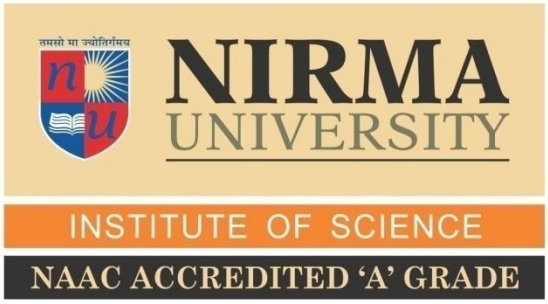
Effect of Recurrent Hypoglycemia on SOD Activity and Neurexin-1 Gene Expression in Streptozotocin Induced Diabetic Rats

A dissertation project
Submitted to
Nirma University
In the partial fulfillment of
Requirement for the Degree of

Masters of Science
In
Biochemistry



Submitted by:

SAJNI NAIK (15MBC015)

DRASHTI SHETH (15MBC017)

Under the guidance of:

Dr. Amee Nair

Institute of Science, Nirma University

Ahmedabad-382481, Gujarat, India

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<u>*Acknowledgement*</u>

"Gratitude is the greatest blossom which springs from the soul."

Words put on paper are mere ink marks, but when they have a purpose there exist a thought behind them. We would like to take this opportunity to express our gratitude towards those individuals who made this project work possible. First and foremost we would like to thank god for always leading us on right path.

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Sajni Naik (15MBC015)

Drashti Sheth (15MBC017)

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ABBREVIATION

C	Control
D	Diabetes
CIIH	Control + insulin induced hypoglycemia
DIIH	Diabetic + insulin induced hypoglycemia
i.v.	Intra-Venous
s.c.	Sub-Cutaneous
ROS	Reactive Oxygen Species
AGEs	Advanced Glycated End products
RNS	Reactive Nitrogen Species
RNA	Ribonucleic Acid
RCS	Reactive Chloride Species
DNA	Deoxyribonucleic Acid
PKC	Protein Kinase C
RBCs	Red Blood Cells
SOD	Superoxide dismutase
CAMs	Cell Adhesion Molecules
NRXN-1	Neurexin-1
LNS	Lamnin, Neurexin Sex Hormone Binding Protein
EGF	Epidermal Growth Factor
PCT	Proximal Convoluted Tubule
PDGF	Platelet Derived Growth Factor
VEGF	Vascular Epidermal Growth Factors
IGF-1	Insulin Growth Factor-1
O-Glyc	O-Glycosylation
CD2AP	CD-2 Associated Protein
PCR	Polymerase Chain Reaction
ANOVA	Analysis of Variance

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ABSTRACT

Administration of insulin beyond the recommended dose in diabetes causes hypoglycemia. During diabetes, generation of oxidative stress increases reactive oxygen species and is known to damage the normal cellular assembly of kidney thereby causing diabetic nephropathy. Diabetes was induced in adult male Wistar rats by using Streptozotocin (50 mg/kg body weight – i.v.) and Hypoglycemia in Control and Diabetic rats by injecting Human Insulin (1.5 IU and 10 IU/kg body weight respectively – s.c.). Generation of ROS causes an alteration in anti-oxidant enzyme levels, of which superoxide dismutase enzyme serves as the first-line of defense against oxidative stress. SOD levels in kidney and spleen of control and experimental group of rats were assessed. Generation of ROS might act as a causative agent to induce morphological alterations in kidneys. These alterations in kidneys of diabetic and experimental group of rats were observed by staining kidney sections by H&E staining procedure. Podocytes, present in kidney are reported to be damaged in diabetic nephropathy. These podocytes have CAMs which are important for cell-cell cross talk. Neurexin-1, a CAM present on podocytes cells is important for maintaining intracellular trafficking within podocytes. To scrutinize this cross-talk, gene expression studies of Neurexin-1 gene was carried out following total RNA isolation and c-DNA synthesis from kidneys of control and experimental group of rats. Further, in spleen due to presence of glycated RBCs in diabetes, recycling of ROS is reported to be altered. To study the status of ROS in diabetic and hypoglycemic conditions, SOD enzyme activity was assessed from spleen also.

1. Introduction

1.1 Diabetes

Diabetes is an endocrine disorder, characterized by inability of the pancreas to secrete enough insulin in order to maintain the blood glucose levels. It is a complex disorder resulting from defects in insulin secretion, insensitivity to insulin receptor and defects in insulin action (Butler et al., 2003). It is a lifelong condition associated with serious complications (Kahn, 1994).

Major areas contributing to diabetic complications includes (1) Hemodynamic changes in Blood pressure and salt/fluid balance (2) Change in glucose metabolism and (3) Genetic factors. These changes further leads to various cellular changes like gene modulation, Protein expression and its modification. This results in cellular dysfunction and death (Ceriello, 2005).

Diabetic symptoms can be divided into two categories: (1) Acute and (2) Chronic. Acute symptoms include Ketoacidosis, Hyperglycemia. Chronic symptoms of diabetes are further divided into two groups: (1) Microvascular and (2) Macrovascular. Microvascular symptoms include retinopathy, neuropathy and nephropathy (Caballero et al, 1999), while macrovascular symptoms include the myocardial infarction, accelerated arteriosclerosis, impaired growth and development (Baynest, 2015).

β-cells, present in the islets of langerhans of pancreas are responsible for secretion of insulin to maintain glucose homeostasis (Jonas et al, 2005). Autoimmune destruction of pancreatic β-cells leads to abnormalities that result in deprivation of insulin secretion (Porte, 1991). Such a destruction, leads to an absolute requirement of insulin therapy. However, administration of insulin beyond the recommended levels leads to various complications. **Hypoglycemia** is one of the majorly feared complications resulting from such a condition during diabetes (Rother, 2007).

1.2 Hypoglycemia

Hypoglycemia occurs when blood glucose levels drop below normal range. It occurs due to the relative insulin excess and compromised glucose counter regulation in diabetes (Shamoon et al., 2002). During hypoglycemia, plasma glucose concentration abnormally falls below 60 mg/dl, which leads to potential harm (McCrimmon and Sherwin, 2010).

Hypoglycemia during diabetes occurs due to incorporation of hyperinsulinemia and compromised defense against falling plasma glucose concentrations (Cryer, 2008).

In diabetes, factors that can trigger hypoglycemia are as follows: (1) Excessive insulin doses (2) Decreased exogenous glucose delivery (3) Increased glucose utilization (4) Decreased endogenous glucose production (5) Decreased insulin clearance (Roth et al., 1963).

Blood glucose levels during hypoglycemia causes an array of symptoms by signaling autonomic nervous system. These symptoms can be divided into two categories: (1) Neurogenic symptoms: anxiety, dry mouth, nervousness, pupil dilation, shakiness, sweating (2) Neuroglycopenic symptoms: ataxia, confusion, coma, difficulty in thinking and speaking, headache, irritability and seizures (Briscoe and Davis, 2006).

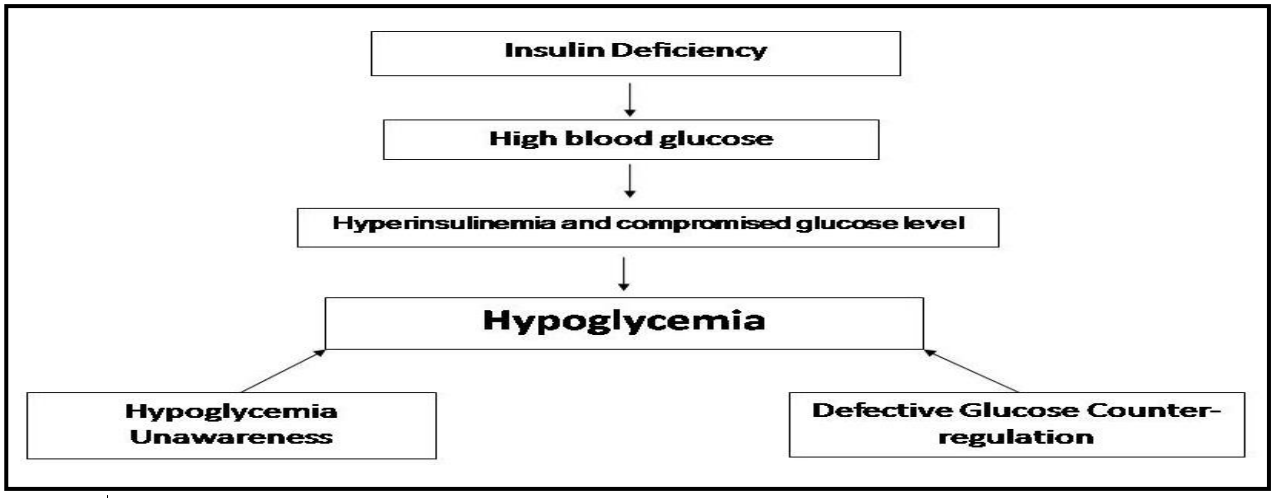


Figure 1: Causes of Hypoglycemia (Cryer and Childs, 2002)

(In diabetes, administration of insulin beyond normal range leads to the hypoglycemia.)

1.3 Oxidative stress, ROS, Diabetes and Hypoglycemia

Oxidative stress occurs due to an alteration in the production of free radical species and antioxidant defense mechanisms, leading to tissue damage. Oxidative stress can cause toxic effects through the free radical species. These free radical species can be divided into different categories: (1) Reactive oxygen species (ROS) (2) Reactive nitrogen species (RNS) and (3) Reactive chloride species (RCS) (Stevens et al., 2005).

ROS are chemically reactive molecules containing oxygen, produced as a natural byproduct of the normal metabolism of oxygen. These molecules are formed due to partial reduction of molecular oxygen that is transient due to its high chemical reactivity (Mates, 2000). The production of mitochondrial superoxide occurs through oxidative phosphorylation via electron transport chain (Monory et al., 2013). ROS, which includes hydroxyl radicals, superoxide anions and hydrogen peroxide, have been associated in oxidative damage inflicted on fatty acids, DNA (Deoxyribo Nucleic Acid) and proteins as well as other cellular components (Chikezie et al., 2015).

Oxidative stress can generate due to development of resistance against insulin, dysfunction of pancreatic β-cells and impaired glucose tolerance. In diabetes, chronic hyperglycemia leads to the production of ROS by various processes like - Glucose oxidation, glucose toxicity and oxidative phosphorylation. Oxidative stress generated due to ROS is responsible for various diabetic complications including **diabetic nephropathy, diabetic neuropathy and diabetic retinopathy** (Silveiro et al, 2005)**.**

During hypoglycemia, activation of insulin receptor also causes the release of hydrogen peroxide (Wiernsperger, 2003). Even though hydrogen peroxide is a non-free radical, it is membrane permeable and can diffuse to sites different from that of its production (Sies, 1991). Hypoglycemia along with impaired antioxidant defense system, leads to inefficient scavenging of hydrogen peroxide. Hydrogen peroxide undergoes reaction, which results in the initiation and propagation of lipid peroxidation. Thus, hypoglycemia via hydrogen peroxide formation increases ROS and contributes to oxidative stress.

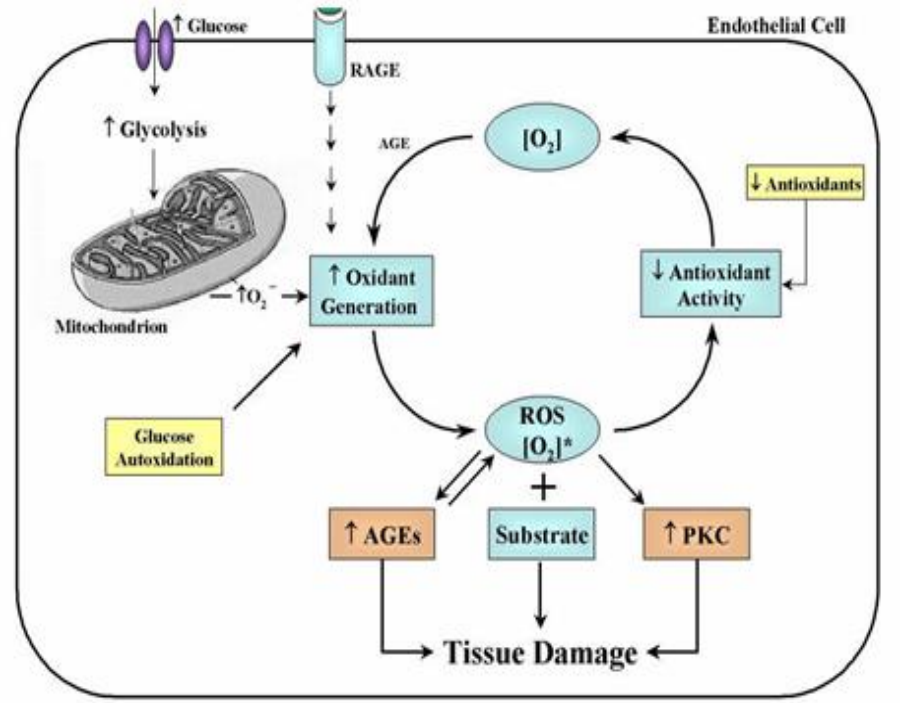


Figure 2: Oxidative stress, ROS and Diabetes (Kim and Loeken, 2004)
(In diabetes, high blood glucose leads to the production of ROS by various processes like - glucose oxidation, glucose toxicity and oxidative phosphorylation. ROS activates different pathways of AGEs, PKC, etc., which leads to the tissue damage)

1.4 Oxidative Stress and Diabetic Nephropathy

Diabetic nephropathy, which can occur due to diabetes, is considered as one of the critical complications of diabetes. In the development of diabetic nephropathy, oxidative stress acts as a major player, (Forbes et al., 2008). Overproduction of mitochondrial superoxide occurs in endothelial cells of large and small vessels in kidney (Cooper, 1998).

Hyperglycemia causes an elevation in plasma free radical concentrations through five major mechanisms: (1) Activation of Polyol pathway (2) Increased intracellular formation of AGEs (Advanced Glycation End products) (3) Increased expression of the receptors for AGEs and its activating ligands (4) Activation of protein kinase C pathway and (5) Activation of hexosamine pathway (Giacco and Brownlee, 2010).

Various cells, which get affected in diabetic nephropathy, include glomerular podocytes, mesengial cells, endothelial cells and tubular epithelia (Kanwar et al., 2008). Diabetic nephropathy related alterations in redox state are caused by persistent hyperglycemic state and increased AGEs. These events affect the renin-angiotensin system leading to chronic inflammation in glomerulus. Hyperglycemia is the driving force for progressive destruction of glomerulus that produces mechanical tension and frictional force caused mainly by hemodynamic changes. This leads to the liberation of numerous cytokines, pro-inflammatory markers and growth factors, which stimulates various pathways of oxidative stress (Dwivedi & Sarkar, 2010).

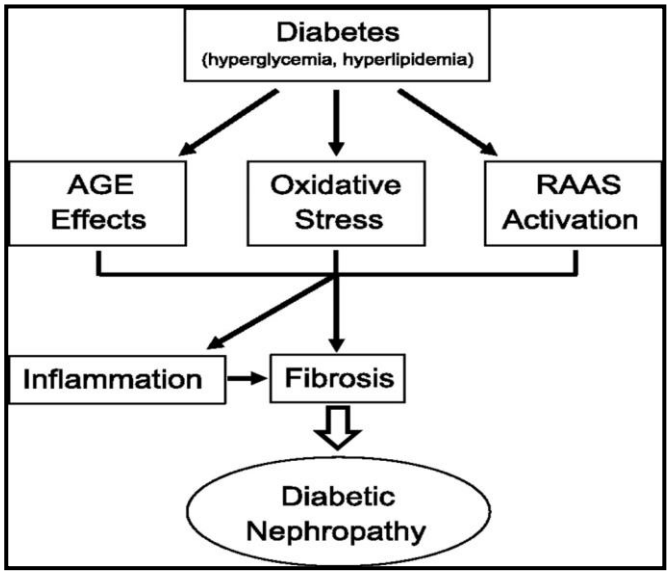


Figure 3: Oxidative stress and Diabetic Nephropathy

(Hyperglycemia is one of the reasons for the formation of AGEs, production of oxidative stress and RAAS activation, which further leads to development of diabetic nephropathy.)

1.5 Oxidative stress and its effect on spleen

Spleen is a secondary lymphoid organ, which acts as an important player in the activity of immune system. Spleen is involved in recycling of RBCs. Free radical species are generally recycled in RBCs. In diabetes, RBCs are glycated due to high blood glucose level. Thus, free radical species recycling is also altered. Due to this, increase in oxidative stress and thereby decrease in the activity of superoxide dismutase occur.

Recurrent hypoglycemia increases oxygen-glucose deprivation induced cell damage. Damaged cells of spleen causes an alteration in the normal recycling of RBCs (Red Blood Cells) and in antigen processing.

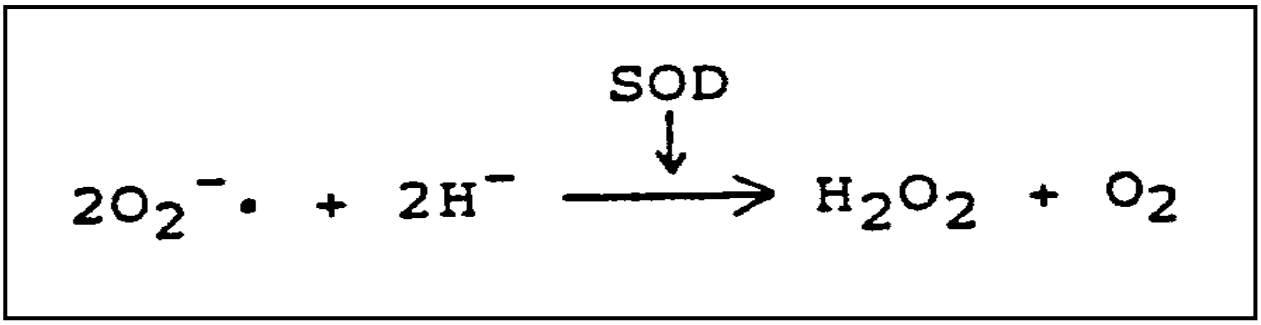
1.6 Antioxidant system and ROS

Antioxidant defense system contains several enzymatic and non-enzymatic antioxidants that are effective in blocking destructive effects of ROS. In antioxidant defense system, primarily three antioxidant enzymes are present, which includes superoxide dismutase (SOD), Catalase, and Glutathione peroxidase (Lee et al., 2013).

1.6.1 Superoxide dismutase

Superoxide dismutase (SOD) acts as a first line of defense against the oxidative stress. It is a major antioxidant defense system against superoxide radicals, which destroys the free radical superoxide by converting it into peroxide. Aerobic respiration produces low levels of superoxide constantly.

There are three isoforms of SOD: (1) Cytoplasmic Cu/Zn SOD (SOD1) (2) Mitochondrial SOD (SOD2) (3) Extracellular SOD (SOD3) (McCord and Fridovich, 1969).



1.7 Cell adhesion molecules in kidney

Podocytes or glomerular visceral epithelial cells are highly specialized epithelial cell which covers the glomerular basement membrane (Pavensta , 2000). Podocytes share some common features with neurons: 1) podocytes and neuron both possesses long and short cell processes, which is equipped with highly organized cytoskeletal system 2) Both the cells shows cytoskeletal segregation 3) In neurons and podocytes, process formation is dependent on various microtubule associated proteins 4) The process formation in both cells is positively regulated by PP2A which is Ser/Thr phosphatase and process is accelerated by laminin which is extracellular protein 5) The elongation of both neuronal dendrites and podocytes processes is supported by Rab8-regulated basolateral type membrane transport 6) Synaptodin is actin associated protein which is expressed in both podocytes processes and neuronal dendrites. It is localized in thin short projections from main shaft (Kobayashi, 2002; Saito et al., 2010).

1.8 Neurexin-1

Neurexins are categorized under the family of cell adhesion molecules. Neurexins were first identified in 1992 by affinity chromatography of brain extract from rat. Neurexin-1 is composed of an N-terminal extracellular sequence containing a signal peptide, 6 LNS domains separated by 3-EGF (Epidermal Growth Factor) like sequences followed by an O-glycosylation region (O-Glyc), a single trans-membrane domain and a short cytoplasmic region.

Neurexin is present in brain, kidney, lung, colon and pancreas. NRXN-1 is the only isoform of neurexin which is present in glomeruli and is restrictedly present at cytoplasmic area around slit diaphragm of glomerular podocytes of kidney. Slit diaphragm is situated between neighbouring foot process of glomerular podocytes. It act as a final barrier to retain plasma protein.

Neurexin-1 is involved in the formation of slit diaphragm and maintatinance of its function. Podocyte specific cell-cell interaction occurs via slit-diaphragm. NRXN is also involved in intracellular trafficking of slit diaphragm components. Neurexin located close to CD2AP (CD2 associated protein) and also interacts with nephrin which constitute extracellular site of the slit diaphragm.

2. Objectives

1. To induce diabetes and hypoglycemia in adult wistar rats
2. To check blood glucose levels, water and food intake and change in body weight of control and experimental group of rats
3. To assess specific activity of superoxide dismutase from kidney of control and experimental group of rats
4. To study histological alterations in kidney of control and experimental group of rats
5. To check the expression of neurexin-1 gene from kidney of control and experimental group of rats
6. To assess specific activity of superoxide dismutase from spleen of control and experimental group of rats

3. Materials and Method

3.1 Chemicals

Streptozotocin (MP Biomedicals, US), Citric acid Monohydrate (Merck Specialities Pvt. Ltd., Mumbai), Tri-sodium citrate dihydrate (S.D. Fine Chemicals Ltd., Mumbai), Human Actrapid insulin (Torrent Pharma, Ahmedabad), Sodium Dihydrogen Phosphate anhydrous (Sisco Research Laboratories Pvt. Ltd., Mumbai), Di-Sodium hydrogen phosphate anhydrous (Merck Specialties Pvt. Ltd., Mumbai), Sodium Chloride (Merck Specialties Pvt. Ltd., Mumbai), Potassium Chloride (Merck Specialties Pvt. Ltd., Mumbai), Potassium dihydrogen Phosphate anhydrous (Merck Specialties Pvt. Ltd., Mumbai), Chloroform (Merck Specialties Pvt. Ltd., Mumbai), Diethyl Ether (Merck Specialties Pvt. Ltd., Mumbai), Paraformaldehyde (Hi-Media, Mumbai), Sodium carbonate (Merck Specialties Pvt. Ltd., Mumbai), EDTA (Merck Specialists Pvt. Ltd., Mumbai), Hydroxylamine Hydrochloride (Central Drug House Pvt. Ltd., New Delhi), Criton-X 100 (Central Drug House Pvt. Ltd., New Delhi), Nitro blue tetrazolium (NBT) (MP Biomedicals, US), Hydrochloric acid (S.D. Fine Chemicals Ltd., Mumbai), Hematoxylin (Hi-Media, Mumbai), Eosin (S.D. Fine Chemicals Ltd., Mumbai), Xylene (S.D. Fine Chemicals Ltd., Mumbai), Absolute Alcohol (Shree Chalthan Vibhag Khand Udhyog Sahkari Mandli Ltd., Surat), QIAzol (QIAGEN sciences, Germany), Isopropanol (Merck Specialties Pvt. Ltd., Mumbai), cDNA synthesis kit (Thermo-scientific, US), PCR Master mix (Thermo-scientific, US), Primer NRXN (Sigma Aldrich, US), DNA ladder (Thermo-scientific, US), Bromophenol blue (Thermo-scientific, US), Agarose (HI-Media, Mumbai), Ethidium bromide (Genetix Biotech, New Delhi).

3.2 Animals

Adult male Wistar rats of 200-250 g body weight were purchased from Bharat Serum Limited, Mumbai. Rats were kept in separate cages under 12-h light and 12-h dark periods. They were maintained on standard food pellets and water. According to the Institutional and National Institute of Health Guide lines, animal care and handling was done.

3.3 Model induction

3.3.1 Induction of diabetes and hypoglycemia:

For experiment, animals were divided into following groups:

Group No.	Animal group	Treatment
1	Control	0.9 % Normal Saline
2	Diabetic	Streptozotocin (50 mg/kg) body weight) i.v.
3	CIIH	1.5 IU/kg body weight, two doses daily (s.c.) to control rats
4	DIIH	10.0 IU/kg body weight, two doses daily (s.c.) to diabetic rats

Table 1: Model Induction

Each experimental group contained 6 animals. Diabetes was induced by a single intravenous (femoral vein) dose (50 mg/kg body weight) of Streptozotocin. CIIH & DIIH group received daily 2 doses: 1.5 IU/Kg body weight in CIIH and 10.0 IU/Kg body weight in DIIH of human insulin (Actrapid) during active phase and inactive phase (Flanagan et al., 2003).

3.4 Glucose Estimation

Glucose estimation was carried out by using i-sense Glucometer and its corresponding care-sense gluco-strips.

3.5 Food and water consumption

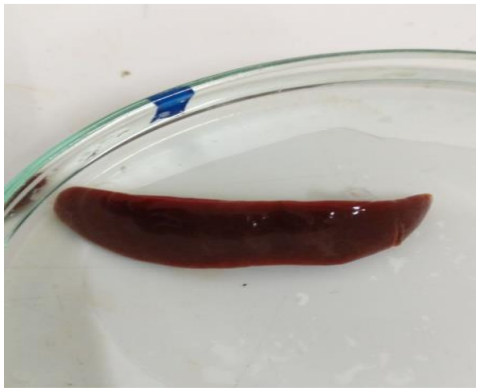
Food and water consumption was recorded throughout the experiments. Food and water consumption of control and experimental group of rats was observed during the active and inactive phase throughout the experiment.

3.6 Body weight measurement

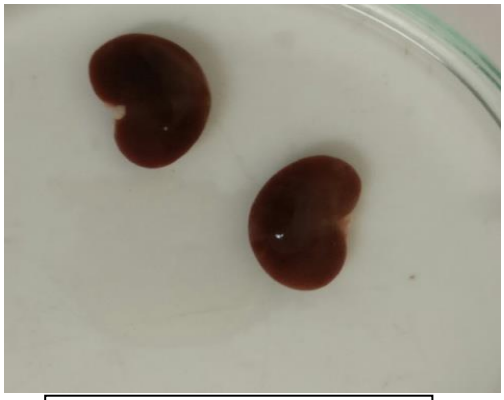
Body-weight was measured everyday throughout the experiment by using standard calibrated weighing balance.

3.7 Tissue Preparations

Rats were sacrificed after 42 days of model induction by cervical dislocation. The body parts like kidney, liver, pancreas, muscle, heart and brain-parts like cerebellum, cerebral cortex, brain stem & hypothalamus were dissected. They were frozen in ice according to the Iversen & Glowinski's procedure. The tissues were stored at -20ºC until assay was carried out.



Spleen



Kidney

Figure 4: Tissues

3.8 Assessment of SOD activity from kidney and spleen

3.8.1 Superoxide dismutase

SOD enzyme assay was carried out from kidney and spleen by the method of Kono et al., 1997.

3.8.2 Superoxide dismutase isolation

By using the method of Kono et al., 1997, 10% homogenate was prepared in 0.1 M sodium phosphate buffer. The homogenate was centrifuged at 1000 rpm at 4ºC for 10 minutes. Then supernatant was collected and collected supernatant was again centrifuged at 10,000 rpm for 30 minutes at 4ºC. The remaining supernatant was used to conduct enzyme assay.

3.8.3 Superoxide dismutase Estimation

The reaction mixture contained 50 mM sodium carbonate buffer, 1 mM hydroxylamine Hydrochloride, 0.6% Criton-x 100, 0.1 mM EDTA, 400 µM NBT. Change in absorbance was

measured at 560 nm for varying concentration of enzyme. Vmax of SOD was determined for varying concentrations of enzymes.

3.9 Histological study

Rats from each experimental group were anesthetized with chloroform and perfused transcardially with 0.9% saline and 4% paraformaldehyde in phosphate buffered saline (PBS). Kidney was dissected out from C, D, CIIH and DIIH rats. Paraffin sections of 3 µm were used for Hematoxylin-eosin staining.

3.9.1 Deparaffinization and rehydration of sections

Sections were deparaffinized by using a microwave oven. They were rehydrated by dipping in Xylene, Absolute alcohol, 95% alcohol, 70% alcohol and Distilled water.

3.9.2 Hematoxylin-eosin staining

Rehydrated paraffinized sections were stained with hematoxylin for 4 minutes followed washing with distilled water. Nucleus of cells was stained with hematoxylin and excess stain was destained with acid-alcohol mixture. Then sections were counter stained with eosin for 2 minutes. Then sections were washed with distilled water, dehydrated with alcohol followed by xylene and mounted with DPX.

3.10 Gene expression

3.10.1 RNA isolation

Total RNA was isolated from the control & experimental group of rat's kidney by using phenol-guanidine thiocynate method with slight variation. Agarose gel electrophoresis method was performed for observing isolated RNA. Purity of RNA is checked by using nanodrop instrument.

3.10.2 cDNA synthesis

Total cDNA synthesis was done from the isolated RNA by using cDNA synthesis kit.

3.10.3 PCR Analysis

PCR analyses were conducted with gene-specific primers which is designed by using Primer quest software and amplified product was observed by agarose gel electrophoresis

Primer designing:

Primer of the gene neurexin was designed by using the PrimerQuest software which is shown below:

Primer	Forward primer	Reverse primer	Tm	GC content	Product length
Neurexin-1	5'GCTTCACACAG GGAAATC3'	5'CATTCACGGG CTCTACTA3'	58	50	18

Table 2: Primer Designing

3.11 Statistical analysis

Statistical analysis was carried out by ANOVA using Graph-pad Prism.

4. Results and discussion

4.1 Blood glucose levels

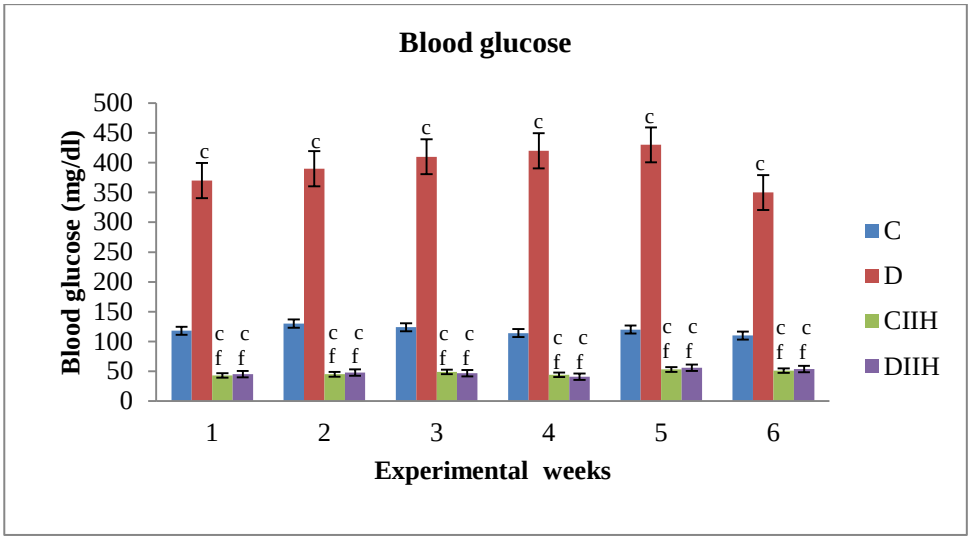


Figure 5: Blood glucose levels in experimental group of rats

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).
ANOVA followed by Student's Newman-Keul's Test
***P<0.001 when compared to C=c ###P<0.001 when compared to D=f

Discussion:

Blood glucose level was high in diabetic group when compared with control. In CIIH and DIIH group blood glucose levels were low compared to control.

Insulin increases glucose transport from the extracellular tissue to the cells. Streptozotocin causes destruction of β-cells, hence insulin is not secreted. Impairment in insulin secretion leads to accumulation of glucose in the body, thereby raising the glucose level in diabetic group (Akbarzadeh et al., 2007).

But excess amount of insulin in the body as compared to glucose concentration causes metabolic imbalance. In case of hypoglycemia, there is no glucose within as well as outside the cells. Hence, there is an excess of insulin as compared to glucose thereby lowering the levels of blood glucose significantly after insulin administration in CIIH and DIIH group (Mahmoud et al., 2009).

4.2 Water consumption

4.2.1 Water consumption during active phase

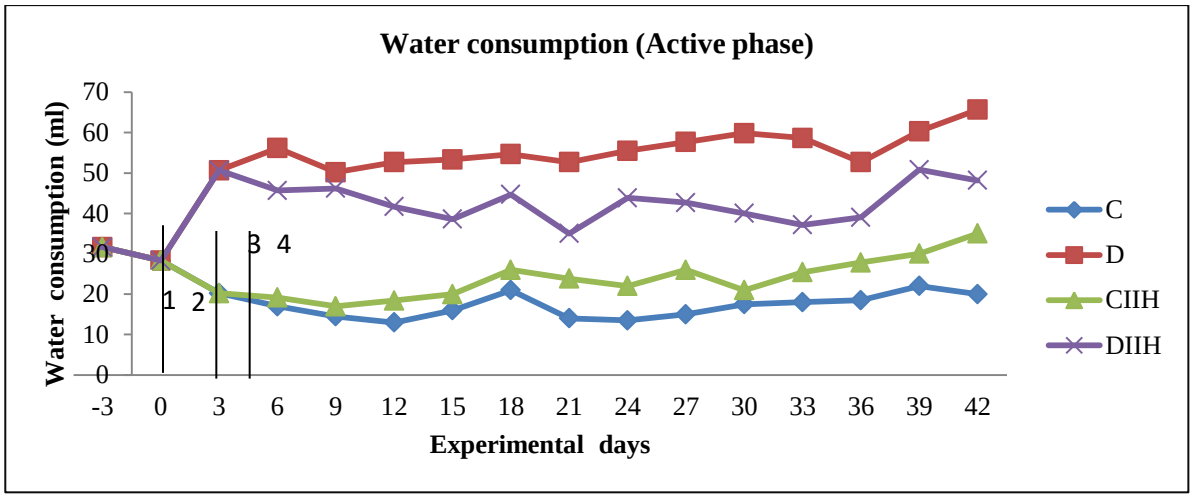


Figure 6: Water consumption during active phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

1-Pre diabetic phase 2-Diabetic manifestation phase

3-Post diabetic phase 4-Hypoglycemic phase

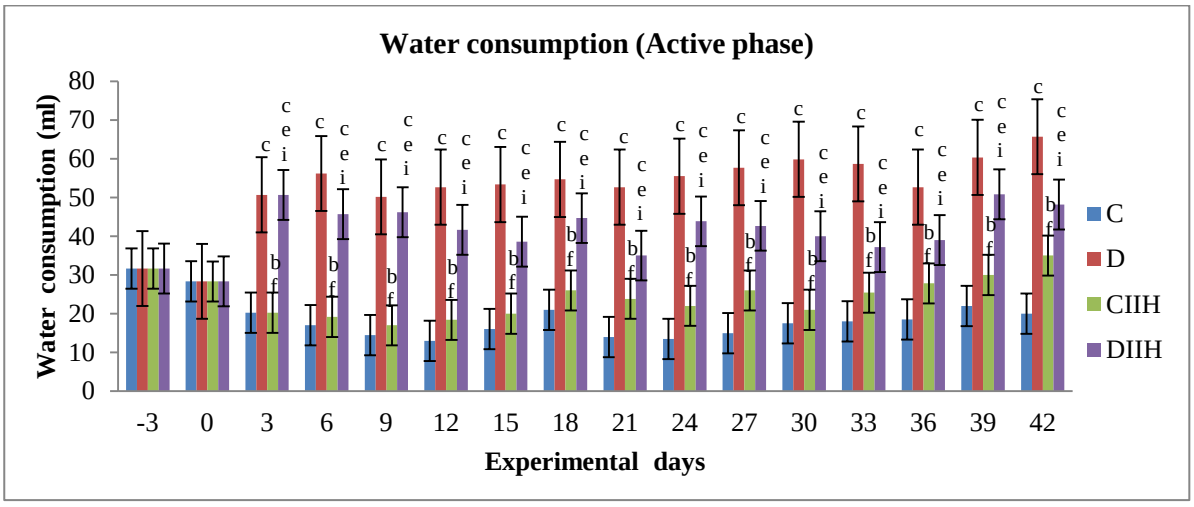


Figure 7: Water consumption during active phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

ANOVA followed by Student's Newman-Keul's test

**P<0.01 when compared to C=b ***P<0.001 when compared to C=c

##P<0.01 when compared to D=e ###P<0.001 when compared to D=f

@@@<0.001 when compared to CIIH=i

4.2.2 Water consumption during inactive phase

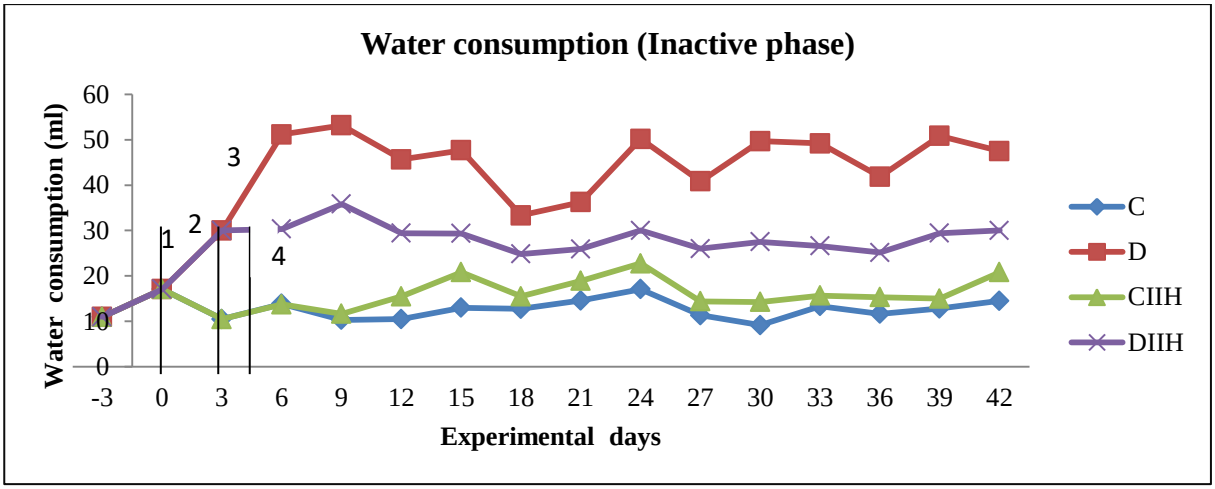


Figure 8: Water consumption during inactive phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

1-Pre diabetic phase 2-Diabetic manifestation phase

3-Post diabetic phase 4-Hypoglycemic phase

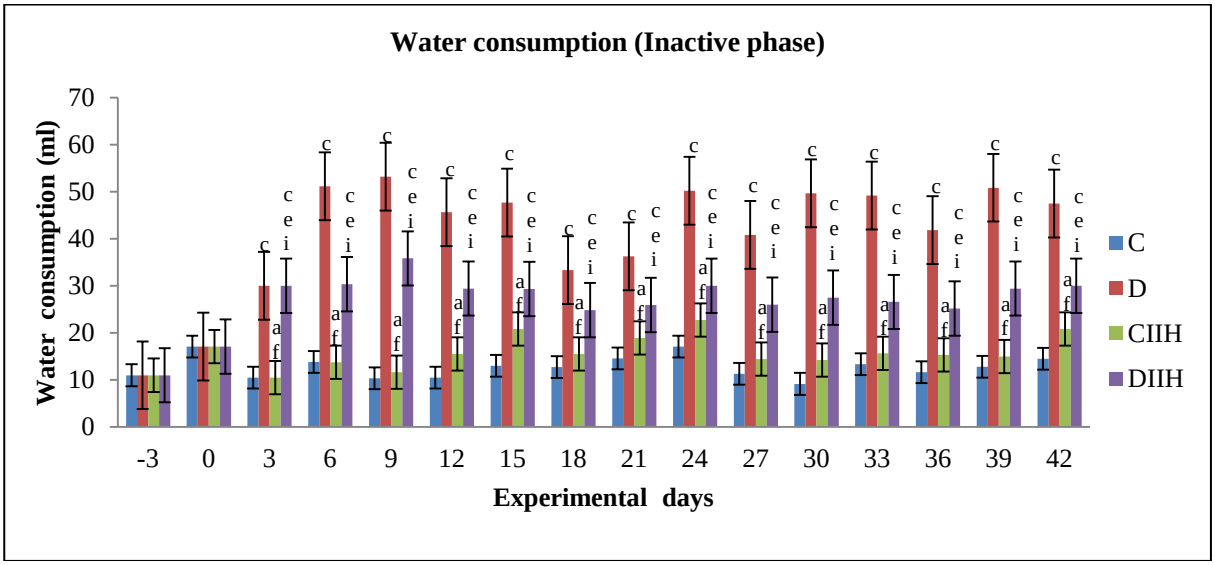


Figure 9: Water consumption during inactive phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

ANOVA followed by Student's Newman-Keul's

*P<0.05 when compared to C=a ***P<0.001 when compared to C=c

##P<0.01 when compared to D=e ###P<0.001 when compared to D=f

@@@P<0.001 when compared to CIIH=i

Discussion

During the pre-induction phase, no significant difference in the water consumption of rats was observed. After 3rd day of model induction, significant increase in amount of water consumption was observed in diabetic rats.

In diabetes, due to high blood glucose levels, electrolyte imbalance was observed and it exerts osmotic pressure by changing osmolarity. This leads to the release of ADH (Anti-Diuretic hormone) which activates thirst center in hypothalamus. Increase in blood glucose levels causes alteration in glomerular filtration rate of kidney, which leads to glycosuria. To dilute concentrated urine, more amount of water is needed. So, tubular secretion of water takes place. Thus, it activates thirst centers in brain to compensate water loss (Akbarzadeh et al., 2007). After the 3rd day of model induction, CIIH and DIIH shows significant increase in water consumption compared to control group. Hypoglycemia causes activation of sympathetic nervous system. It causes increased activity of HPA axis which results into increased secretion of epinephrine, which is responsible for activation of thirst center. (Dennis et al., 1982)

4.3 Food consumption

4.3.1 Food consumption during active phase

/

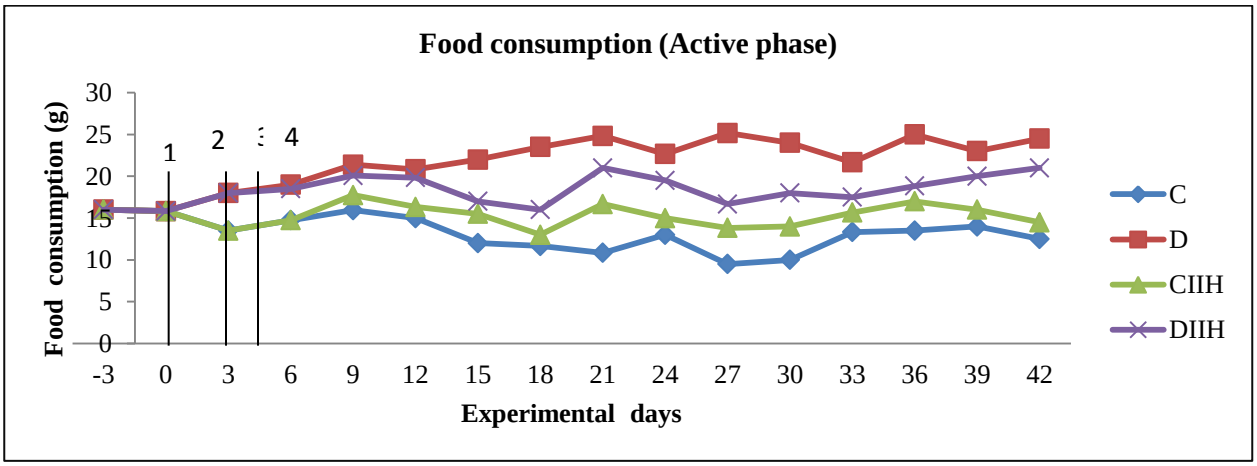


Figure 10: Food consumption during active phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

1-Pre diabetic phase 2-Diabetic manifestation phase

3-Post diabetic phase 4-Hypoglycemic phase

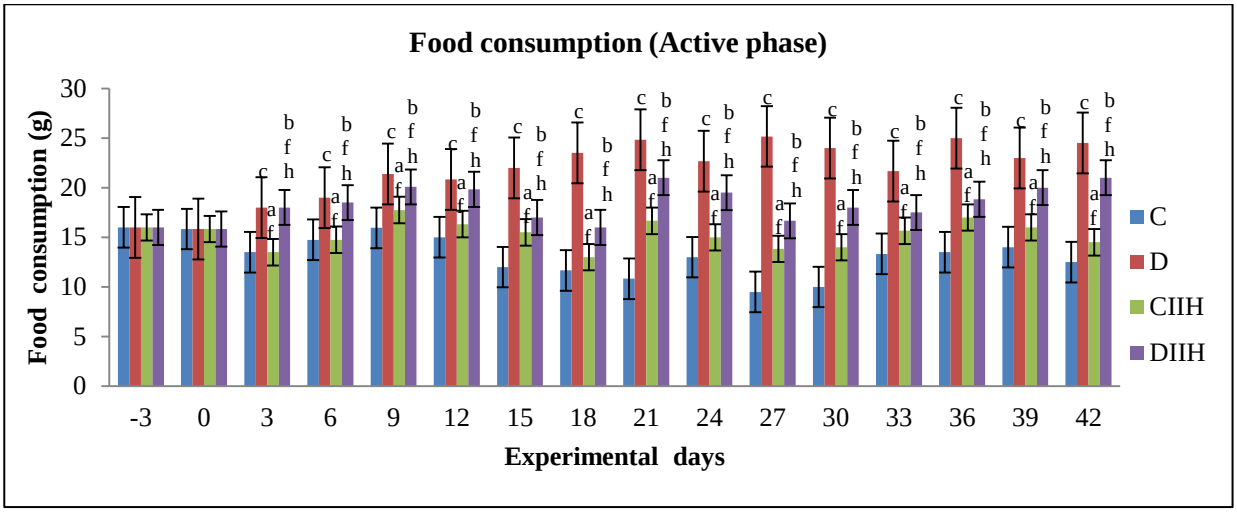


Figure 11: Food consumption during active phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

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

*P<0.05 when compared to C=a

**P<0.01 when compared to C=b

***P<0.001 when compared to C=c

###P<0.001 when compared to D=f

@@P<0.01 when compared to CIIH=h

4.3.2 Food consumption during inactive phase

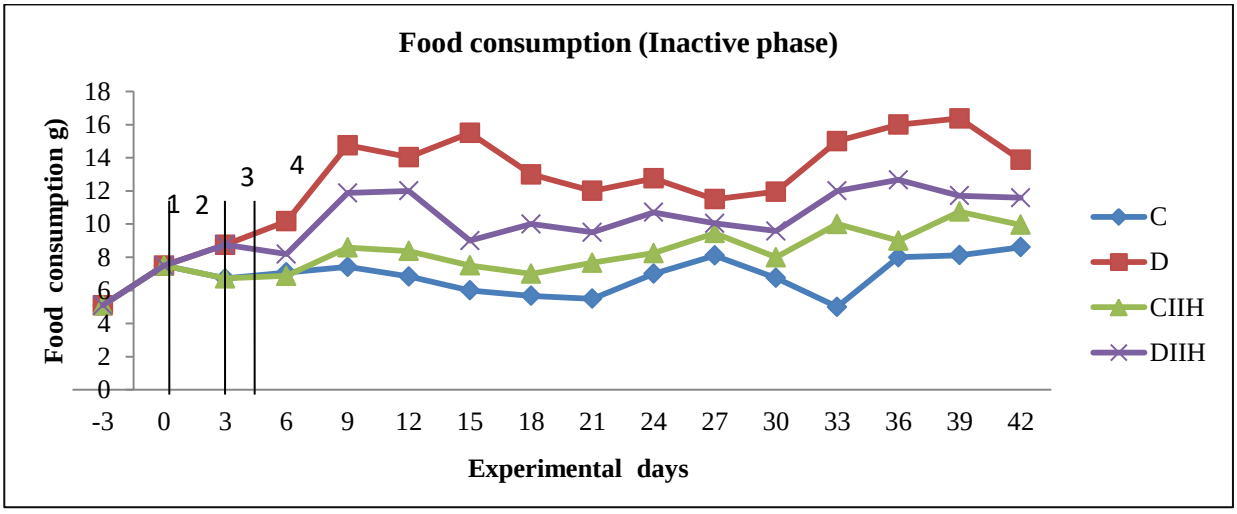


Figure 12: Food consumption during inactive phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

1-Pre diabetic phase

2-Diabetic manifestation phase

3-Post diabetic phase

4-Hypoglycemic phase

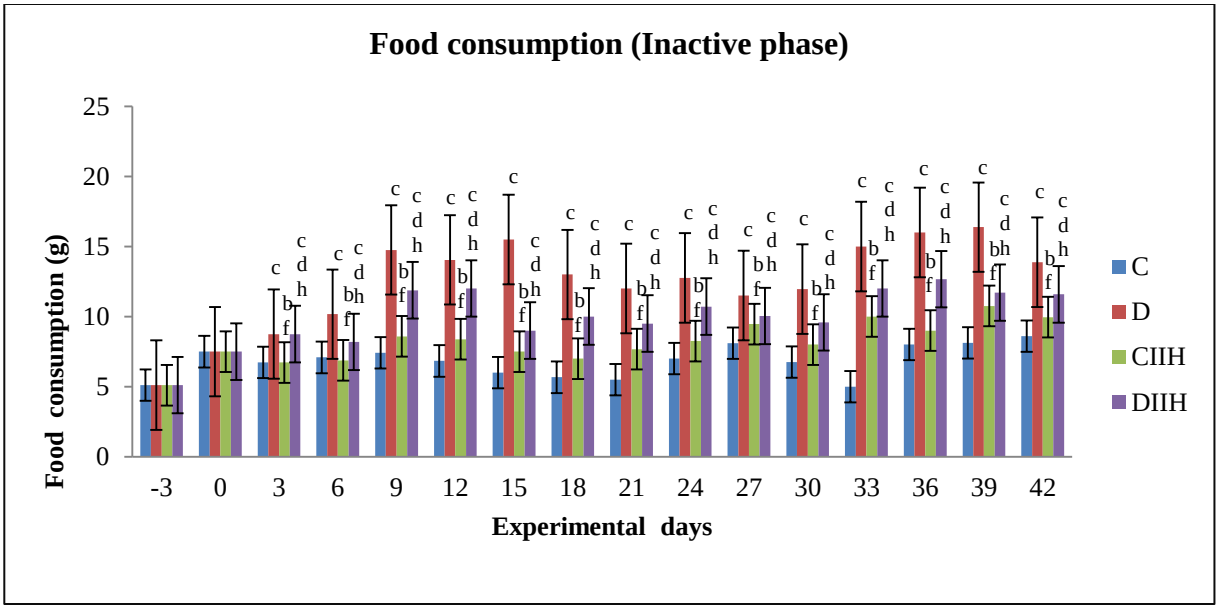


Figure 13: Food consumption during inactive phase

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

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

**P<0.01 when compared to C=b

***P<0.001 when compared to C=c

#P<0.05 when compared to D=d

###P<0.001 when compared to D=f

@@P<0.05 when compared to CIIH=h

Discussion

During the pre-induction phase, there was no difference in the food consumption of rats. A significant variation was observed in the food intake pattern in diabetic and hypoglycemic rats as compared to that of the control rats from day 3 of model induction. In diabetic group of rats, significant increase in amount of food intake was observed compared to control group. Diabetic rats consume excess food due to Polyphagia. In diabetes, peripheral blood glucose level is very high but glucose levels inside the cell are low due to lack of insulin. This impairs insulin mediated glucose transport inside the cell and leads to activation of hunger center in hypothalamus (Roth et al., 2016). A significant increase in food intake was observed in hypoglycemic rats (CIIH and DIIH) compared to control group. Hypoglycemia shows low glucose inside and outside of the cells. Low blood glucose is able to initiate glycogenolysis and gluconeogenesis pathway via release of glucagon and adrenalin that increase leptin to some extent, resulting in activation of the feeding center in hypoglycemia (Jiang and Zhang, 2003).

4.4 Change in body weight

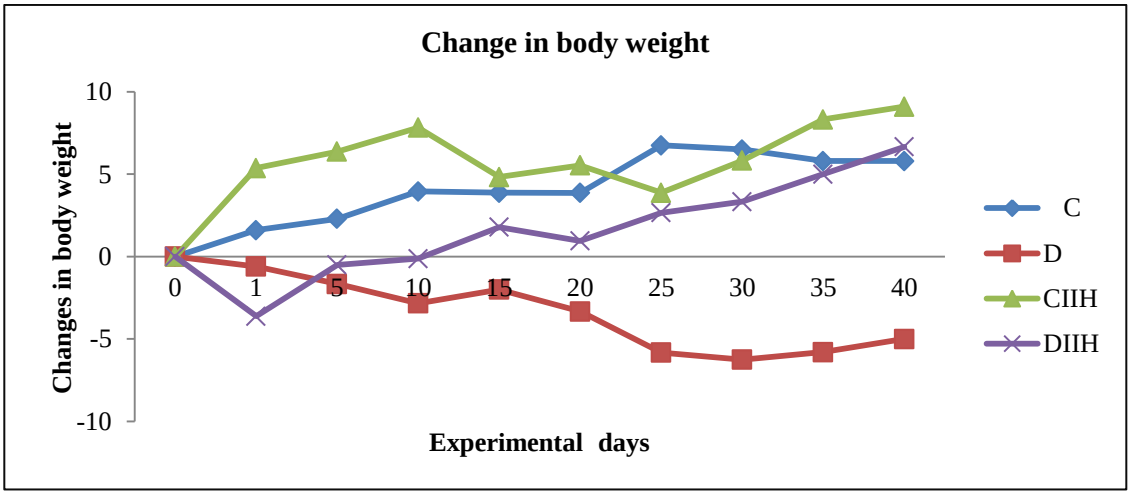


Figure 14: Change in body weight

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).

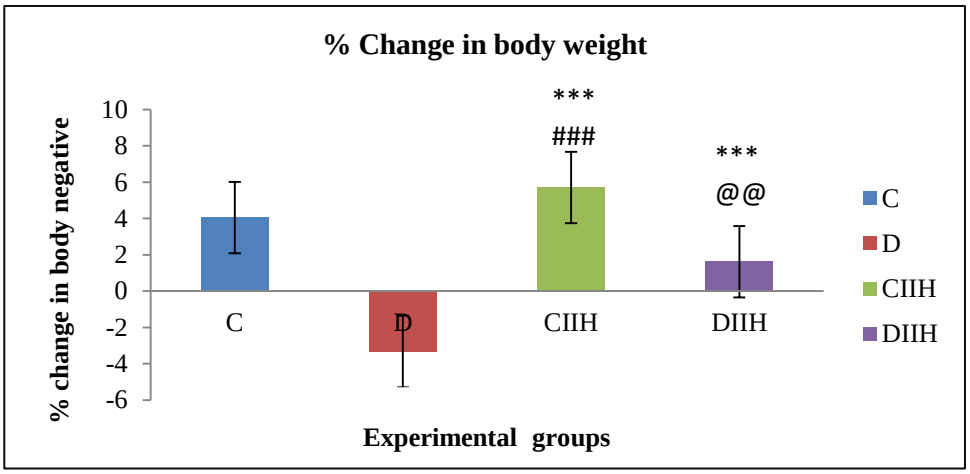


Figure 15: % change in body weight

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/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 CIIH

Discussion

From the 3rd day of model induction, a significant decrease in body-weight of diabetic rats was observed compared to the control rats. It occurs corresponding to high blood glucose level, resulting in Polyuria, which causes dehydration and loss of body fluids and electrolytes. During

diabetes, lack of insulin is prevalent in the body cells. So, insulin mediated transport of glucose is affected (Mahmoud et al., 2009). It leads to activation of alternative mechanism for glucose production such as glycogenolysis and gluconeogenesis. Glycogenolysis causes depletion of glycogen and lipids which leads to weight loss in diabetic group. In hypoglycemic group of rats (CIIH and DIIH), body weight increases in comparison to diabetic group of rats but less than control group of rats. In CIIH group, body weight increases during initial phase, but during treatment phase, it decreases in comparison to control group, which is mainly due to insulin, which prevents excessive fat metabolism (Chiarelli et al., 1999).

4.5 Enzyme assay

4.5.1 Superoxide dismutase assay in kidney

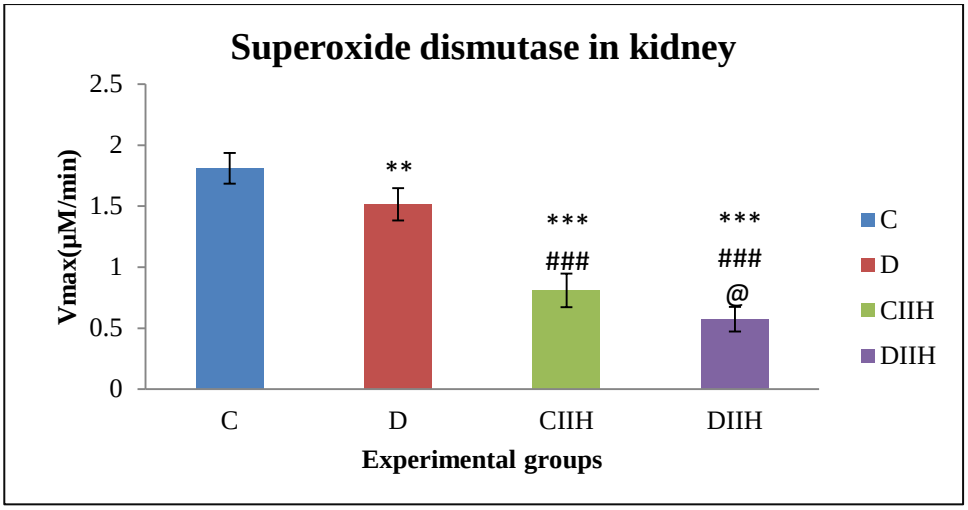


Figure 16: Superoxide dismutase assay in kidney

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).
ANOVA followed by Student's Newman-Keul's Test
**P<0.01 when compared to C ***P<0.001 when compared to D
###P<0.001 when compared to D @P<0.05 when compared to CIIH

Discussion

In diabetes, metabolic abnormalities causes mitochondrial superoxide overproduction and decreased levels of antioxidant enzymes. High blood glucose levels and the oxidative stress caused thereby, leads to renal damage which in turn causes diabetic nephropathy. Therefore, we assessed

superoxide dismutase activity from kidney (Sies, 1993). SOD activity in diabetic group of rats was significantly low ($P<0.01$) than control group of rats. This is mainly because of high levels of oxidative stress, which causes metabolic and hemodynamic changes during diabetes. This further leads to the activation of various pathways of oxidative stress. Thus, oxidative stress and ROS production is high during diabetic nephropathy.

In hypoglycemic group of rats (CIIH and DIIH), SOD activity is low compared to diabetic group. During hypoglycemia, blood glucose level is decreased. This causes an impairment in glycogenolysis and gluconeogenesis. Thus, insufficient glucose causes an adverse effect on kidney which leads to the generation of oxidative stress (Fujita et al., 2009).

4.6 Histological study

4.6.1 Alterations in RBC levels in glomerulus and change in thickness of glomerular membrane

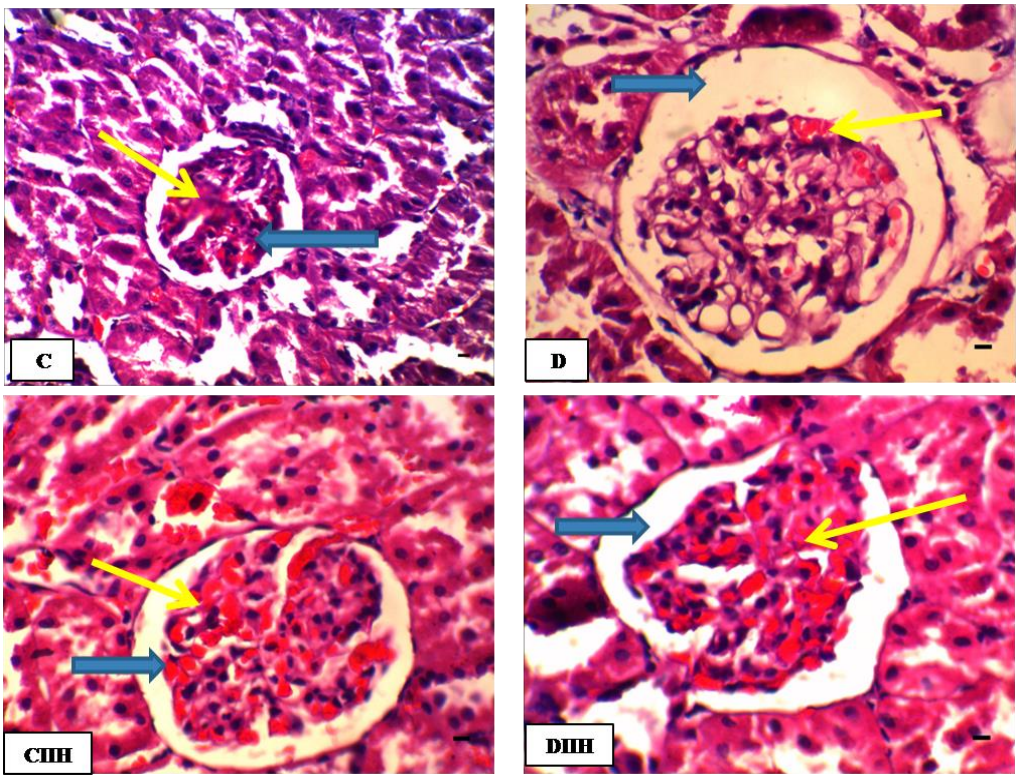


Figure 17: alterations in RBC level and Change in thickness of glomerular membrane

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. → =alterations in RBC level. → =Change in thickness of glomerular membrane.

Discussion

During diabetes, RBC levels are decreased in glomerulus of kidney. Hormone erythropoietin is responsible for the production of RBCs. This erythropoietin is produced by the kidney. In diabetes, due to renal damage, erythropoietin production is affected. Thus, during diabetes RBC levels are decreased in glomerulus (McGill et al, 2006). In Hypoglycemia, that is CIIH and DIIH groups, RBC levels are increased. During hypoglycemia, hypoxia can occur. Hypoxia is a condition in which oxygen levels are decreased. To compensate oxygen levels hormone erythropoietin increases RBC production. Thus, during hypoglycemia RBC level is increased in glomerulus.

The thickening of glomerular membrane during diabetes takes place due to protein synthesis such as collagen, laminin and fibronectin. These proteins are glycated during diabetes, which leads to increases protease resistance and thereby, get accumulated on the membrane of the glomeruli. In hypoglycemic condition, these proteins are not glycated and protease resistance does not occur. Hece, membrane of glomeruli is normal because accumulation of these proteins does not occur (Kolset et al., 2012).

4.6.2 Mesengial cell expansion in glomerulus

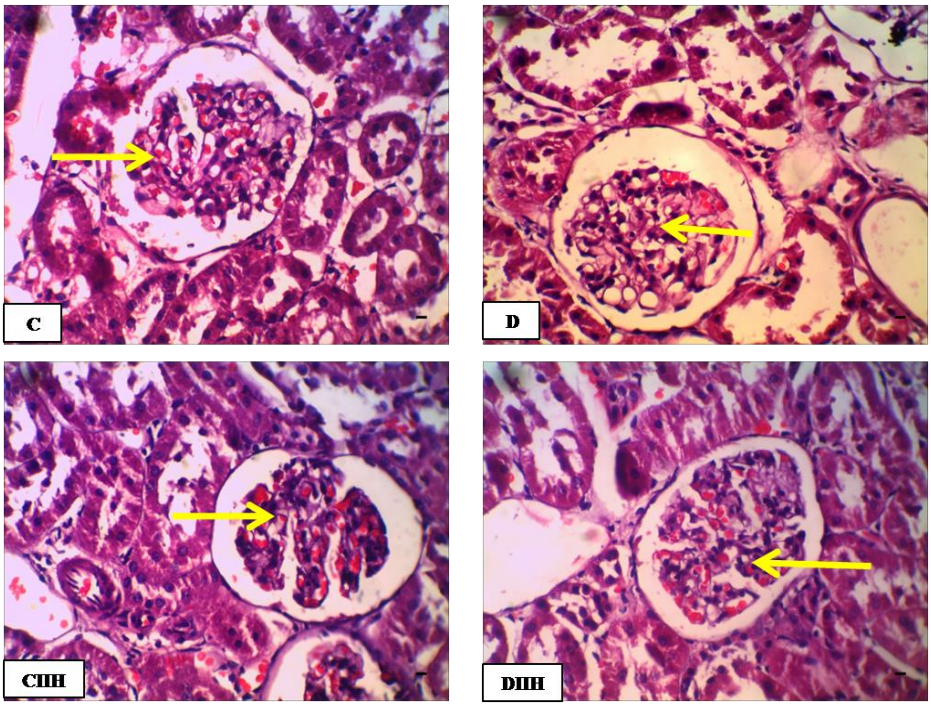


Figure 18: Mesengial cell expansion in glomerulus

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. ⟶ =Mesengial cell expansion in glomerulus.

Discussion

Messengial cells are specialized pericytes located among the glomerular capillaries inside the glomerulus. The expansion of these cells is one of the characteristic of diabetic nephropathy.

These mesengial cells expand through cell proliferation via increased deposition of collagen, fibronectin and laminin. In diabetic nephropathy, renin-angiotensin system is activated in mesengial cells and angiotensin stimulates matrix growth through activation of Type-2 receptors, which secondarily up regulate growth factors (Abrass, 1995).

Glomerular hypertension causes mesengial cells to stretch which causes induced expression of GLUT1 leading to increased cellular glucose. The repetition of stretching of mesengial cells due to hypertension increases mesengial cell proliferation (Kawasaki et al, 2007).

During hypoglycemia, production of collagen, fibronectin and laminin is normal. Due to low blood glucose level hypertension does not occur in glomeruli. Hence, mesengial cell expansion does not occur.

4.6.3 Thickening of renal arteries

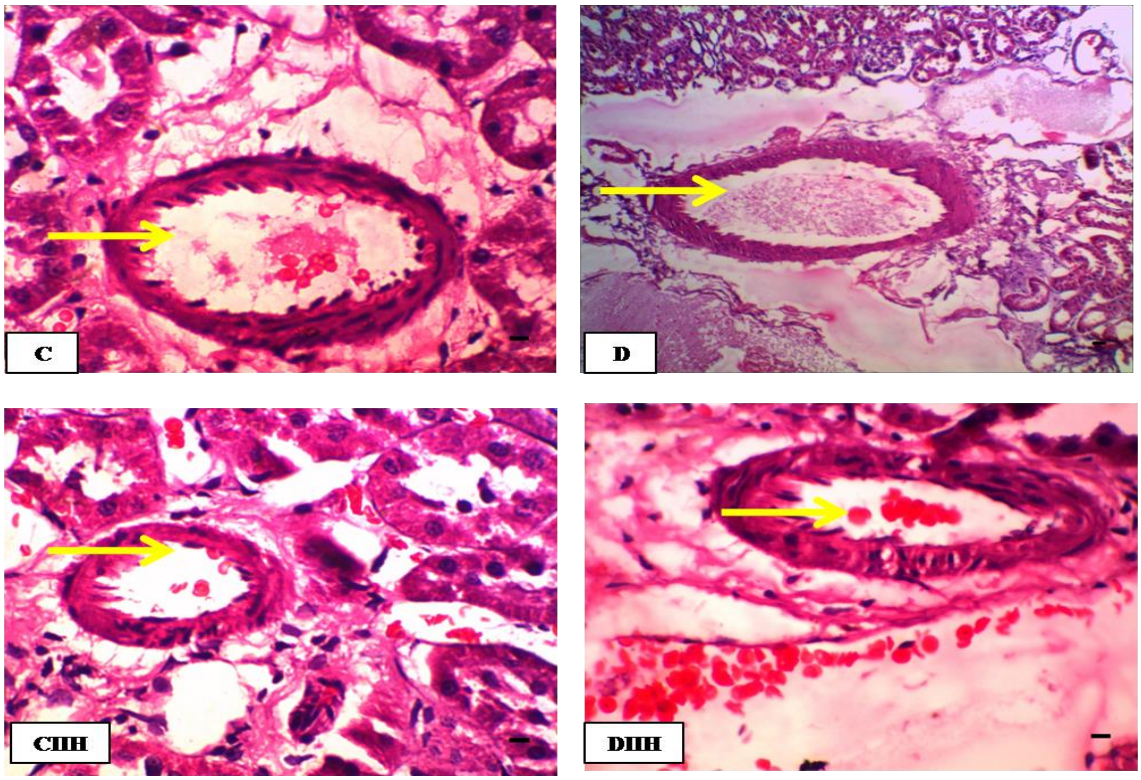


Figure 19: Thickening of renal arteries

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. ⟶ =Thickening of renal arteries.

Discussion

Renal fibrosis is a characteristic of diabetic nephropathy. Eosinophilic deposition is an early sign of the renal fibrosis due to glycation of extra-cellular matrix and loosening of the balance of extracellular matrix protein synthesis and degradation. Various growth factors which are increased in diabetic nephropathy are responsible for the renal damage. Advanced glycation end products (AGEs) can induce renal damage by stimulation of growth factors through AGE receptors. In hypoglycemia (CIIH and DIIH), thickening of renal arteries does not occur (Abrass, 1995).

In hypoglycemia, due to low blood glucose levels AGEs formation does not takes place. So, renal damage cannot occur because AGEs are mainly responsible for the renal thickening. Thus, thickening of renal arteries does no takes place during hypoglycemia (Kawasaki et al, 2007).

4.6.4 PCT proliferation

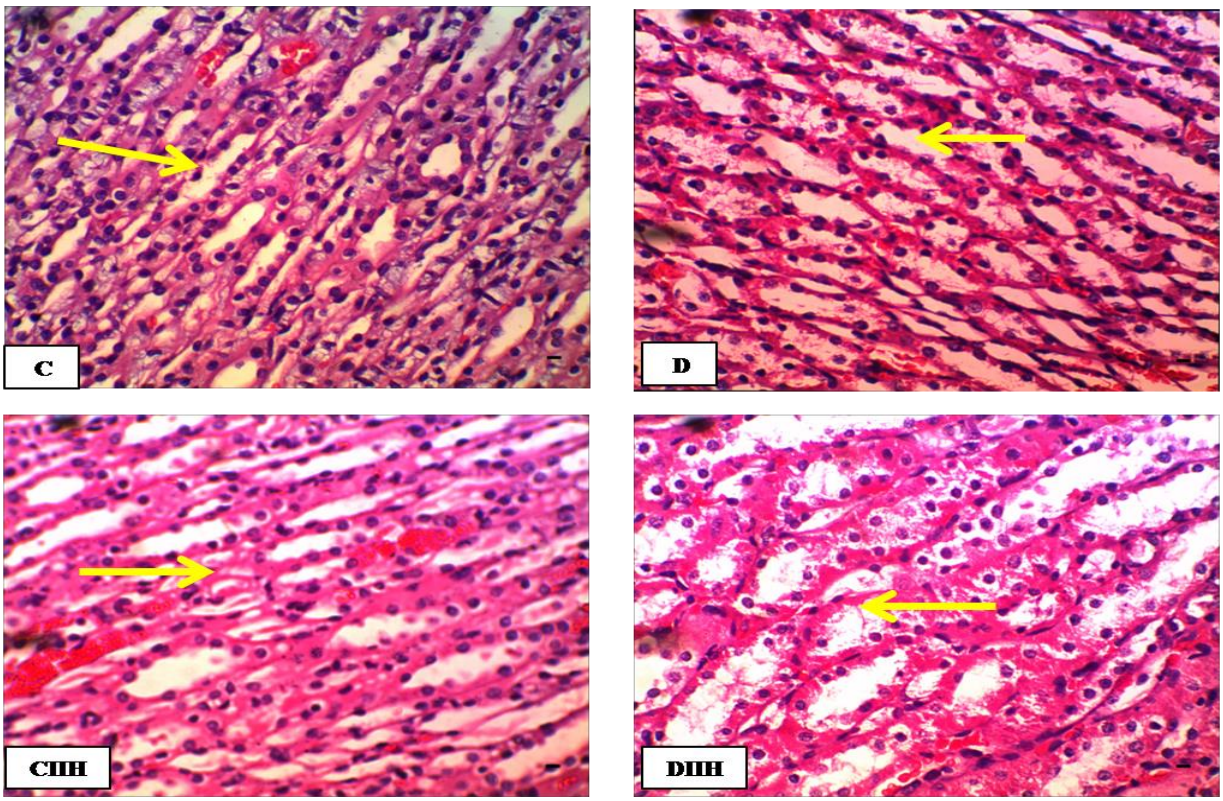


Figure 20: PCT proliferation

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. ⟶ =PCT proliferation

Discussion

Proximal convoluted tubule (PCT) is a portion of nephron, which is functional unit of kidney. It is the major site for reabsorbtion of glucose and ions. Glucose is co-transported with sodium ions into the proximal convoluted tubule walls via the SGL2 co-transporter. Numerous growth factors such as, insulin like growth factor-1 (IGF-1), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) contribute to proliferation of cell (Abrass, 1995).

In hypoglycemia, blood glucose level is low. So, rate of glucose absorption in PCT is low compared to diabetic group. Therefore, proliferation of PCT is less than that in diabetes (Kawasaki et al, 2007).

4.7 Neurexin-1 gene expression

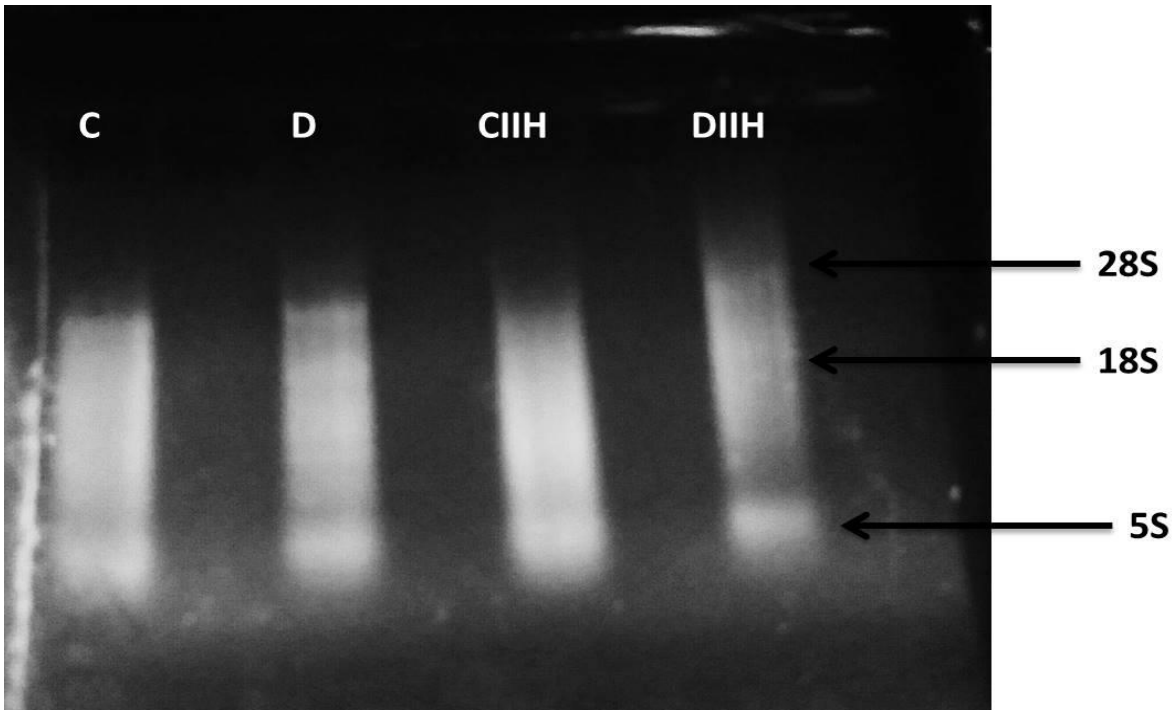


Figure 21: Total RNA Isolated from Kidney as expressed on an agarose gel

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemic rats, DIIH-Insulin induced diabetes hypoglycemic rats

Total RNA was isolated from the kidney of control and experimental group of rats using Tri reagent. Total RNA isolation from experimental rats was carried out and was checked on agarose gel to assess the integrity of RNA and then quantified using nanodrop.

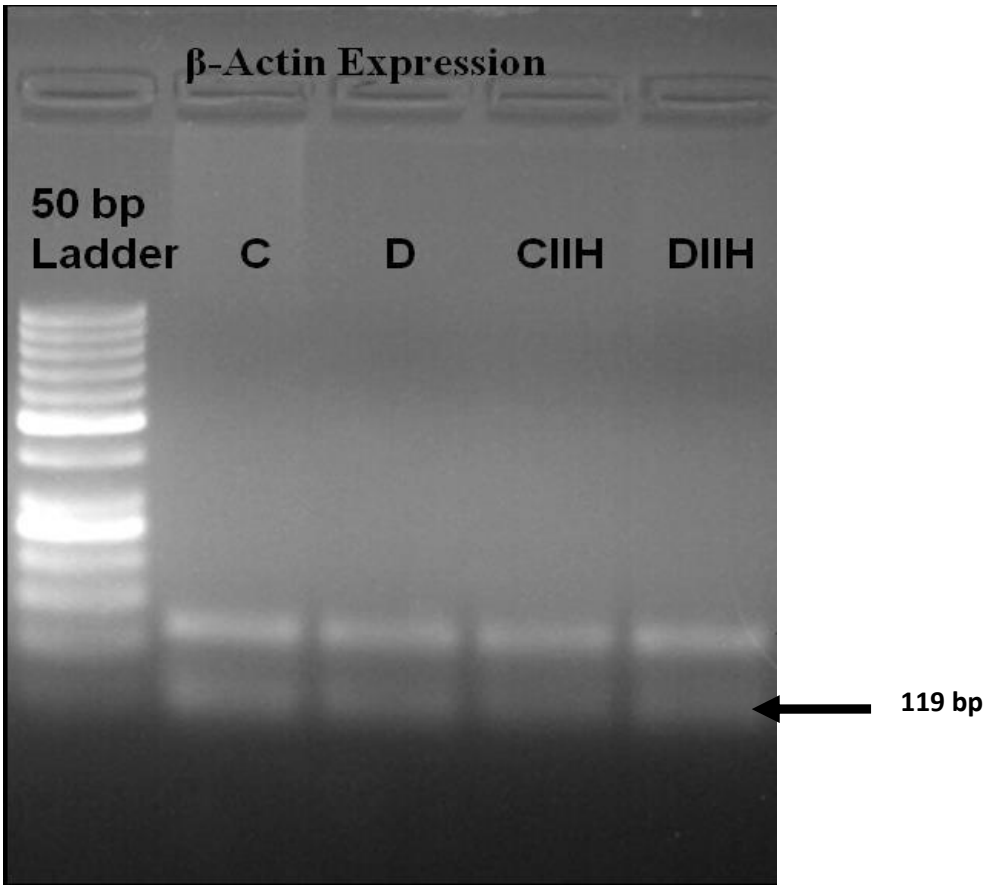


Figure 22: β-Actin expresssion in Kidney as expressed on an agarose gel

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats

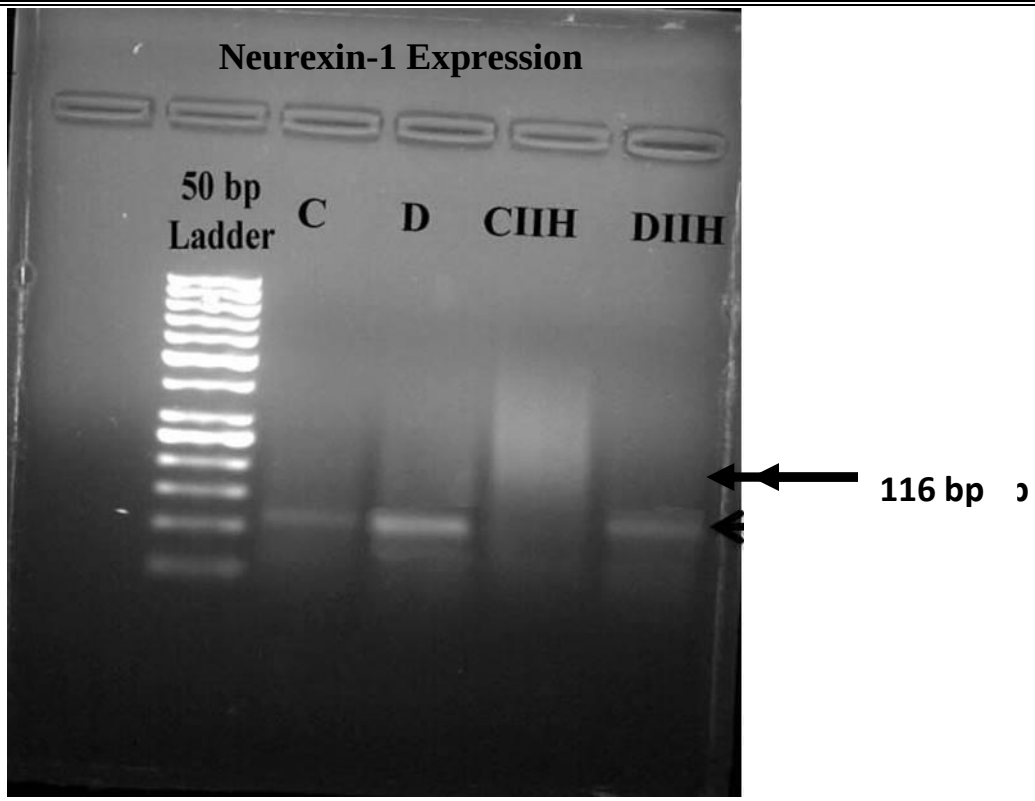


Figure 23: Neurexin-1 expression in Kidney as expressed on an agarose gel

The above results were obtained by performing PCR from RNA isolated from kidney of experimental rats. This experiment was performed only once hence, conclusion regarding Neurexin-1 expression cannot be drawn from the results obtained. For this, the experiment needs to be repeated.

4.8 Superoxide dismutase assay in spleen

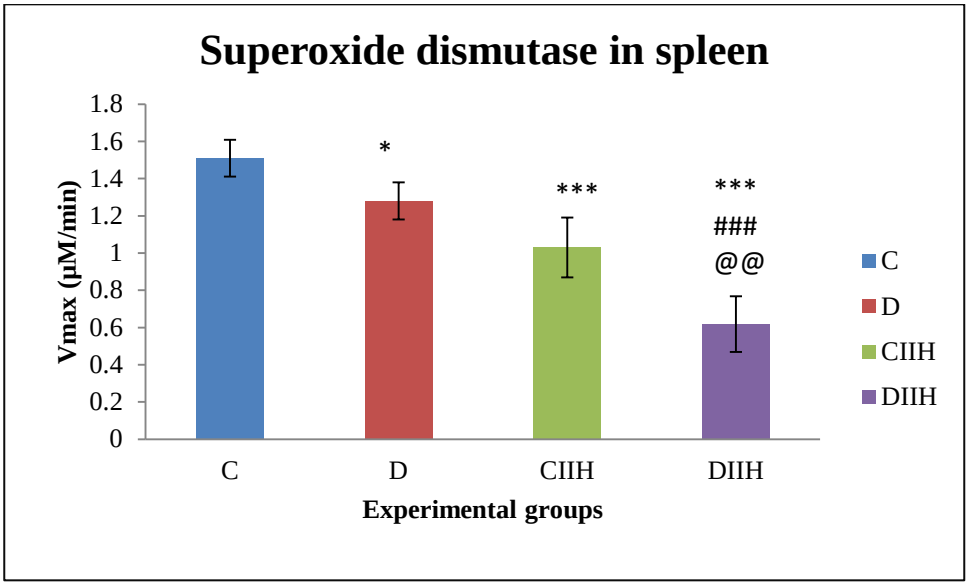


Figure 24: Superoxide dismutase assay in spleen

C-Control rats, D-Diabetic rats, CIIH-Insulin induced hypoglycemia in control rats and DIIH-Insulin induced hypoglycemia in diabetic rats. Values are mean ±SD for 3 separate experiments (n=6 rats/group).
ANOVA followed by Student's Newman-Keul's Test
*P<0.05 when compared to C
***P<0.001 when compared to C
###P<0.001 when compared to D
@@P<0.01 when compared to CIIH

Discussion

During diabetes, high blood glucose levels affect the immune system of the body. Innate immune system, which is the first line of defense, gets affected during hyperglycemia. Spleen is the major component of the immune system. So we assessed the SOD activity from the spleen. During diabetes, glycated RBCs leads to alteration in free radical species alterations, which leads to the generation of oxidative stress. This leads to decrease in activity of superoxide dismutase activity.

SUMMARY

Diabetes is a complex disorder which results in various complications like, Nephropathy, Neuropathy and Retinopathy. During diabetes, high dose of insulin leads to progression of hypoglycemia. Single Intrafemoral dose of streptozotocin (50 mg/kg body weight) was administered to induce diabetes. Hypoglycemia was induced by giving daily two doses to rats subcutaneously in CIIH (1.5 IU/Kg) & DIIH (10 IU/Kg). Blood glucose, food-water & body weight measurement was done during the experimental days. Significant increase in blood glucose level of diabetic group compared to control group was observed ($P<0.001$). Significant decrease in blood glucose level of CIIH and DIIH group in comparison to control was observed ($P<0.001$). There was significant increase in food & water intake of diabetic group compared to control ($P<0.001$).There was significant increase in food & water intake of CIIH & DIIH was observed compared to control. Significant decrease in body weight of diabetic group was observed compared to control ($P< 0.001$). Significant increase in body weight of CIIH & DIIH was observed compared to control group ($P< 0.01$). To study the effect of oxidative stress mediated damage in kidney, the activity of enzyme superoxide dismutase (SOD) was performed. Enzyme activity of SOD of diabetic group was low in comparison to control group ($P<0.01$) Enzyme activity of SOD of CIIH & DIIH group was low in comparison to control ($P<0.001$). To check the expression of NRXN-1 gene, total RNS was isolated from the kidney and converted to cDNA by reverse transcriptase assay and PCR was carried out to check the expression of Neurexin-1 gene expression.

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