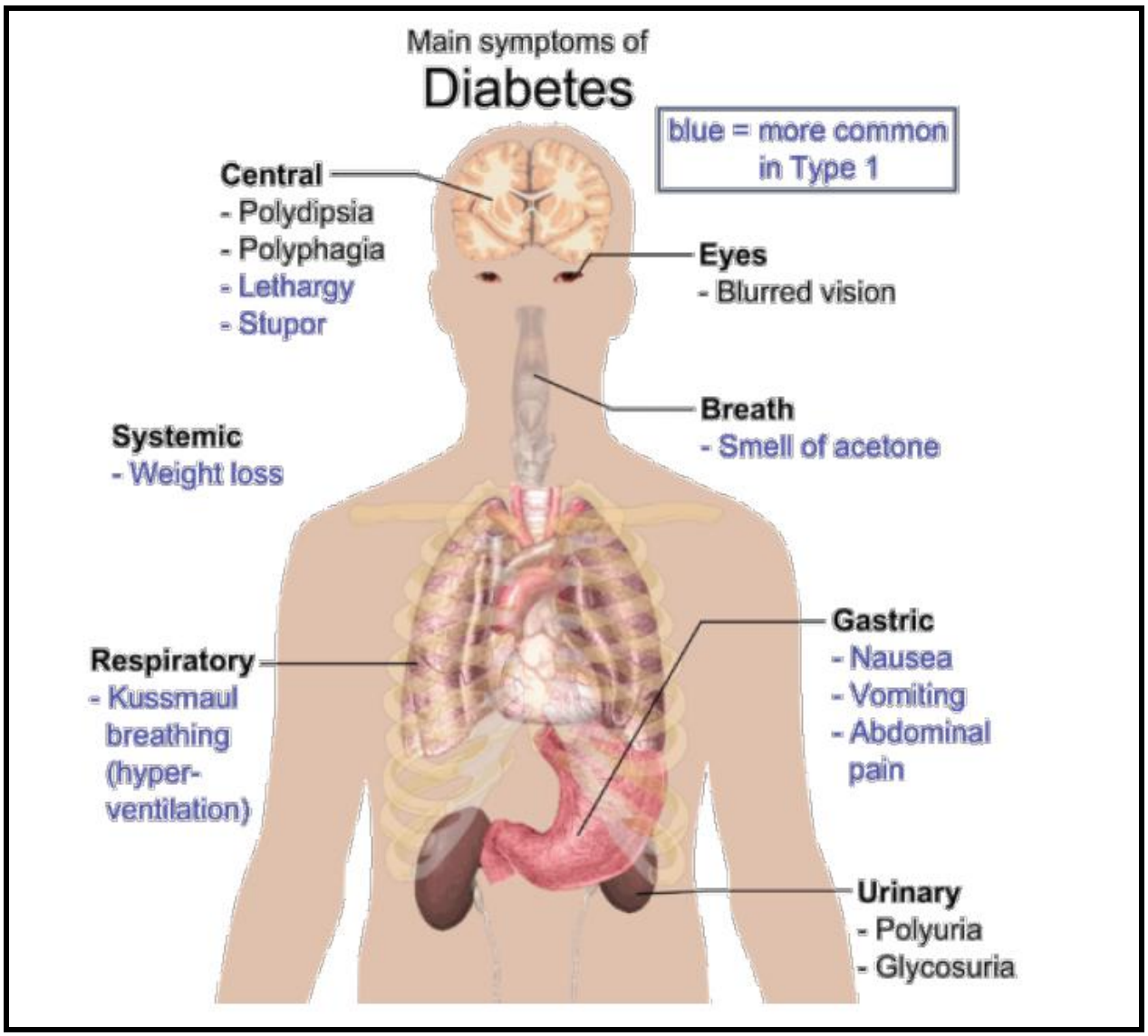
Abnormal increase in appetite: In a diabetic state the insulin level in the blood is not normal and thus the body cells are not able to get essential amount of energy to perform daily activities resulting in unusual hunger in order to fulfill the lack of energy.

Sudden loss in weight: An abnormal deviation in the weight is observed without any effort if one is suffering from diabetes. This is due to inability of body to absorb glucose and frequent urination (Atalay *et. al.,* 2002).

Mental exertion or Fatigue: Due to inability of glucose to enter the body cells, there is lack of supply of energy in the body leading to irritation and tiredness in the patient.

Slow rate of healing wounds: It takes a long time for a patient suffering from diabetes to heal even a minor wound due to weak immune system. This can be commonly observed in case of bladder and vaginal infections in woman (Laaksonen *et. al.*, 2002).

Fig. 3 Diabetic Symptoms (www.foods-healing-power.com)



Blurry vision: Due to abnormal increase in the levels of glucose, the blood vessels get damaged resulting in blurred vision or sometimes even blindness if ignored.

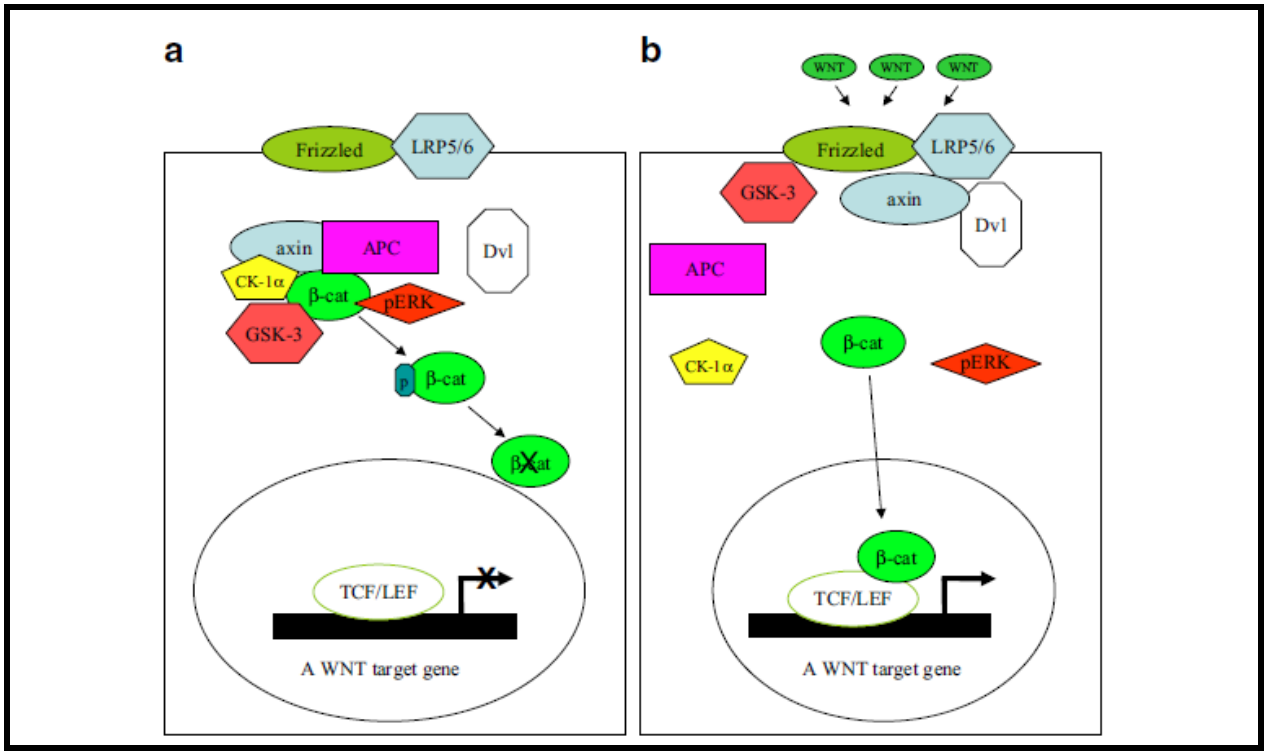
Dryness of skin: Due to peripheral neuropathy, the circulation and proper functioning of sweat gland is hampered resulting in dry and itchy skin.

Tingling or Numbness in Hands, Legs or Feet: Due to increase in the sugar level of blood in the body, the blood vessels get damaged leading to loss of sensation in hands and feet. In addition to that, there is a burning sensation in the arms, hands, legs and feet due to the damage occurred in motor nerve fiber.

2.2 Wnt/ β-catenin Pathway

The Wnt/Wingless signaling transduction pathway plays an important role in both embryonic development and tumorigenesis. B-Catenin, a key component of the Wnt signaling pathway, interacts with the TCF/LEF family of transcription factors and activates transcription of Wnt target genes. Recent studies have revealed that a number of proteins such as, tumor suppressor APC and Axin are involved in the regulation of the Wnt signaling pathway. Furthermore, mutations in APC or β- catenin have been found to be responsible for the genesis of human cancers (Akiyama *et. al.*, 2000).

Fig. 4 Wnt Pathway (T. Jin.Diabetologia (2008) 51:1771–1780)



The Wnt signaling transduction pathway plays an important role in a number of developmental processes, including body axis formation, development of the central nervous system, axial speciation in limb development and mouse mammary gland development.

The Wnt signaling pathway is an ancient and evolutionarily conserved pathway that regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development. The Wnts are secreted glycoproteins and comprise a large family of nineteen proteins in humans hinting to a daunting complexity of signaling regulation, function and biological output. Till date major signaling branches downstream of the Fzd receptor have been identified including a canonical or Wnt/β-catenin dependent pathway and the non-canonical or β-catenin-independent pathway which can

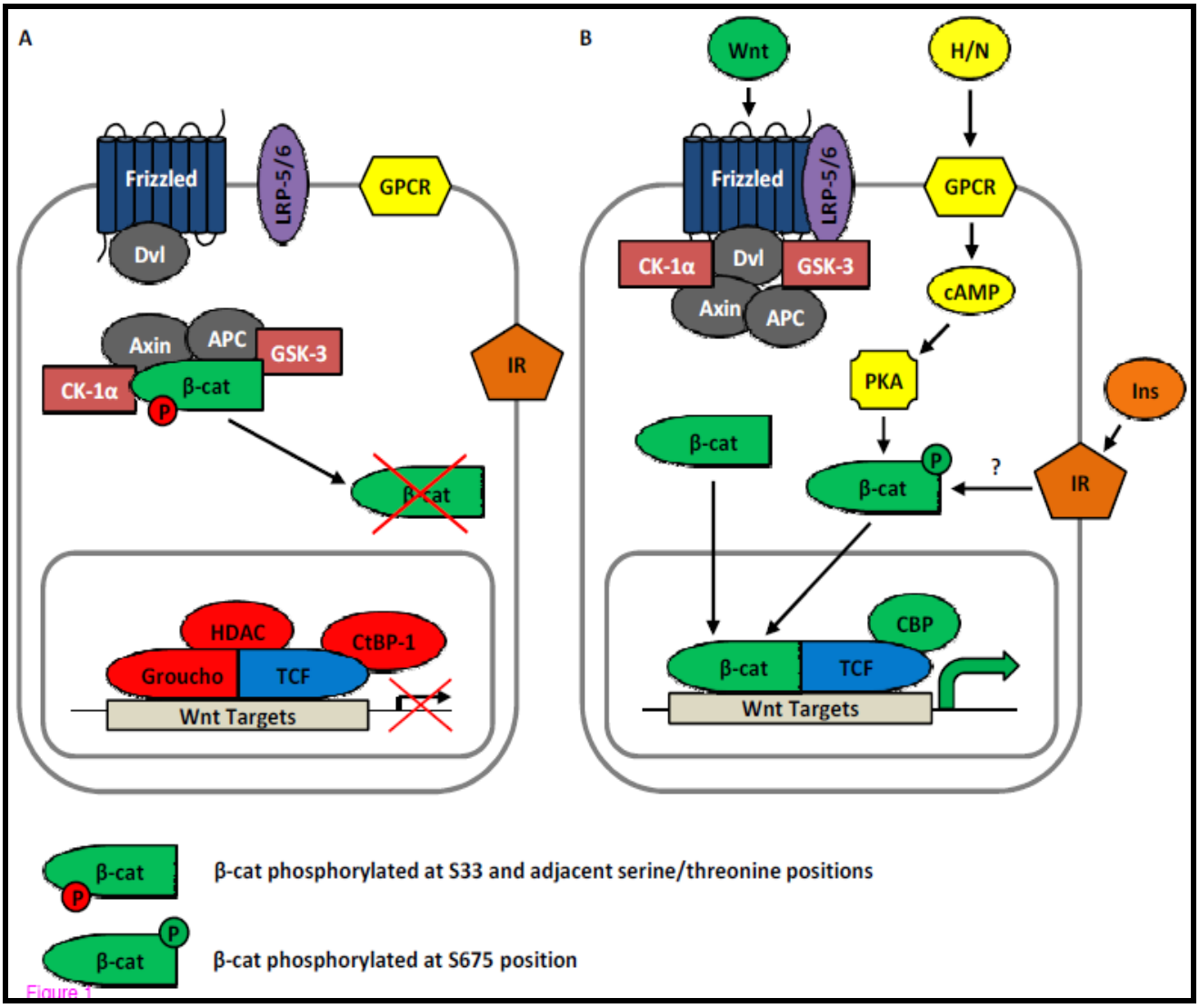
be further divided into the Planar Cell Polarity and the Wnt/Ca2+ pathways (Komiya *et. al.*, 2008).

Tight control of cell-cell communication is essential for the generation of a normally patterned embryo. A critical mediator of key cell-cell signaling events during embryogenesis is the highly conserved Wnt family of secreted proteins. Recent biochemical and genetic analyses have greatly enriched our understanding of how Wnt signal, and the list of canonical Wnt signaling components has exploded. The data reveal that multiple extracellular, cytoplasmic, and nuclear regulators intricately modulate Wnt signaling levels. In addition, receptor-ligand specificity and feedback loops help to determine Wnt signaling outputs (Wang *et. al*., 1997).

The Wnt family of proteins consists of more than 15 closely related secreted glyco-proteins. Receptors for the Wnt proteins are members of the frizzled family of transmembrane proteins, and the Wnt signal is transduced to a cytoplasmic protein, Dishevelled (Dvl). Upon activation by the Wnt signal, Dvl then inhibits the activity of glycogen synthase kinase-3β (GSK-3β). In the absence of the Wnt signal, GSK-3β is thought to phosphorylate and consequently induce the degradation of β-catenin. Therefore, the Wnt signal stabilizes and causes the accumulation of β-catenin, which in turn associates with TCF/LEF family transcription factors, ultimately altering the expression of Wnt signaling target genes.

Sequence comparisons revealed that FZD3, encoding a 591 amino acid protein, is a novel member of a seven transmembrane domain receptor family that are mammalian homologs of the Drosophila tissue polarity gene *frizzled.* FZD3 is expressed predominantly in brain, testis, eye, skeletal muscle and kidney. Recently, *frizzled* has been identified as the receptor for the *wingless (wg)* protein (Wang *et. al*., 1997).

Fig.5 Wnt Pathway Frizzled Function. (Positive Wnt ligand) (www.cellandbioscience.com)



In the absence of Wnt-signal, β-catenin, an integral cell-cell adhesion adaptor protein and transcriptional co-regulator, is targeted by coordinated phosphorylation by CK1 and the APC/Axin/GSK-3β-complex leading to ubiquitination and proteasomal degradation of β-catenin. In the presence of Wnt ligand, the co-receptor LRP5/6 is brought in complex with Wnt-bound Frizzled. This leads to activation of Dvl by sequential phosphorylation, poly-ubiquitination, and polymerization, which displaces GSK-3β from APC/Axin through an unclear mechanism that may involve substrate trapping and/ or endosome sequestration. GSK-3β is involved in glycogen

metabolism and other signaling pathways, which has made its inhibition relevant to diabetes and neurodegenerative disorders.

Wnt signaling is known to play a key role in neurogenesis and the fate of neural progenitors (Lee *et. al.,* 2004; Gao *et. al.,* 2007). Moreover, it has been identified as a major regulator of neuronal circuit formation during development, involved in neuron positioning, polarization, axon and dendrite development, and synaptogenesis (Salinas and Zou, 2008). In the adult brain, Wnt signaling continues supporting the proper functioning and viability of neurons (Toledo *et. al*., 2008).

Myelin sheaths are important for the efficient conduction of action potentials and support the integrity of axons in the vertebrate nervous system. The myelination of axons is performed during development by Schwann cells in the peripheral nervous system (PNS) and oligodendrocytes in central nervous system (CNS). Myelination involves the extension of large sheaths of membranes and their wrapping around axons, accompanying synthesis of a variety of myelin components, including basic myelin proteins (ffrench-Constant *et. al.,* 2004; Jessen and Mirsky, 2005). Wnt/β-catenin signaling also plays a major role in the development of the nervous system and contributes to neuronal plasticity (Makoukji *et, al.,* 2011).

However, its role in myelination still remains unclear. Wnt/β-catenin signalling plays a key role in expression of myelin sheath, both in the peripheral and central nervous systems. As reported, inhibition of Wnt/β-catenin signaling has indeed resulted in hypomyelination, without affecting Schwann cell and oligodendrocyte generation or axonal integrity. The present findings attribute to Wnt/β-catenin pathway components an essential role in myelin gene expression and myelinogenesis (Received Aug. 16, 2010; revised Dec. 14, 2010; accepted Dec. 30, 2010).

The progressive loss of CNS myelin in multiple sclerosis (MS) has been proposed to result from the combined effects of damage to oligodendrocytes and failure of remyelination. A common feature of demyelinated lesions is the presence of oligodendrocyte precursors (OLPs) blocked at a premyelinating stage (Fancy *et. al.,* 2009).

The report also informed that β-catenin signaling is active during oligodendrocyte development and remyelination in vivo. Moreover, elevated levels of Wnt pathway mRNA transcripts and proteins within MS lesions were observed, indicating activation of the pathway in this pathological context. Dysregulation of Wnt/β-catenin signaling in OLPs results in profound delay of both developmental myelination and remyelination, based on (1) conditional activation of β-catenin in the oligodendrocyte lineage *in vivo* and (2) findings from APC in mice, which lack one functional copy of the endogenous Wnt pathway inhibitor APC. Together, findings indicate that dysregulated Wnt/β-catenin signaling inhibits myelination/remyelination in the mammalian CNS. Evidence of Wnt pathway activity in human MS lesions suggests that its dysregulation might contribute to inefficient myelin repair in human neurological disorders (Fancy *et. al.*, 2009).

Our discussion will be mainly focussed on frizzled receptors of Wnt/β-catenin pathway. Genetic and biochemical data have demonstrated that the Fz proteins are the primary receptors for the Wnts (Bhanot et al. 1996). Fzd are seventransmembrane receptors with a long N-terminal extension called a cysteine-rich domain (CRD). Wnt proteins bind directly to the Fz CRD (Bhanot *et. al.,* 1996, Dann *et. al.,* 2001, Hsieh *et. al.,* 1999). Fzd3, encoding a 591 amino acid protein, is a novel member of a seven transmembrane domain receptor family that are mammalian homologs of the Drosophila tissue polarity gene *frizzled.* FZD3 is expressed predominantly in brain, testis, eye, skeletal muscle and kidney. FZD3 has a potential function in transmitting a Wnt protein signal in the human brain and other tissue.Though its role in diabetic neuropathy is still unknown (Umbhauer *et. al.*, 2003).

Fzd3 was latest reported in context of Williams syndrome (WS) which is a developmental disorder with a characteristic personality and cognitive profile (ref). The well studied transcription factor 7-like 2 (TCF7L2) gene is part of the Wnt/β-catenin signaling pathway and plays a critical role in cell development and growth regulation. Single nucleotide polymorphisms (SNPs) in transcription factor 7-like 2 (TCF7L2) are strongly associated with the risk of type 2 diabetes (Shao *et. al.*, 2012). TCF7L2 variants rs12255372 and rs7903146 have been reported.

Genome-wide scans of diabetic populations have uncovered several genes associated with susceptibility to type 2 diabetes and a number of them are part of the Wnt signaling. β-Catenin, a Wnt downstream effector participates in pancreatic development, however, little is known about its action in mature β-cells. Deletion of β-Catenin in Pdx1 pancreatic progenitors leads to a decreased β-cell mass and impaired glucose tolerance. β-Catenin in the regulation of metabolism and energy homeostasis and suggest that Wnt signaling modulates the susceptibility to diabetes by acting on different tissues.

Several genes involved in canonical Wnt signaling as potential drivers of benign neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs). As reported, In human neurofibromas and MPNSTs, activation of Wnt signaling increases with tumor grade and is associated with down-regulation of β-catenin destruction complex members. Induction of Wnt signaling was sufficient to induce transformed properties to immortalized human Schwann cells, and down-regulation of this pathway was sufficient to reduce the tumorigenic phenotype of human MPNST cell lines (Watson *et. al.*, 2013).

Wnt-pathway being important in neuronal system, its role in diabetic neuropathy is still unknown. This integration attempts to the effect of Fzd3 gene expression in the diabetic model and the experimental group treated with insulin.

2.3 Succinate Dehydrogenase

In eukaryotes, succinate dehydrogenase is tightly bound to the inner mitochondrial membrane; in prokaryotes, to the plasma membrane. The enzyme contains three different iron-sulfur clusters and one molecule of covalently bound FAD. Electrons pass from succinate through the FAD and iron-sulfur centers before entering the FAD and iron-sulfur centers before entering the chain of electron carriers in the mitochondrial inner membrane (or the plasma membrane in bacteria). Electron flow from succinate through these carriers to the final electron acceptor, O_2, is coupled to the synthesis of about 1.5 ATP molecules per pair of electrons (respiration-linked phosphorylation). Malonate, an analog of succinate not normally present in cell, is a strong competitive inhibitor of succinate dehydrogenase and its addition to mitochondrial blocks the activity of the citric acid cycle.

(Chapter 16 the Citric Acid Cycle@ Lehninger Biochemistry 4e)

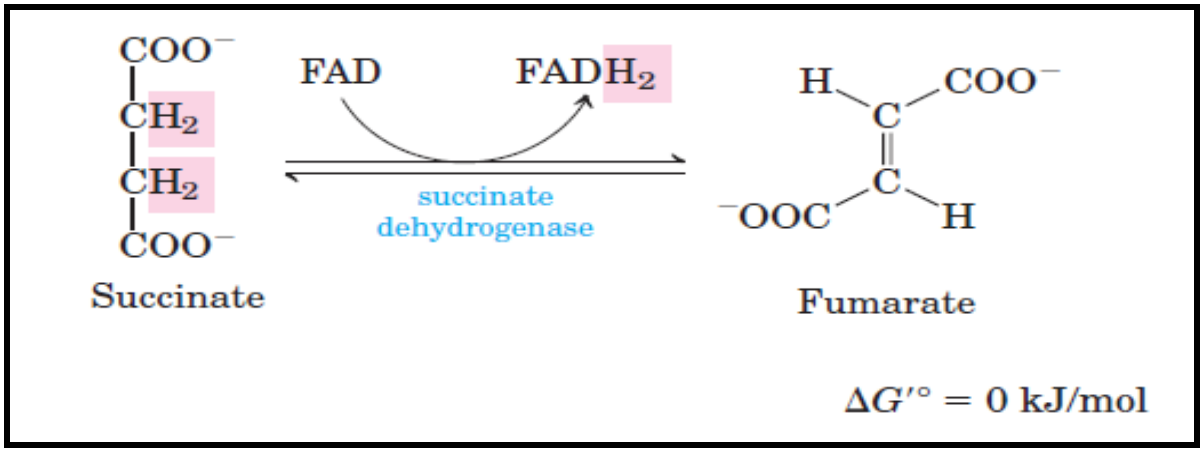
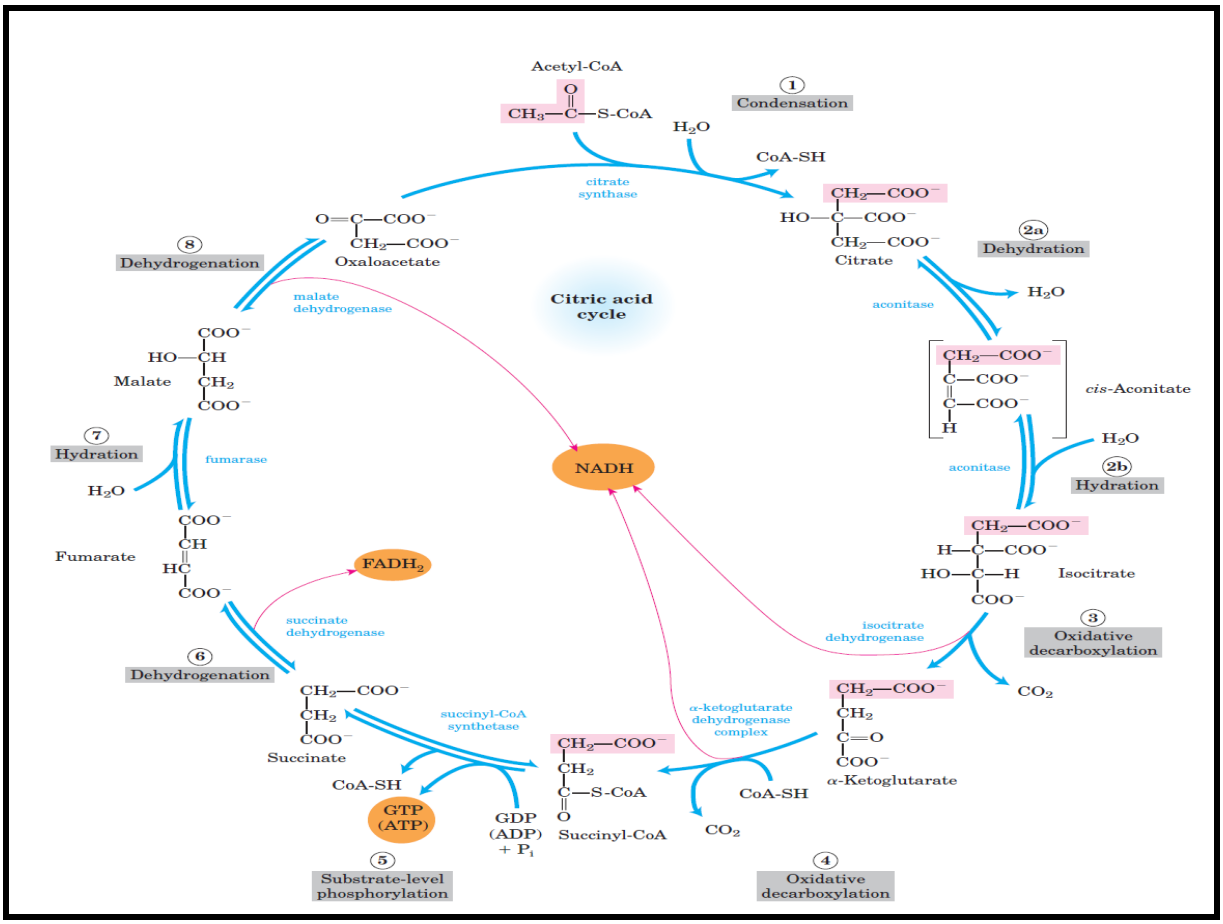


Fig. 6 Citric Acid Cycle (Chapter 16 the Citric Acid Cycle@ Lehninger Biochemistry 4e)



2.4 Behavioral study

2.4.1 Modified Karl Lashley's Maze

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

Maze running involves many parts of the brain. It involves vision (remembering the sight of correct pathways), spatial sense (remembering the direction to turn), olfaction (smelling the food and moving toward the more powerful odour), and kinesthesis (the feeling of arms and legs running a certain direction). If one type of clue is eliminated, there are many others remaining, allowing the rat to guide itself to the end of the maze. The maze was modified by adding doors and compartments.

Modified maze was compartmentalized into 3 sections. Each compartment contained doors and pseudo doors. . Compartment 1: contained 1 door. Compartment 2: contained 2 doors from which one was pseudo door. Compartment 3: contained 3 doors which had 3 doors amongst which 2 were pseudo door. The rat was trained without the doors to remember the path. After training doors and pseudo doors were attached to each compartment.

Modified form of Karl Lashley's maze helped us to check the memory, cognitive effects, time taken by each group to solve the maze and the false hits they did while solving the maze along with the above discussed parameters in the STZ- induced diabetic model. The differences in the behaviour of the experimental groups were checked.

2.4.2 Staircase

The "staircase" test is designed for measurement of side-specific deficits in coordinated paw movements in rats during skilled reaching and grasping tasks. It has been shown to reveal impairments on the contralateral side following unilateral lesions in a wide range of motor structures of the brain.

Brain damage, stroke and neurodegenerative diseases such as Parkinson's or Huntington's disease can cause severe motor deficits in skilled forelimb use in both humans and rats. These deficits are typically analyzed in a reach-to-eat paradigm. Skilled reaching in rats has been found to be a good model of human skilled reaching. Therefore, rats serve as an excellent tool to monitor the development of deficits after neurological insults or changes after medical intervention. The following protocols comprise two different tests of rat skilled reaching. The single pellet reaching test is a paradigm that involves detailed rating and analysis of qualitative aspects of the reaching movement itself. The staircase test is an objective, high-throughput reaching task that allows reaching success (number of pellets eaten) to be investigated in multiple rats at the same time. Both tests have been used extensively to investigate motor deficits and effects of treatment (Klein *et. al.,* 2012).

Differences between mice and rats in normal performance of the task are noted. The staircase test provides a simple objective test of skilled motor function that allows measurement of lateralised effects without unduly constraining the animal, and which may prove as useful for mice as has previously been demonstrated in rats (Baird *et. al.*,1994).

3. Materials and Method

3.1) Demographic studies

3.2) Model induction

3.3) Biochemical tests

3.4) Behaviour studies

3.5) Succinate dehydrogenase enzyme assay

3.6) PCR Analysis of Fzd3 receptor

3.7) Statistics

MATERIALS

Streptozotocin (Sigma Aldrich), EDTA (Merck Specialists Pvt. Ltd.), Glucose estimation kit (Lab Care Diagnostics Pvt. Ltd.,Gujarat), Actrapid human insulin (Novo Nordisk India Pvt. Ltd, Banglore), Cupric sulphate ($CuSO_4$; S.D. Fines), Sodium potassium tartarate ($NaKC_4H_4O_6$; FINAR Chemicals), Sodium hydroxide (NaOH; Sisco Research Lab, Mumbai), Bovine serum albumin (Central Drug House, Mumbai), Sodium Carbonate(RANKEM) , Sodium Phosphate Monobasic Anhydrous(Sisco Research Lab, Mumbai), Di-Sodium hydrogen phosphate anhydrous(Merck Specialists Pvt. Ltd), Folin Ciocalteu Reagent (Sisco Research Lab, Mumbai), Na glycinate (Sigma Aldrich)Na succinate (Himedia laboratories Pvt. Ltd.), *Nitro blue tetrazolium* (NBT) (Himedia laboratories Pvt. Ltd.),Tris HCl (Spectrochem Pvt. Ltd,Mumbai), Sucrose (S.D. Fines), Tri reagent (Sigma Aldrich), DEPC water (Himedia laboratories pvt. Ltd.), Chloroform (Sisco research Lab, Mumbai), cDNA synthesis kit (Fermentas, USA), PCR Master mix (Fermentas, USA), Primers Fzd3 (IDT, USA), Bromophenol blue (Central Drug House, Mumbai), Agarose (Sisco Research Lab, Mumbai), Ethidium bromide (Central Drug House, Mumbai), DNA ladder (Fermentas, USA). All biochemicals used in the present study were of analytical grade.

ANIMALS

Adult male Wistar rats of 200-250 gm body weight were purchased from Haffkine Biopharmaceuticals Ltd., Mumbai and used for all experiments. They were housed in separate cages

under 12-h light and 12-h dark periods and were maintained on standard food pellets and water at 25±3 °C. Animal care and procedures were done according to the Institutional and National Institute of Health Guide lines.

3.1 Demographic studies

A cross-sectional survey study in Surat was conducted to describe diabetes management, diabetes control and late complication status among patients managed in primary health care clinics and hospitals. All the information and data contained in the survey has been collected from home to home, Civil Hospital (Surat), Nandanvan Hospital (Surat) under gudience of Dr. Amee Nair, Dr. Ashvin Bhojani and Jatin Mavani. The patients were interviewed and the medical records such as management practice, glycemic control and complications were retrospectively reviewed for a period of six month. All data were entered in the case record forms and analyzed statistically. Eighty six patients, including 61 males and 25 females, were included in the present study. Maximum patients were in the age group of 50-60, and also in 65-85.

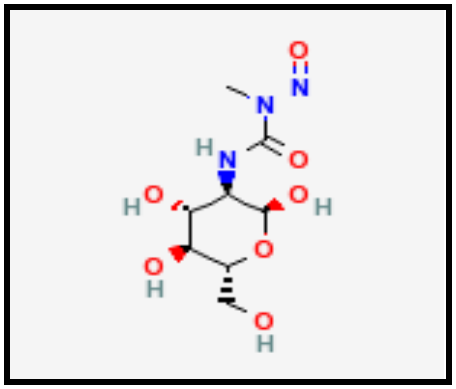
3.2 Model induction

3.2.1 Chemicals

Chemical used for diabetes model induction was Streptozotocin (Qualigens fine chemicals, Mumbai, India). Streptozotocin (STZ) is a synthetic antineoplastic agent that is classically an anti-tumor antibiotic and chemically is related to other nitrosureas used in cancer chemotherapy. Streptozotocin (STZ) (2-deoxy-2-({[methyl (nitroso)amino]carbonyl}amino)-β-D glucopyranose) is a naturally occurring compound, produced by the bacterium Streptomyces achromogenes, that exhibits broad spectrum antibacterial properties Streptozotocin is a mixture of α- and β-stereoisomers that appear as a pale yellow or off-white crystalline powder. STZ is very soluble in water, ketones, and lower alcohols and only slightly soluble in polar organic solvents. Streptozotocin sterile powders are available and can be prepared as a chemotherapy agent. Each vial of sterilized streptozotocin powder contains 1 g of Streptozotocin active ingredient with the chemical name, 2-Deoxy-2-[[(methylnitrosoamino)-carbonyl] amino]-D-

glucopyranose and 200 mg. citric acid. Streptozotocin is available for intravenous use as a dry-frozen, pale yellow, sterilized product. Pure streptozotocin has alkaline pH. When it is dissolved inside the vial in distilled water as instructed, the pH in the solution inside the vial will be 3.5-4.5 because of the presence of citric acid. This material can be prepared in 1g vials and kept in cold store and refrigerator temperature (2-8 °C) away from light (Akbarzadeh *et. al*., 2007).

Fig. 7 Structure of Streptozotocin



3.2.2 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) synthetase during DNA reparation. The low levels of NAD^+ also produce a decrease in intracellular ATP levels. On the other side, the participation of nitric oxide (NO) and reactive oxygen species (ROS) in cytotoxic effects of streptozotocin was proposed. As a conclusion, Okamoto proposes a common mechanism of action of Alloxan and streptozotocin toxicity suggesting that beta cells, trying to repair the damaged DNA, produce a suicidal response. The initial injury is produced by different causes: Alloxan acts mainly by production of reactive oxygen species and Streptozotocin by DNA alkylation (Rigalli *et. al*., 2009).

3.2.3 Induction of diabetes

Animals were divided into following groups as-

1.) Control [C]

2.) Diabetic [D]

3.) Diabetic +Insulin [D+I]

Each group consisted of 3 animals. Diabetes was induced by a single intrafemoral dose (50mg/kg body weight) of Streptozotocin prepared in citrate buffer, pH 4.5 (Arison *et al*., 1967; Hohenegger and Rudas *et al*., 1971). Blood glucose was estimated by Glucose estimation kit (Lab Care diagnostics Pvt. Ltd., Gujarat) using glucose oxidase-peroxidase method. D+I group received daily 2 doses (1-1.5 IU/Kg body weight) of regular human insulin (Actrapid) (Flanagan *et al*., 2003). D+I was given daily two episodes of insulin for 11 days. Control rats were injected with citrate buffer.

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

3.2.4 Tissue preparation

Rats were sacrificed by cervical dislocation and then decapitation on the 15th day of the experiment (Robinson *et. al*., 2009). Brain (cerebellum, cerebral cortex, corpus straitum and brain stem) and body parts (liver, heart, kidney, muscle and pancreas) were dissected out (Iversen & Glowinski *et. al*., 1966). The tissues were stored at -80 °C until assay.

3.3 Biochemical Assay

3.3.1 Glucose estimation

Blood glucose was estimated by Glucose estimation kit (Lab Care diagnostics Pvt. Ltd., Gujarat) using glucose oxidase-peroxidase method. Glucose is oxidized 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. Intensity of the colour formed is directly proportional to the amount of glucose concentration measured at 505 nm.

Glucose Oxidase

$$Glucose + O_2 + 2H_2O \text{ ----------> } Gluconate + H_2O_2$$

Peroxidase

$$2H_2O_2 + 4\text{-Aminoantipyrene ----------> Phenol Quinoneimine dye (red)}$$

3.3.2 Protein estimation

Protien was measured by method of Lowry *et al*. (1951) using bovine serum albumin as standard. The intensity of purple blue colour was proportional to the amount of protein, which was read in a U.V spectrophotometer (Shimadzu 1800) at 660 nm (Remya et *al*., 2009).

3.4 Behavioral Study

3.4.1 Staircase Test

Long-term prevalence of diabetes has been recorded as brain damaging. Brain damage can cause motor deficits and neurological disturbances in animal model. This test was performed to test motor impairment and cognitive activity in STZ-induced diabetic rats. Differences between the time taken by experimental groups to reach the end point of staircase was noted.

In our modified apparatus, Adult Wistar Rats (10-12 weeks) were placed in a narrower compartment of length 75 cm, creating a 4.5 cm wide trough on either side. Steps of stair case were of length 9cm and height 3 cm each. They were starved for 15 minutes before the test was conducted. A hungry animal would likely collect pellets by reaching at the end point of the

staircase. The animals were tested in a quiet room with a low level of background illumination (Inserm *et. al.*, 1994).

Fig. 8 Staircase Model

3.4.2 Modification of Karl Lashley's maze test

In this maze, three compartments were done. Each contained a door to move from one compartment to other. For adding to the complexity of the maze, pseudo doors were added to the second and third compartment. Experimental groups of rats were trained without doors. After the training sessions, during the testing period, they were kept on fasting condition for 10-15min. Total time and immobile time taken by the rat to solve the maze were recorded. False hits (attempts done to hit the pseudo door) were calculated.

In our modified apparatus, length of apparatus was 72cm, width was 51cm. All the three compartments were 22 cm long and width was 51cm. The length and width of door and pseudo door was 7cm and 10cm respectively.

Fig. 9 Modified Karl Lashley's Maze Model Without doors

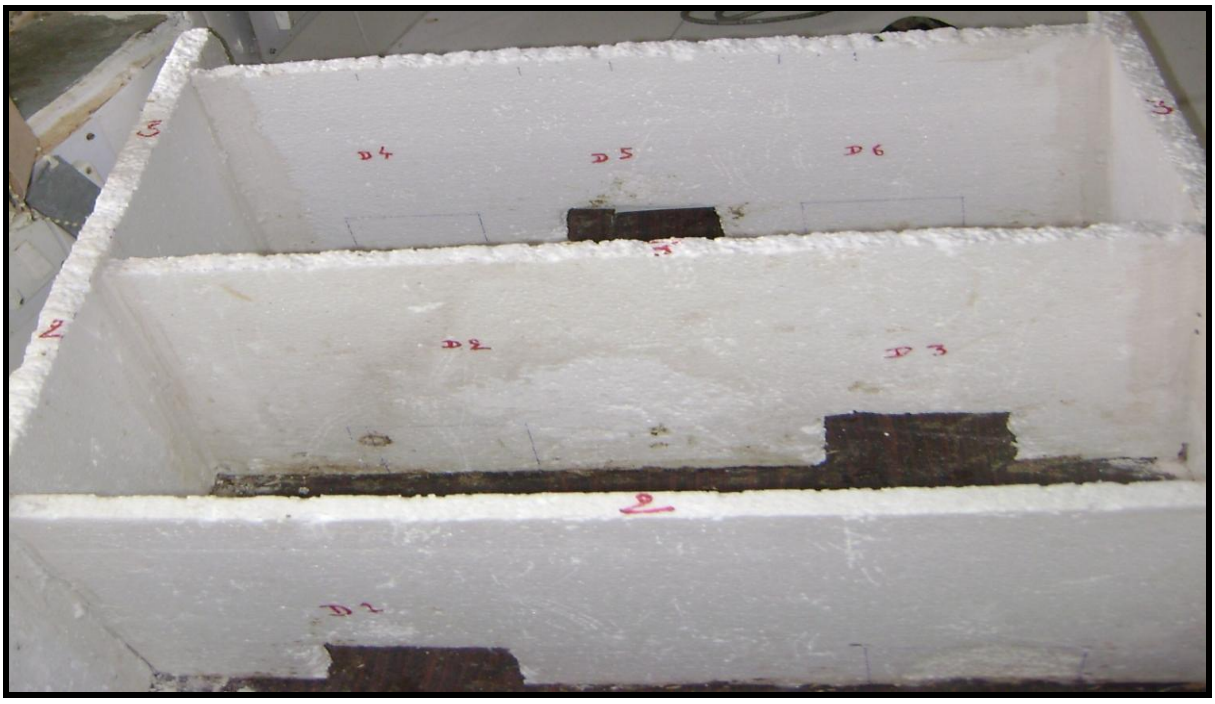


Fig. 10 Modified Karl Lashley's Maze with Doors

3.5 Succinate Dehydrogenase

3.5.1 Materials

Na-glycinate (5mM)

Sucrose (100mM)

1% Triton X 100

Na-succinate(200mM)

Nitro blue tetrazolium (NBT 2.5mg/ml)

Sodium dodecyl sulphate (2%SDS)

Phosphate Buffer (200mM, 7.4 pH)

3.5.2 Method

Succinate dehydrogenase enzyme assay in liver, kidney and muscle was done using spectrophotometric method of (Dooley *et.al*., 1979). Homogenate (20%) of liver, kidney and muscle was prepared in 100 mM of sucrose and 5mM Na-glycinate (homogenate buffer) it was centrifuged at 800g for 15 minutes followed by centrifugation at 7500g for 15 minutes at 4 °C. The enzyme activity was measured in supernatant. The reaction mixture contains 200mM phosphate buffer at pH 7.4 (37°C), 100 ul of enzyme sample extract of appropriate concentrations, Na-succinate of different concentrations 50 mM-250 mM were used as substrate. The reaction mixture of 750 ul volume and after 30min incubation reaction stop adding 2% SDS and it was assayed at 500 nm using Shimadzu 1800 UV spectrophotometer.In this assay, succinate is used as a substrate and nitroblue tetrazolium (NBT) as an artificial electron acceptor which changes to purple color when it accepts electrons. Thus, the formation of purple color is directly proportional to enzyme activity.

3.6 PCR analysis of Fzd3

Total Ribonucleic acid (RNA) was isolated from the Cerebral cortex 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 of NCBI.

RNA isolation protocol: Frozen brain tissue was homogenized in Tri Reagent standard protocol (Chomczynski *et. al*., 1995).

Sequence of Gene Specific Primers

PRIMERS	**FORWARD**	**REVERSE**	**PRODUCT SIZE**
Fzd3	5’AACAGAGTTCGGATTGAG 3’	5’GGACAAGGTAGAGAAT GC3’	100

3.7 Statistics

Statistical evaluations were done with ANOVA followed by Student–Newman–Keuls Test using Microsoft Excel.

4. RESULTS

4.1 Demographic studies

Diabetes is now one of the most common disorders. It is the leading cause of death in most developed countries. Diabetes education, a controlled diet and physical exercise are all important components of the management of NIDDM.

The main aim of this study was to estimate the major factors affecting diabetes patients afflicted with diabetes in Surat city of Gujarat. This data was collected from health care clinics and pathology laboratories. The data reported here is of type 2 (NIDDM) diabetes patients. 100 diabetics were selected for this study of which, total males afflicted by were 60 and 40 females were found diabetic. The data presented here is for individuals in 1-80 years of age group.

Fig. 11 Diabetic population recorded in sample population studied (N=100) M-60 and F-40

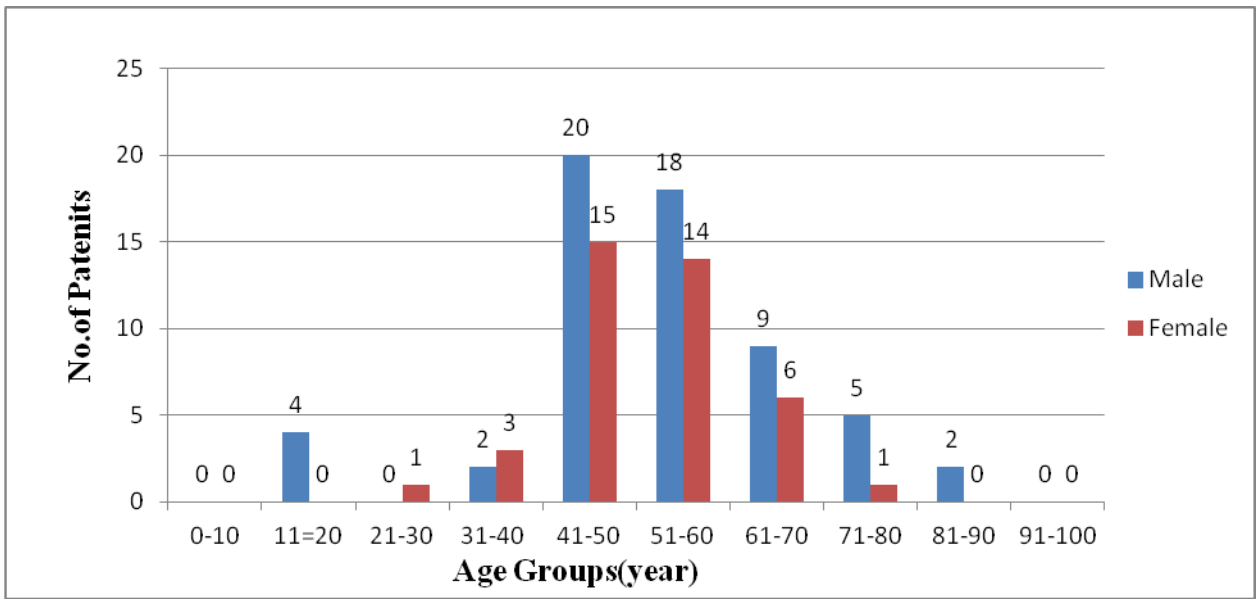


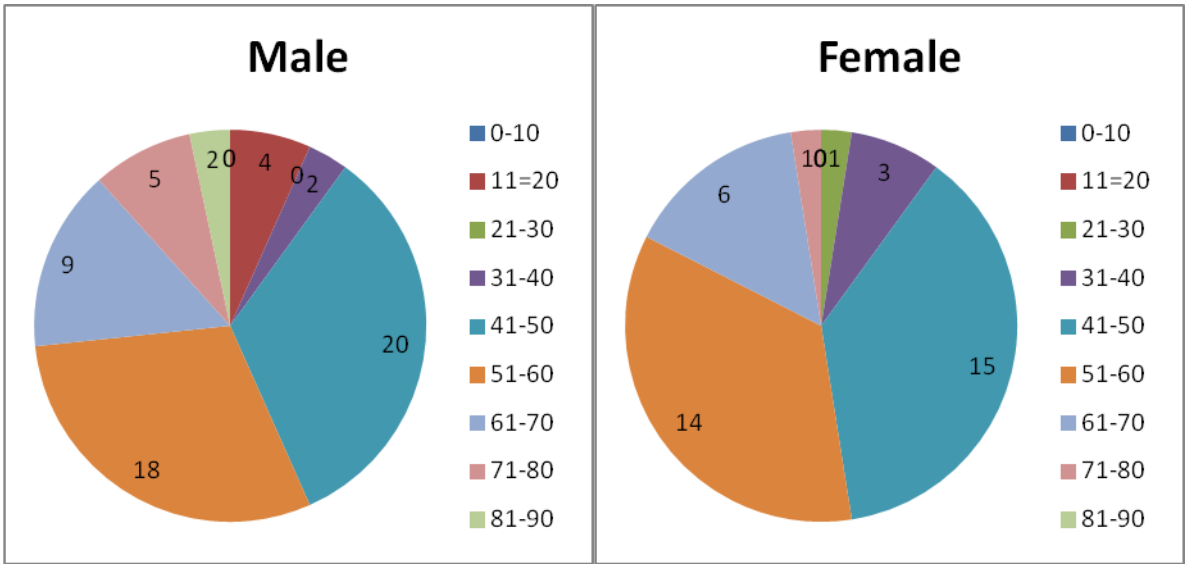
Table: 1 Diabetic population recorded in sample population studied (N=100)

Age group (years)	No. of Males Afflicted	No. of Females Afflicted
0-10	0	0
11-20	4	0
21-30	0	1
31-40	2	3
41-50	20	15
51-60	18	14
61-70	9	6
71-80	5	1
81-90	2	0
Total	**60**	**40**

From a randomly selected diabetic population of 100 patients, males afflicted by diabetes were 60, and females were 40. The data presented here is for individuals in 0-90 years of age group. Maximum patients were in age group 41-50, 51-60 and 61-70. In the age group 11-20, there were 4 male and no female patient reported during the study. In the age group 21-30, there were no males and 1 female patient reported during the study. In the age group 31-40, there were 2 males and 3 female patients reported during the study. In the age group 41-50, there were 20 males and 15 female patients reported during the study. In the age group 51-60, there were 18 males and 14 female patients reported during the study. In the age group 61-70, there were 9

males and 6 female patient reported during the study. In the age group 71-80, there was 5 male and 1 female patient reported during the study. In the age group 81-90, there were 2 male and no female patient reported during the study.

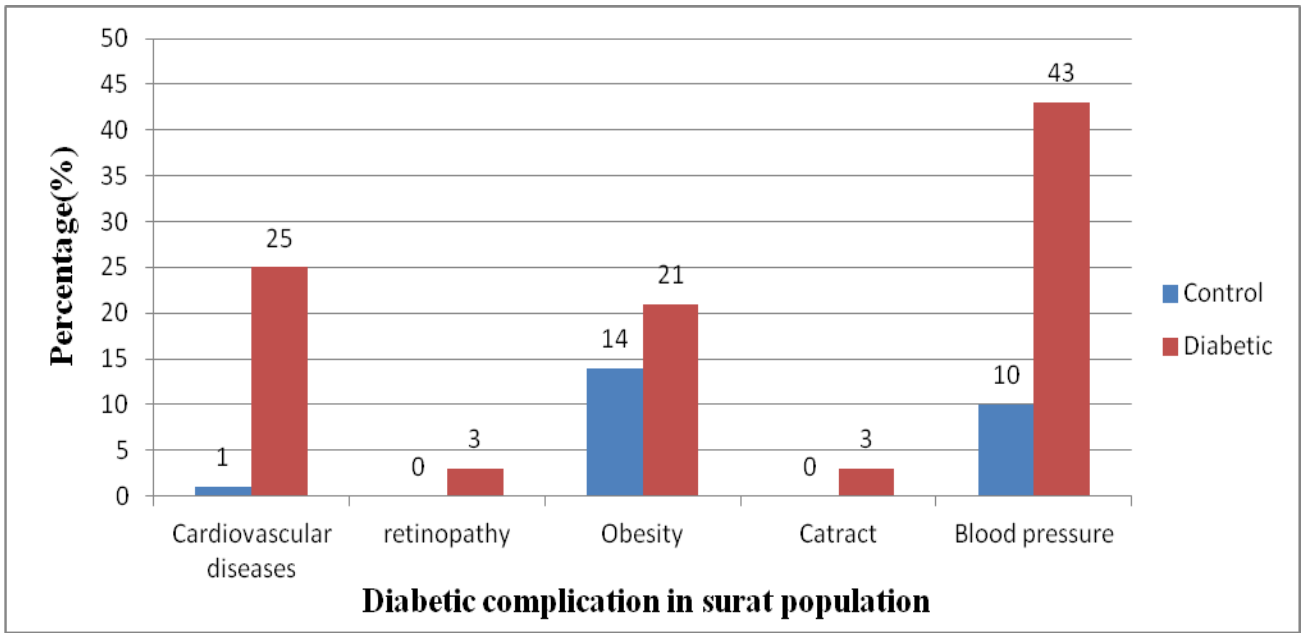
Fig. 12 Diabetic population is categorized into Male and Female. (N=100)



From a randomly selected diabetic population of 100 patients, males afflicted by diabetes were 60, and females were 40. The data presented here is for individuals in 1-90 years of age group. Maximum patients were in age group 41-50. In the age group 11-20, there were only 4 male and no female patient reported during the study. In the age group 31-40, there were 2 males and 3 female patient reported during the study. In the age group 41-50, there were 20 males and 15 female patients reported during the study. In the age group 51-60, there were 18 males and 14 female patient reported during study. In the age group 61-70, there were 9 males and 6 female patients reported during the study. In the age group 71-80, there were 5 males and 1 female patient was reported during the study. In the age group 81-90, there were 2 males and 0 female patients, reported during the study. Several complex and interrelated factors are at work in bringing about the rise in diabetes prevalence.

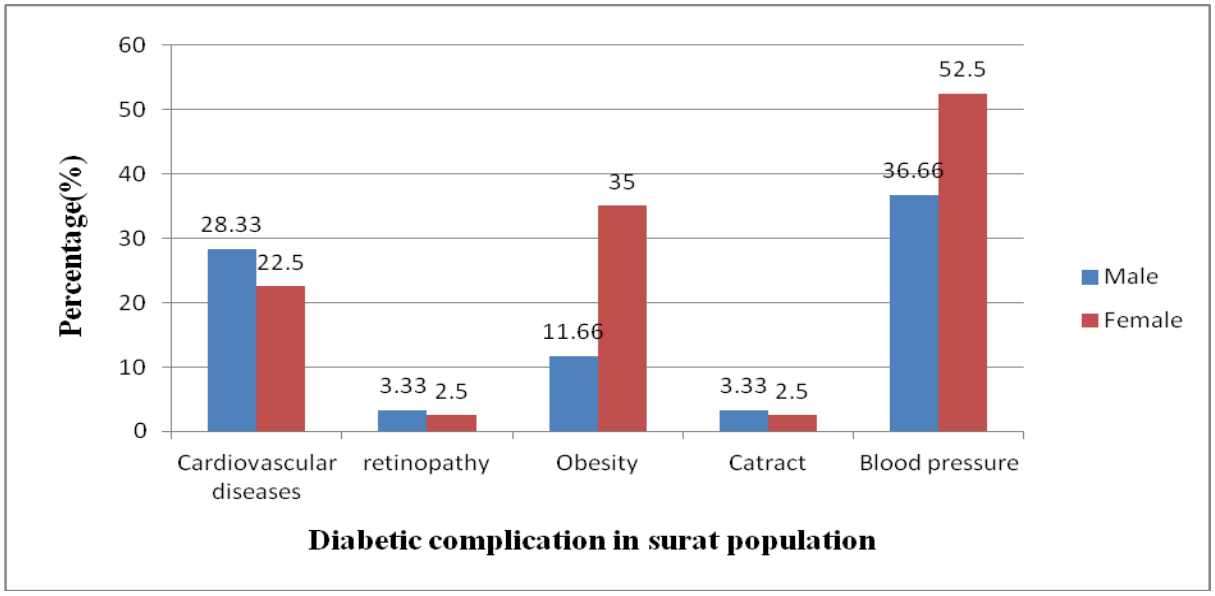
Due to busy lifestyle, irregular food habits, sedimentary jobs, lack of physical activity increase the probability of diabetes in urban territories. These factors including many others combine to produce large numbers of people with NIDDM (Toumilehto *et. al*., 2001).

Fig. 13 Complications in Control population (N=100) and Diabetic population (N=100)



A total of 100 patients each were interrogated from Surat, out of which 43 % were affected by blood pressure and 10% from the control population, 3 % in diabetic population were afflicted by cataract and retinopathy while none in the control population had symptoms of diabetic retinopathy and cataract, 21% in diabetic population and 14 % in control population were obese, while no patient was found in control population, 25% in diabetic population and 1% in control population had cardiovascular diseases.

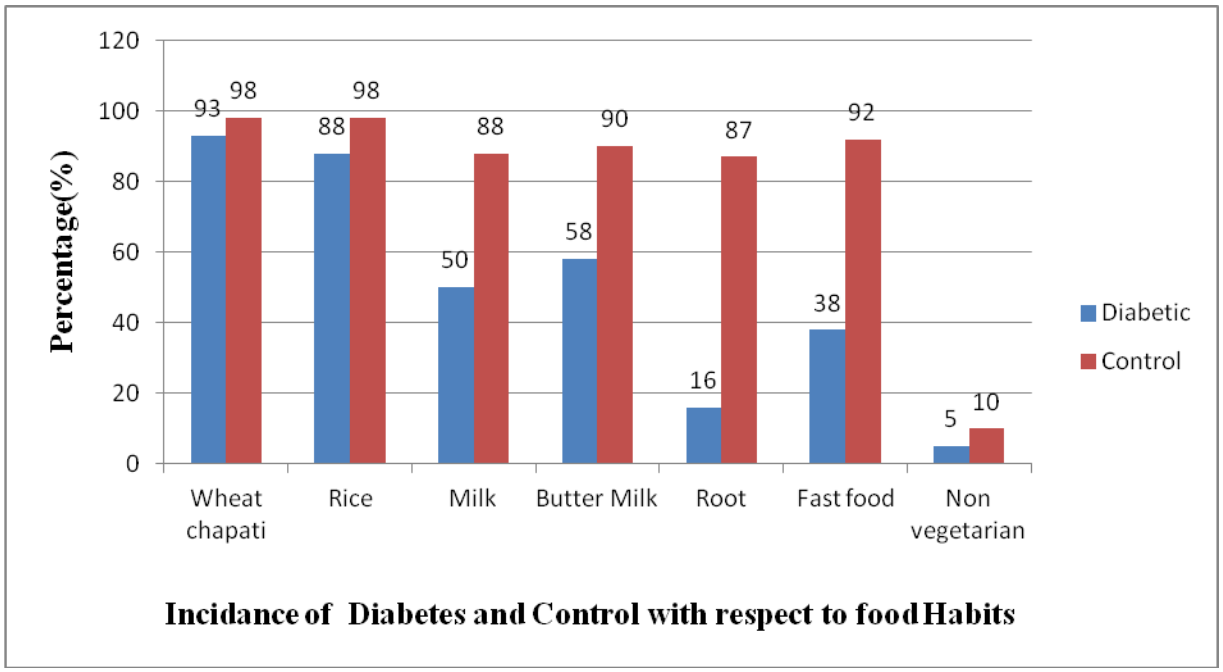
Fig.14 Diabetic complications in Males and Females of Surat (N=100)



A total of 100 patients each were interrogated from Surat, of which 36.66% males and 52.5% females in Surat had blood pressure, 3.33% males and 2.5% females had symptoms of diabetic retinopathy, 11.66% males and 35% females were obese, 3.33% males and 2.5% females were affected by cataract, 28.33% males and 22.5% females had cardiovascular diseases.

This gives a glimpse rising prevalence of diabetes and its complications. Ailments associated with diabetes are coronary artery and peripheral vascular disease, stroke, amputations, nephropathy and retinopathy, are resulting in increasing disability and reduced life expectancy. Hyperglycemia is a significant risk factor which can cause diabetic neuropathy. Foot ulceration and amputation are among the most costly diabetic complications. Diabetic neuropathy can reduce sensation in the feet and can result in foot ulceration and amputation. Regular inspection and good care of the foot by healthcare professionals can prevent foot ulceration and amputation (Ramachandran *et. al.*, 2001). Obesity which is increasing in prevalence as well is a significant risk factor.

Fig. 15 Dietary habit of Diabetic population (N=100) and Control population (N=100) in Surat

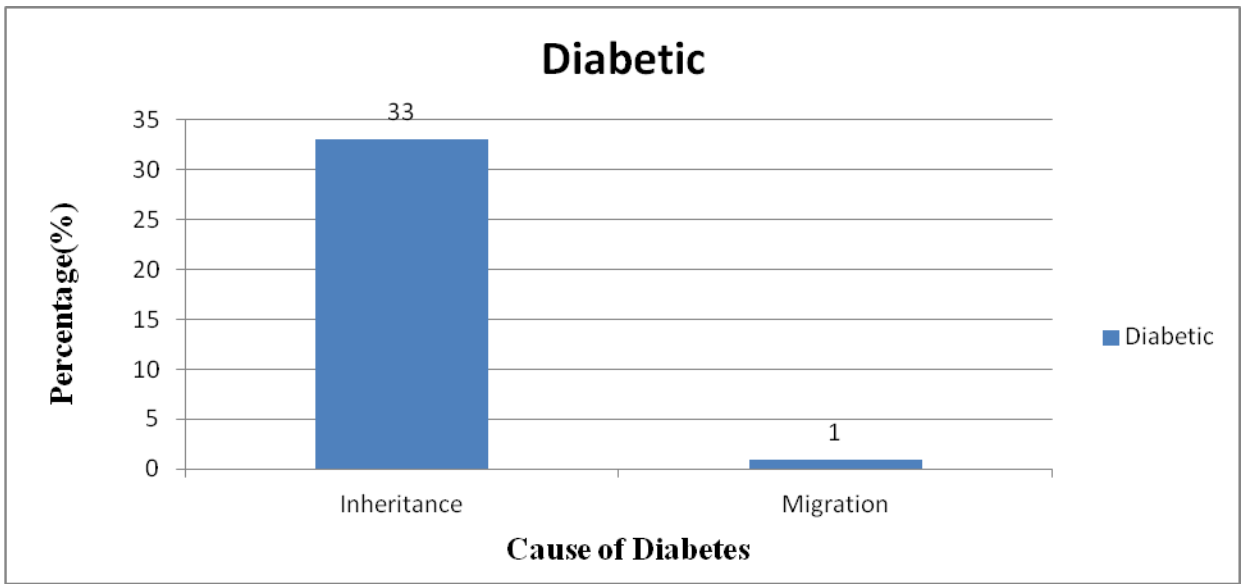


Diet plays an important role in metabolic disorder. Out of 100 diabetic patients, 93% in Surat diabetic population and 98% control population were found to have wheat chapatti in food, rice intake was 88% for the diabetic population and 98% in control population, 50% in diabetic population and 88% in control population had milk, 58% in diabetic population and 90% in control population were found to take butter milk, 16% in diabetic population and 87% in control population were found to take root in food, 38% in diabetic population and 92% in control population were found to have a liking towards fast food, where as 5% in Surat diabetic population and 10% in control population had non vegetarian food.

Also, Surat is fast moving towards culture of metropolitan city like Ahmedabad, Mumbai and Delhi. This could account for the high consumption fastfood habit which is one of the potential causes for the increase in number of diabetics in Surat. In Surat, which is comparatively rural city, Bajara and Makai chapattis are consumed more frequently than wheat chapattis. Milk and buttermilk consumption was high in Surat since it is an agrarian society where also fastfood

habits were comparatively high. Newer technologies and mechanised farming have made the physical activity much reduced and farming easier.

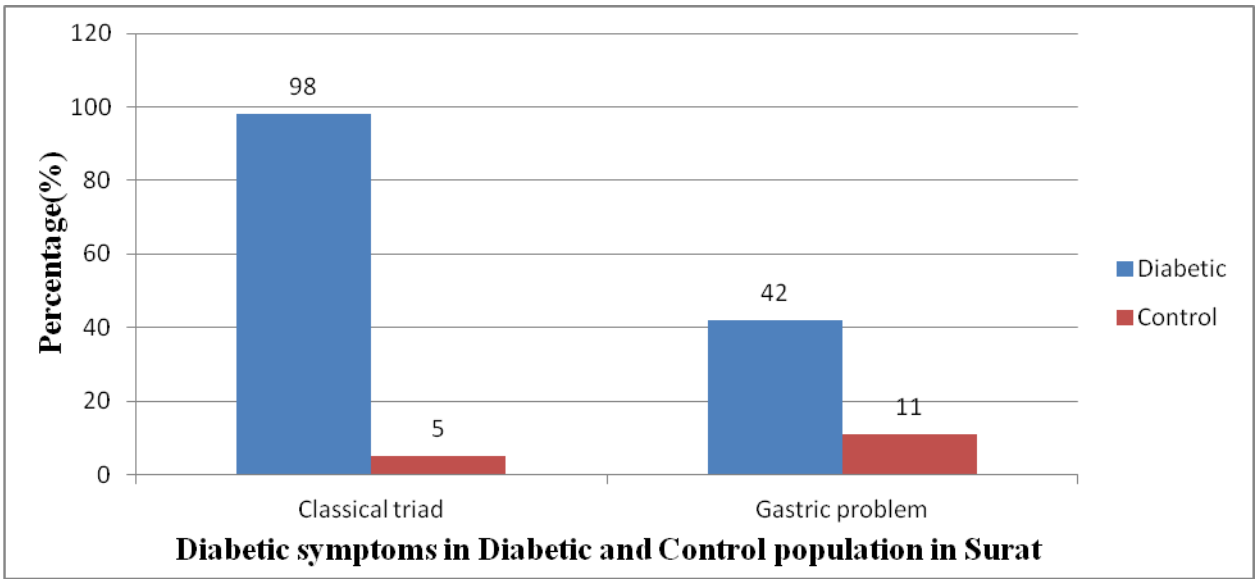
Fig.16 Rate of Inheritance and Migration amongst the Diabetic population In Surat (N=100)



Out of 100 diabetic patients, 33% in Surat were found to get diabetes in inheritance whereas, 1% in Surat was found to get diabetes because of migration from other state.

Diabetes also results from a genetic predisposition and from lifestyle factors, especially those of the so-called western lifestyle, characterized by high calorie intake and little exercise (Jared Diamond *et. al*., 2001).Such higher inherited diabetes in Surat population support higher genetic predisposition of disease in Surat. Because type 2 diabetes is a complex condition involving a combination of genetic and environmental factors, DNA testing for susceptibility genes is not yet warranted. However, because family history reflects genetic susceptibility in addition to other factors, it may be a useful public health tool for disease prevention (Harrison *et. al.*, 2003).

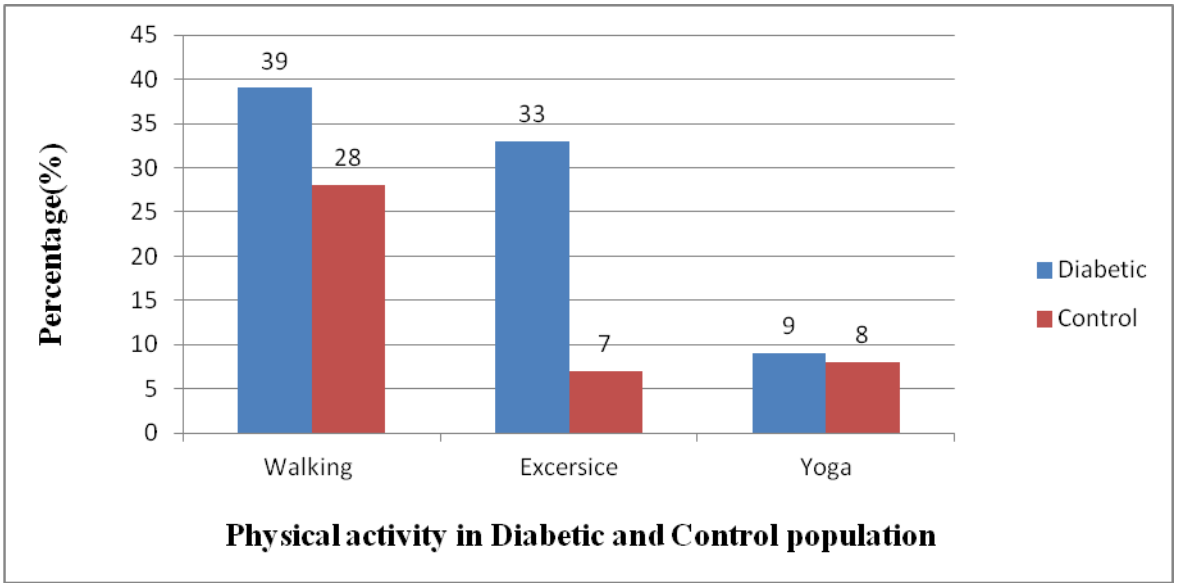
Fig. 17 Comparative account of symptoms associated with Diabetes in Control population (N=100) and Diabetic population (N=100) of Surat



A total of 200 people were interrogated, 100 control and 100 diabetic patients in Surat, of which 5% controls and 98% diabetic patients in had classical symptoms of diabetes (polyuria, polyphagia, and polydipsia) where as 42% controls and 11% diabetic patients had gastric problems.

Diabetes mellitus, caused by the malfunction of insulin-dependent glucose and lipid metabolism, presents with the classical triad of symptoms: polydipsia, polyuria, and polyphagia which are often accompanied by chronic fatigue and loss of weight (Soskolne *et. al.*, 2001).

Fig.18 Percentage of Diabetic population (N=100) and Control population (N=100) engaged in physical activity in Surat

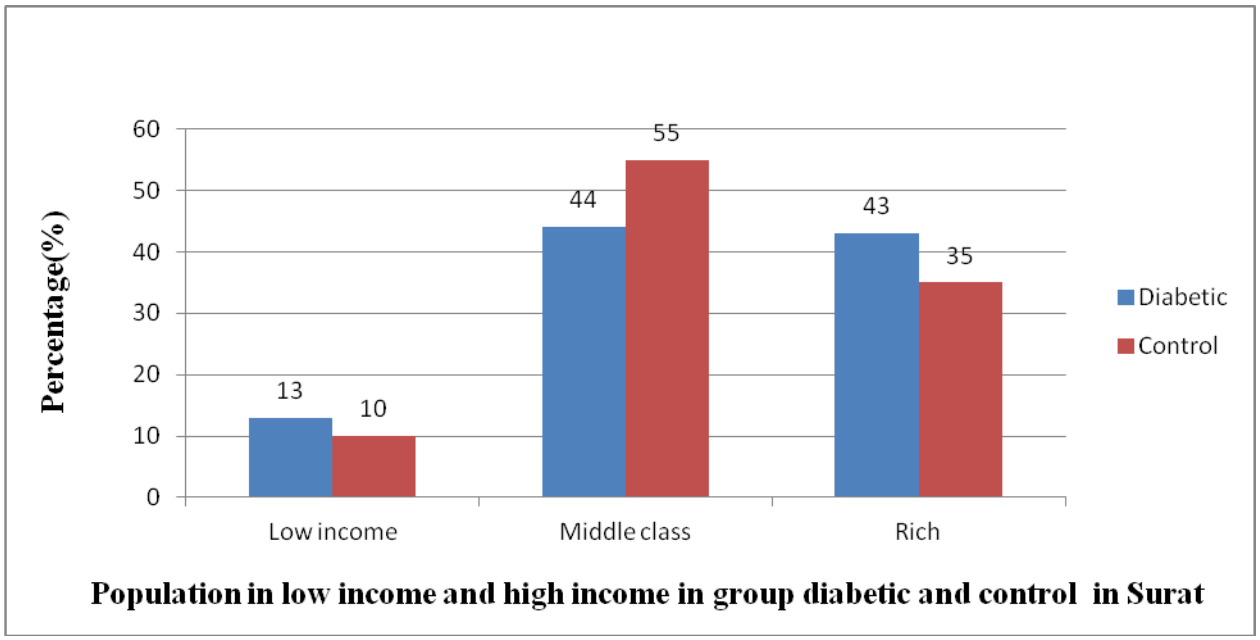


In diabetic population (N=100) and control population (N=100), 39% diabetic people and 28% control people in Surat were found to do walking regularly, 33% diabetic people and 7% control people in Surat were found to do regular exercise, where as 9% diabetic people and 8% control people in Surat were found to do yoga regularly.

Different physical activities like walking, Yoga and regular exercise is generally preferred by diabetic patients to reduce calorie. Above results suggests that walking is best preferred and suggested exercise by physician for diabetic control. People also use regular exercise and yoga to reduce extra calorie, a lack of physical activity and unhealthy eating habits as a result of urbanization combine to produce large numbers of people with NIDDM (Toumilehto *et. al*., 2001).

Target areas which sought to improve the quality of life of people with diabetes are improving the detection and control of diabetes, raising public awareness of the opportunities of prevention of diabetes and its complications, promotion of self-care for people with diabetes, supporting centers of excellence in diabetes education and research.

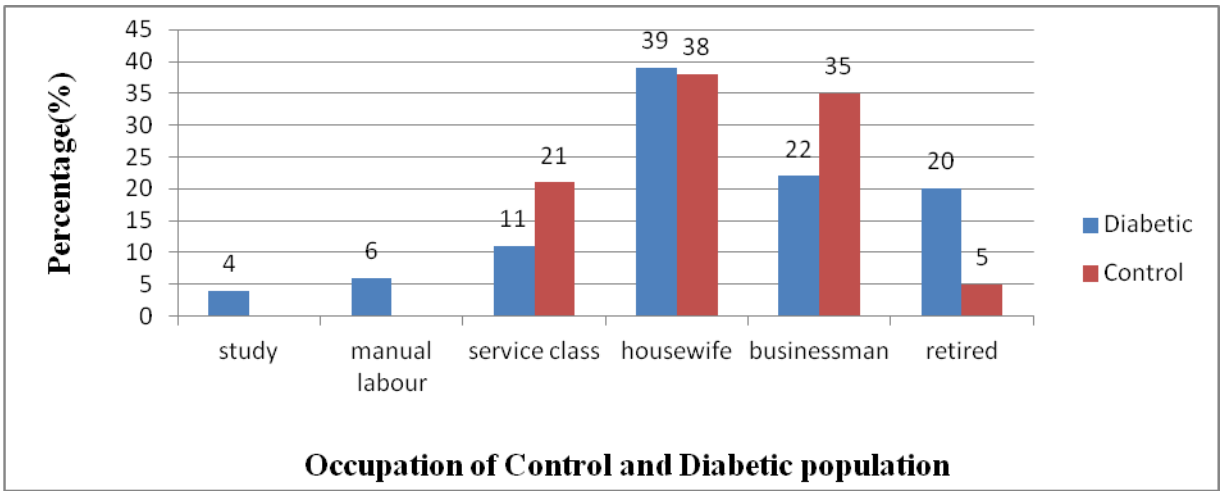
Fig. 19 Economic status of Control population (N=100) and Diabetic Population (N=100) of in Surat



In control population (N=100), 100 each from Surat, 43% diabetic people and 35% control people in Surat were found to be rich, where as 44% diabetic people and 55% control people in Surat were found to belong to middle class, Where as 13% diabetic people and 10% control people in Surat were found to belong to lower middle class.

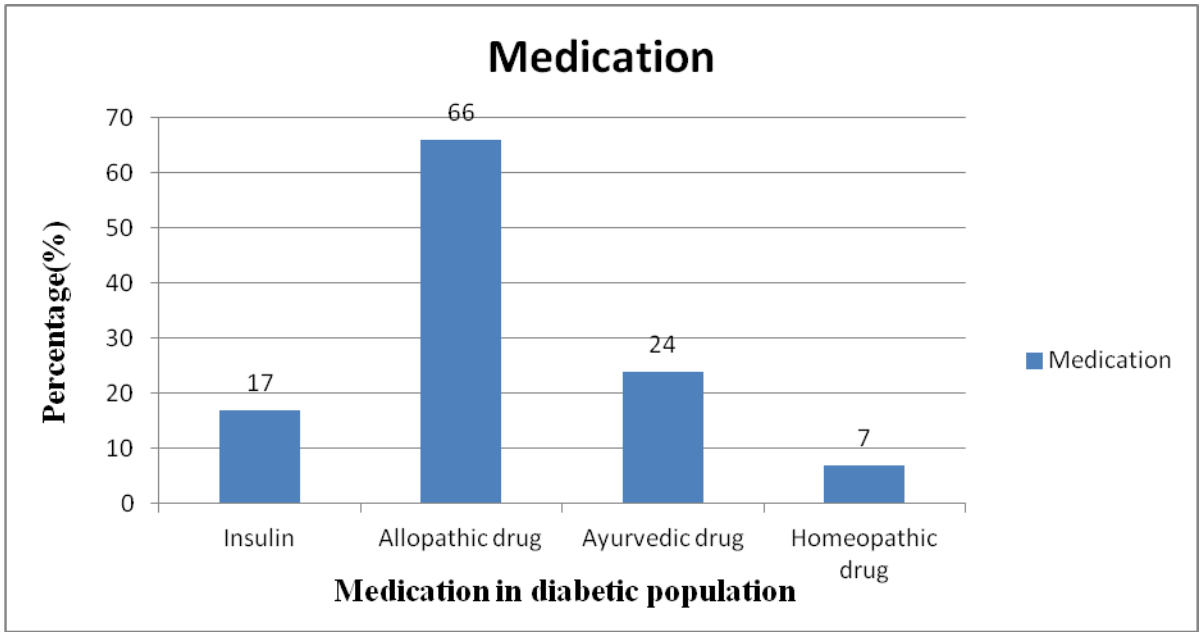
Diabetes has been always known as lifestyle disease. Surat showed higher number of rich class people and had higher incidence of diabetes too compared to the middle class population. Our finding indicates that diabetes is now spreading from cities to rural areas too.

Fig.20 Occupation of Control (N=100) and Diabetic population (N=100) in Surat



In diabetic and control population of 100 each from Surat, 6% diabetic people and no any control people in Surat were repoted during studies manual labourers, where as 11% diabetic people and 21% control people in Surat belong to service class. Housewives comprised of 39% diabetic people and 38% control people in Surat. 22% diabetic people and 35% control people in Surat were business class.20% diabetic people and 5% control people in Surat were retired.

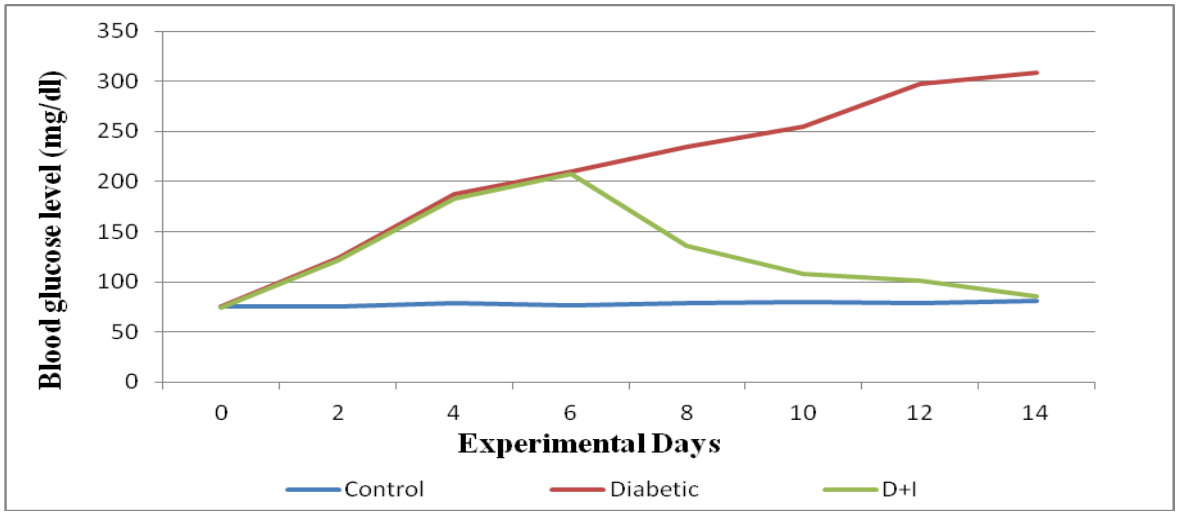
Fig. 21 Medical treatment preferred by Diabetic population (N=100)



Out of 100 diabetic patients, 17% in Surat were found to take insulin treatment, 66% in Surat were found to take allopathic drugs, where as 24% in Surat were found to take ayurvedic drugs, 7% in Surat were found to take homeopathic drug, where as interestingly, and few population in Surat were found to take herbal drug powder such as Karela,turmeric, Methi and jamboon powder along with their prescribed drugs.

4.2 Blood glucose level

Fig. 22 Blood glucose level in control and experimental group of Rat

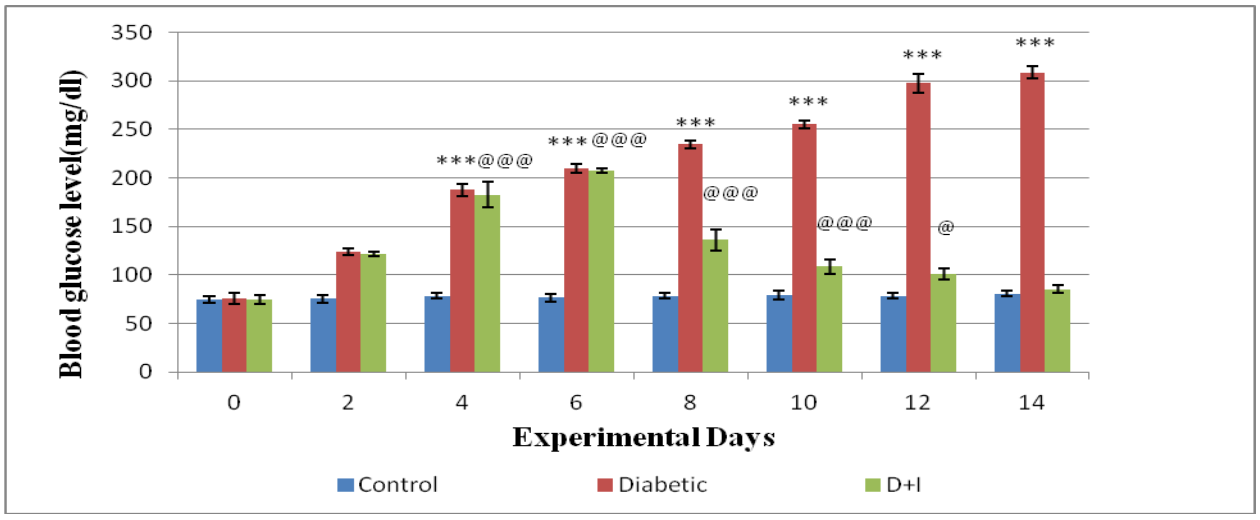


Values are Mean±SD of 3 separate experiments (n=3 rats per group).

C-Control, D- Diabetic, D+I-Diabetic+Insulin

A significant increase (P<0.001) in blood glucose level of D was observed as compared to C on 8th and 14th day of experiment. In D+I group there was a significant decrease (P<0.001) in blood glucose level as compared to D on 8th and 14th day of experiment.

Fig. 23 Blood glucose level in control and experimental group of Rat



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

*<P0.05 when compared to C @P<0.05 when compared to D

C-Control, D-Diabetic, D+I-Diabetic+Insulin

Table: 2 Blood glucose level in control and experimental group of rats

Experimental groups	Blood glucose level(mg/dL)		
	Day 2	**Day 8**	**Day 14**
C	75.5±3.87	78.5±2.64	81±2.94
D	124±3.91	234.5±4.20***	308.75±6.29***
D+I	121.75±2.36	136±11.34@@@	85.25±4.11

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

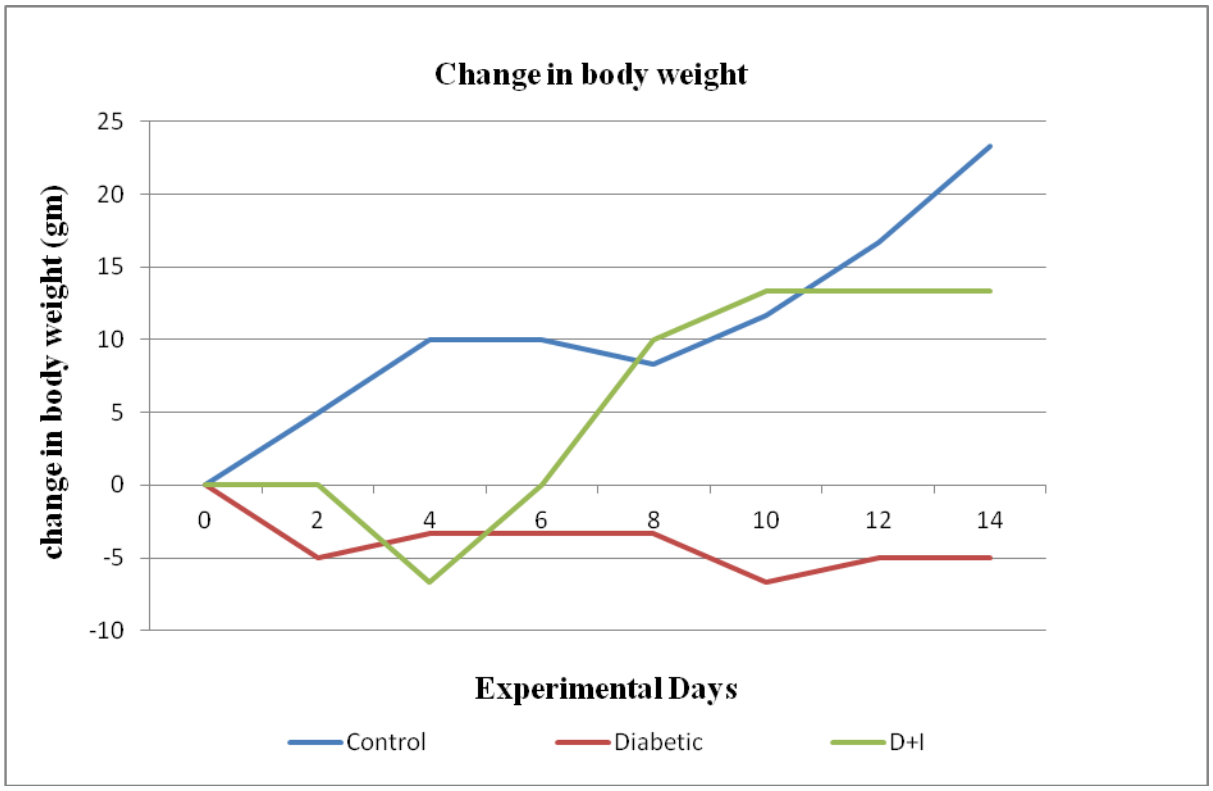
C-Control, D-Diabetic, D+I-Diabetic+Insulin

A significant increase (P<0.001) in blood glucose level of D was observed on 3rd day compared to C. D+I group showed an increase in glucose level when compared to C (P<0.001) on 3rd day of experiment. From day 3rd onwards a significant increase (P<0.001) in blood glucose level of D was observed compared to C, till 14th day of experiment. In D+I, there was a significant decrease (P<0.001) in blood glucose level compared with D from 4th day to 14th day of experiment.

A significant increase in blood glucose level of D was observed from 3rd day of model induction with streptozotocin when compared to C. This elevated level continued till 14th day of experiment. D+I group showed an increase in glucose level when compared to C on 3rd day of experiment, when D+I group was actually diabetic since insulin treatment was started on 4th day. D+I group also showed significant increase in glucose level when compared to D on 3rd day of experiment suggesting that rats in D+I group became hyperglycemic faster as compared to D. In D+I group there was a significant decrease in blood glucose level when compared to D from 4th

to 14th day of experiment post insulin treatment as anti hyperglycemic effect of insulin is well known (Adewole *et al*., 2007; Akbarzadeh *et al*., 2007; Mahmoud *et al*., 2009). Blood glucose level of D group was 200mg/dL-350mg/dL and blood glucose level of D+I group was 80-120 mg/dL.

Fig. 24 Body weight of control and experimental group of rats



Values are Mean of 2 separate experiments (n=3 rats per group).

C - Control, D - Diabetic, D+I - Diabetic+Insulin

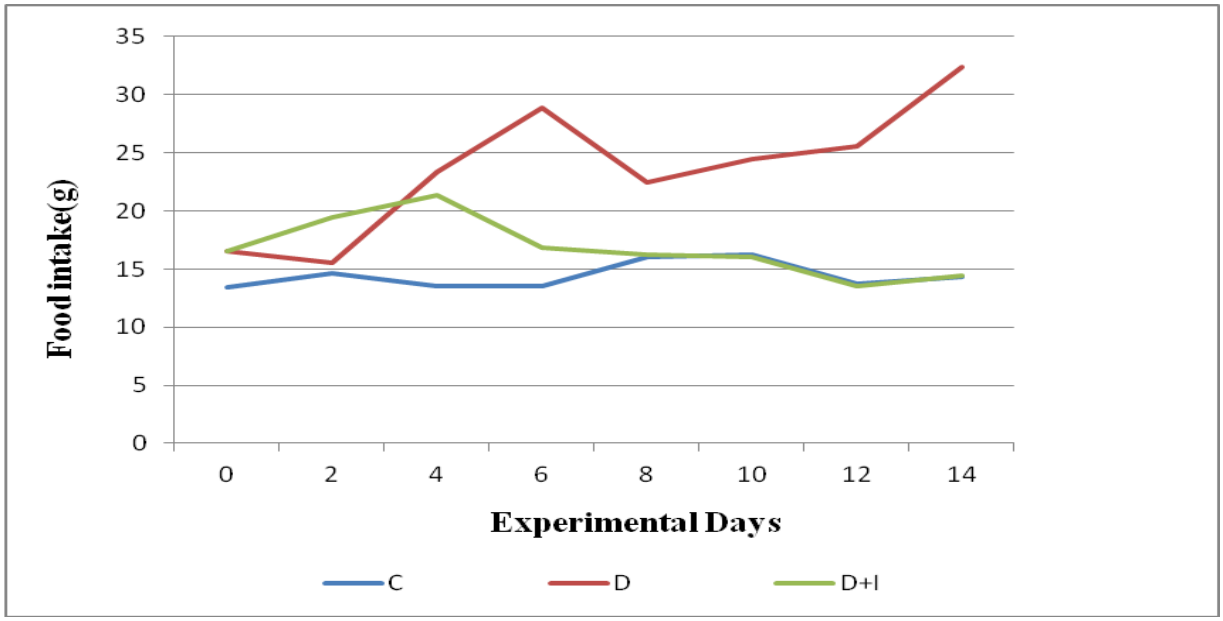
A decrease in body weight of D was observed compared with C from 3rd to 14th day of experiment. In D+I group there was an increase in body weight compared to D from 4th to 14th day experiment.

A significant decrease in body weight of D was observed when compared to C from 3rd to 14th day of experiment corresponding to high blood sugar resulting in polyuria which led to dehydration and loss of body fluids and electrolytes. During diabetes, lack of insulin prevents

transporters mediated glucose entry into the cell.Hence initiating alternative mechanism for glucose production in cell via glycogenolysis and gluconeogenesis. Glycogenolysis leads to depletion of glycogen reserves signaling for lipolysis causing this unhealthy weight loss in D group (Wood *et al.*, 2004). Insulin treatment prevents excessive fat metabolism and thus an increase in body weight of D+I group when compared to D from 4th to 14th day experiment was observed. A similar effect of insulin has previously been reported by the studies of Mahmoud *et al.*, (2009) and Akbarzadeh *et al.*, (2007).

4.3 Food consumption

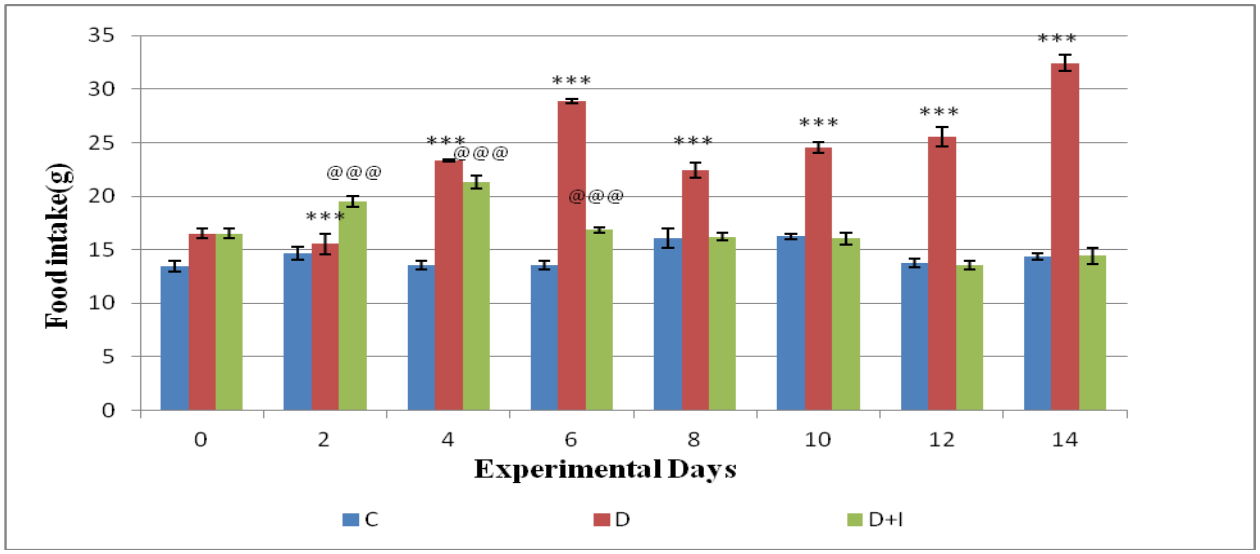
Fig. 25 Total food consumption after model induction in control and experimental group of rats (Active phase)



Values are Mean of 2 separate experiments (n=3 rats per group).

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Fig. 26 Total food consumption after model induction in control and experimental group of rats (Active phase)



Values are Mean±SD of 2 separate experiments (n=3 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

C - Control, D - Diabetic, D+I - Diabetic+Insulin

A significant increase (P<0.001) in food consumption of D was observed on 3rd day when compared to C. D+I group showed an increase (P<0.001) in food consumption compared to C on 3rd day of experiment. From day 3rd onwards a significant increase (P<0.001) in food consumption of D was observed compared to C, till 7th day of experiment. In D+I there was a significant decrease (P<0.001), in food consumption compared with D from 4th day to 7th day of experiment.

Table: 3 Total food consumption after model induction in control and experimental group of rats (Active phase)

Experimetal groups	Food inatake after model induction(g)		
	Day 2	**Day 8**	**Day14**
C	14.66±0.57	16.066±0.92	14.33±0.288
D	15.53±0.92	22.433±0.75***	32.43±0.75***
D+I	19.5±0.5	16.2±0.34	14.43±0.75

Values are Mean±SD of 2 separate experiments (n=3 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
C - Control, D - Diabetic, D+I - Diabetic+Insulin

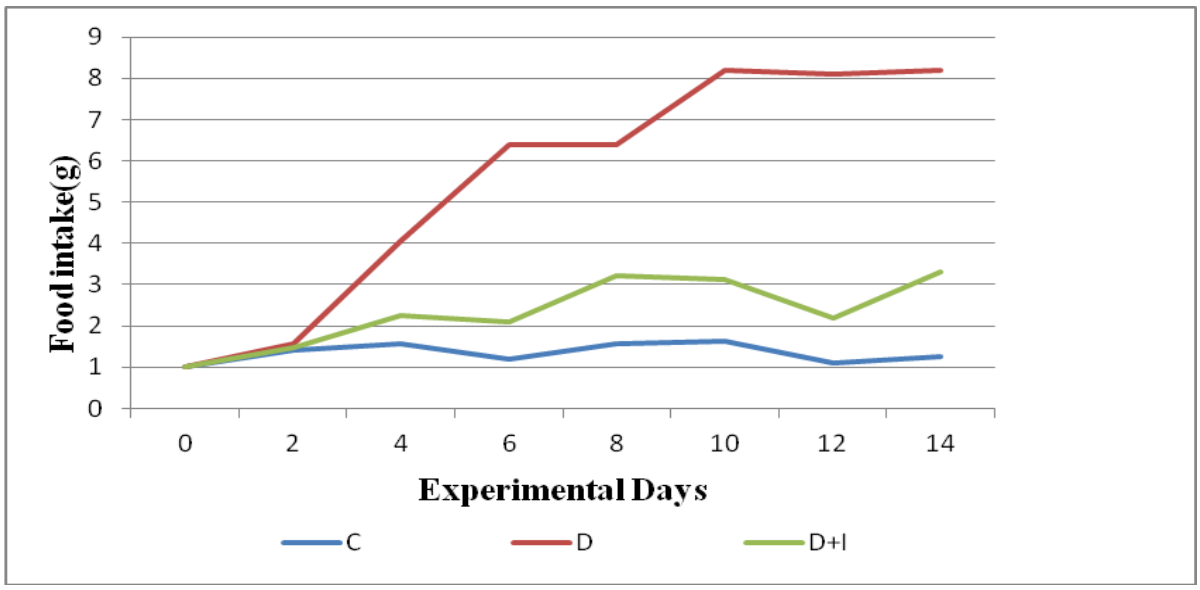
A significant increase (P<0.001) in food consumption of D was observed as compared to C on 7^{th} and 14^{th} day of experiment. In D+I group there was a significant decrease (P<0.001) in food consumption as compared to D on 7^{th} and 14^{th} day of experiment.

A significant increase in food consumption of D (P<0.001) was observed compared to C on 8^{th} day of experiment. A significant increase in food consumption of D (P<0.01) was observed compared to C on 9^{th} and 10^{th} day of experiment. A significant increase in food consumption of D (P<0.001) was observed compared to C on 11^{th} day of experiment. A significant increase in food consumption of D (P<0.05) was observed compared to C on 12^{th} day of experiment. A significant increase in food consumption of D (P<0.01) was observed compared to C on 13^{th} day of experiment. A significant increase in food consumption of D (P<0.001) was observed compared to C on 14^{th} day of experiment.

In D+I (P<0.01), there was a significant decrease in food consumption compared with D on 8^{th}, 9^{th} and 10^{th} day of experiment. In D+I (P<0.001), there was a significant decrease in food

consumption compared to D on 11th day of experiment. In D+I ($P<0.05$), there was a significant decrease in food consumption compared with D on 12th and 13th day of experiment. In D+I ($P<0.01$), there was a significant decrease in food consumption compared with D on 14th day of experiment.

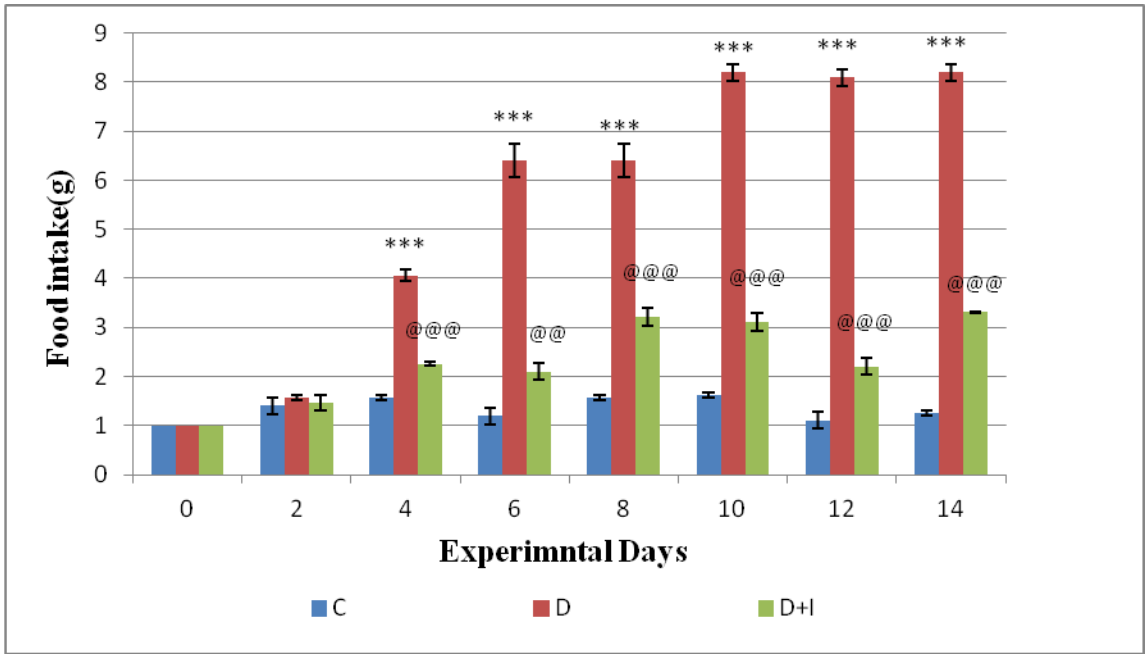
Fig. 27 Total food consumption after model induction in control and experimental group of rats (Inactive phase)



Values are Mean of 2 separate experiments (n=3 rats per group).

C- Control, D - Diabetic, D+I - Diabetic+Insulin

Fig. 28 Total food consumption after model induction in control and experimental group of rats (Inactive phase)



Values are Mean±SD of 2 separate experiments (n=3 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

** P<0.01 when compared to C @@ P<0.01 when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 4 Total food consumption after model induction in control and experimental group of rats (Inactive phase)

Experimetal Group	Total Food intake(g)		
	Day 2	Day 8	Day 14
C	1.4±0.17	1.56±0.05	1.26±0.05
D	1.56±0.05	6.4±0.34***	8.2±0.17***
D+I	1.46±0.15	3.22±0.19$^{@@@}$	3.31±0.017$^{@@@}$

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

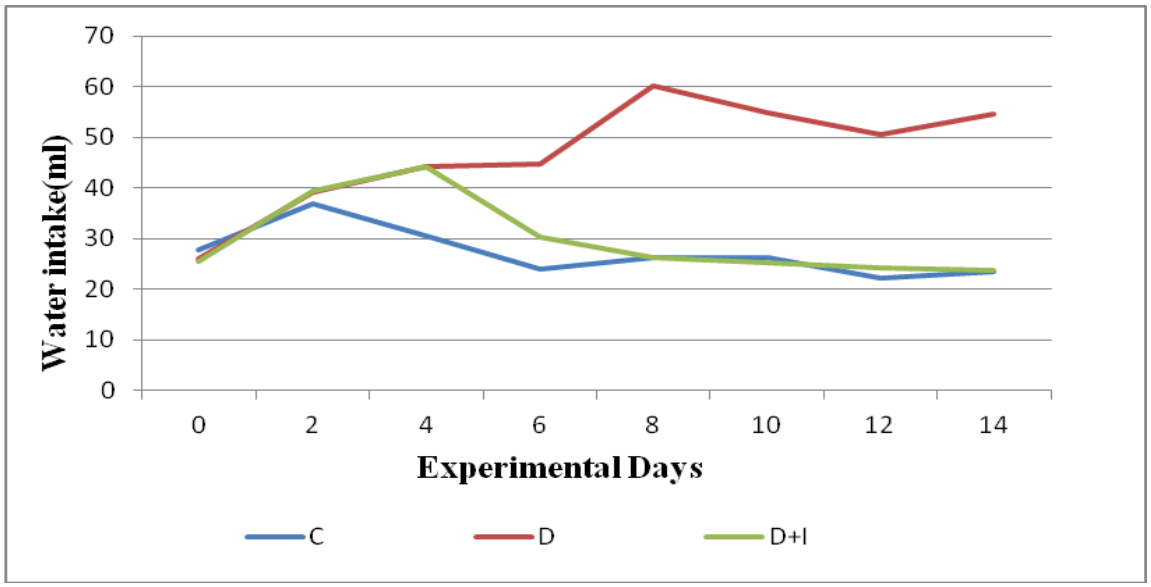
*** P<0.001 when compared to C @@@ P<0.001 when compared to D

** P<0.01 when compared to C @@ P<0.01 when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

4.3.1 Water intake

Fig. 29 Total Water intake after model induction in control and experimental group of rats (Active phase)

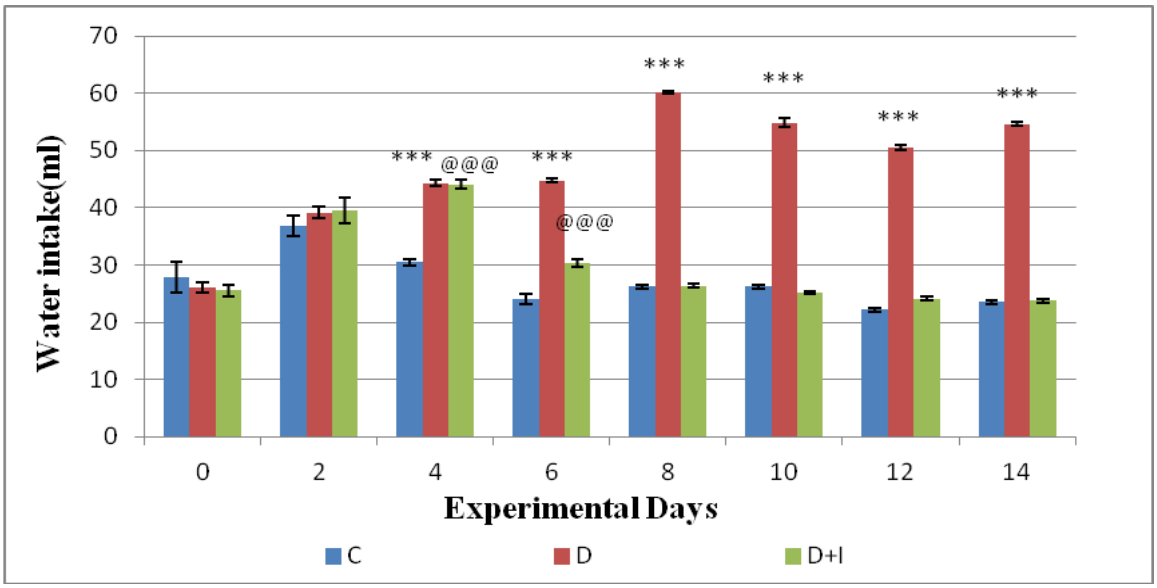


Values are Mean of 2 separate experiments (n=3 rats per group).

C - Control, D - Diabetic, D+I - Diabetic+Insulin

A significant increase in water consumption of D (P<0.001) was observed on 3rd day compared to C.D+I (P<0.001) group showed an increase in water consumption compared to C on 3rd day of experiment. From day 3rd onwards a significant increase in water consumption of D (P<0.001) was observed compared to C, till 14th day of experiment. In D+I (P<0.001), there was a significant decrease in water consumption compared with D from 4th day to 14th day of experiment.

Fig. 30 Total Water intake after model induction in control and experimental group of rats (Active phase)



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 5 Total Water intake after model induction in control and experimental group of rats (Active phase)

Experimental Groups	Total Water intake(ml)		
	Day 2	Day 8	Day 14
C	36.86±1.8	26.2±0.34	23.53±0.4
D	39.16±1.04	60.16±0.288***	54.66±0.28***
D+I	39.5±2.17	26.4±0.34	23.76±0.4

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

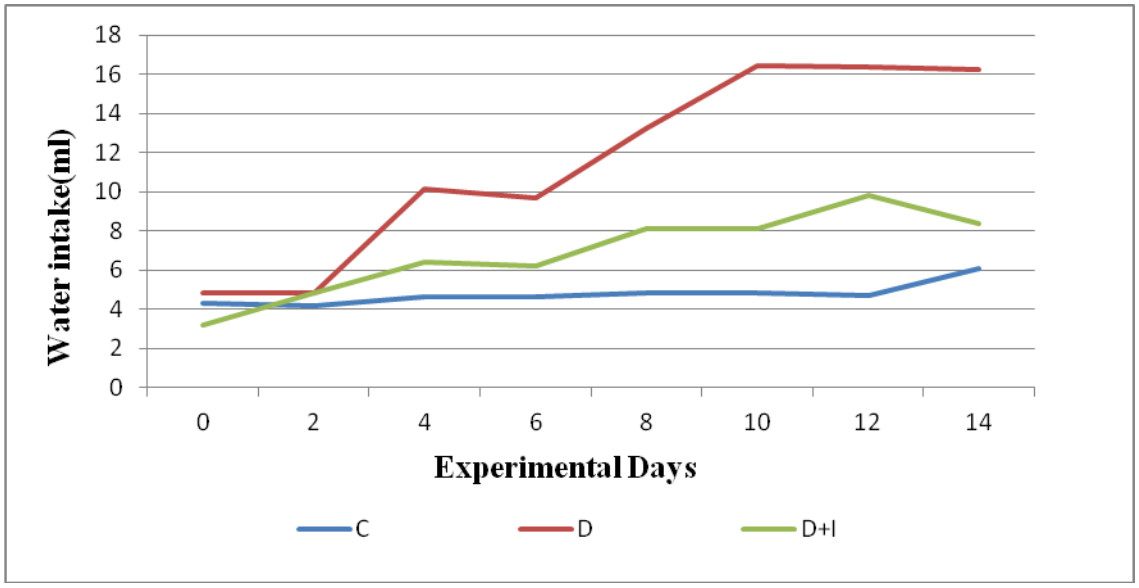
ANOVA followed by Student's-Newman-Keul's Test.

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

A significant increase in water consumption of D (P<0.001) was observed compared to C from 4th to 14th day of experiment. In D+I (P<0.001), there was a significant decrease in water consumption compared to D from 4th to 14th day of experiment.

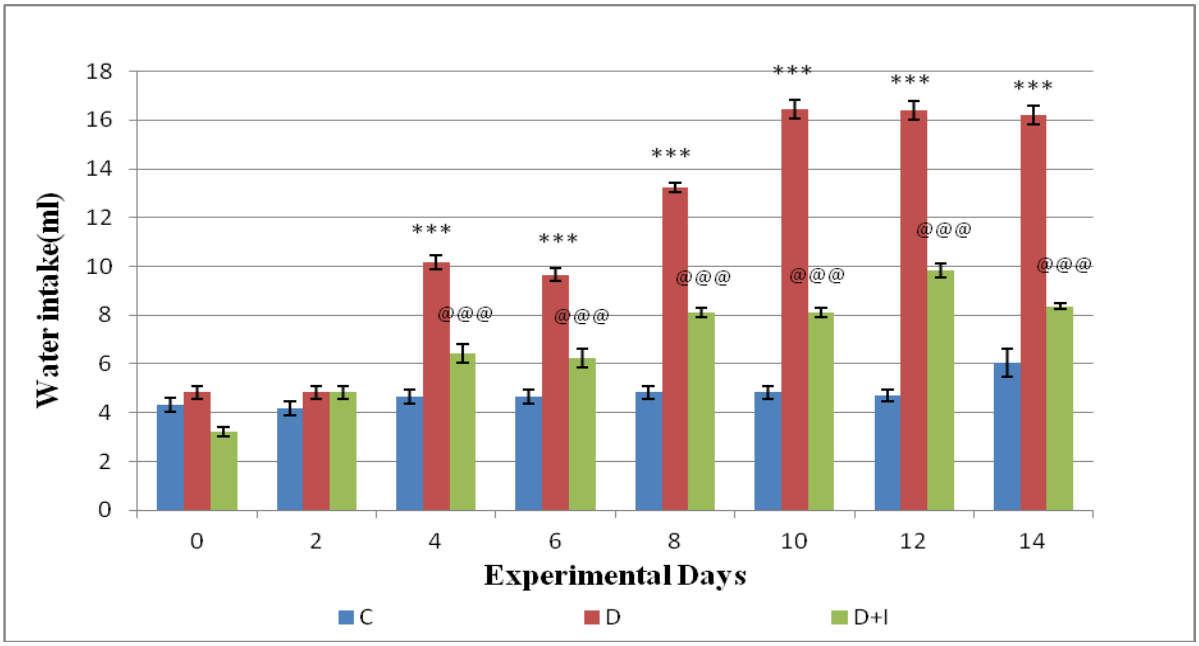
Fig. 31 Total Water intake after model induction in control and experimental group of rats (Inactive phase)



Values are Mean of 2 separate experiments (n=3 rats per group).

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Fig. 32 Total Water intake after model induction in control and experimental group of rats (Inactive phase)



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 6 Total Water intake after model induction in control and experimental group of rats (Inactive phase)

Experimental group	Total water intake(ml)		
	Day 2	Day 8	Day 14
C	4.16±0.26	4.83±0.28	6.05±0.58
D	4.83±0.28	13.22±0.17***	16.22±0.38***
D+I	4.83±0.18	8.11±0.19$^{@@@}$	8.37±0.1$^{@@@}$

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

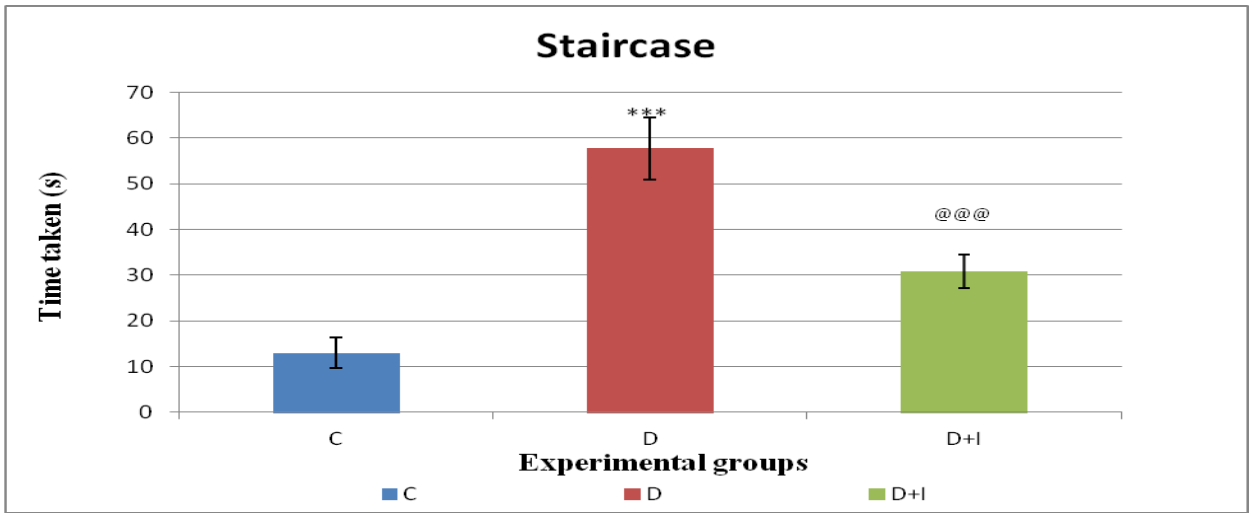
C - Control, D - Diabetic, D+I - Diabetic+Insulin

4.4 Behavioural study

Hyperglycaemia appears to play an important role in cerebral dysfunction. Diabetic mice with demonstrated cognitive impairment have been found to have increased expression of RAGEs (receptors of AGEs) in neurons and glial cells and damage to white matter and myelin, suggesting a possible role of RAGEs in the development of cerebral dysfunction. Still, the role of AGEs and receptors for AGE (RAGEs) in the development of cerebral complications of diabetes remains uncertain. (Christopher T. Kodl and Elizabeth R. Seaquist., 2008)

4.4.1 Staircase Test

Fig. 33 Staircase Test in Control and Experimental group of rats



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 7 Staircase test in control and experimental group of rats

Experimental groups	Total time taken (sec)
C	12.98±3.39
D	57.74±6.76***
D+I	30.83±3.58@@@

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

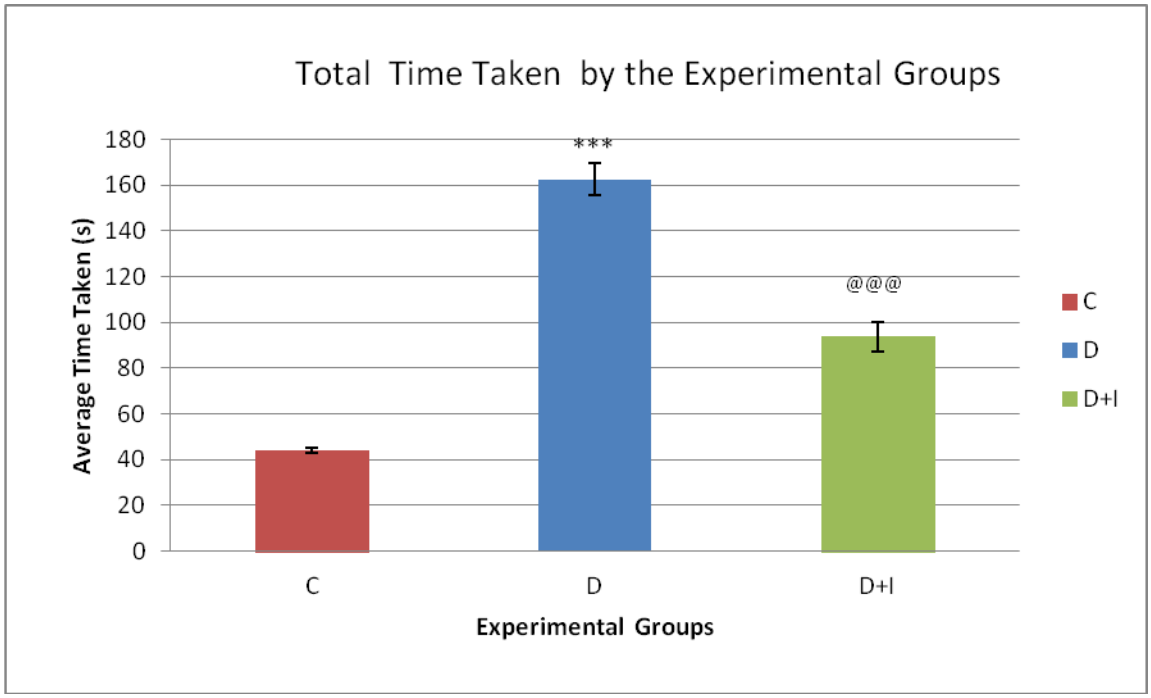
C - Control, D - Diabetic, D+I - Diabetic+Insulin

Staircase test when control compared to diabetic (P<0.001) showed significant increase in taken time on stair case in diabetic compare to control and when diabetic compare to diabetic + insulin (P<0.001) showed a significant decrease in the taken time on the stair case. Insulin treatment to diabetic rats (D+I) significantly decreased the retention time in stair case (P<0.001) compared to D. There was significant alteration in time taken by rat on stair case in C, D and D+I group. Insulin treatment to diabetic rats (D+I) significantly reverses the time near to C.

4.4.2 Karl Lashley's Maze

Diabetic Neuropathy is symptomatic behaviour commonly noticed in hyperglycaemia. To check the effect of memory and cognitive ability in diabetic rats they were assigned to solve the maze after training sessions. Three parameters were checked by the test: 1)total time taken by the maze 2) Immobile time taken by the groups 3) False hits.

Fig. 34 Total time taken by the Experimental groups to solve the maze



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 8 Total time taken by the Experimental groups to solve the maze

Experimental groups	Total time taken by Experimental group(sec)
C	44.08±1.19
D	162.58±7.07***
D+I	93.75±6.45$^{@@@}$

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

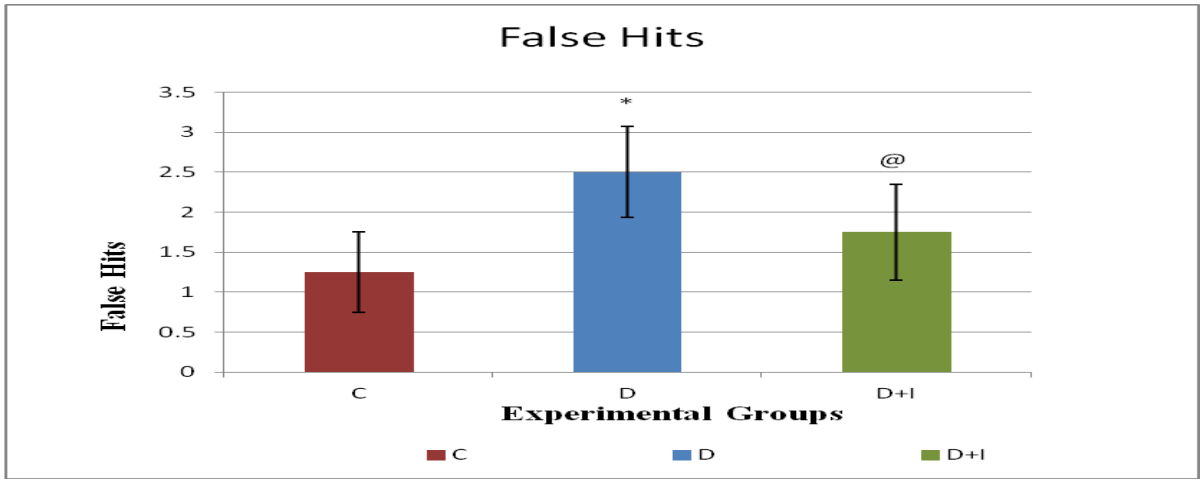
ANOVA followed by Student's-Newman-Keul's Test.

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

As per the results obtained in the test, Diabetic rat took maximum time to reach the end point. It was less active during the test and also was not able to recollect the doors. Time taken in each compartment was recorded. It concluded that the rats took major time in clearing the most difficult compartment which contained two pseudo doors and a single exit.

Fig. 35 Total number of False Hits performed by the Experimental groups



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

* P<0.05 when compared to C @ P<0.05 when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 9 Total number of False Hits performed by the Experimental groups

Experimental group	False Hits
C	1.25±0.5
D	2.75±0.6*
D+i	1.25±0.9@

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

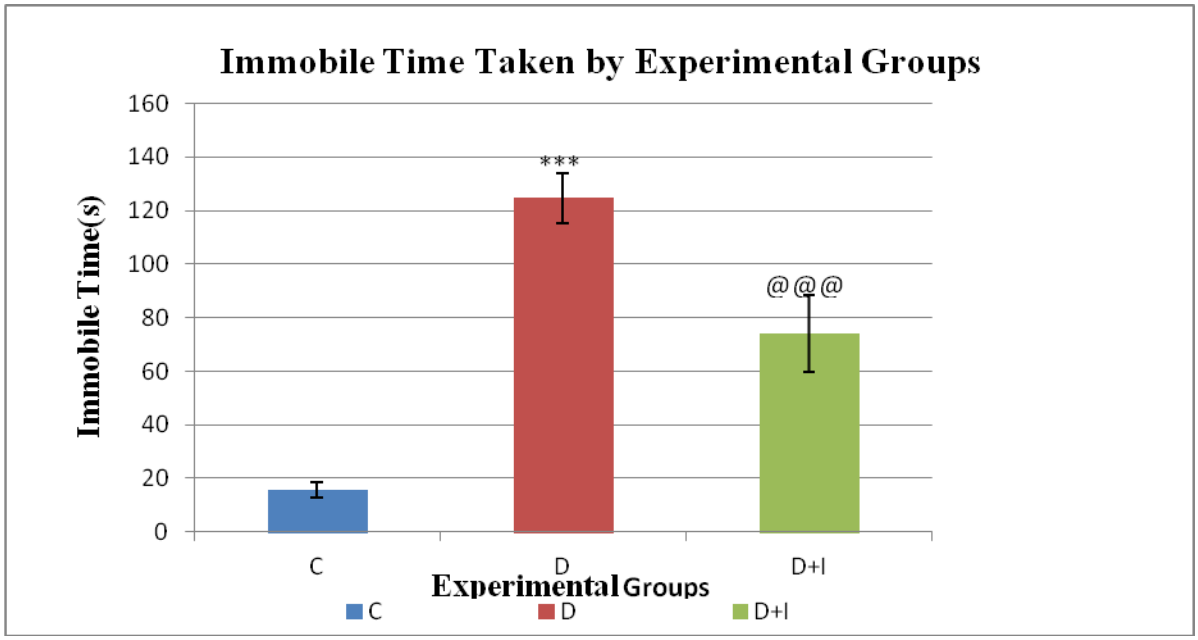
ANOVA followed by Student's-Newman-Keul's Test.

* P<0.05 when compared to C @ P<0.05 when compared to D

C-Control, D-Diabetic, D+I-Diabetic+Insulin

The third parameter checked was of false hits. The attempts done by a rat to escape by a pseudo door was recorded. Diabetic group committed maximum no. of false hits. Though there was not a significant difference between all the experimental groups. This showed defective cognition as they could not differentiate between the real and the pseudo door also after training sessions.

Fig. 36 Immobile time taken by experimental groups while solving the maze



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 10 Immobile time taken by Experimental groups while solving the maze

Experimental groups	Immobile Time taken by rats complete to task(sec)
C	15.83±2.88
D	124.75±9.28***
D+I	74±14.51@@@

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

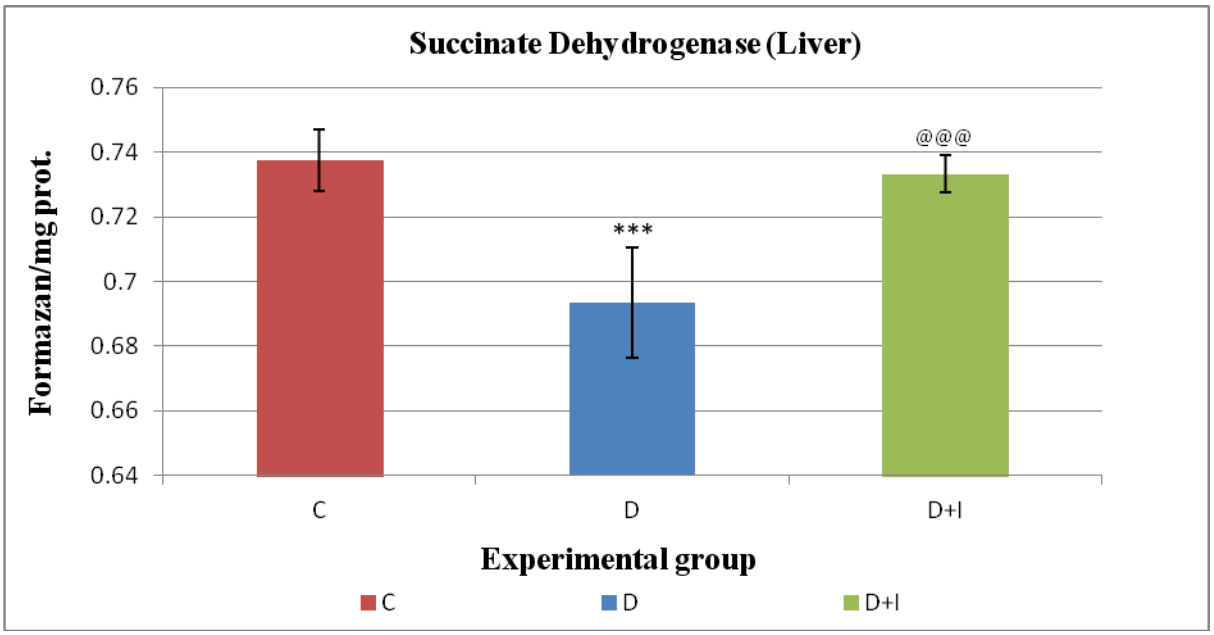
C-Control, D-Diabetic, D+I- Diabetic+Insulin

The rats showed a higher anxiety levels and immobility by continuous grooming behaviour. They did not try to find the door for escape. Diabetes reduced alertness and also affected the mobility. Increased urination was observed. Compared to the control rats diabetic did not try to find the clues to escape the maze and remained dormant.

4.5 Succinate Dehydrogenase

4.5.1 Enzyme Activity of Succinate Dehydrogenase in Liver

Fig. 37 Succinate Dehydrogenase in Liver



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I- Diabetic+Insulin

Table: 11 Enzyme Activity of Succinate Dehydrogenase in Liver

Experimental Group	Value
C	0.753±0.009
D	0.693±0.017***
D+I	0.733±0.005@@@

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C-Control, D-Diabetic, D+I- Diabetic+Insulin

A significant decrease in Diabetic group was observed. Insulin treatment could not restore the levels of succinate in Diabetic+Insulin Treated group but its activity was revived to an extent compared to the diabetic group.

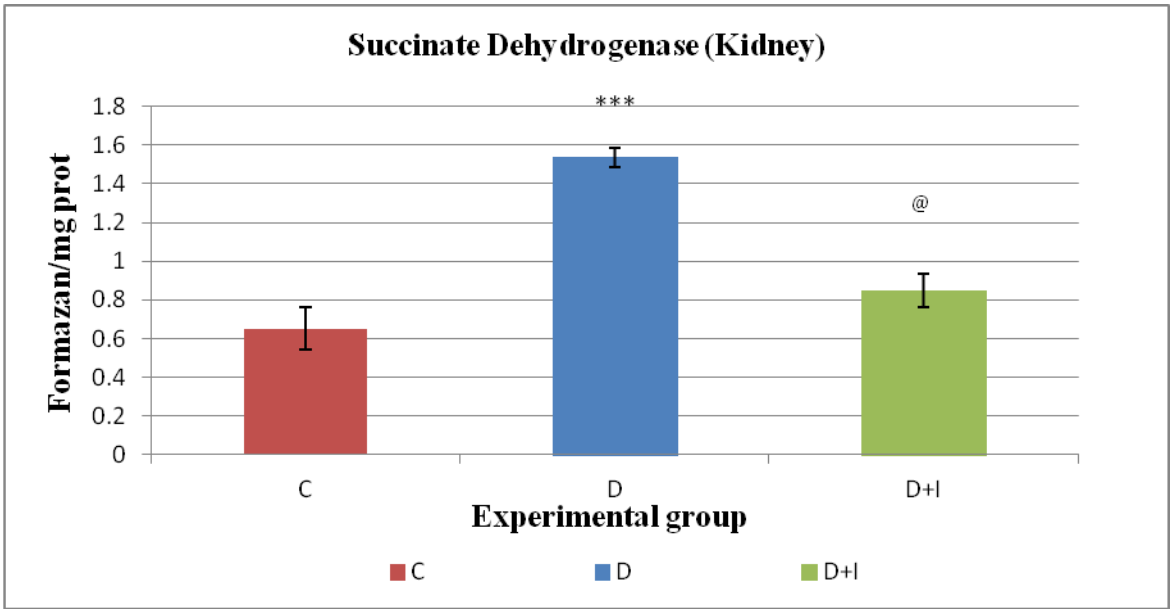
Discussion

In liver tissue, decrease in diabetic group was observed in diabetic model. Insulin treatment showed a positive effect on the diabetic group when compared with control.

Hyperglycemia increases electron flux through the mitochondrial electron transport chain which increases ATP/ADP ratio and hyperpolarization of the mitochondrial membrane potential as reviewed. In turn, this drives partial reduction of O_2, resulting in generation of free radical anion superoxide (Nishikawa et al., 2000; Brownlee,2001). Generation of ROS, is believed to be the fundamental source for mitochondrial dysfunction that plays a critical role in diabetes-related metabolic disorders and tissue histopathology. Also UCPs (uncoupling proteins) are able to modulate the coupling between the respiratory electron transport chain and ATP synthesis. UCP-induced proton leakiness causes partial depolarization of the mitochondrial transmembrane potential during type 2 diabetes (Dullo and Samec, 2001). Thus reduced activity of SDH was observed in our results.

4.5.2 Enzyme Activity of Succinate Dehyrogenase in Kidney

Fig. 38 Succinate Dehydrogenase in Kidney



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

*P<0.001 when compared to C @P<0.01 when compared to D

C-Control, D-Diabetic, D+I- Diabetic+Insulin

Table: 12 Enzyme Activity of Succinate Dehudrogenase in Kidney

Experimental Group	Value
C	0.652±0.11
D	1.536±0.05***
D+I	0.848±0.08$^{@}$

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C-Control, D-Diabetic, D+I- Diabetic+Insulin

Discussion

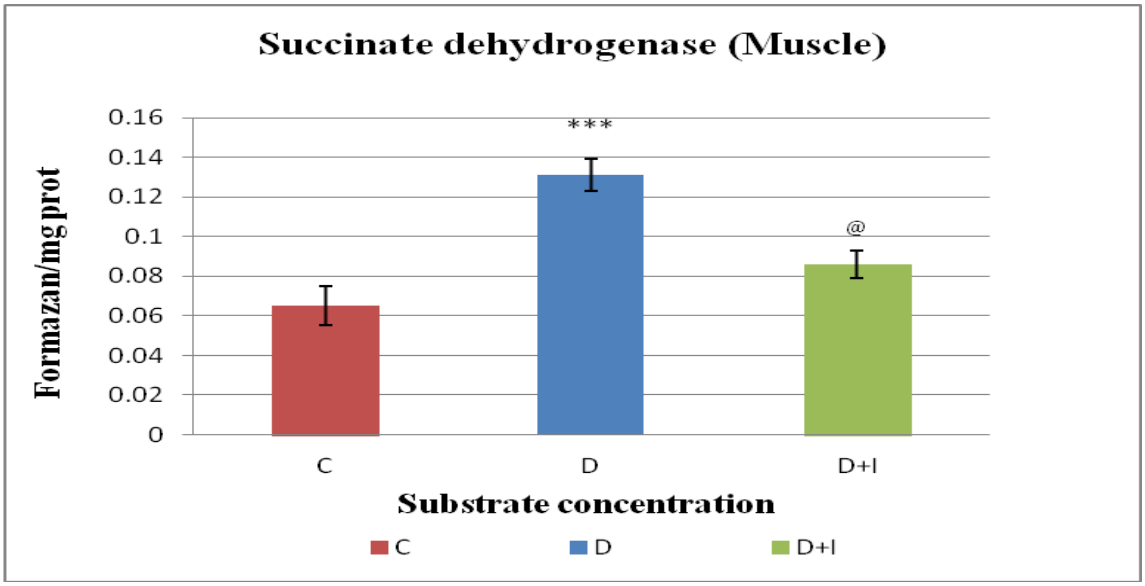
In Kidney, a steep increase in the enzyme activity of SDH (succinate dehydrgenase) was measured in diabetic group when compared to control. Insulin treatment was proved efficient in reducing the effect of damage on kidney.

Hyperglycemia is clearly associated with microvascular complications in many organs including the kidney, and diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) (Ritz E and Dikow R., 2006). In addition, diabetes may lead to other vascular complications, including systemic hypertension. Recent reports show kidney-specific effects of hyperglycemia on the activation of the local, intrarenal renin–angiotensin system (RAS) as a strong candidate for the core abnormality that leads to renal tissue injury. TCA cycle is a metabolically important cycle, functional in many tissues of the body and serves as a direct link between succinate dehydrogenase and hyperglycemia in kidney.

In kidney, it was reported that high glucose levels directly triggered the release of the prohypertensive hormone rennin. Increase in glucose levels resulted in accumulation of metabolic intermediate succinate, in plasma and local interstitium. Succinate acts as a ligand for GPR91 receptor in endothelial cytosol that stimulates production of calcium along with NO and Prostanglandin. These factors result in vasodilation and hyperpolarization that acts as a major development for glomerular hyperfilteration (Peti-Peterdi *et al*., 2008). Also, increase of SDH activity in kidney is reported. In insulin treated groups, It has been recently reported that insulin lispro, a rapid-acting insulin analogue, prevented glomerular hyperfiltration and offset the renal effects of meal-associated hyperglycaemia in diabetic patients with overt nephropathy (Iglesias et al., 2007). These literatures support our results as SDH activity was highest in diabetic group and reduced after insulin treatment.

4.5.3 Enzyme activity of succinate dehydrogenase in Muscle

Fig. 39 Succinate Dehydrogenase in Muscle



Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Table: 13 Enzyme Activity of Succinate Dehydrogenase in Muscle

Experimental group	Value
C	0.065±0.009
D	0.131±0.008***
D+I	0.086±0.007@

Values are Mean±SD of 2 separate experiments (n=3 rats per group).

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

***P<0.001 when compared to C @@@P<0.001when compared to D

C - Control, D - Diabetic, D+I - Diabetic+Insulin

Discussion

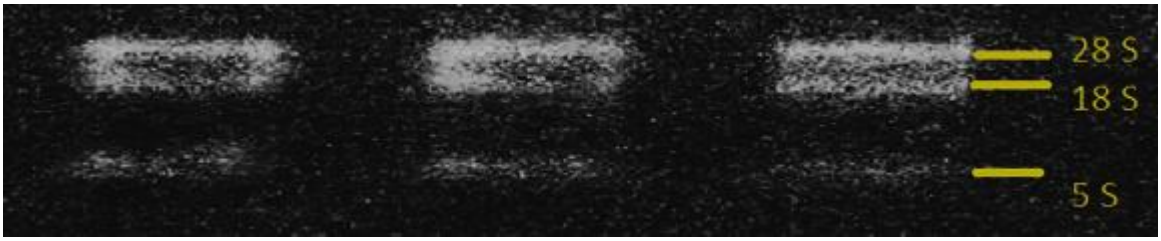
In muscle, significant increase in enzyme activity was observed in diabetic group than in control and insulin treated.

Kern and co-workers (1999) observed that loss in body weight resulted in increased capillarity/ fibre ratio i.e., muscle capillarization and SDH activity, suggesting an increase in muscle oxidative capacity with no change in muscle fibre type or cross-sectional area (Kern P. A. *et al*., 1999). In our investigation, diabetic rats showed a significant decrease in body weight and a corresponding increase in the activity of SDH enzyme. Post Insulin treatment, diabetic rats showed a gradual but significant increase in body weight compared to diabetic rats. Endurance training have been reported to alter metabolic characteristics in skeletal muscle by increasing the activity of some Krebs cycle enzymes including SDH in mitochondrial. Physical training resulted in increase of red muscles (type I) in expense to white muscles (type II) (Kirkendall *et al*., 1998). Type I fibre is small, has a low tension output, but is highly resistant to fatigue because type I fibre have numerous, large mitochondria that contain the enzymes of the Krebs cycle and the electron transport chain (Kirkendall *et al*., 1998). Our groups were also exposed to many tests which made them remain active where their motor activity was checked. This training may be involved in increasing SDH activity in our experimental groups, can be inferred.

4.6 PCR Gene expression

4.6.1 Total RNA isolation

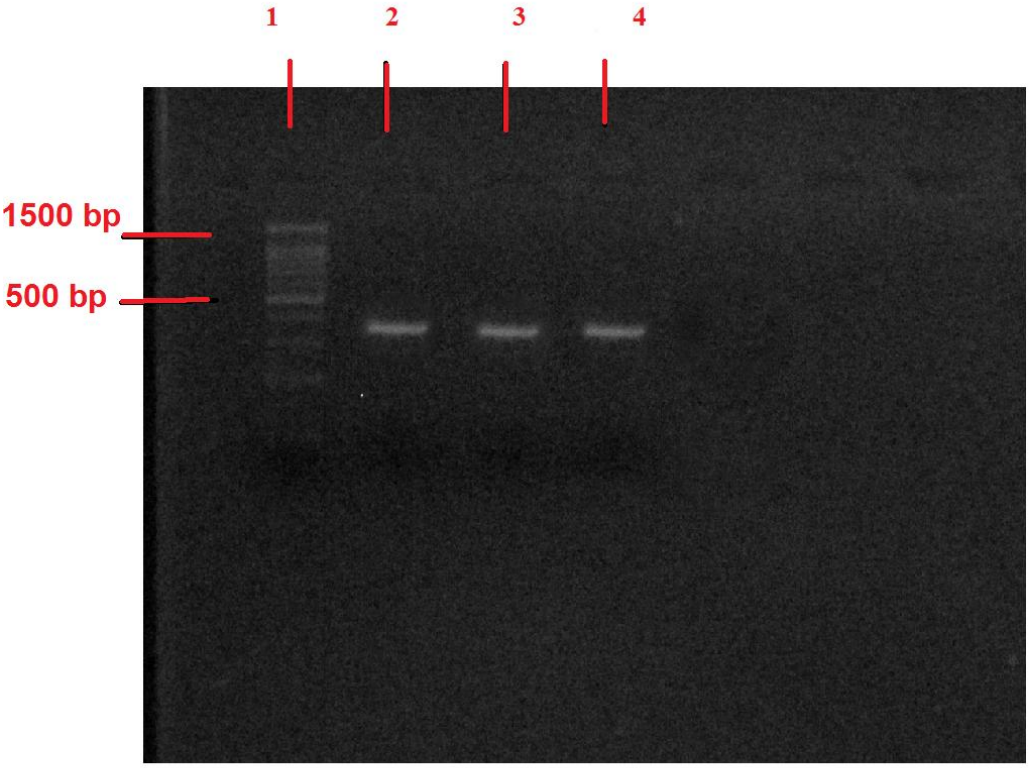
Fig. 40 Total RNA isolation in Control, Diabetic and Diabetic+Insulin group



Total RNA isolated is good enough to work upon.

4.6.2 β – actin gene expression (Positive control)

Fig. 41 β – actin in Control, Diabetic and Diabetic + Insulin group



Lane 1: Ladder

Lane2: C

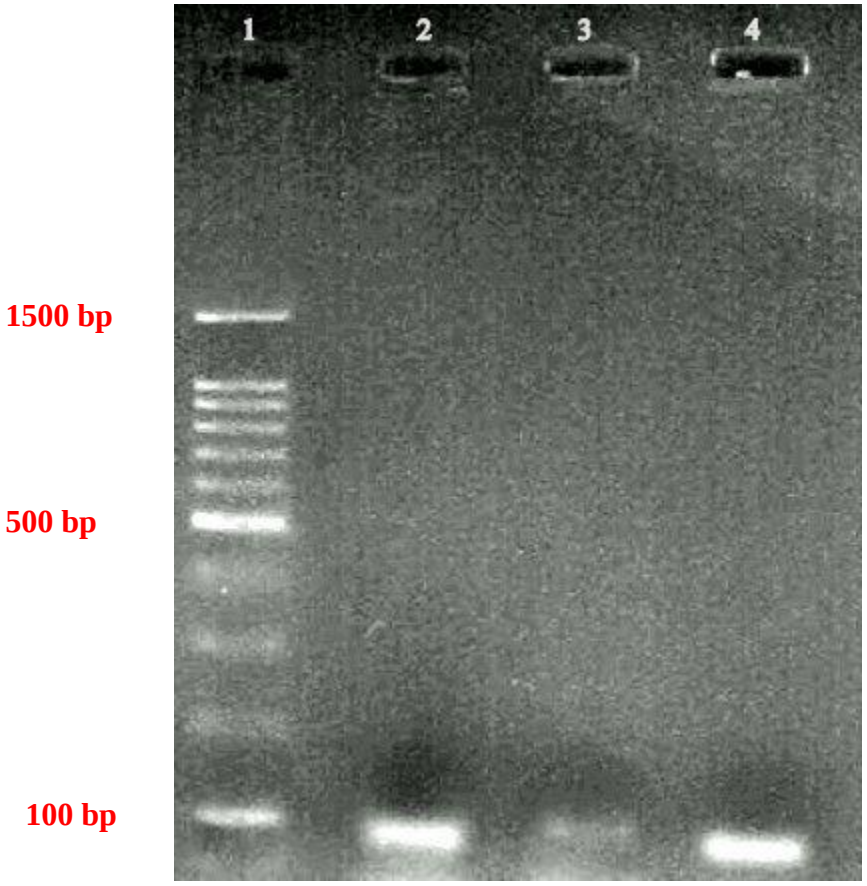
Lane3: D

Lane 4: D+I

As this is a house keeping gene. It is expressed in C, D and D+I groups. The intensity of bands using β-actin primer shows functional cDNA.

4.6.3 Fzd 3 gene expression

Fig. 42 Fzd 3 gene expression Control, Diabetic and Diabetic+Insulin group



Lane 1: ladder

Lane2: C

Lane3: D

Lane4: D+I

Fzd 3 gene expression

Functional primers were obtained but expression of frizzled 3 gene is still under standardization.

Summary

Our findings suggest that, neuropathy is one of the most common and debilitating complication of diabetes and results in motor dysfunction. Hyperglycemia induces oxidative stress in diabetic neurons and results in activation of multiple biochemical pathways. Insulin treatment was successful in our model. As Insulin receptors are found in the PNS on Schwann cells, pericytes, endothelial cells and neurons, especially sensory neurons. Our data shows that insulin treatment reverses the motor dysfunction in diabetes. Inefficient insulin secretion leads to many metabolic abnormalities which retards both mild and advance micro vascular complications in patients. Insulin-deficient rat models of diabetes appear to have severe noticeable neuropathy suggesting insulin deficiency contributes to the development of neuropathy. Defects in insulin secretion also affects body organs differently was recorded. Local delivery of insulin to streptozotocin treated rats improved nerve condition velocity at a level that does not cure hyperglycemia but able to decrease signs of mitochondrial distress in sensory neurons.

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