Effect of Hyperglycemia on Nogo Receptor Mediated Demyelination And Ornithine Transcarbamylase in Streptozotocin Induced Diabetic Rats

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Dedicated

to

My parents

And

My God

Acknowledgement

First and foremost I dedicate my sincere thanks to the Almighty for providing me a pure heart and a divine intervention. I shall seek his blessing in successfully going through the most challenging days of my graduate life.

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Ankita Mishra

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ABREVIATIONS

ANOVA: Analysis of Variance

BLAST : Basic local alignment search tool

CC: Cerebral Cortex

CPS: Carbamyl phosphate Synthase

DEPC : Diethyl pyrocarbonate

DPN: Diabetic peripheral Neuropathy

DNA : Deoxy ribonucleic acid

IDDM: Insulin Dependent Diabetes Mellitus

MAL : Myelin and lymphocyte associated glycoprotein

MIT: Mitocondria

NCBI: National Centre for Biotechnology information

NIDDM: Non Insulin Dependent Diabetes Mellitus

NO: Nitric Oxide

OTC: Ornithine transcarbamylase

PCR: Polymerase Chain Reaction

PKC-Protein Kinase C

PNS: Peripheral Nervous System

CNS: Central Nervous System

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RNA: Ribonucleic Acid

C: Control

D: Diabetes

D+I: Diabetes + Insulin

ABSTRACT

Neuropathy is the most common and debilitating complication of diabetes. Hyperglycemia induces oxidative stress in diabetic neurons and results in activation of multiple biochemical pathways. A significant increase was observed in the blood glucose, food consumption and water consumption in D group of rats as compare to control where as significant decrease in the body weight was observed in D compared to C. Enzyme assay of urea cycle enzymes such as ornithine transcarbamoylase (OTC) were studied. The enzyme activity of OTC was estimated at different substrate concentrations in liver and Kidney. A significant decrease in enzyme activity was observed in liver and kidney D as compared to C. Good quality RNA was isolated from cerebral cortex which was used for the cDNA preparation. Nogo receptor specific primer was designed to study its expression. There was no significant difference was obtained for Nogo receptor expression in experimental group of rats.

INTRODUCTION

1.1 Diabetes:

Diabetes mellitus is a diseases which is caused by insulin secretion or action, it affect metabolism of glucose, lipid and protein (Mayfield, 1998; Kim et al., 2006). It is associated with long-term damage, palsy, and failure of normal functioning of various organs, principally the eyes, kidneys, nerves, heart, and blood vessels (Paneni et al., 2013). Diabetes specific microvascular disease is the most leading cause of blindness, renal failure, and nerve damage (Cade, 2008). Diabetes is not only a single disease but also a group of disorders with glucose intolerance in common. It is a metabolic disorder characterized by hyperglycemia and results from defective insulin production, secretion, and utilization. The main source of fuel for the body is glucose. After digestion, glucose passes into the bloodstream, which is used by cells for growth and energy. The hallmark of diabetes is a disturbance in glucose homeostasis resulting in increased blood glucose levels because the body either does not release or use insulin adequately (Varanauskiene et al., 2006).

1.2 Diabetic Neuropathy:

Diabetic neuropathy is a long term complication of diabetes mellitus combined with neuronal damage (Bril et al., 2011). Hyperglycemia induces oxidative stress and results in activation of multiple metabolic pathways. These activated pathways are a major source of damage and are potential therapeutic targets in diabetic neuropathy due to oxidative stress (James et al., 2008). Thus axonal degeneration is hallmark of diabetic neuropathy but demyelination in CNS is not much studied hence this proposal intend to study inhibitory signal of CNS during diabetic condition. People with diabetes may develop nerve damage throughout the body without having any symptoms. Others may have symptoms such as pain, tingling, and numbness- loss of feeling-in the arms, hands, legs, feet. Nerve problems can occur in every organ system, including heart, digestive tract and sex organs. About 60 to 70% of people with diabetes have some form of neuropathy (James et al., 2008).

1.3 Urea Cycle:

The Urea Cycle (also known as the ornithine cycle) is a cycle of biochemical reactions that occurs in many animals which produces urea from ammonia (NH3). This cycle was the first metabolic cycle discovered (Hans Krebs and Kurt Henseleit, 1932). The urea cycle takes place primarily in the liver, and to a lesser extent in the kidney in mammals. Urea and uric acids are much less toxic than ammonia (Alton, 2010).

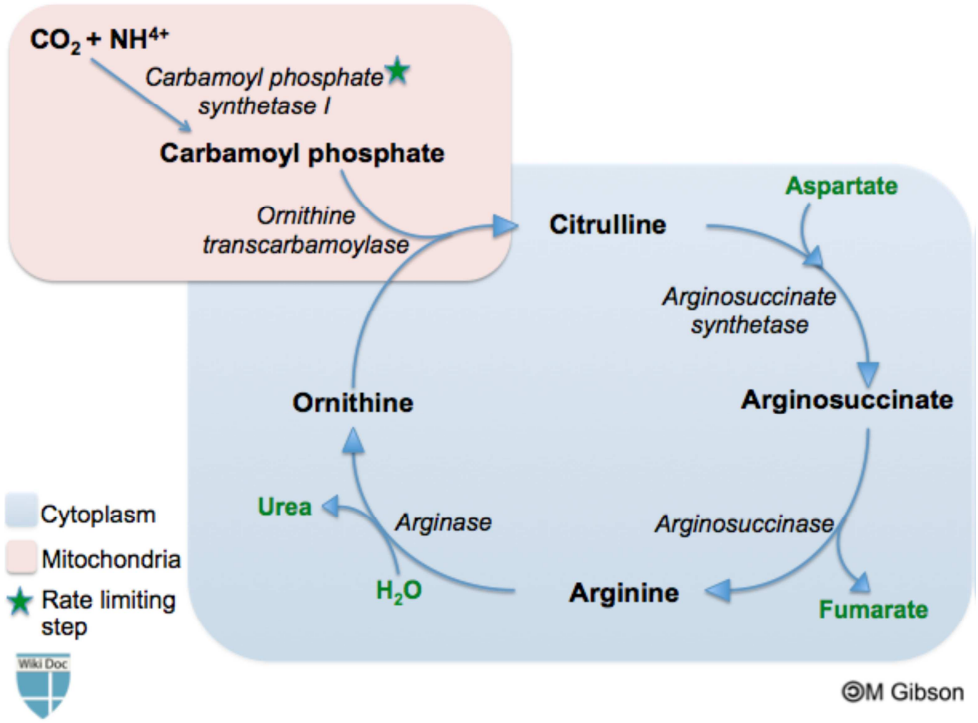


Fig. 1: The different steps of the Urea cycle

Insufficiency of the urea cycle occurs in some genetic disorders (inborn errors of metabolism), and in liver failure. The result of liver failure is accumulation of nitrogenous waste, in which mainly ammonia, that leads to hepatic encephalopathy.

1.3.1 Ornithine Transcarbamylase:-

OTC is a mitochondrial matrix enzyme, which catalyzes ornithine and carbamyl phosphate and results in citrulline. The corresponding gene of OTC is located on Xp11.4 Chromosome (Tuchman et al., 1994). Deficiency of this enzyme may lead to dysfunction urea cycle resulting in accumulation of ammonia that may add to neurological and metabolic problems. OCT has been used to be a sensitive serum marker of hepatic damage in diabetic rats (Hamaoki et al., 2009).

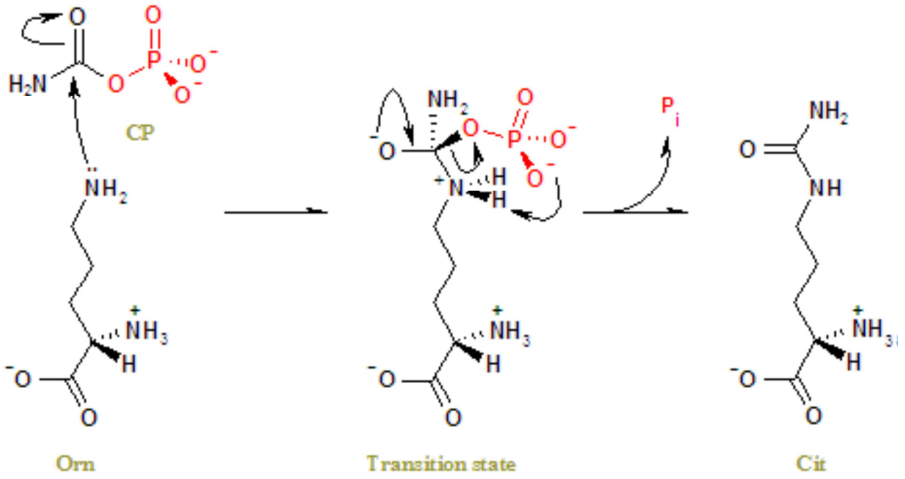


Fig.2 : Conversion of Ornithine to Citrulline by OTC enzyme

1.4 Oxidative Stress:-

Oxidative stress can be defined as a measure of the steady-state level of reactive oxygen or oxygen radicals in a biological system. Oxidative stress plays a major role in the development of

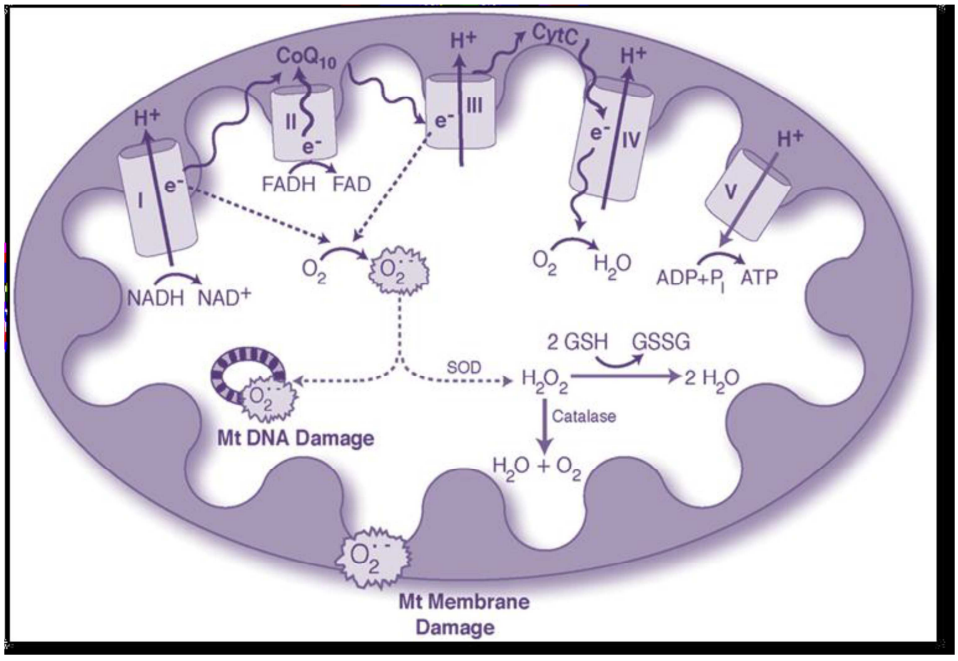


Fig.3: Oxidative stress and mitochondrial dysfunction (Lehninger et al., 2006)

diabetes complications, both microvascular and cardiovascular. The metabolic abnormalities of diabetes cause mitochondrial overproduction of superoxide in endothelial cells in both large and small vessels, as well as in the myocardium (Pellerin and Magistretti, 2002).

This increased superoxide production causes the activation of 5 major pathways involved in the pathogenesis of complications like Polyol pathway flux, increased formation of AGEs (advanced glycation end products), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C isoforms, over activity of the hexosamine pathway.

1.5 Introduction to Nogo Pathway

The Nogo receptor (NgR) plays a central role in mediating growth inhibitory activities of myelin-derived proteins, thereby severely limiting axonal regeneration after injury of the adult mammalian central nervous system (CNS). The inhibitory proteins Nogo, myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) are the major ligands which bind to the extracellular leucine rich repeat (LRR) domain of NgR, which provides a large molecular surface for protein–protein interactions. Ngr interacts with p75NTR to transduce the inhibitory signal across the membrane (McDonald et al., 2011) The inability of mammalian central nervous system (CNS) axons to regenerate may be attributed to growth-inhibitory proteins present in CNS myelin. Three myelin proteins –MAG, NOGO, and OMGP that are capable of inducing growth cone collapse and inhibiting neurite outgrowth *in-vitro*. Nogo occurs in three alternative splicing forms, termed Nogo-A, Nogo-Band Nogo-C, each of which share a common C-terminal portion composed of two transmembrane domains and a short 66 amino acid residue extracellular domain (Nogo-66). Nogo-66 has been shown to be at least partly responsible for the inhibitory activity of Nogo-A. MAG is a transmembrane protein whose extracellular region is composed of five immunoglobulin (Ig)-like domains. OMGP is a glycosylphosphatidylinositol anchored receptor with a LRR domain of eight repeats, followed by a serine/threonine-rich region. Even though these three inhibitory proteins do not share structural similarities based on primary sequence, each binds to the NgR.

1.6 HYPOTHESIS:

Based on these facts my aim for this project is to check, Effect of hyperglycemia on the expression of Nogo receptor mediated demyelination in streptozotocin induced diabetic rats.

1.7 Objective

- To study Nogo and its receptor function in cerebral cortex of brain inexperimental group of rats (C, D, D+I).
- To assess the alteration specific activity of Ornithine Transcarbamylase in control & experimental group of rats (C, D, D+I)

Aims to Attain Objectives

- To induce diabetes in adult Wistar rat by Streptozotocin.
- To check food, water consumption, change in body weight and blood glucose level in control and experimental group of rats (C, D, D+I).
- To study Nogo and its receptor function in cerebral cortex of brain inexperimental group of rats (C, D, D+I).
- To assess the alteration specific activity of Ornithine Transcarbamylase in control & experimental group of rats (C, D, D+I).

REVIEW OF LITERATURE

2.1 Diabetes

Diabetes mellitus is condition which requires a medical treatment and certain degree of health management. An estimated 346 million people worldwide live with diabetes, resulting in 3.4 million deaths in 2004, with more than 80% of these deaths occurring in low- and middle income countries (Qaseem, 2012).

The term diabetes describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with imbalance of carbohydrate, protein and cholesterol metabolism resulting from lack in insulin secretion, insulin action, or both. The result of diabetes include long–term damage, defective and failure of various organs (Report of a WHO,1985). Patient with diabetes shows with characteristic symptoms such as thirst, renal disorder, blurring of vision and weight loss. Often symptoms are not intense, or may be introuvable and consequently hyperglycemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made (Report of a WHO Consultation, 1999).

The effects of diabetes mellitus include long-term damage, decayed, and failure of various organs, especially the eyes (retinopathy), kidneys (nephropathy),heart (cardiomyopathy), and blood vessels (blood pressure) (Muranyi et. al., 2006). Diabetes presents symptoms such as Polydypsia, Polyuria, blurring of vision, weight loss, and polyphagia, which, in the absence of effective treatment, can even be fatal (Joslin's, 2000).

2.2 Types of Diabetes:

2.2.1 Type 1 Diabetes:

In this type of diabetes, insulin sensitivity does not play a major role so it can be also called as Insulin Independent Diabetes. Instead of insulin sensitivity the B cell dysfunction play a major role and due to B cell dysfunction insulin does not secret properly. This type of diabetes is immune mediated diabetes in which cellular mediated autoimmune destruction of the cells of the pancreas occur (Cade, 2008).

Type 2 Diabetes:

Type 2 Diabetes is a another form of diabetes which is commonly called as Hyperglycaemia associated with insulin resistance, decreased insulin signalling and β cell dysfunction. This cause abnormal glucose and lipid metabolism, increased oxidative stress and inflammation. Long term pathogenic condition of this type of diabetes includes micro and macro vascular complications, neuropathy, retinopathy, nephropathy (Santaguida et al., 2002; Evans et al., 2003; Spranger et al., 1998).

2.2.3. Gestational diabetes mellitus:

For many years, GDM has been defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Although most cases resolve with delivery, the definition applied whether or not the condition persisted after pregnancy and did not exclude the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy. This definition facilitated a uniform strategy for detection and classification of GDM, but its limitations were recognized for many years. As the ongoing epidemic of obesity and diabetes has led to more type 2 diabetes in women of childbearing age, the number of pregnant women with undiagnosed type 2 diabetes has increased (Dobrowsky et al., 2004). According to a 2014 analysis by the Centers for Disease Control and Prevention, the prevalence of gestational diabetes is as high as 9.2% (Dobrowsky et al., 2004).

2.2.4 Latent autoimmune diabetes of adults (LADA):

Latent autoimmune diabetes of adults (LADA) more closely resembles type 1 diabetes, it can often be misdiagnosed as type 2 diabetes. The main clinical difference is that the requirement for insulin is delayed in LADA. The term latent autoimmune diabetes of the adult (LADA) was introduced in 1995 to define the subgroup of adult diabetes patients who are classified clinically type 2 diabetes subjects but tested positive for GAD or other islet autoantibodies. Five years after diagnosis, 80% of LADA patients progress to insulin dependence (Irvine et.al., 1976).

2.3 Symptoms

Polydipsia and Polyurea : One of the most common symptoms of diabetes is an urge of excessive thirst and frequent urination. This condition arises due to enormous 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).

Polyphagia : 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 (Joslin's, 2000).

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 inefficiency of body to absorb glucose and frequent urination (Atalay et. al., 2002).

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 (Muranyi et. al., 2006).

Dryness of skin: Due to peripheral neuropathy, the circulation and proper functioning of sweat gland is hampered resulting in dry and itchy skin (Varanauskiene et al., 2006).

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 (Muranyi et. al., 2006).

2.4 Etiology

Diabetes is a chronic disease characterized by relative or absolute deficiency of insulin, causing glucose intolerance. The classic symptoms of diabetes mellitus result from abnormal glucose metabolism due to insulin resistance. 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." 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).

IDDM is due to destruction of pancreatic β cells. The cause of cell destruction is unknown. A few cases have followed viral infections, most commonly with coxsakievirus B or mumps virus. Autoimmunity is believed to be the major mechanism involved. NIDDM is due to a impaired insulin release. Basal secretion of insulin is often normal, but the rapid release of insulin follows a meal is greatly impaired, resulting in failure of normal handling of a carbohydrate load. In most patients, some level of insulin secretion is maintained, so that the abnormality of glucose metabolism is limited and Ketoacidosis is uncommon. In these patients, insulin secretion can be stimulated by drugs such as sulfonylurea. Exogenous insulin is therefore not essential in treatment (Muranyi et. al., 2006).

2.5 Diabetic Neuropathy:

Diabetic neuropathy is neuronal complications caused by/ through diabetes. Several types of diabetic neuropathies are recognized, It was classified by Leyden in 1893, as hypersthetic (painful), paralytic (motor) and ataxic neuropathies. Affected nerves during diabetic neuropathy are large fiber sensory, small-fiber sensory, autonomic, and motor. Syndrome of diabetic neuropathy are also varied and totally dependent on pathology of the disease, in which some can produce progressive disorders and others can lead to monophasic illness (Leyden, 1893). Diabetic neuropathy is the leading cause of non traumatic limb amputation (Thomas et al., 1999).

Hyperglycemia leads to increased mitochondrial activity, raising reactive oxygen species (ROS) production of mitochondrial peroxynitrite, the primary reactive nitrogen species (RNS) is formed by the reaction of superoxide and nitric oxide (NO). RNS induces a number of cytotoxic effects including protein nitrosylation and activation of poly ADP ribose polymerase (PARP) (Obrosova et al., 2005; Edwards et al., 2008).

2.6 Myelination

The myelin sheath is produced by glial cell present around axons (oligodendrocytes in the CNS). Myelin sheath produce large membranous extensions that ensheath the axons in successive layers that are then compacted by exclusion of cytoplasm to form the myelin sheath. The density of the myelin sheath (the number of wraps around the axon) is proportional to the axon's diameter (Seigel, 2006). Myelination is the process by which glial cells ensheath the

axons of neurons in layers of myelin. Myelin ensures the rapid navigation of electrical impulses in the nervous system. Myelin sheaths are formed by the ample membranous extensions of glial cell like oligodendrocytes in the CNS (Grandpre et al., 2000).

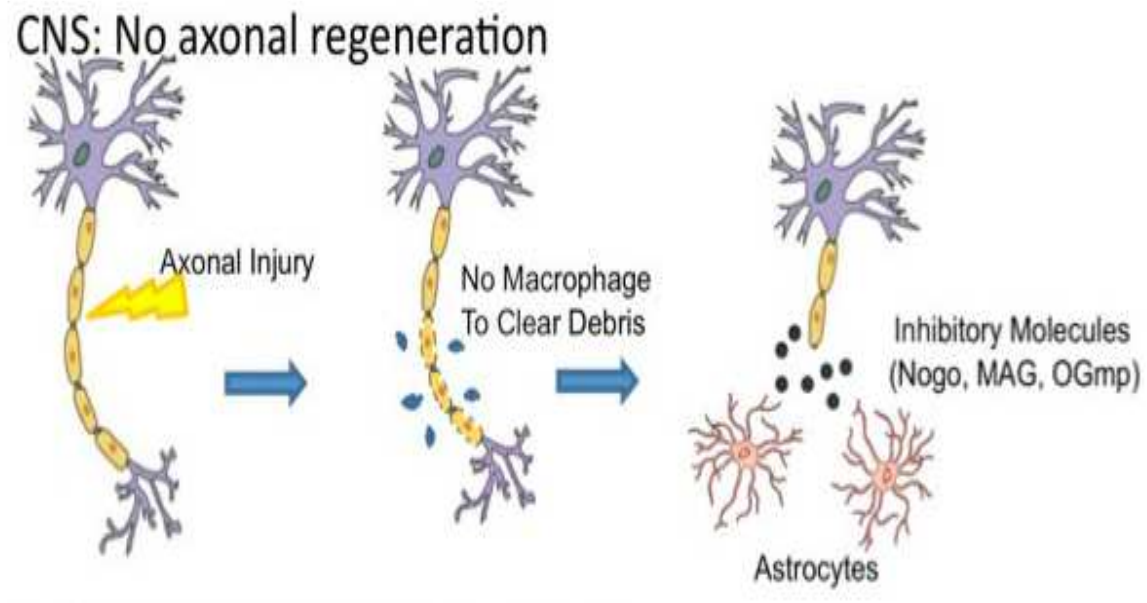


Fig. 4 CNS: No axonal regeneration (Grandpre et al., 2000)

In the CNS, macrophages are not present to clear the debris impeding neuronal repair. In addition, CNS damage attracts astrocytes, which form a glial scar that prevents regeneration. Regeneration of CNS neuron is also hindered by inhibitory components in the myelin such as Nogo, MAG, and OMgp, all of which inhibit neuronal sprouting. Hence, myelin damage in CNS usually do not recover substantially from their ailment (Muranyi et. al., 2006). In PNS inhibitory molecules are absent.

2.8 Oxidative stress and ROS formation during Diabetic Neuropathy

Oxidative stress arises due to disturbed equilibrium between pro-oxidant/ antioxidant homeostasis. Generation of ROS and free radicals those are potentially toxic for neuronal cells (Rosen et al., 2004). Brain contains various types of glial cell and these are involved in anatomic support and biochemical requirement. The endothelial cells encompassing the glial cells are less permeable for uptake of protective cells and various molecules viz. macrophages compared to

other endothelial cells in the body (Simonian and Coyle, 1996). ROS are active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, whose metabolism is factory of reactive oxygen species, this are exclusive to the brain and serve as sources of oxidative stress. ROS offensive glial cells and neurons, which are post-mitotic cells and for it, they are individually sensitive to ROS, leading to neuronal damage (Gilgun-Sherki Y et al., 2001).

The toxic oxygen species produce serious derangement in cell metabolism, including DNA destruction, modification of proteins and also induced damage to membrane ion transporters and/or other specific proteins. Hydrogen peroxide synthesized in the course of oxidative stress participates in the development of diabetic vascular complications (Packer, 1993).

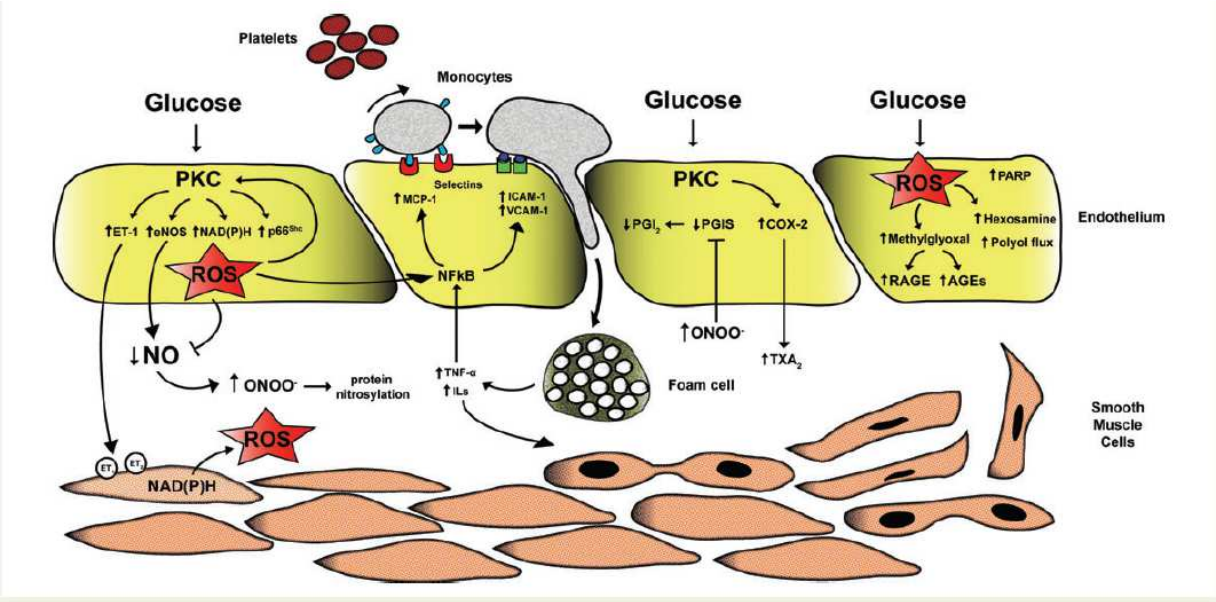


Fig.5: Mechanisms of hyperglycemia-induced vascular damage (Paneni et al.,2013)

Mechanisms of hyperglycemia-induced vascular damage has been shown in fig 10. High intracellular glucose concentrations lead to PKC activation and subsequent ROS production by NADPH oxidase and p66Shc adaptor protein. Increased oxidative stress rapidly inactivates NO leading to formation of the pro-oxidant responsible for protein nitrosylation. Reduced NO availability is also due to PKC-dependent eNOS deregulation. Indeed, PKC triggers enzyme up-regulation thus enhancing eNOS uncoupling and leading to a further accumulation of free radicals. On the other hand, hyperglycemia reduces eNOS activity blunting activatory

phosphorylation at Ser1177. Together with the lack of NO, glucose-induced PKC activation causes increased synthesis of ET-1 favouring vasoconstriction and platelet aggregation. Accumulation of superoxide anion also triggers up-regulation of pro-inflammatory genes MCP-1, VCAM-1, and ICAM-1 via activation of NF-kB signalling. These events lead to monocyte adhesion, rolling, and diapedesis with formation of foam cells in the sub-endothelial layer. Foam cell-derived inflammatory cytockines maintain vascular inflammation as well as proliferation of smooth muscle cells, accelerating the atherosclerotic process. Endothelial dysfunction in diabetes also derives from increased synthesis of TXA2 via up-regulation of COX-2 and inactivation of PGIS by increased nitrosylation. Furthermore, ROS increase the synthesis of glucose metabolite methylglyoxal leading to activation ofAGE/RAGEsignalling and the pro-oxidanthexosamine and polyolpathway flux. PKC, protein kinase C; eNOS, endothelial nitric oxide synthase; ET1, endothelin 1; ROS, reactive oxygen species; NO, nitric oxide; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intracellular cell adhesion molecule-1; AGE, advanced glycation end product (Paneni et al.,2013).

As in similar conditions, oxidative stress in diabetes mellitus stems mainly from increased or excess production of free radicals and/or a sharp reduction of antioxidant resistance. It is well known that superoxide anion is the primary radical formed by the reduction of molecular oxygen that may lead to secondary radicals or reactive oxygen species (ROS), Then again, there is confirmation that diabetes induces changes in the activities of antioxidant enzymes in various tissues (Ahmed, 2005).

2.9 Nogo Pathway

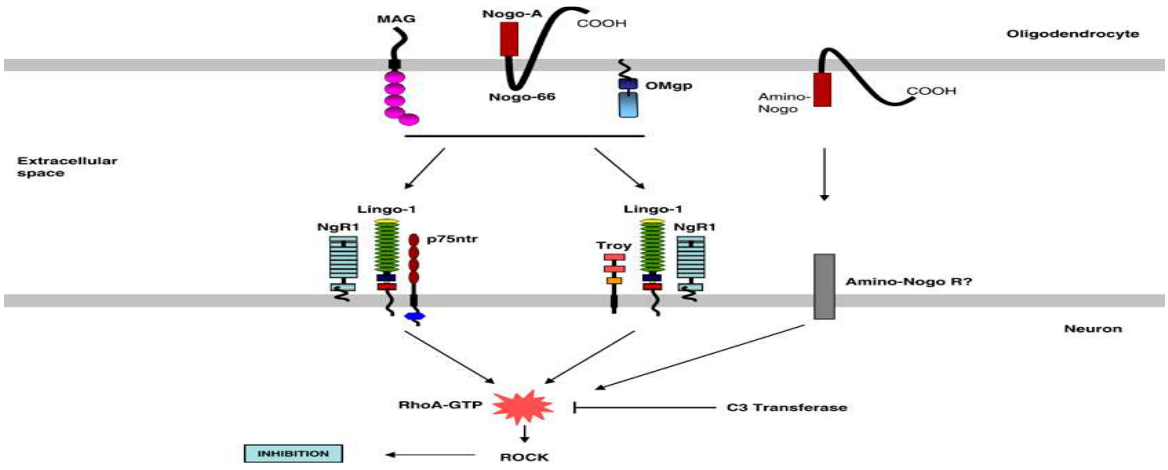


Fig.6 Oligodendrocyte Myelin Components Inhibit Axonal Regeneration (Fournier et al., 2001)

The Nogo pathway, TLR-ASK1-p38 pathway, Calpain-caspase pathway, MEK Erk pathway are major pathways of axonal demyelination. Injury to the central nervous system (CNS) has long been known to cause fatal and irreversible damage to axons and neurons. A number of physical and molecular inhibitory factors demonstrated by neurons, astrocytes, and oligodendrocytes serve to maintain the architecture of the mature CNS, but at the same time donate to the shortage of repair mechanisms following damage. Axons are essential for neuronal communication but they do not regenerate after injury to the adult mammalian brain or spinal cord. Unsuccessful regeneration is due in part to the production of a potent axonal growth inhibitor, Nogo, by myelinating cells. The finding of a high affinity axonal receptor for the extracellular domain of Nogo provides the first insight into the basis of Nogo action. Interupting the interaction of Nogo with the Nogo-66 receptor may facilitate axonal regeneration *in vivo* (Fournier et al., 2001).

The Nogo receptor (NgR) plays a central role in mediating growth inhibitory activities of myelin-derived proteins, because of that soberly limiting axonal regeneration after injury of the adult mammalian central nervous system (CNS). The inhibitory proteins Nogo, myelinassociated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMGP) are the major ligands which bind to the extracellular leucine rich repeat (LRR) domain of NgR, this domain provides a large molecular surface for protein–protein interactions (Wang et al., 2002). Even so, epitopes within the LRR domain of NgR for binding Nogo, MAG and OMGP have not still been disclose.

Ngr interacts with p75NTR to transduce the inhibitory signal across the membrane. The receptor for amino-Nogo is not known. Withal, amino-Nogo, Nogo-66, MAG and OMGP all activate Rho to bring about inhibition. MAG has been shown to inactivate Rac (McDonald et al., 2011).

The inability of mammalian CNS axons to regenerate may be attributed to growth-inhibitory proteins present in CNS myelin. Three myelin proteins – MAG, NOGO, and OMGP that are adequate of inducing growth cone collapse and inhibiting neurite outgrowth *in-vitro*. It remains to be seen further

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS characterized by sharply demarcated areas of demyelination and axonal loss or damage, resulting in a numerosity of neurological deficits. So far, MS has been regarded as a primary demyelinating disorder, and much effort has been devoted to investigating the relationship between the evolution of lesions and clinical progression in terms of myelin destruction and repair. Axonal damage in the pathogenesis of MS shows that there is a strong correlation among the extent of axonal damage in the spinal cord, palsy and loss of ambulation (Redowicz, 1999). Unlike the PNS regenerative nerve fiber growth and structural plasticity are limited in the adult CNS after insult. In many cases, it has been shown that rather than the absence of growth-promoting molecules in the CNS, it is the bearing of axon outgrowth inhibitors (NOGO), including components of both CNS myelin and astroglial scars, that limits regeneration (Karnezis et al., 2004). It remains to be seen whether Nogo pathway is activated during diabetes.

2.9.1. Receptor NgR

NgR is a glycosyl phosphatidyl-inositol-anchored protein and lacks transmembrane or cytosolic domains, it was predicted to require a signal-transducing molecule to effect inhibition (Yamashita *et al.*, 2002). Nogo-66 receptor and Nogo-66 interact directly to induce growth cone collapse *in vitro*. When NgR was expressed in neurons that were normally unresponsive to Nogo-66, they became responsive (Fournier *et al.*, 2001). NgR as a receptor for MAG and showed that application of soluble NgR or dominant-negative forms of NgR can effectively block the inhibitory effects of MAG (Domeniconi *et al.*, 2002).

2.10 Chemicals agent used for Diabetes induction in experimental animals

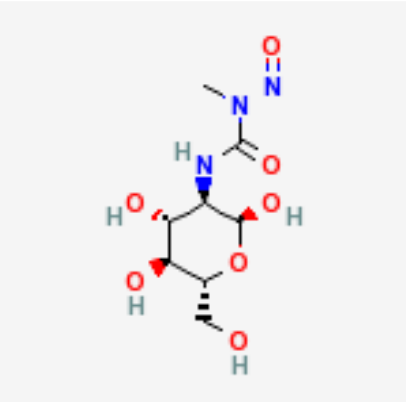
Streptozotocin (Qualigens fine chemicals, Mumbai, India) was used for diabetes model induction in rats. Streptozotocin (STZ) is a synthetic antitumor agent that is classically an anti-tumor antibiotic and chemically is related to other nitrosureas used in cancer chemotherapy. Streptozotocin sterile powders are available and can be prepared as a chemotherapy agent. Each vial of sterilized STZ powder contains 1g of STZ active ingredient and 200 mg of citric acid. STZ is available for intravenous use as a dry-frozen, pale yellow product. Pure STZ has alkalic pH. When Streptozotocin is dissolved inside the vial in distilled water, the pH in the solution inside the vial will be 3.5-4.5 due to 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, Rigalli et al., 2009).

Properties of Streptozotocin (Sivajothi et al.,2007) :

Table No. 1: Properties of Streptozotocin

Chemical name	2 Deoxy 2 methylnitrosoamino, carbonyl amino - D glucopyranose
Chemical structure	Cytotoxic methylnitrosourea moiety(Nmethyl-N-nitrosourea) attached to the glucose molecule;glucosamine derivative
Chemical Properties	Hydrophilic, beta cell-toxic glucose Analogue Relatively stable at pH7.4 and 37^{o} C (at least for up to 1 h)
Chemical reactivities	DNA alkylating agent, Protein alkylatin agent,NO donor
Mode of toxicity	DNA alkylation

Structure of Streptozotocin



The mechanism of STZ action

STZ action in B cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. These changes in blood glucose and insulin concentrations reflect abnormalities in B cell function. STZ impairs glucose oxidation (Bedoya et al., 1996) and decreases insulin biogenesis and secretion (Bolaffi et al., 1987, Nukatsuka et al., 1990). Reports have shown that STZ at first abolished the B cell response to glucose. Recent experiments have proved that the main reason for the STZ-induced B cell death is alkylation of DNA (Delaney et al., 1995, Elsner et al., 2000).

STZ is well studied as a nitric oxide (NO) donor which, brings about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced DNA damage (Kröncke et al. 1995, Morgan et al. 1994). Pancreatic B cells exposed to STZ manifested changes characteristic for NO action, i.e. increased activity of guanylyl cyclase and enhanced formation of cGMP (Turk et al. 1993 However, the results of several experiments provide the evidence that NO is not the only molecule responsible for the cytotoxic effect of STZ. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells (Takasu et al. 1991b, Bedoya et al. 1996). The formation of superoxide anions results from both STZ action on mitochondria and increased activity of xanthine oxidase (EC 1.1.3.22). It was demonstrated that STZ inhibits the Krebs cycle (Turk et al. 1993) and substantially decreases oxygen consumption by mitochondria (Nukatsuka et al. 1990b). These effects strongly limit mitochondrial ATP production and cause depletion of this nucleotide in B cells (Nukatsuka et al., 1990b, Sofue et al., 1991). It can be stated that potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrate. Therefore intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity (Szkudelski T, 2001).

MATERIALS AND METHODS

3.1 Chemicals:

Streptozotocin (Sigma Aldrich), EDTA (Merck Specialists Pvt. Ltd.), Tri reagent (Sigma Aldrich), DEPC water (Himedia Laboratories Pvt. Ltd.), cDNA synthesis kit (Thermo scientific, USA), PCR Master mix (Thermo scientific USA), Sucrose (S.D.Fines), , Agarose (Sisco Research Lab, Mumbai), Ethidium bromide (Central Drug House, Mumbai), DNA ladder (Fermentas, USA), Primers (Sigma), Sulphuric acid (Merck Specialists Pvt. Ltd), Sodium Carbonate (RANKEM), potassium sodium tartrate (FINAR Chemicals), Cupric sulphate (S D fine – chem. limited), copper sulphate (CuSO4; RFCL New Delhi), Sodium Hydroxide (NaOH; Sisco Research Lab, Mumbai), Bovine Serum Albumin (Central Drug House, Mumbai), Folin Ciocalteu Reagent (Sisco Research Lab, Mumbai), carbomyl phosphate (Sigma Aldrich), N-acetyl L-glutamine (SRL), Hydroxylamine (RANKEM), sodium glycinate (Sigma Aldrich), Triton X 100 (CDH Pvt. Ltd.), L-ornithine (S D fines), Tris HCl (Himedia Laboratories Pvt. Ltd.), Trichloroacetic acid((Merck Specialists Pvt. Ltd), ferrous ammonium sulphate (Merck Specialists Pvt. Ltd), ferric ammonium sulphate (Merck Specialists Pvt. Ltd), Butanedione monoxime (S D fine – chem. Limited) etc . All chemicals used in the present study were of analytical grade.

3.2 Animals:

Adult male Wistar rats of 200-250 g body weight were purchased from Bharat Serum Limited, Mumbai and used for all experiments. They were housed in separate cages under 12-h light and 12-h dark periods and were maintained on standard food pellets and water. Animal care and procedures were done according to the Institutional and National Institute of Health Guide lines.

3.3 Model induction:

Induction of diabetes

Animals were divided into following experimental groups as:

1.) Control [C]

2.) Diabetic [D]

3.) Diabetic +Insulin [D+I]

Each group consisted of 3-4 animals. Diabetes was induced by a single intrafemoral dose (55 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). Blood glucose was estimated by Glucometer using Accue Check strips. D+I group received daily 2 doses (1 IU/Kg body weight) of regular human insulin (Actrapid) (Flanagan et al., 2003). D+I was given daily two episodes of insulin for 18 days. Control rats were injected with citrate buffer.

Table No. 2: Model induction in Experimental groups rats

Group no.	Experimental group	Treatment
1	Control	Saline treatment
2	Diabetic	Streptozotocin (i.v) 55 mg/kg body weight
3	Diabetic + Insulin	Streptozotocin (i.v) 55 mg/kg body weight and Insulin (Subcutaneous) 1IU/kg body weight, Two doses daily.

3.4 Water, Food consumption and Body Weight measurement:

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

3.5 Glucose Estimation:

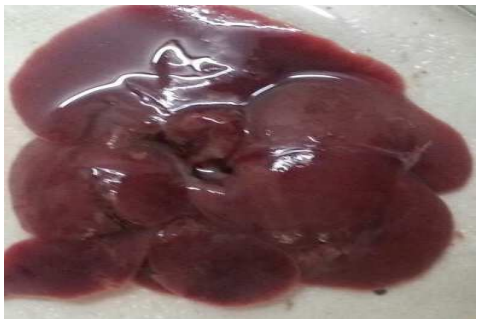
Glucometer:

Glucose estimation was carried out by Onetouch Glucometer. (Johnsons & Johnsons, USA)

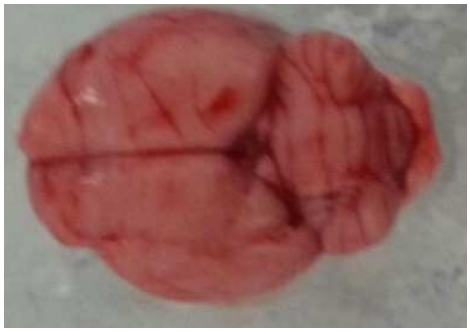
Blood reacts with enzyme glucose oxidase to produce gluconic acid from the glucose of blood. At the other end of the test strip, the meter transfers a current to the test strip. The test strip has electric terminals which allow the meter to measure the current between the terminals. The current between the terminals changes depending on the level of gluconic acid that has been produced.

3.6 Tissue Preparations:

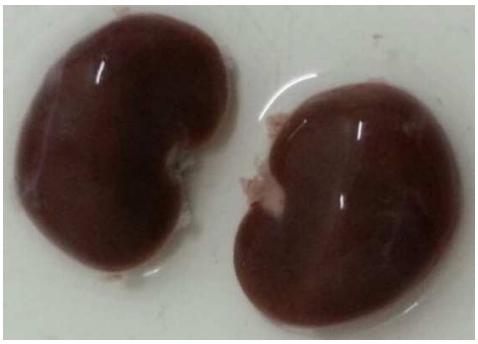
Rats were sacrificed on the 21st day of model induction by cervical dislocation. The cerebral cortex, and liver, kidney were quickly dissected out and frozen in ice according to the procedure of (Iversen & Glowinski, 1966). The tissues were stored at -80°C until assay.



LIVER



BRAIN



KIDNEY

3.7 Ornithine Transcarbamylase (OTC) assay:

Isolation:

10% liver homogenate was prepared in 100mM sucrose and 5mM sodium glycinate buffer, centrifuged at 800g for 15minutes. The supernatant was collected and further centrifuged at 7500g for 15 minutes at 4°C twice. Finally the supernatant was used for enzyme assay (Padh H, 1992).

Estimation:

Ornithine transcarbamylase (OTC) enzyme assay was performed from liver by colorimetric method. Carbamyl phosphate was used as substrate in different concentration ranges from 250µM - 2000µM. L-Citrulline produced in the reaction was measured at 490nm in UV spectrophotometer. Protein estimation was measured by using bovine serum albumin as standard (Lowry et al., 1951).

Protein estimation

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

3.8 Nogo Receptor gene expression

The brain was dissected from which cerebral cortex was taken for total RNA isolation from Cerebral Cortex was done using the Tri-reagent. This was used in the preparation of cDNA using cDNA synthesis kit of Thermo Scientific. Gene specific primers were designed by using Primer 3, Primer quest and the selected primer sequence was analyzed using oligo analyzer and oligo calc .

	Primer	GC content	Tm value	Amplicon length
Sense Primer	CTCTCTTCCTTCTCTATC	44.5 %	46.9 ºC	492 bp
Anti-Sense Primer	CTTCCCTCATAAGTATCT	38.8 %	47.4 ºC	

RESULT
AND
DISCUSSION

4.Results and Discussion:

4.1 Food Consumption in Control and Experimental Groups of Rats

4.1.1 Active phase food consumption in control and experimental group of rats

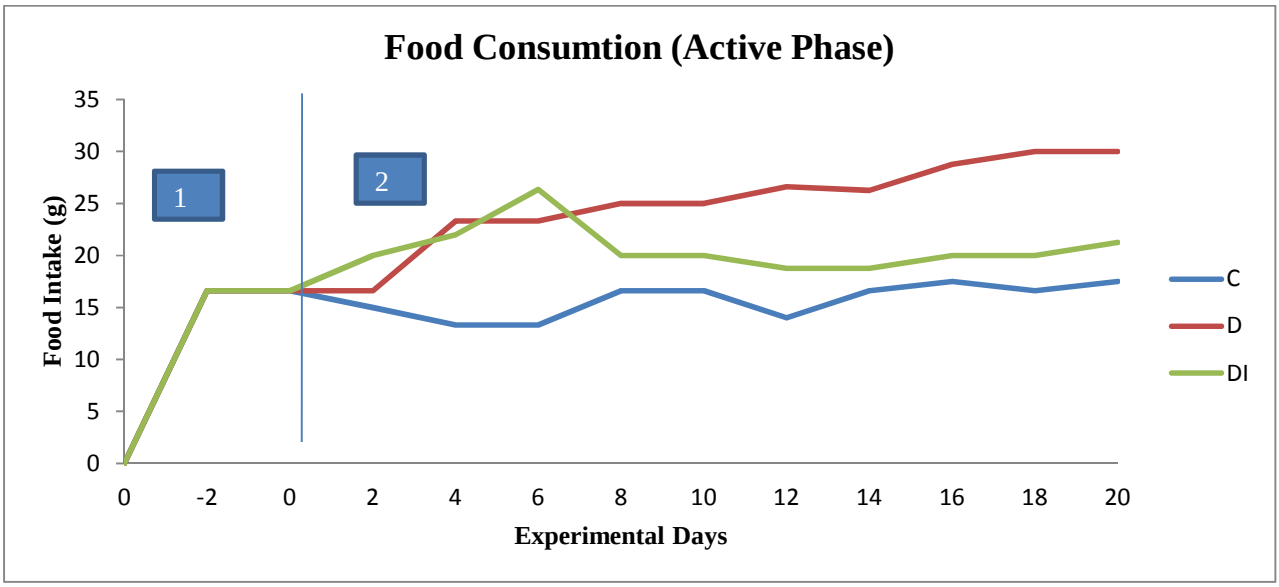


Fig.8: Food consumption in active phase

C- Control, D-Diabetic, D+I- Diabetic + Insulin
1-Pre Diabetic Phase, 2- Post Diabetic Phase

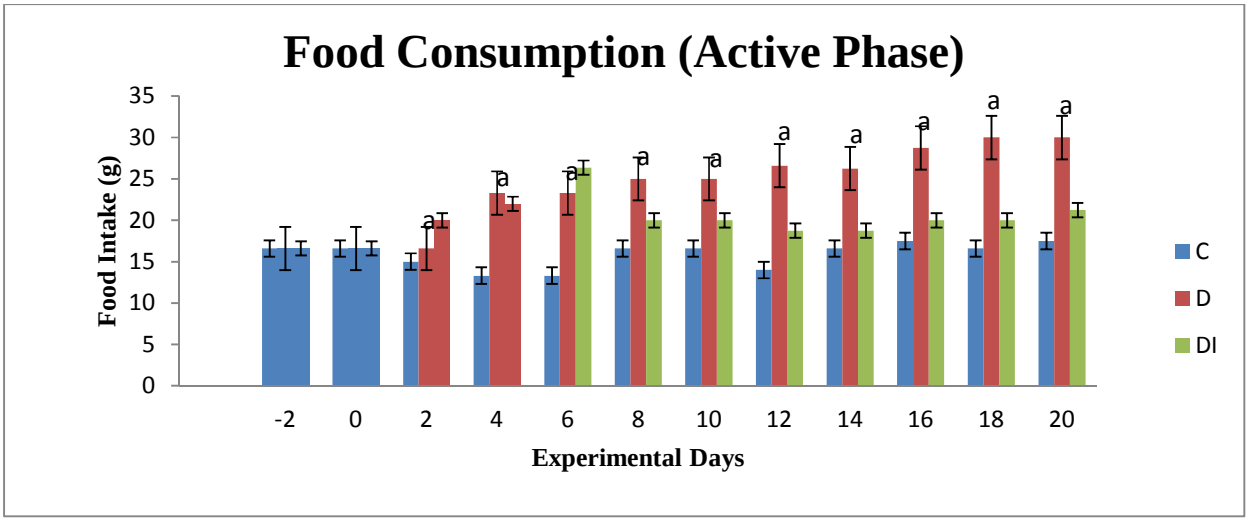


Fig.9: Food consumption in active phase

C- Control, D-Diabetic, D+I- Diabetic + Insulin
1-Pre Diabetic Phase, 2- Post Diabetic Phase
ANOVA followed by Student's-Newman-Keul's Test.
a -***<0.001 When Compare to C.

4.1.2 Inactive phase food consumption in control and experimental group of rats

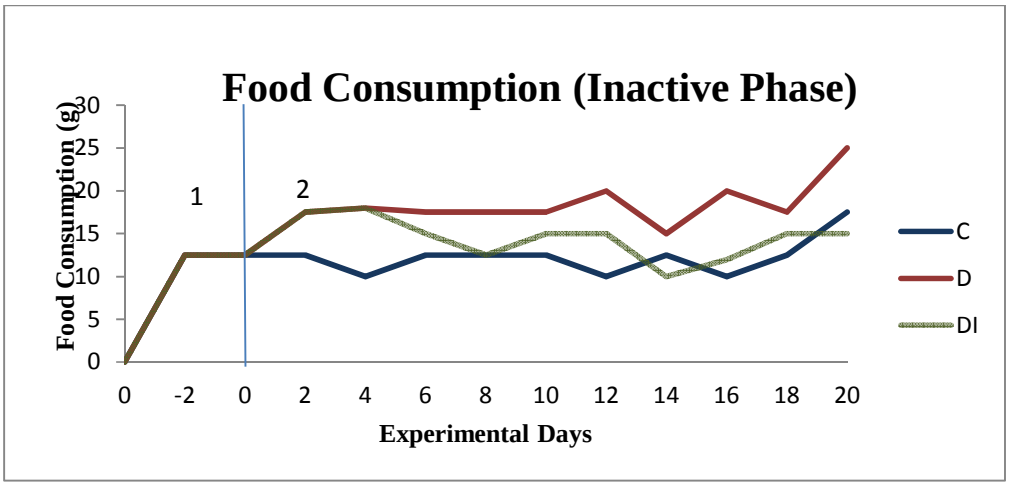


Fig.10: Food consumption in inactive phase

C- Control, D-Diabetic, D+I- Diabetic + Insulin
1- Pre Diabetic Phase, 2- Post Diabetic Phase

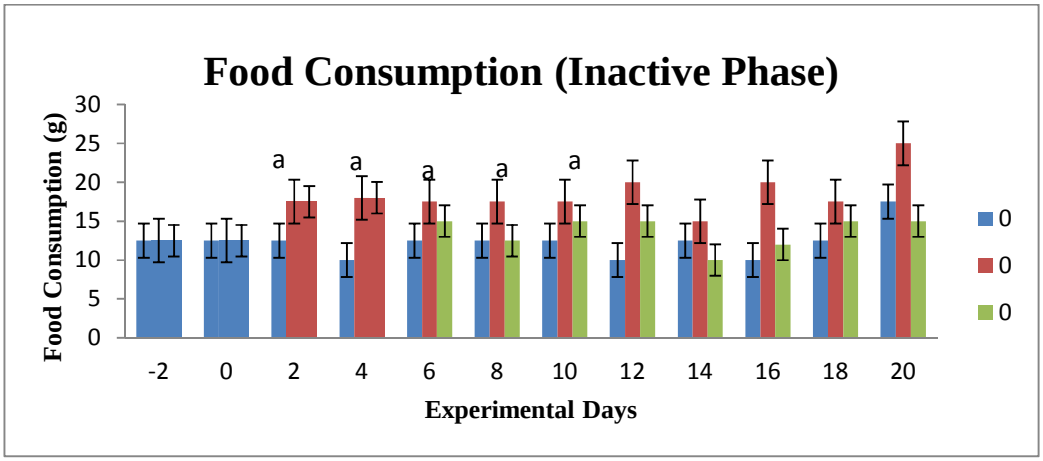


Fig.11: Food consumption in active phase

C- Control, D-Diabetic, D+I- Diabetic + Insulin
1-Pre Diabetic Phase, 2- Post Diabetic Phase

ANOVA followed by Student's-Newman-Keul's Test.
a -***<0.001 When Compare to C.

Discussion:

There is significant increase in food consumption of D group as compared to Control and D+I group in both active and inactive phase. Increase in food consumption in D group is mainly because of lack of glucose in cells, that prevents the entry of glucose inside the cell thus low or no energy is obtained by cells to perform cellular activities. This causes the appetite centre in brain to send out hunger signals causing the body to crave more food thus increasing food consumption (Akbarzadeh et al., 2007). Food consumption in insulin treated rats gradually decreases as compared to D.

4.2 Water Consumption in Control and Experimental Group of Rats

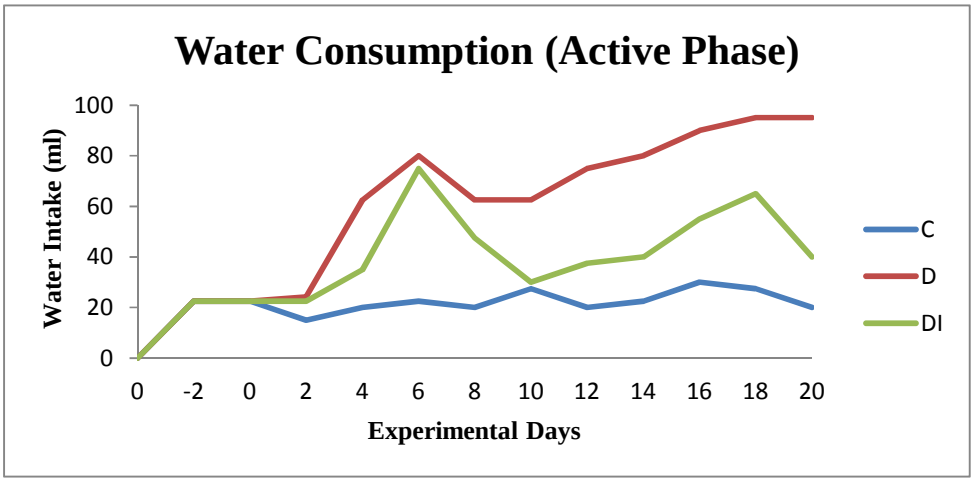


Fig.12: Water consumption in active phase

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

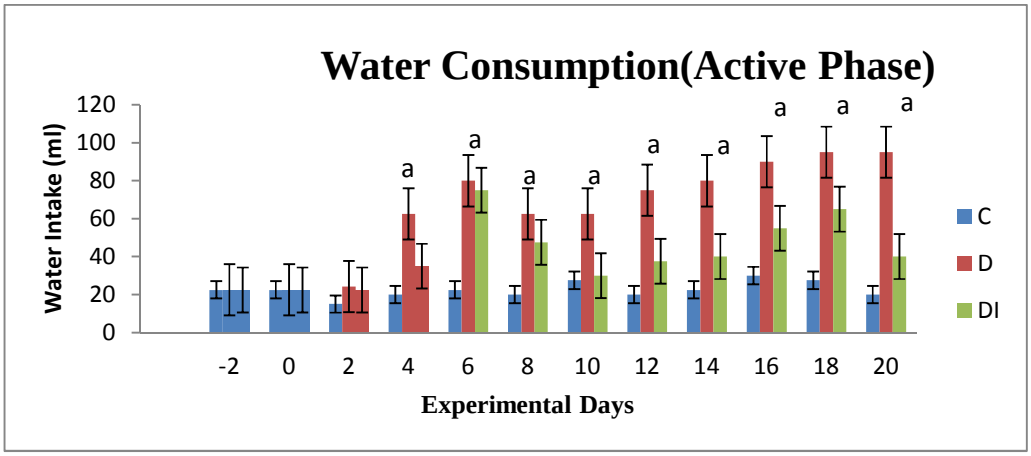


Fig.13: Water consumption in active phase

C- Control, D-Diabetic, D+I- Diabetic + Insulin,
ANOVA followed by Student's-Newman-Keul's Test.
a -***<0.001 When Compare to C.

4.2.2 Inactive phase Water consumption in control and experimental group of rats

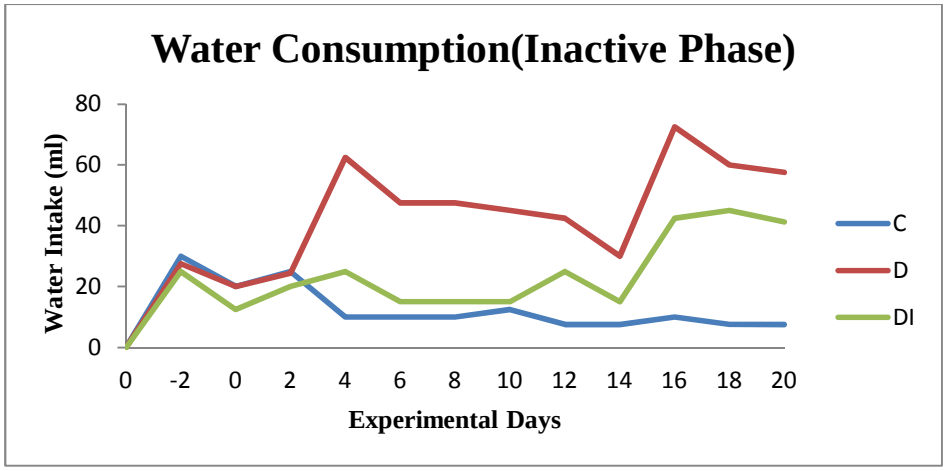


Fig.14: Water consumption in Inactive phase

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

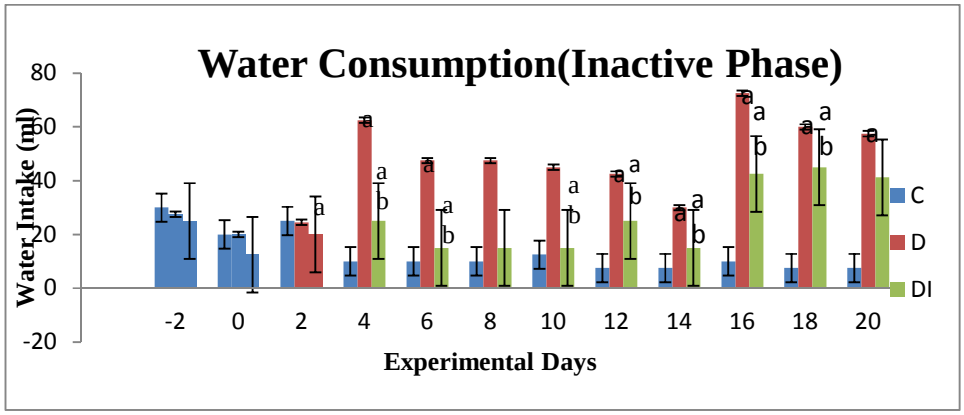


Fig.15: Water consumption in Inactive phase

C- Control, D-Diabetic, D+I- Diabetic + Insulin
ANOVA followed by Student's-Newman-Keul's Test
a -***<0.001 When Compare to C, b-***<0.001 When Compare to D.

Discussion:

It was observed that there is significant increase in water consumption of Diabetic group as compared to Control and D+I group in both active (fig.1) and inactive phase (fig.2). Elevated blood glucose alters the glomerular filtration rate of the kidney. Excess glucose leads to glycosuria i.e. high glucose content in urine. Glycosuria decreases blood glucose levels by excreting excessive glucose in urine. To dilute the concentrated urine, tubular secretion of water takes place in tubular region of nephrons (Akbarzadeh et al., 2007). This activates thirst centre in brain to compensate for water loss thus increasing water consumption.

4.3 Change in Body weight in Control and Experimental Groups of Rats

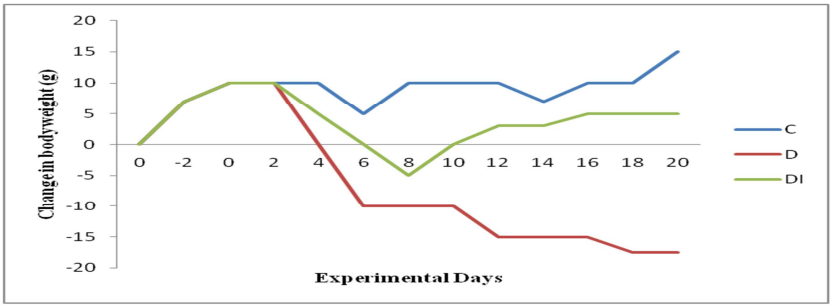


Fig.16: Body weight of control and experimental group of rats

Discussion:

A significant decrease in body weight of Diabetic group as compared to Control group. In diabetes, lack of insulin prevents transporters to mediated glucose entry into the cell, thus 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 therefore increase in body weight of D+I group when compared to Diabetic group.

4.4 Blood glucose level in control and experimental group of rats

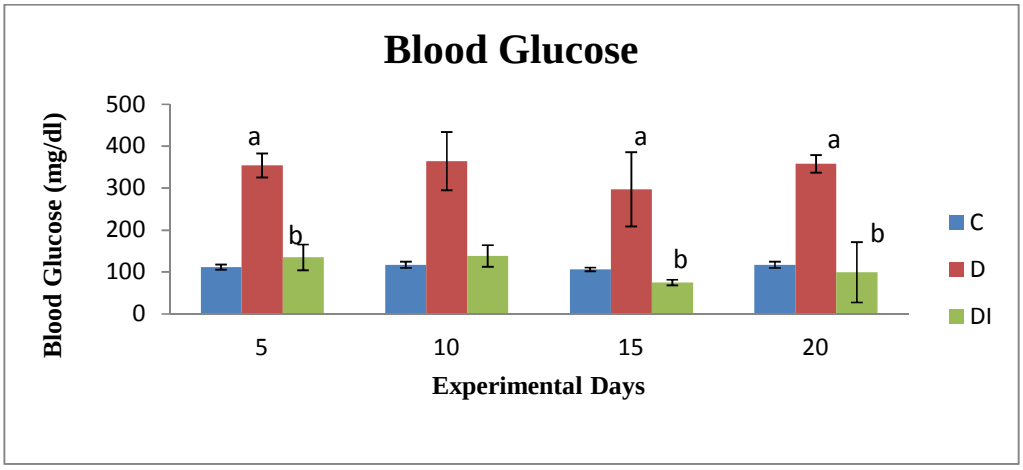


Fig.17: Blood glucose level

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

Table No. 3 : Blood glucose level in control and experimental group of rats

Experimental Groups	Experimental Days			
	5th Day (mg/dl)	10thDay (mg/dl)	15thDay (mg/dl)	20thDay (mg/dl)
C	111.7±6.3	117.2±7.2	106.2±4.3	117.2±7.2
D	354±28.7^{a}	364.5±69.6 ^{a}	297±88.1 ^{a}	358±20.7 ^{a}
DI	134.7±31.1^{b}	138.5±25.7 ^{b}	74.7±6.8 ^{b}	99.25±72 ^{b}

Values are Mean ± SD of 4 separate experiments (n=4rats per group).
C- Control, D-Diabetic, D+I- Diabetic + Insulin
ANOVA followed by Student's-Newman-Keul's Test.
a -***<0.001 When Compare to C, b-***<0.001 When Compare to D.

Discussion:

Insulin activates transport of glucose into muscle and adipose tissue, and also promotes synthesis of glycogen and triglycerides (Saul Genuth, 2001) But in absence of insulin glucose can't enter cells (Mahmoud et al., 2009) and thus blood glucose levels gradually increases in Diabetic group. In D+I group, due to insulin treatment, blood glucose levels gradually lowers down than Diabetic Group of rats.

4.5 Ornithine transcarbamylase enzyme assay

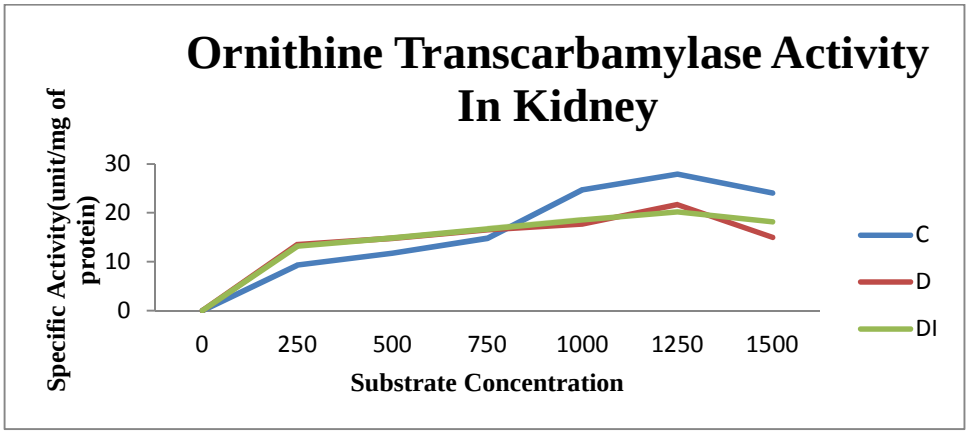


Fig.18: Specific Activity of Ornithine Transcarbamylase

C-Control, D-Diabetic, D+I- Insuline treated Diabetic Rats

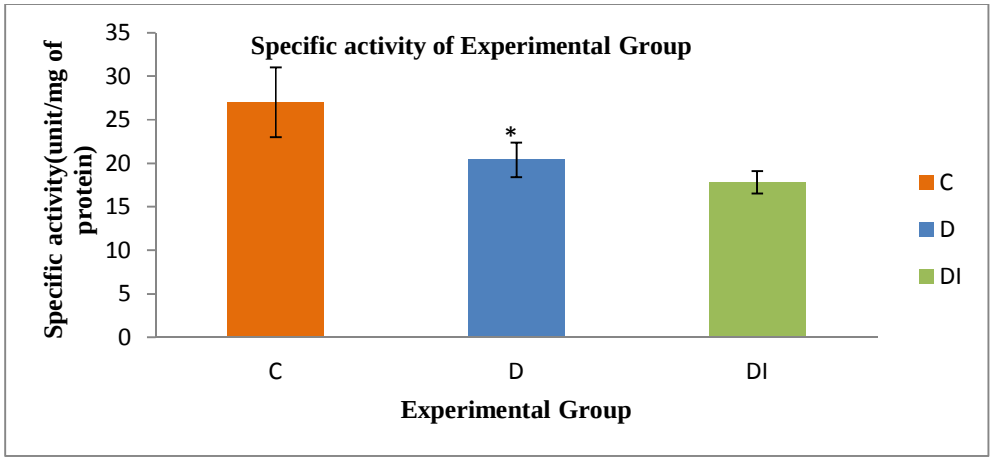


Fig19: Kinetic parameters Vmax of ornithine transcarbamylase in the Kidney of control and experimental group of rats

C-Control, D-Diabetic, D+I- Insuline treated Diabetic Rats
ANOVA followed by Student's-Newman-Keul's Test.
*<0.01 when compared to C

Table No. 4: kinetic parameters of OTC in the Kidney of control and experimental group of rats

Experimental Groups	Vmax	Km
Control	27±4	533.5± 31.18
Diabetic	20.4±2.6*	451.5± 36.93*

D+I	17.8 ± 1.3*	390.5± 32.76*

Values are Mean ± SD of 5 separate experiments (n=3 rats per group)
ANOVA followed by Student's-Newman-Keul's Test.
*<0.01 when compared to C

Discussion:

Ornithine transcarbamylase, is the most commonly studied urea cycle enzyme. In diabetes, electron flux increases through the mitochondrial electron transport chain which increases ATP/ADP ratio. As the in ATP/ADP ratio rises, this will partly reduce 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 and hyperglycemic related metabolic disorders (Murayama et al., 2010). The increased presence of ROS and NO seems to be increasing the urea levels in blood. There is significance observed between experimental groups. There is significant result is obtained in D when compare to C in experimental group of rats.

4.5.2 Ornithine Transcarbamylase Activity In Liver

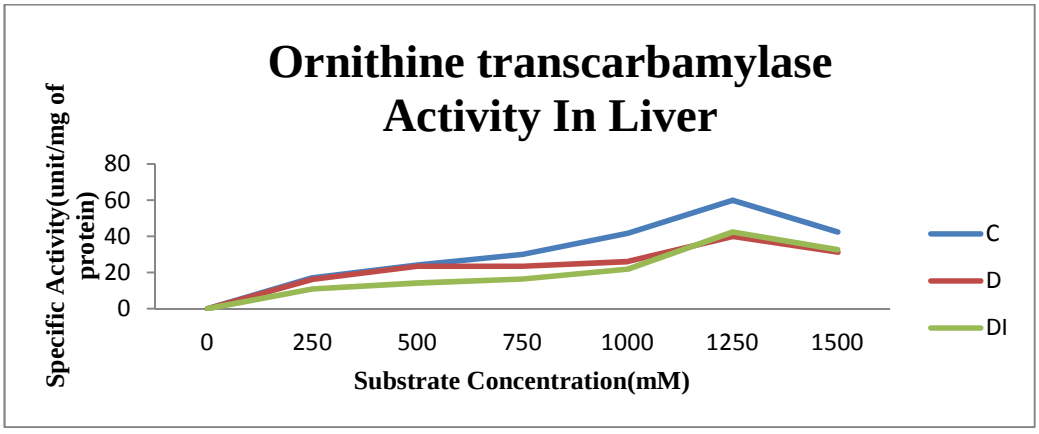


Fig.20: Specific Activity of Ornithine Transcarbamylase

C-Control, D-Diabetic, D+I- Insuline treated Diabetic Rats

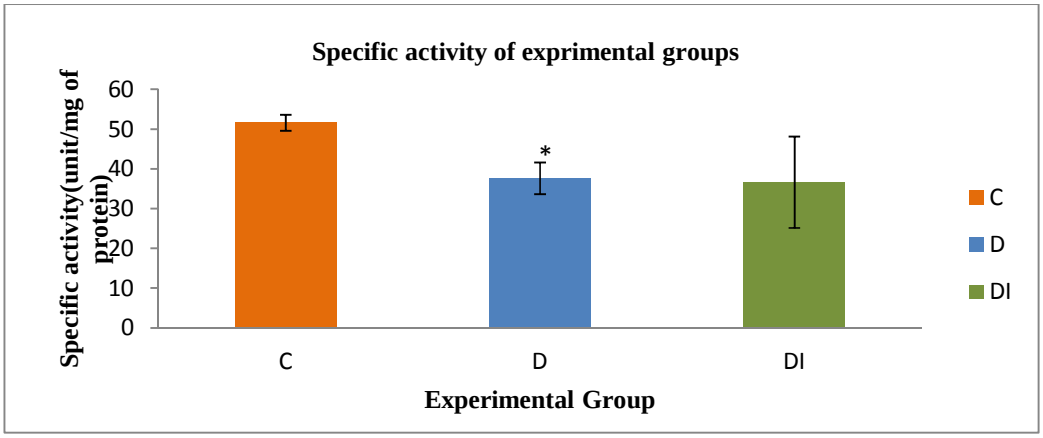


Fig.21: Kinetic parameters Vmax of ornithine transcarbamylase in the Liver of control and experimental group of rats
C-Control, D-Diabetic, D+I- Insuline treated Diabetic Rats
ANOVA followed by Student's-Newman-Keul's Test.
*<0.01 when compared to C

Table No.5 : kinetic parameters of OTC in Liver of control and experimental group of rats

Experimental Groups	Vmax	Km
Control	51.6±2	533.5± 31.18
Diabetic	37.66±4*	451.5± 36.93*
D+I	36.66± 11.5	390.5± 32.76

Values are Mean ± SD of 4 separate experiments (n=3 rats per group)
ANOVA followed by Student's-Newman-Keul's Test.
*<0.01 when compared to C

Discussion:

As anticipated, diabetic rats excreted more urea and had lower liver and body weight. The increased urea excretion was the result of increased ammonia availability to the urea cycle enzymes (Murayama et al.,2010) Citrulline synthesis is the result of the sequential action of CPS and OTC, two mitochondrial enzymes of the cycle (Mahmoud et al., 2009). The rate of citrulline synthesis by intact mitochondria is determined by the levels of active CPS, which in turn depends on the amounts of enzymatic protein. There is significant decrease is observed in D when compare to C in experimental group of rats.

4.6 Integrity of total RNA isolated from CC on Agarose gel electrophoresis

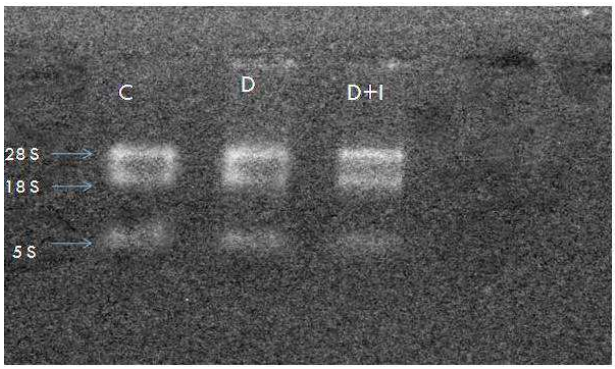


Fig.27: Total RNA isolated

4.7 Nogo Receptor gene Expression

The thermal profile was standardized using different temperatures and time (s/mins).

The following was the final thermal profile which provided the gene expressions.

Table No. 6:- Thermal profile provided for gene expression

Steps	Temperature(ºC)	Time	No of Cycles
Initial Denaturation	94	3 mins	1
Denaturation	94	30 sec	35
Annealing	45	1 min	
	72	45 sec	
Extension	72	10 mins	

Sense Primer: CTC TCT TCC TTC TCT ATC

Anti-Sense Primer: CTT CCC TCA TAA GTA TCT

Amplicon length: 492bp

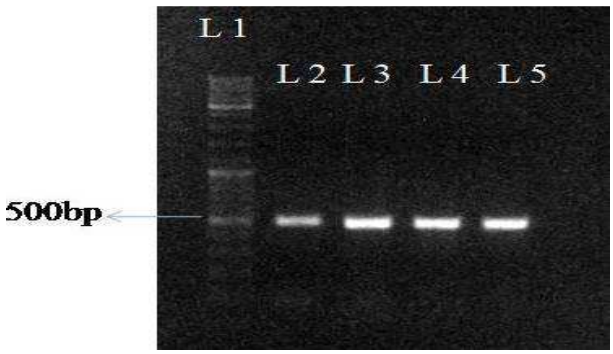


Fig 28: Expression of Nogo Receptor

Lane 1- 100 bp DNA ladder.
Lane 2 - GAPDH.
Lane 3 - amplified product of Nogo Receptor from C.
Lane 4 - amplified product of Nogo Receptor from D.
Lane 5 - amplified product of Nogo Receptor from C+I group of rats.

Discussion:

Receptor gene Expression

Nogo Receptor gene was amplified from the cDNA of the cerebral cortex of experimental group of rats. There is a no significant difference is observed in the expression of Nogo receptor between experimental group of rats. This is the result of only one experiment hence, the result cannot concluded.

SUMMARY

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. These activated pathways are a major source of damage and are potential therapeutic targets in diabetic neuropathy. From the present study, it was observed that there was a significant increase in the blood glucose level as compare to C and D+I where as D+I had shown significance change in blood glucose when compared to D indicating successful rat model induction. In liver and kidney, a significant decrease in activity of OTC is seen in D, when compare to C. A decreased OTC leads to accumulation of ammonia in cells. The Nogo receptor gene expression study shows no significant difference between control and other experimental group of rats.

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