Identification Of Interlinked Signaling Pathways In Mouse Model Of Diabetes Mellitus And Hepatocellular Inflammation

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ISLAMABAD

December, 2018

Identification Of Interlinked Signaling Pathways In Mouse Model Of Diabetes Mellitus And Hepatocellular Inflammation

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

MS Biomedical Sciences

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Acknowledgements

I am thankful to my Creator Allah Subhana-Watala to have guided me throughout this work at every step and for every new thought which You setup in my mind to improve it. Indeed I could have done nothing without Your priceless help and guidance. Whosoever helped me throughout the course of my thesis, whether my parents or any other individual was Your will, so indeed none be worthy of praise but You.

I am profusely thankful to my beloved parents who raised me when I was not capable of walking and continued to support me throughout in every sector of my life.

I would also like to express special thanks to my supervisor Dr. Adeeb Shehzad for his help throughout my thesis and also for courses which he has taught me. I can safely say that I haven't learned any other subject in such depth than the ones which he has taught.

I would also like to pay special thanks to Shagufta Rehmat for her tremendous support and cooperation. I appreciate her patience and guidance throughout the whole thesis. I must express my gratitude to Ma'm Muneeba Qureshi for her continuous support, encouragement and guidance. A special thanks to each and every member of Oncology Lab who taught me a great deal about both scientific research and life in general.

Finally, my sincere regards to my GEC members, principal SMME and administration for providing a great platform for carrying out my research.

Tehmeena Mazhar

Dedicated to my exceptional parents and adored sibling

Abstract

Diabetes Mellitus (DM), a metabolic disorder characterized by hyperglycemia and insulin resistance is the leading chronic disease and has emerged a big socio-economic burden in Pakistan. Chronic liver diseases on the other hand make Pakistan stand on 2nd position only after Egypt. Liver inflammation ranges from hepatic steatosis to progressive forms of diseases include liver injury, fibrosis, cirrhosis and carcinoma. Liver plays a central role in glucose and lipid metabolism and number of meta-analysis and observational studies shows a strong association between diabetes and chronic liver inflammation. The purpose of present study is to investigate the mutual factors between both pathological conditions. Variance in body weights, lipid profile, level of alanine aminotransferase (ALT), alkaline phosphatase (AST), Bilirubin, blood glucose and anti-oxidant activities were found more abnormal in balb/c model that developed both liver injury and diabetic condition. Histopathology of liver reveals a positive association between diabetic and liver insulted mice model. Western blot analysis revealed the presence of agents of pro-inflammatory pathways i.eNFκB, COX-2 and Akt. Level of these pro-inflammatory agents is found higher in mice group induced with both diabetes and liver injury than the group induced with either of the diseases. These investigations reveal the severity of pathogenesis in the presence of both diseases and also single out potential predictors of hepatic inflammation in diabetes mellitus.

Key Words: *Diabetes Mellitus, Hepatocellular inflammation, Diethylnitrosamine, Pro-inflammatory pathway, NFκB, COX-2, Akt*

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CHAPTER 1: INTRODUCTION

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is one of the major concerns of public health all over the world. It is a group of variant metabolic disorders including insulin resistance, hyperglycemia and various other related pathophysiological complications such as retinopathy, chronic kidney and heart diseases and hepatopathy etc. Despite of accelerating development in the field of public health and clinical sciences, diabetes mellitus is still a life prone and incurable malady that is prevailing equally among all genders of all age groups. Globally diabetes mellitus is affecting a vast proportion of population of each country but the occurrence is much more frightening in Middle East and South Asian countries [1]. In South Asian region, Pakistan is one of the developing and populous countries whose economy is swinging in transition. Though majority of inhabitants reside in rural areas but increasing urban life trend has led people to sedentary lifestyle, high caloric food intake, lack of exercise and taxing and traumatic conditions drive towards metabolic disorders including diabetes mellitus. According to world health organization's 2011 report on diabetes mellitus, 13 million people has fallen a prey to diabetes among them only 10 million people are diagnosed. WHO also projected that by 2030 diabetes mellitus will be the 7^{th} leading cause of death by increasing the number of patients from 13 to 15 million [2]. In 2015 only, 1.6 million deaths were reported caused by this malady.

1.2 Liver Inflammation

Liver is the largest human body gland. Its main role in body is to perform metabolic functions and maintain immunological processes. Recently data on liver functions reveals the presence of a distinct immune-microenvironment due to abundant blood supply to liver. Numbers of pro-inflammatory agents are also present in liver to modulate the immunological processes. Kupffer cell is another histological entity of liver that averts the foreign body attack through phagocytosis. Natural killer cells and natural killer T cells are the elements of innate immunity which are also present in abundance in liver. Lastly liver is also source of various acute phase proteins which are causes of inflammatory reactions. All these entities

make liver a strong immunological organ of body. But in spite of this strong defensive mechanism any of the liver diseases including viral hepatitis, alcoholic/ non alcoholic fatty liver diseases, fibrosis, cirrhosis, toxin-induced liver damage can cause a serious disturbances to normal immune system of liver [3]. When the liver is under attack by any circumstances a number of signaling cascades play important role in either to protect the liver from harm or others that drive liver to more destruction. Cell necrosis occurs in the presence of continual infectivity and inflammation denoted by massive inflammatory cell infiltration in organ vasculature. This trend was noticed by Virchow in early 19^{th} century and he claimed a relation between inflammation and certain types of cancers [4]. Same phenomenon can be studied in liver inflammation. NFκB and COX-2 are two signaling pathways that lead to activation of certain pro-inflammatory agents including growth factors, chemokines, cytokines etc in response to accidental cell death. Activation of reactive oxygen species (ROS) and reactive nitrogen species by these inflammatory agents can also alter the DNA programming. And if the damage results in uninterrupted activation and inactivation of oncogene or tumor-suppressor gene respectively, carcinogenesis is favored.

1.3 Crossover of Diabetes Mellitus and Liver Inflammation

Role of Inflammation: With the advancement in research in clinical and biomedical sciences fields, enormous data and evidences are available that support arises of infections and inflammation due to complications in Diabetes Mellitus (DM). This DM induced inflammation not only plays a critical role in instigation of release of pro-inflammatory cytokines, tumor necrosis factors (TNF-α), Interleukin-1β and Interleukin-6 to protect the cell from injury. But chronic inflammation causes the cellular damage and leads the cell fate towards carcinogenesis [5].

*Role of Fatty Liver Diseases:*One of the main causes of disturbance in liver functions is the complication that arises due to different metabolic disorders. Early liver or hepatic steatosis was accounted due to alcohol consumption but now hepatic steatosis is reported to be found in non-alcoholic persons known as non-alcoholic hepatic steatosis (NASH). Obesity is a threat in developing this fat adorned steatosis which has ability to result in the form of liver inflammation, fibrosis, cirrhosis and ultimately hepatocellular carcinoma.

Role of Signaling Pathway: Nuclear factor kappa B (NFκB) known to have its interaction

with κ-light chain of immunoglobulin in B cells. NFκB is found in all cells including hepatocytes where it administers the proliferation and regeneration of liver epithelium [5]. NF-κBavctivation can be triggered by release of various inflammatory and non-inflammatory mediators e.g insulin resistance, oxidative strain, C-reactive protein, protein kinase C (PKC) etc[6]. This activation of NFκB further enhances the release of pro-inflammatory cytokines in diabetic patients and hepatocytes encounter with lethal consequences. NFκB also triggers the production of ROS & NOS through Tumor Necrosis Factor Alpha (TNF-α) pathway that further accelerates tissue damage [7]. Hence NFκB can be proved an apt target for not only the treatment of DM but also for hepatic injuries [8]. The other pathway that is proposed to be mutually shared between DM and liver injury is of Akt signaling pathway. Akt/PKB/PKC signaling cascade is activated by receptor tyrosine kinase which upstream regulated by insulin growth factor by phosphorylation of receptor tyrosine kinase. Activated Akt executes an assortment of cellular functions cell survival, proliferation, differentiation and migration. In downstream, Akt is also a regulator of NFκB and regulate necrosis, apoptosis and inflammation resulted by hyperglycemic conditions [9].

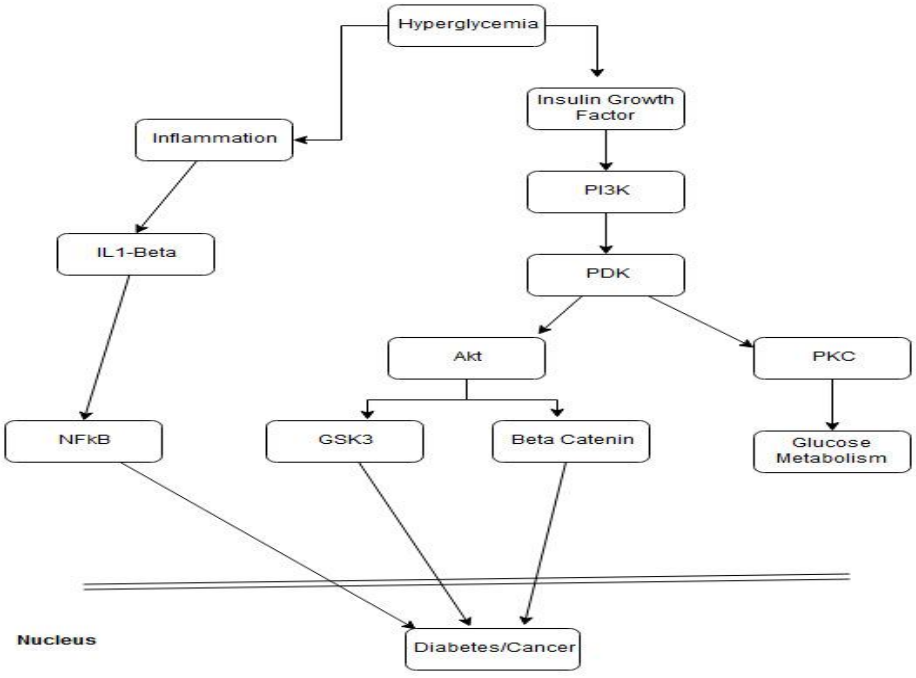

Figure 1.1: Hypothesized Schematic Representation Of Relation Between Diabetes And Cancer

1.4 Scope of Study

The present study was designed to evaluate the mutual signaling link between two most common diseases of time i.e. diabetes mellitus and hepatocellular inflammation. Number of statistical analysis and meta-analysis are available online that provide the link between these two maladies but in actual there is no any in-vivo experiment which investigate both diseases in a single mice model. Hence one of the novelties of this research is development of mice model induced with two diseases. Both diseases independently has proven their selves very fatal for health and here we postulate that there must be a mutual signaling pathway or more integrated pathways that trigger side by side that can have deleterious effect far worse than in patients sicken by a single disease. So, the scope was to investigate that either pathophysiology of mice model of both disease was significantly worse than mice model of either of the disease or not. To check the mutual link between two diseases, expression level of Akt, Cox-2, NF-κ B and IκB were calculated through western blotting. Level of Cox-2 and NF-κ B were significantly high in mice model of dual diseases giving us insight between the two very different diseases. Identification of mutually activated molecular entities are giving us prospective predictors of both diseases and potential targets for treatment of patients prey of both diseases.

1.5 Objectives of Study

1. The study will help in understanding the possible signaling mechanism involve in and mutual with both; diabetes and liver fibrosis (inflammation).
2. The study will be helpful in finding potential treatment targets for liver diseases and diabetes.
3. The conducted study will help in determining whether patients with insulin-dependent diabetes mellitus and those with non-insulin-dependent diabetes mellitus differ in their risk for primary liver inflammation or whether the liver damage risk is affected by the type of diabetes treatment.
4. Results of experiment can benefit medics and researchers working in the fields of diabetes and oncology to curb the recent worldwide metabolic syndrome epidemic and to reduce the liver damage burden.

Chapter 2 Literature Review

Although the world is unaware of the exact signaling mechanism between diabetes and hepatic inflammation, literature proposed a strong overlapping correlation between the two. Non-alcoholic fatty liver disease (NAFLD) is one of the most well known hepatic diseases that is correlated with mal-nutritious conditions and metabolic disorders. Chronic NAFLD leads to non-alcoholic steatohepatitis (NASH), hepatic fibrosis, and liver cirrhosis hence makingNAFLD a contender of spreading 25% chronic liver diseases[10]accelerating the rate of HCC in western civilization [11][12]. Fracanzani and coworkers [12] accounted the association of T2DM, insulin resistance with liver inflammation in patients who expressed normal liver enzymes level.

Kwok and collaborators initiated a cohort study of 2466 patients and found interesting relations between diabetes and liver disorders. About 62% patients were found to have liver fibrosis while 10% others also had advance liver diseases. Among patients who were on diabetic medications, those who usedThiazolidinediones found to have improve liver histology early effected by NASH, metformin users showed decreased casualty rate in cirrhotic patients while patients prey of cirrhosis also found insulin resistant [13]. Koehler in another research used elastography to measure liver stiffness. Results showed an advance rate of fibrosis in 17% patients who also suffered with diabetes [14].

Arachidonic acid (AA) which is the activator of COX-2, has presented itself as a strong linker between obesity and body's defense mechanism. Three enzymatic pathways are involved in activation of AA. (i) Cytochrome P-450 converts AA intoepoxy-eicosatrienoic acids (EETs)[15] (ii) Lipooxygenases also acts on AA to convert it into leukotrienes and 12-hydroxyeicosatetraenoic acid (12- HETE) [16] and (iii) AA is also mediated by cyclooxygenases (COX) to synthesize prostaglandins (PGs) [17] including PGD2, PGI2, PGF2 and PGE2. [18]. Literature shows the activity of PGE2 in impeding the discharge of insulin from β cells of pancreas by dysfunctioning and destroying them and PGI2 causes the insulin sensitivity in diabetic patients [19]. Konheim and coworkers showed the activation of rs20417 variant of COX2 in 30% patients of T2DM in a study on Pima Indians. Shanta et al, [20]found an increased Cyclo-oxygenase (COX-2) mRNA levels in human islets of langerhans in superglycemic environment but the same form was found recessive in freshly isolated healthy langerhans. COX-2 was found activated prostaglandin which further established proinflammatory reactions. Katori

and coworkers found COX-2 inhibitors as a potent anti-inflammatory agent [21].

Inflamed liver is the key cause behind the liver injury succession into fibrosis and cirrhosis with vivid physiological alterations like elevated hepatic enzymes, increased oxidative stress and inflammatory entities and dysregulated hepatic function.PI3K/Akt/mTOR is an intercellular signaling pathway that involves in regulation of cell cycle by controlling cell proliferation, cancer mechanism and cell permanency. Akt is activated by phosphorylating by PI3K and downstream regulates (i) activation of CREB[22] (ii) inhibit FOXO1 (iii) activates phosphatidyl inositol 3-phosphate (PIP3) and (iv) activates mammalian target of Rapamycin (mTOR). PI3K is activated by number of ligands among which insulin is an important inducer.As PI3K/Akt pathway involves in cell-cycle survival by initiating cascade of inflammatory cytokines and cell immunity so any fault in normal pathway route may lead to enhance the mal-liver conditions. PTEN which is the negative regulator of PI3K/Akt also downregulates the insulin signaling at insulin receptors (IRS) hence involve in insulin resistance and diabetic conditions. Downregulation of PTEN was found to start increased Akt activity, GLUT4 translocation, increased synthesis of triglycerides and fatty acid uptake in hepatocytes [23]. However one study ironically reported the presence of hepatic carcinoma in PTEN deleted mice models [24].

NF-κB being a mediator of inflammatory pathway expresses in all body cells including hepatic epithelium where it functions to maintain cell proliferation, regeneration and development. In stress conditions various inflammatory and non-inflammatory mediators trigger the release of NF-κB. Diabetic conditions are now known to generate low-grade chronic inflammation can cause the initiation of production of NF-κB be dephosphorylating IKKB. NF-κB promotes the formation of various pro-apoptotic cytokines, cell degeneration, production of oxidative species and increased liver tissue damage. While IKKB alongwith activation of NF-κB also blocks the insulin receptors hence causing insulin resistance that also leads to fibrosis, cirrhosis and end-stage liver damage. The whole loop of PI3K/Akt and NF-κB involving diabetes and tissue damage that we proposed is vividly explain in figure 2.1.

Another study hypothesized and explained the dysregulation in multiple signaling pathways that triumphed in escaping programmed cell death and resulted in formation of lymphomas [25]. Bay11-7085 being an NF-Kb inhibitor when added with Primary effusion lymphoma (PEL) cell lines, it not only hindered theNFκB mediated anti-apoptotic role by targeting its p65 subunit but also caused inactivation of PI3-kinase/AKT pathway. Conversely reduction of Akt by siRNA

also resulted in inactivation of IkkB which assures the cross talk between two pathways [26].

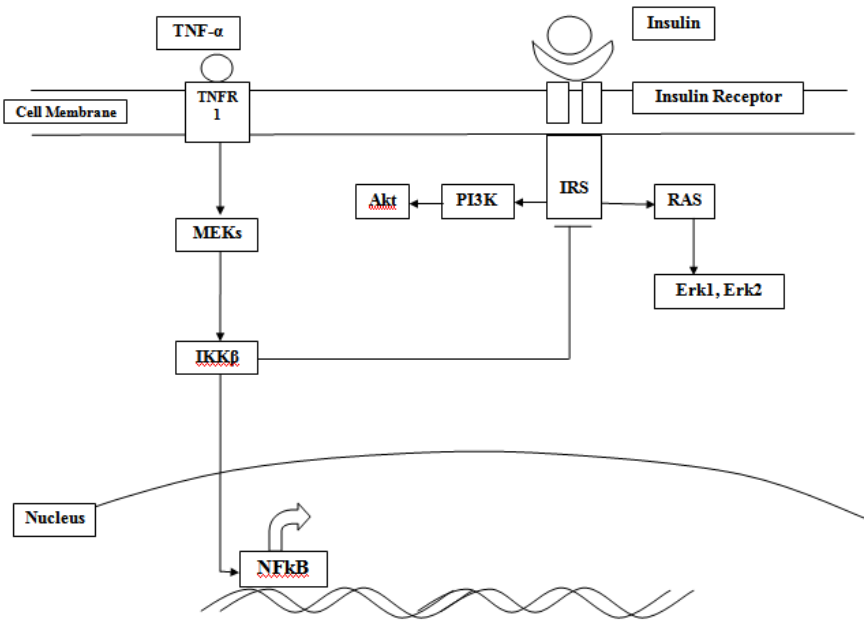

Figure 2.1: Proposed Schematic Representation Of Liver Damage With Insulin Resistance

Literature depicts the combined treatment with NF-kB and p38 MAPK inhibitors downregulated TNF-α that was involved in the release of PGE2 in murine cementoblast cell lines. Here the author linked the PGE2 induction by TNF-α with either NF-kB or p38 MAPK signalling pathway[27].

Insulin resistance which is the major contributor to T2DM causes oxidative stress and aggravated itself by inflammatory response in positive feedback loop. Liver is the major body organ that plays its role in body's detoxification and glucose homeostasis. Presence of reactive oxygen species like superoxides, peroxides, hydroxyls etc take another route to liver damage. Kupffer cells which are liver phagocytic cells are very susceptible to these ROS species and starts deteriorating the hepatocytes. Although liver is also well armed with number of antioxidants ascatalases (CAT), dismutases (SOD) and glutathione enzyme (GSH) but Parveen and colleagues indicated the decline in these anti-oxidants during hyperglycaemic conditions which further enhance the oxidative stress and ultimately oxidation-induced liver damage[28]. Novo in his work evidently showed the involvement of ROS in pro-fibrinogenic processes in hepatic myofibroblasts that leads to terminal liver conditions. He with his colleagues interpreted the therapeutic role of antioxidants to circumvent fibrosis and ROS-attenuating effect[29].

Despite of being deficient in knowledge of mutual pathways of liver damage and T2DM, literature suggests a strong correlation between two. Hence it is rational to enquire the mutual connection to elucidate the risk rate of one disease or its treatment on the other and vice versa.

CHAPTER 3: METHODOLOGY

3.1 Development of Mice Model:

3.1.1 Animal Acquisition:

For the development of mice model of diabetes mellitus and liver injury, a mice strain- Balb/C was acquired from National Institute of Health Islamabad (NIH). 20 male mice of 2-3 weeks old were purchased from the institute. Mice were weighed and parted into 4 groups in such a way that each group received mice of relative weights. Average of weights of each group as further used to calculate the dose of Diethylnitrosamine (DEN), carbon tetrachloride (CCl_4) and Strptozotocin (STZ).

3.1.2 Room Condition

Animals were kept in animal house whose temperature was maintained in the range of 25℃ ± 2℃. A 12 hours light-Dark cycle was kept in animal house. Mice were fed with regular mice diet and purified water. Animals were kept confined in well ventilated polypropylene cages with chipboard bedding. We kept changing the bedding for every week. Mice were given free access to food and water

3.1.3 Animal Grouping

As described earlier animals were divided into 4 groups. These groups named as Control, STZ, DEN+CCl_4 and DEN+CCl_4+STZ.

Groups	Group Size	Label	Description
Group 1	5	Control	Normal group
Group 2	5	STZ	Diabetic group
Group 3	5	DEN+CCl_4	Hepatocellular inflammation group
Group 4	5	DEN+CCl_4+STZ.	Diabetic & Hepatocellular inflammation group

Table 3.1: Animal Grouping Based upon Drug Induction

3.1.4 Drug Dosage

Liver Inflammation Model: To induce liver inflammation, mice of group 3 and group 4 were injected with two different chemicals. First is a carcinogenic and mutagenic Diethyl nitrosamine

(DEN) and second is Carbon tetrachloride (CCl4) in Olive Oil. Both of these are attributed to cause neutrophilic infiltration, bile duct proliferation, fibrosis and cell necrosis. Mode of administration of both chemical was intraperitoneal. DEN was given in a quantity of 30mg/kg of mice weight twice a week for continuous 7 weeks. 10% CCl_4 prepared in olive oil was given per kg of mice weight.

Group	Drug Dosage	Duration
Group 3	DEN = 30mg/kg CCl_4 in olive oil = 10%/kg	Twice A Week For 7 Weeks Once A Week For 7 Weeks
Group 4	DEN = 30mg/kg CCl_4 in olive oil =10%/kg	Twice A Week For 7 Weeks Once A Week For 7 Weeks

Table 3.2: Drug Dosage and Duration For Induction Of Liver Inflammation Model

Diabetic Model: To induce diabetes mellitus, mice were injected with streptozotocin (STZ) which is a cytotoxic for pancreatic islet insulin-producing β cells. 60mg/kg STZ dissolved in 0.1M sodium citrate buffer was given to group 2 and group 4 for 5 consecutive days on 5th week of experiment to prepare diabetic mouse model.

Group	Drug Dosage	Duration
Group 2	STZ= 60m/kg	5 consecutive days on 5th week of experiment
Group 4	STZ= 60m/kg	5 consecutive days on 5th week of experiment

Table 3.3: Drug Dosage and Duration For Induction Of Diabetic Model

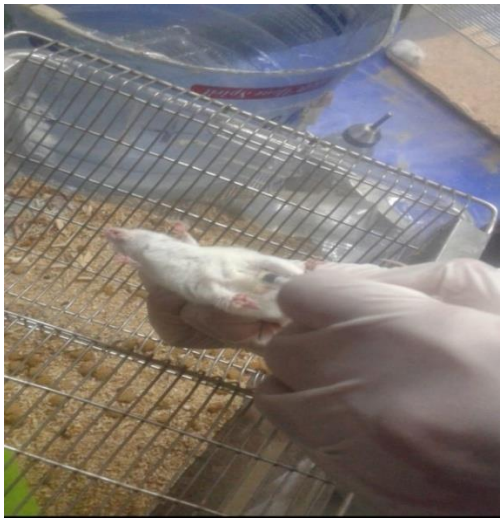

Figure 3.1: Drug Induction Through Intrapepritoneal Injection

3.1.5 Observations

During drug induction, weights of mice were recorded every week to observe the effect of drug. Calculated amount of food was given to mice each day. On every next day the quantity of leftover food gave the idea of change in appetite on the course of animal model preparation. Rate of urine recurrences and colour of urine was observed to confirm the occurrence of diabetes in mice. Fasting blood glucose level was also noted down every alternate day from a week before STZ treatment till 7^{th} day of STZ treatment. Animal was kept on fast for 4 hours. Tail was cleaned properly and pricked with lancet directly in caudal vein (tail vein). Blood was collected on test strip and immediately measured the glucose value on glucometer by "On Call EZ II".

Figure 3.2: Blood Glucose Level Measurement From Caudal Vein

3.2 Animal Sacrification

Once the protocol for model development completed, animals were prepared for sacrification for organ collection. Anesthesia was prepared by mixing 30µl Xylaz in 1ml Ketamax and making the volume upto 10 ml by adding distill water. 400 µl was injected to mouse of weight ranging between 25-30 grams. Once the animal was anesthetized, with quick hands it was pinned down on surgical platform. A long abdominal incision up till throat was given and immediately blood collected through heart puncture when the mouse was still breathing. Blood collection resulted in mouse death. After mouse death required organs as liver and pancreas separated from mouse.

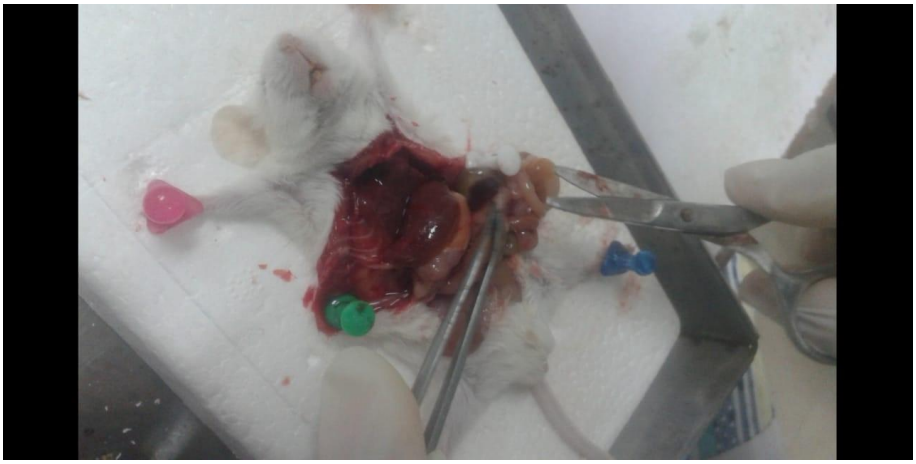

Figure 3.3: Surgical Procedure For Tissue Collection

3.3 Tissue Preservation

Blood collected from heart centrifuged at 5000 rpm for 10 minutes at 4 °C to collect the serum. Serum was stored at -80°C in cryofreezer. Organ separated from mouse body was washed with ice cold phosphate buffer saline (PBS) and weighted while kept on ice. A fraction of tissue snap freeze and stored at -80°C for biochemical assays. While remaining part was stored in Neutral Buffer Formalin (NBF) at room temperature for histological analysis.

Figure 3.4: (a) Liver

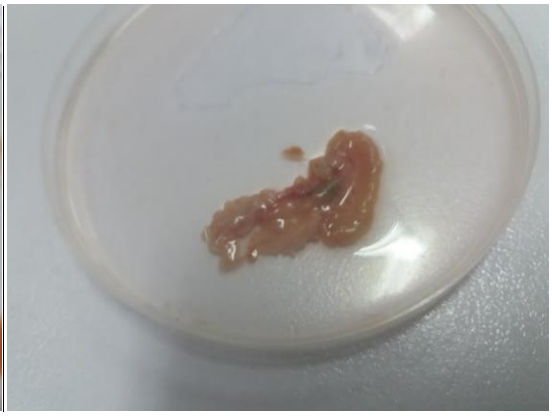

Figure 3.4: (b) Pancreas

3.4 Tissue Lysate Preparation

0.2 grams of tissue was weighed and dissolved in 1ml ice cold tris buffer while kept on ice. Dissolution involves continuous pipetting for complete cellular rupture and sonication for obtaining a fine homogenate. This homogenate then centrifuged at 10,000 rpm for 15 minutes at 4 °C. Supernatant contained cell lysate separated, aliquoted and stored at -80°C.

3.5 Antioxidant Assays

Antioxidant assays were performed to evaluate the antioxidant status of liver homogenate and to access the antioxidant comeback against the free radicals produced during liver injury and diabetes.

3.5.1 Antilipid Peroxidase Assay

Principle: Malondialdehyde (MDA) is a compound that releases when polyunsaturated fats in cellular membranes are get oxidized by the attack of reactive oxidation species. This MDA on exposure to thiobarbituric acid (TBA) produces a pink coloured compound which gives characteristic absorbance at 535 nm.

Procedure: 250µl tissue lysate mixed with 250µl of trichloroacetic acid (TCA) and centrifuged at room temperature for 10 minutes at 3000 rpm. Add 250µl 0.67% thiobarbituric acid and kept the mixture on boiling water for 15 minutes. Optical density of pink coloured mixture was measured at 535nm. Concentration was measured by using molar extinction coefficient of 0.641×10^{-5}.

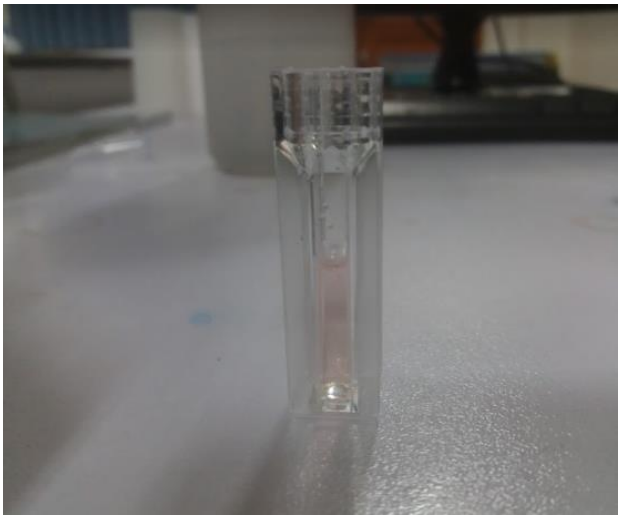

Figure 3.5: Antilipid Peroxidase Assay

3.5.2 Superoxide Dismutase Assay

Principle: Superoxide Dismutase (SOD) is an enzyme that dismutates the toxic superoxides into low risk oxides and peroxides radicals. Level of SOD is measured by calculating the extent with which enzymes inhibits superoxides produced by pyrogallol.

Procedure: Tris-EDTA buffer solution was prepared by adding 50Mm Tris into 1mM EDTA and maintaining the pH at 8.5. 0.1 ml of this solution was dissolved with 0.1 ml of 20mM of pyrogallol and 0.1 ml of tissue sample. Blank was prepared by replacing 0.1 ml of tissue sample with double distilled water. Solutions were incubated for 15-20 minutes and checked their optical density at 420nm. Percentage inhibition of pyrogallol was calculated by using following relation:

$$\% \text{ Inhibition of Pyrogallol} = \frac{\text{OD control} - \text{OD test}}{\text{OD control}} \times 100$$

SOD activity was calculated through following equation.

$$\text{SOD activity} = \frac{\% \text{ Inhibition of Pyrogallol}}{50}$$

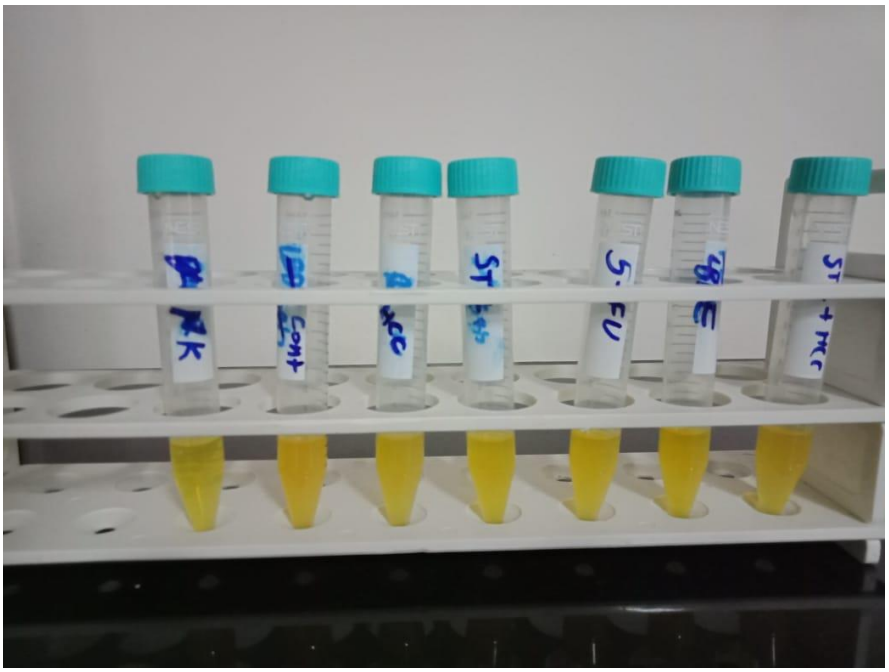

Figure 3.6: Superoxide Dismutase Assay

3.6 Serum Analysis

Serum collected from mouse blood is sent to Atta-ur-Rahman School of Biological Sciences Diagnostic Lab for serum pathology examination. Following liver function tests were performed for a complete picture of liver pathology.

3.6.1 Liver Function Tests

Alanine aminotransferase (ALT)

Alkaline Phosphatase (ALP)

Total Billirubin

3.7 Histopathology Analysis

For histopathology analysis, tissue samples stored in NBF were sent to Islamabad Diagnostic Center, Islamabad.

3.8 Western Blotting

To spot the presence of proteins of interest western blot analysis was carried out. Mini Trans-Blot cell from BIO-RAD was used. Bradford assay was performed to quantify the proteins in tissue lysate. Absorbance values were used to calculate the concentration of protein in sample and regression line equation was obtained to reckon the loading sample size.

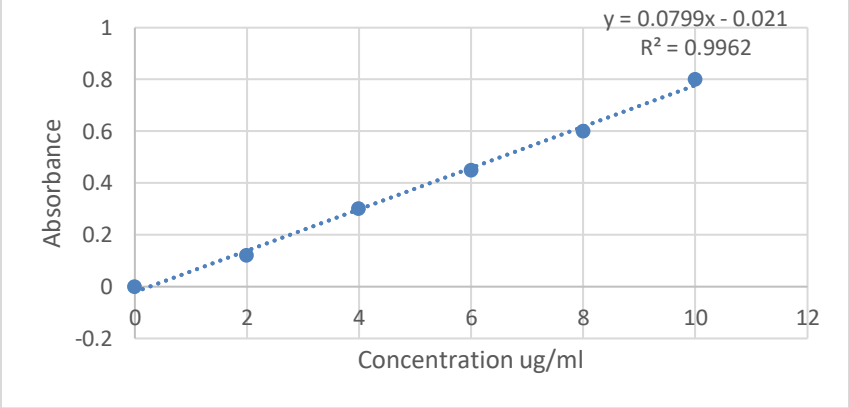

Figure 3.7: Regression Line To Calculate Loading Sample

3.9 Protein Preparation and Loading

Loading sample was prepared by mixing tissue sample with 4X buffer and water in the following ratio.

	abs	2ug	1ug	20	H_2O	4X	Total
Control	0.29	3.8	1.9	10.5	7.5	6	24
DEN+CCl_4	0.19	2.6	1.3	15.3	2.7	6	24

STZ	0.22	3.02	1.51	13.3	4.7	6	24
DEN+CCl_4+STZ	0.39	5.14	2.57	8	10	6	24

Table 3.4: Loading Sample for Western Blot

After mixing sample was centrifuged and boiled for 5 minutes. Sample was again centrifuged and stored at 4 until loading time.

3.10 SDS PAGE Electrophoresis

3.10.1 Gel Preparation and Running

The constituency of separating gel was optimized to use at 10%. Gel was prepared just before the time of pouring into cassettes. After polymerization of the separating gel, stacking gel of 5% constituency was made and poured immediately over separating gel along with the placement of comb for wells formation. After the formation of both gels, both gels were fixed in blotting apparatus and filled with 1X running buffer. The prepared protein material was loaded in the 1X buffer filled wells along with protein marker from Santa Cruz and 4X buffer with following sequence.

4X 24ul	Protein Marker 24ul	Control 24ul	STZ 24ul	DEN+CCl_4 24ul	DEN+CCl_4+STZ 24ul	4X 24ul	4X 24ul	4X 24ul

The voltages were set to 90V and allowed the gel to run for a sufficient time of 2-2.5 hrs. Once the indicating dye line reached the bottom of the gel, cassettes were removed from running apparatus and prepared for transfer.

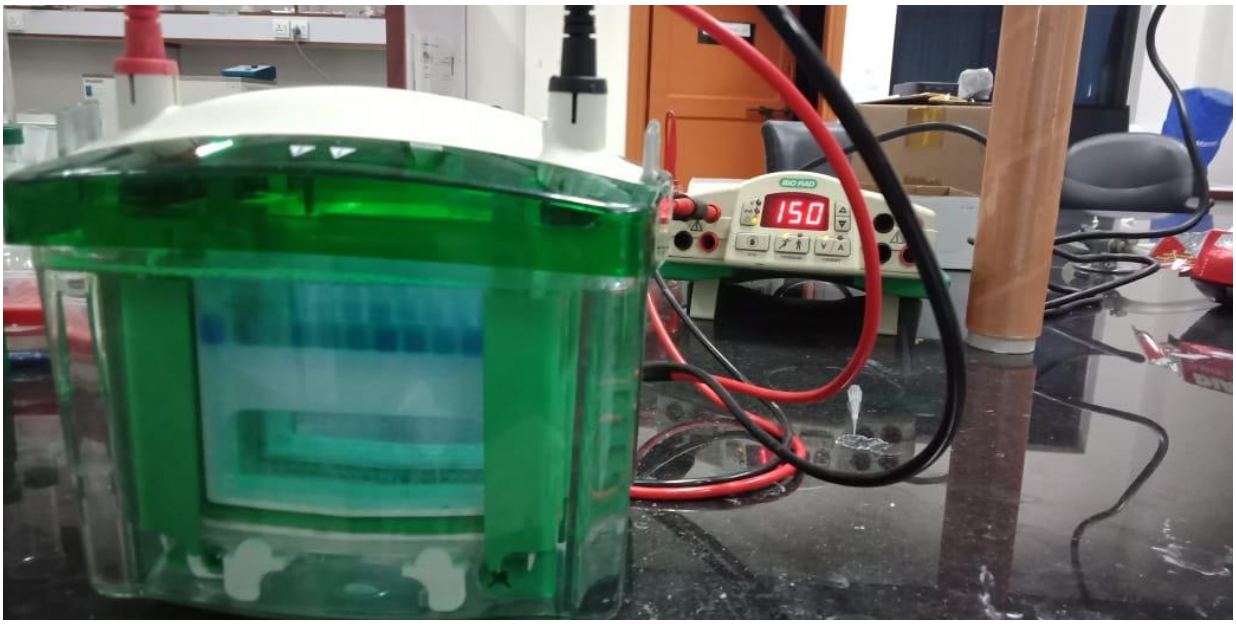

Figure 3.8: Western Blot Apparatus Separating Proteins Loaded on Gel

3.10.2 Transfer to NC Membrane

After separating proteins on the basis of molecular weight, transfer on Nitrocellulose membrane (NC membrane) was carried out. For this transfer, given below sequence was followed.

Sponge	Filter paper	Gel	NC membrane	Filter paper	Sponge

This setup then shifted to transfer apparatus filled with transfer buffer. Transferring was performed at 90 volts for 50 minutes for 2 gels while switching their positions after 25 minutes.

3.10.3 Ponceau Staining

After transfer, NC membrane was put in 1X ponceau stain to make the protein bands visible. This process helped in marking the position of protein markers and their respective molecular weight protein of interest. Positions were properly marked and NC membrane was cut into strips before moving to the next step.

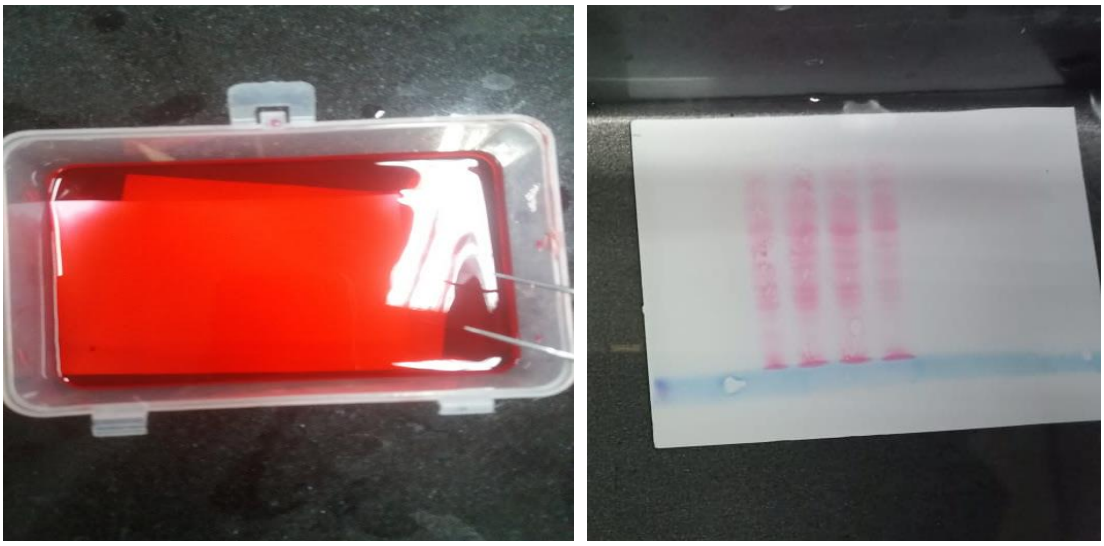

Figure 3.9: Ponceau Staining

3.10.4 Blocking of Proteins on NC Membrane

In this step, the stripped NC membranes were added into blocking agent which was the 5% non-fat milk prepared in 1X tris buffered saline (TBS). The membranes were then kept at 4 °C for 2 hours on continuous shaking. This blocking is done to avoid non-specific binding of antibodies with proteins on NC membrane which can cause false results in background.

3.10.5 Primary Antibody Incubation:

After 2 hours of blocking, strips were removed from blocking agent and placed in primary antibody solution for antibody incubation. Primary antibodies of NF-κB, IκB, COX-2, Akt and actin from rabbit source were used and diluted in 1X TBS in the ratio of 1:1000. Strips were left afloat in solution at 4°C overnight on continuous shaking. After 14-16 hours of primary antibody incubation, strips were washed with solution of tris buffer saline and tween-20 (TBST) for 40 minutes in time interval of 5, 5, 10, 10 and 10 minutes.

3.10.6 Secondary Antibody Incubation

Anti-rabbit secondary antibodies were used to probe the primary antibodies. Secondary antibodies were diluted in the ratio of 1:2000 with TBS buffer, added to wash strips and kept for 2 hours at 4°C on constant shaking. On completion of secondary antibody incubation, strips were

again washed with TBST for 40 minutes for 5, 5, 10, 10 and 10 minutes before heading for blot detection.

3.10.7 Signal Detection using X-ray development:

Blot was detected by enhanced chemiluminescence method. Western blot luminol reagents from Santa Cruz were used in equal ratio. Washed strips dried on paper towel and placed on clean plastic sheet. Western blot luminol reagents mixed in equal ratio poured on strips and wait for 5 minutes until it is absorbed by strips. Strips then fixed into X-Ray cassette and covered by a transparent sheet. X-Ray sheets placed over transparent sheet in dark and closed the cassette. After 30-40 minutes of ECL exposure, X-Ray sheets were develope by using developer and fixer until obtained required results.

Figure 3.10: Setting NC Membrane in X-Ray Cassette

Chapter 4: Results

4.1 Histopathological Analysis of Liver Tissue

Histological examination of liver section of a control mouse reveals normal sinusoidal pattern and regular hepatic parenchyma. Intact cytoplasm with a distinguished nucleus, clear central vein and a compact arrangement of hepatocytes with no fatty lobules characterize the hisotology of liver of control mouse (1a). In contrast mice liver treated with DEN+$CCl_4$are showing cellular necrosis and cell dysplasia. Inflammatory cells also seen dispersed in sinusoids but mostly in central region (1b). Scanty portal tract inflammation and meek degeneration of cellular structure is seen in STZ only treated mice (1c). However the liver section of mice treated with DEN+CCl_4+STZ to induce both liver injury and diabetes exhibits high grade dysplasia with high nucleus/cytoplasm ratio, hyperchromatic nuclei and eosinophillic cytoplasm and confirms the hypothesis that presence of diabetes can ruin extensively liver diseases with acceleration.

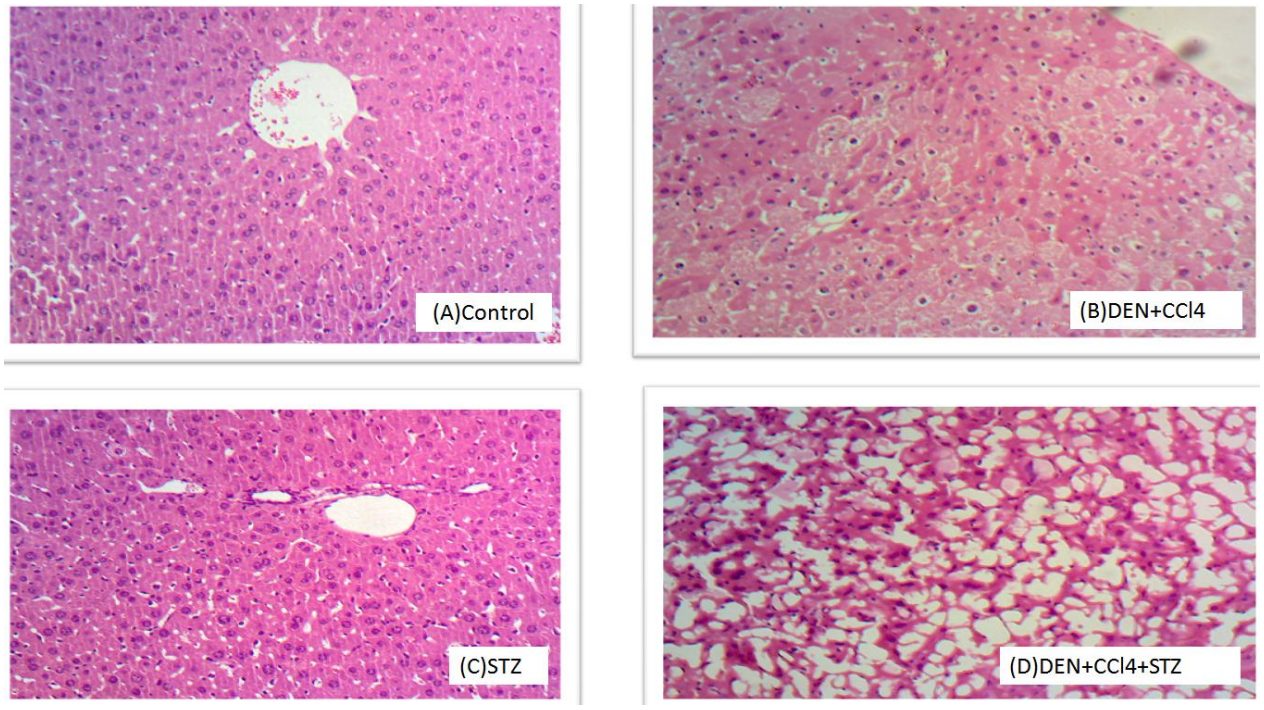

Figure 4.1:Histopathological Analysis of Liver Tissue(a) Liver section of a normal control mouse showing normal architecture (b) Liver section of a DEN+CCl_4 treated mouse showing a massive presence of inflammatory cells and cellular necrosis(c) Liver section of a mouse treated with STZ showing mild necrosis and mild presence of inflammatory cells (d) Liver section of a DEN+CCl_4+STZ treated mouse showing extensively degenerated tissue with high grade dysplasia. **(Magnification is 40X)**

4.2 Histopathological Analysis of Pancreatic Tissue

Control group is showing normal pancreatic structure. The exocrines are closely packed and islets are seen interspersed and appearing lightly stained as compared to surrounding cells (2a). The architecture of pancreatic tissues of DEN+CCl_4treated mice is very close to control group with normal structure of Islets of Langerhans and acinar cells (2b). Histology of diabetic rats treated with STZ is representing severe cellular atrophy and entire loss of Islet β-cells (2c). In DEN+CCl_4+STZ treated mice the histology of pancreatic cell is worst than only STZ treated mice. Extensive pathological changes can be seen in exocrine and endocrine parts. Acinar cells and β-cells are seen distorted due to presence of vacuoles.

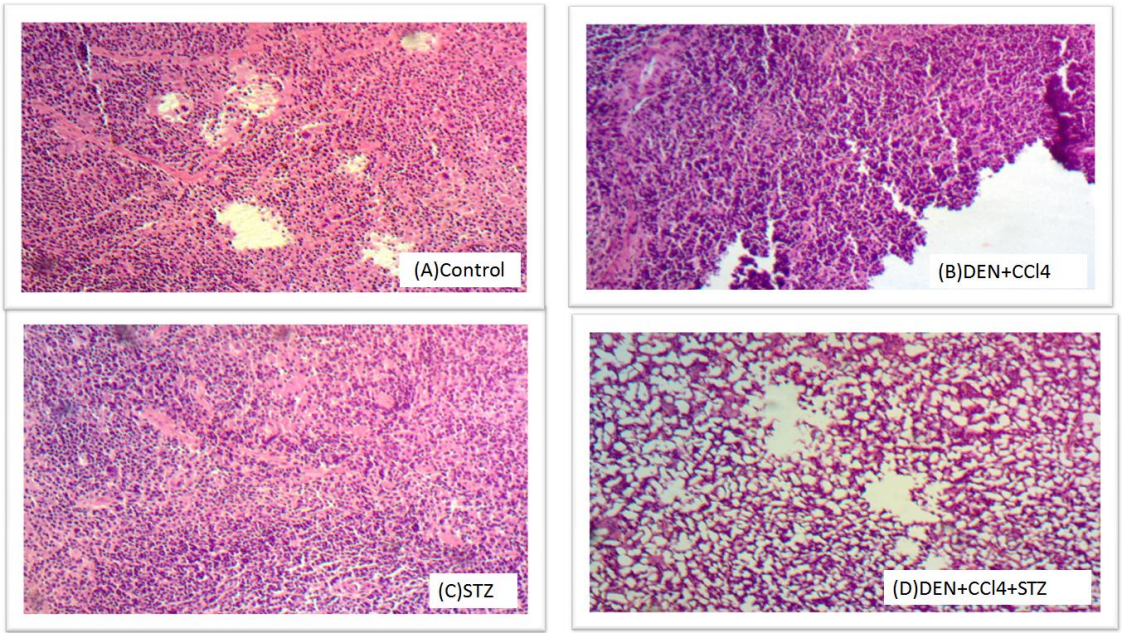

Figure 4.2: Histopathological Analysis of Pancreatic Tissue (a) Pancreas of a normal control mouse showing normal architecture (b) Pancreas of a DEN+CCl_4 treated mouse showing a massive presence of inflammatory cells and cellular necrosis(c) Pancreas of a mouse treated with STZ showing mild necrosis and mild presence of inflammatory cells (d)Pancreas of a DEN+CCl_4+STZ treated mouse showing extensively degenerated tissue with high grade dysplasia.(Magnification is 40X)

4.3 Body Weight Statistics

Induction of DEN+CCl_4 and STZ has adversely affected the body weights of mice as shown in figure 4.3. There is a regular increasing trend in body weights of mice in control group upto the 7^{th} week of experiment as they were given proper diet and environment (blue line). Body weights of DEN+CCl_4 treated mice showed a slight up and down in body weights but weights noted were very low as compared to control group's weights (red line). STZ treatment was started in 5^{th} week of experiment. So uptill 5^{th} week we can see a continuous increase in weights compared to control group. But then there is a sudden fall after induction of diabetes due to STZ toxicity (green line) and indicates significant body weight reduction ($P<0.05$). Peculiarly DEN+CCl_4+STZ (purple line) group did not show a sudden drop which can be reckoned as body is already under stress and full of toxicity due to DEN+CCl_4 treatment hence there was not significant body weight reduction in DEN+CCl_4+STZ group .

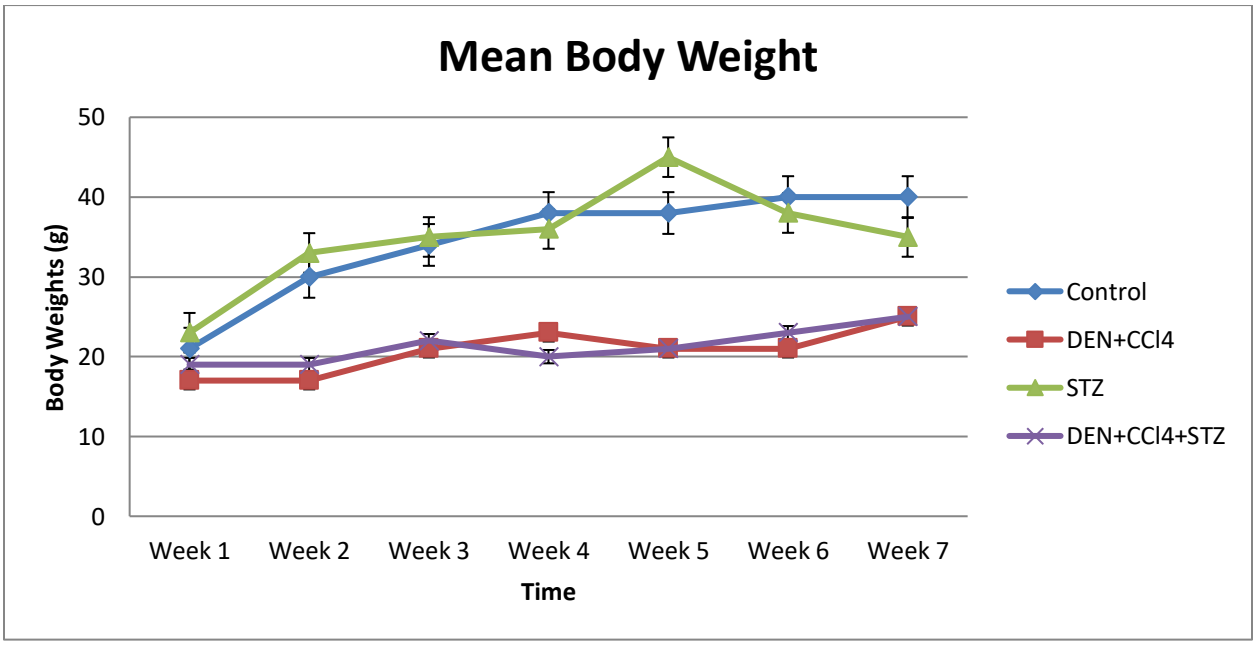

Figure 4.3: Mean Body Weights Effect of diabetes and liver inflammation on mice body weights. Data is represented as mean±1 SE

4.4 Blood Glucose Level

Before STZ treatment blood glucose level (BGL) of all groups lied within normal range. We can observe a significant rise in BGL of mice induced with DEN+CCl_4+STZ and STZ only at day 1, day 3 and day 5 after treating with STZ. Rise in BGL in DEN+CCl_4+STZ group is more significant than STZ because in former not only β-cells had destroyed but also liver is not stabilizing the conditions after being injured.

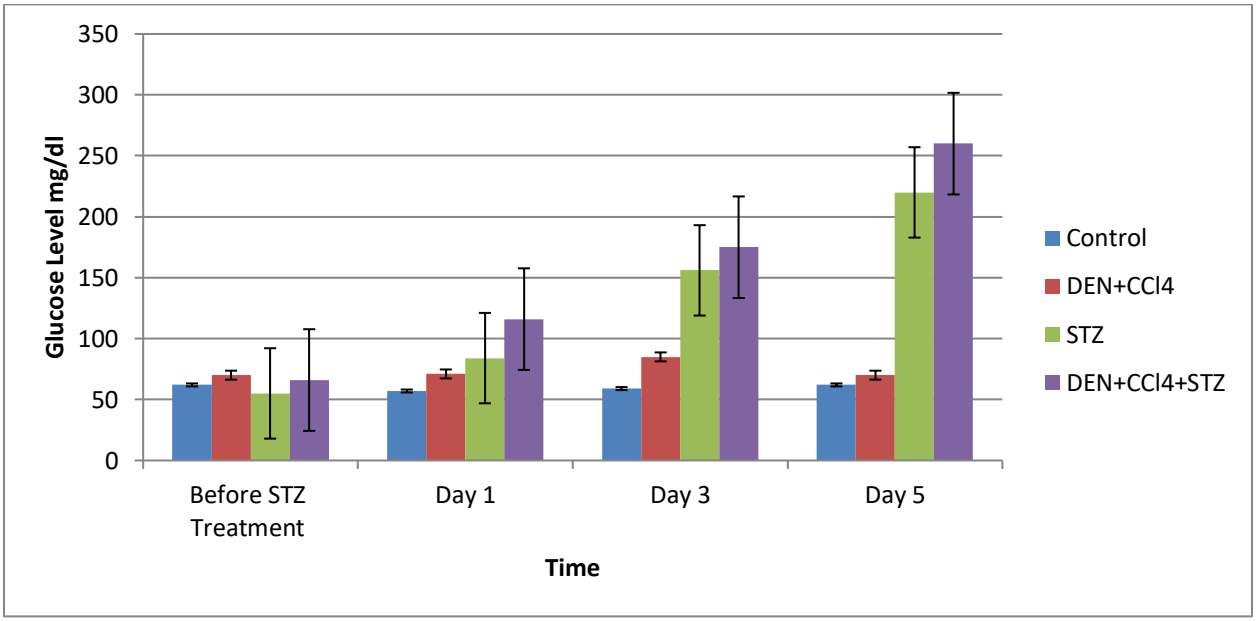

Figure 4.4: Blood Glucose Level Variance in blood glucose level in mice model of diabetes and liver inflammation. Data is represented as mean±1 SE

4.5 Liver Function Serum Markers

As shown in figure 4.5, treatment with DEN+$CCl_4$and STZ resulted in striking elevation of liver function serum marker.

4.5.1 Alanine Transaminase (ALT)

Alanine transaminase (ALT) is an enzyme releases from liver in small amount and present in body even in normal physiological conditions. In the consequence of liver injury, level of ALT increases in blood which is a biomarker for liver diseases. As shown in the figure 4.5, ALT level is less than 50 international unit per liter (IU/L) in control group. ALT level is comparatively higher in DEN+CCl_4treated group than control and is almost equal to control in STZ treated

group. Due to hepato-toxicity ALT level is observed significantly highest ($p<0.05$) in DEN+CCl_4+STZ treated group among all other experimental groups.

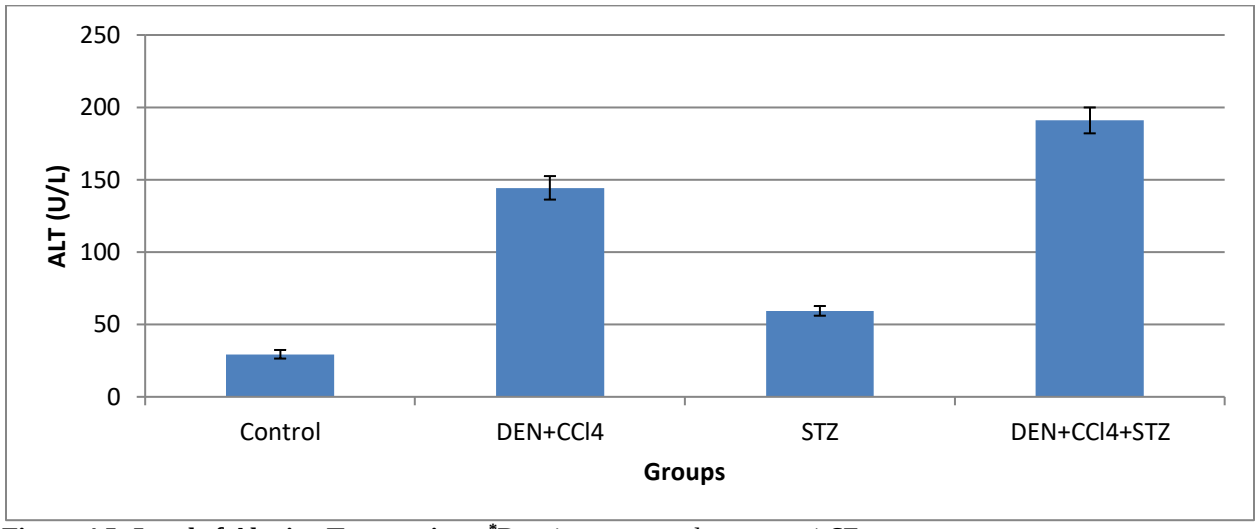

Figure 4.5: Level of Alanine Transaminase[*]Data is represented as mean±1 SE

4.5.2 Alkaline Phosphatase (ALP)

Like ALT, ALP is also an enzyme releases from bile duct lining of liver and functions in lipid transposition in small intestine. During infiltrative diseases, level of ALP increases in blood serum. Figure 4.6 well describes the ALP level in all experimental groups. Both groups induced with DEN+CCl_4 and DEN+CCl_4+STZ exhibited significantly high levels of ALP. Slightly high level of ALP in STZ treated group indicates mild liver injury in diabetic group.

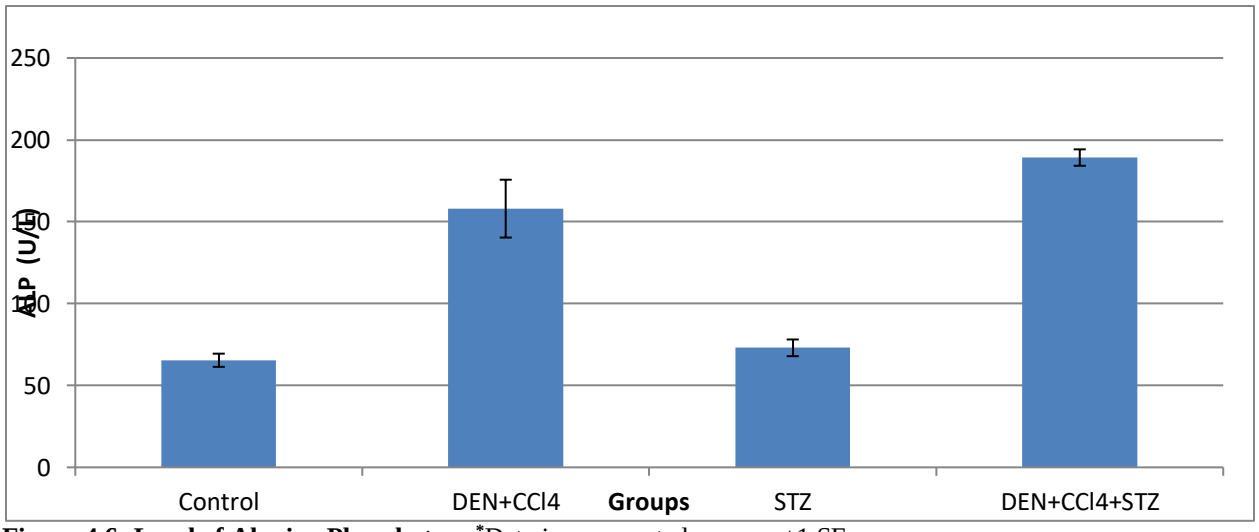

Figure 4.6: Level of Alanine Phosphatase [*]Data is represented as mean±1 SE

4.5.3 Bilirubin

A pigmented compound released by breakdown of red blood cells, transported to liver and stored in gall bladder called bilirubin which is often used as biomarker of liver health. Both groups induced with DEN+CCl_4 and DEN+CCl_4+STZ exhibited significantly high levels of bilirubin as shown in figure 4.7. Slightly high level of bilirubin in STZ treated group than control group indicates mild liver injury also in diabetic group.

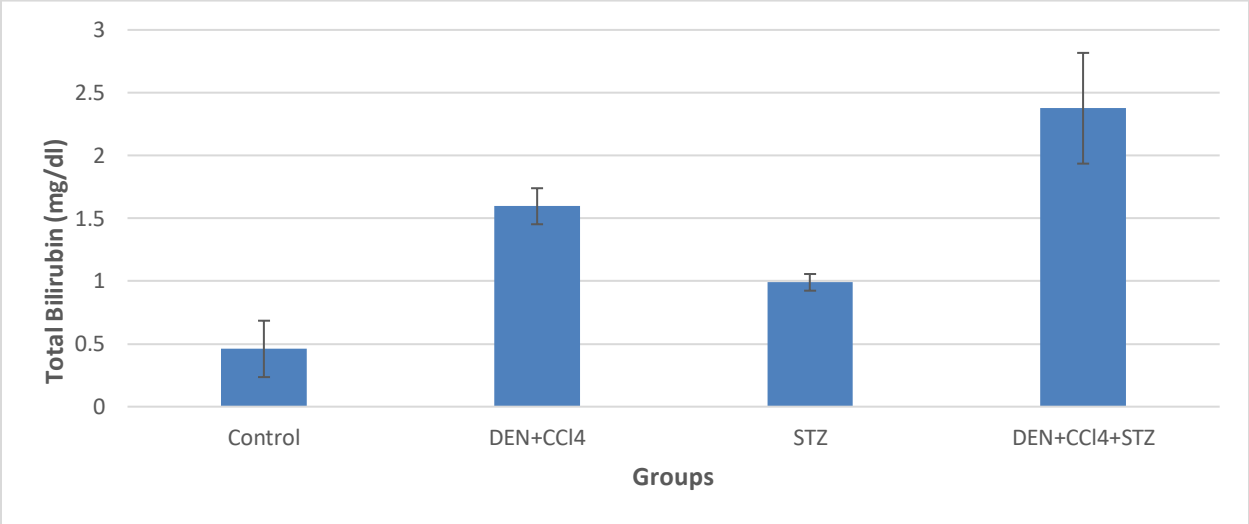

Figure 4.7: Level of Total Bilirubin *Data is represented as mean±1 SE

Oxidative Stress

Level of MDA: Cellular stress was found to be increased in DEN+$CCl_4$and STZ group than in control group. In DEN+CCl_4+STZ level was significantly highest amongst all due to excess damage as shown in figure 4.8.

Superoxide Dismutase level: Level of SOD in liver homogenate of DEN+$CCl_4$and STZ group was seemed to be significantly decreased than from control. DEN+CCl_4+STZ group showed amplified hepatotoxicity as compared to all other groups as shown in figure 4.9.

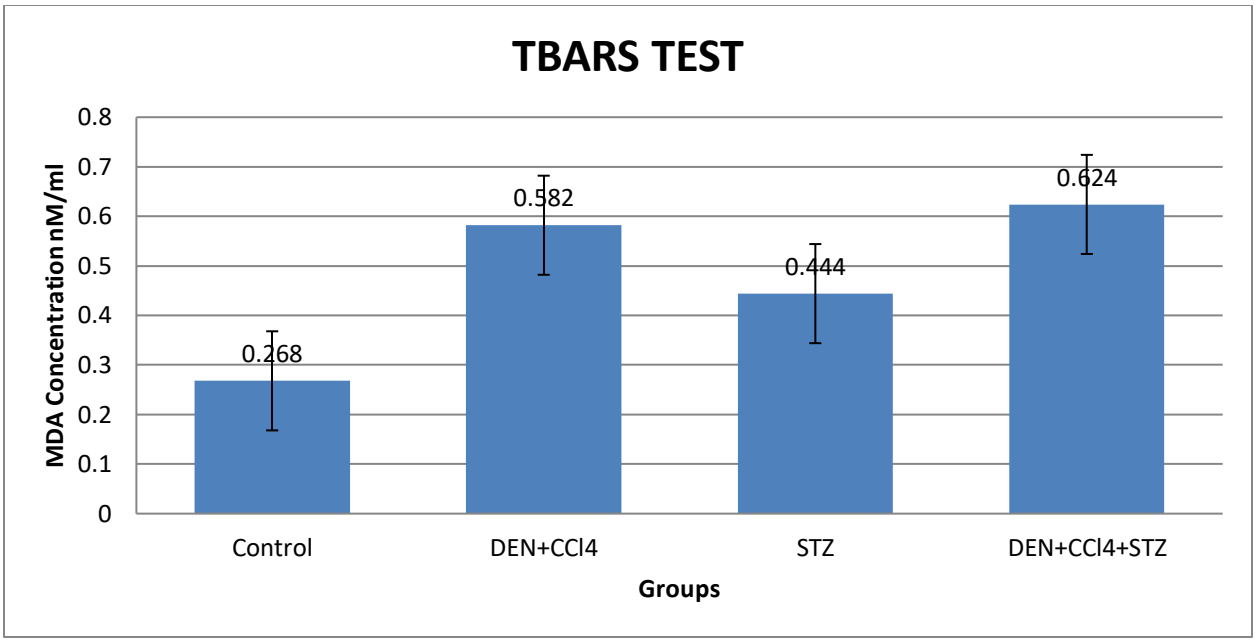

Figure 4.8: Level of Malondialdehyde (MDA)/ Anti-lipid Peroxidation

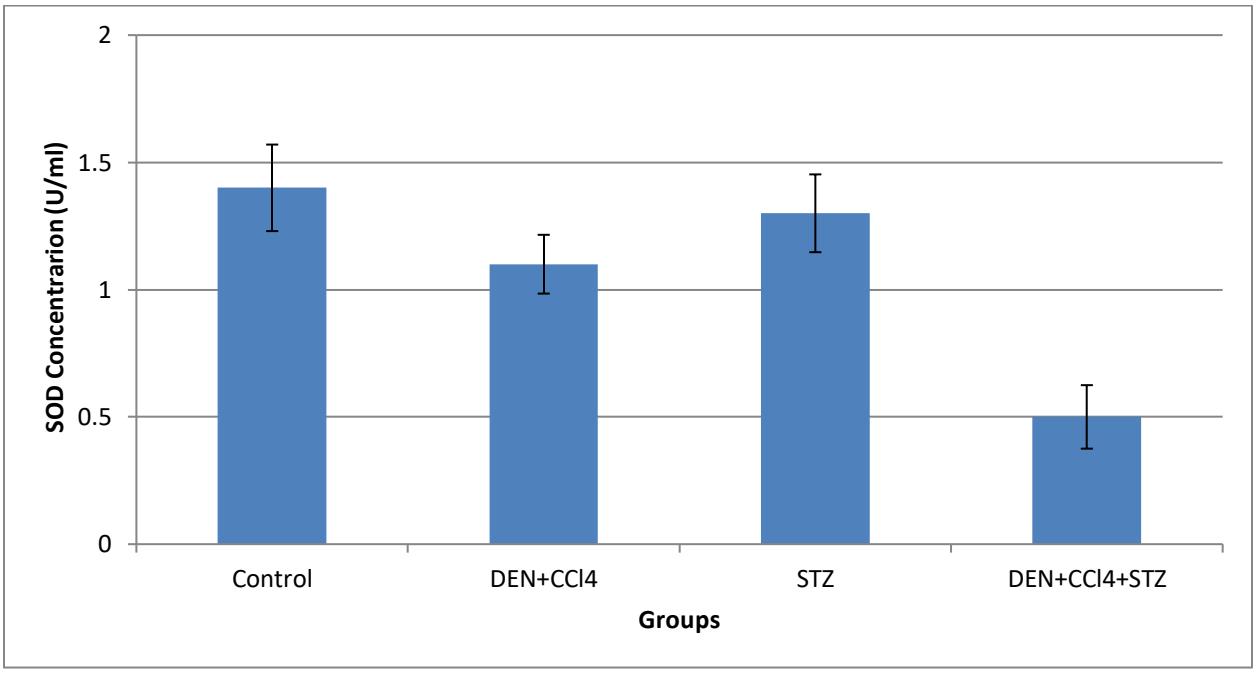

Figure 4.9: Level of Superoxide Dismutase

Western Blotting

Beta-Actin protein (A house keeping protein) was used as positive control to ensure the loading level of other protein would be same throughout in gel. NFκB is a pro-inflammatory protein whose level must be high in injured tissues. That was found true by observing the western blot results. In control group expression of NFκB was appeared light that indicated absence of inflammation. Conversely in STZ and DEN+CCl_4groupsdarker bands interpreted the presence of inflammation in cells. In DEN+CCl_4+STZ these bands were darker than all other groups because of both diabetic and hepatic injury stimulus. IκB is inhibitor of NFκB and expression was appeared reversed on NC membrane. Cyclooxygenase-2 (COX-2) formerly called prostaglandin G/H synthase is also an indication of inflammation. Expression of COX-2 appeared darkest in DEN+CCl_4+STZ group signaled the enormous level of inflammation while the expression was comparatively reduced in other groups that were meant relatively less inflammation. Protein kinsae B (Akt) ia usually induced by high glucose level and positively regulated by NFκB was appeared to be elevated in DEN+CCl_4+STZ group than DEN+$CCl_4$and STZ groups.

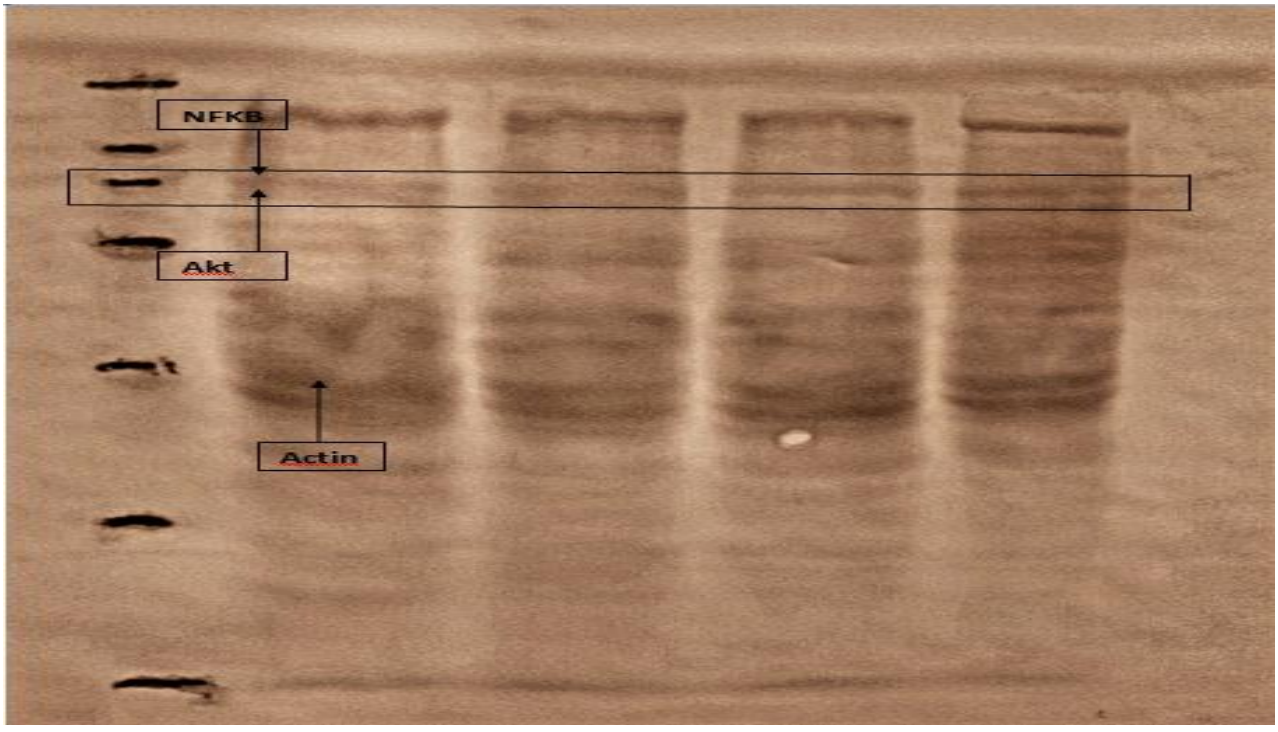

Figure 4.10: Western Blot NC Membrane Analysis.

CHAPTER 5: DISCUSSION& CONCLUSION

The present study was conducted to identify the link between hepatocellular inflammation and diabetes and potential mutual predictors of both diseases. By comparing both diseases through various biochemical assays, serum analysis and western blot analysis; our proposed hypothesis is properly substantiated. Several meta-analysis and cohort studies available that point towards the link between two diseases but there are no in-vivo one to associate both conditions with one another at molecular level. In the above study, three animal models were prepared of diabetes, hepatocellular inflammation and diabetes+hepatic inflammation to evaluate the factors that affect the person victim of both diseases. First of all histopathology of liver and pancreas confirms the relation of both conditions as the state of liver and pancreas was worst in liver of mice induced with both disease. Secondly, the changes in weights of mice and blood glucose level were seen higher in mice with diabetes+hepatic inflammation. It can be hypothesized that factors of liver function tests; ALT, ALP, Bilirubin must be higher in mice induced with liver injury (Group 3&6) but interestingly these enzymes were also found slightly higher in diabetic group that second our hypothesis that there must be a relation in the above mentioned diseases.

The relation between the two was also confirmed by the signaling pathways activated by both of the diseases. Insulin plays an important role in activation of a very well documented signaling pathway; PI3K/Akt pathway. After insulin binding, Akt is phosphorylated downstream and regulates the synthesis of glycogen by inhibiting GSK3β (Glycogen Synthase Kinase-3β) thus plays an important role in regulating diabetes. Treatment with STZ destroyed β pancreatic cells, decreased the normal insulin circulating level and induced hyperglycemia hence set up stage for DM type 2. This interfered with IRS (Insulin Receptor Substrate) function and also downstream signaling by reducing the Akt level in diabetic mice. In diabetic+hepatic injured mice, both insulin deficiency and ROS played a crucial role in decelerating the level of Akt. Decreased Akt level is not available now to reduce the oxidation by ROS (through HIF-1α)[30]and injured liver further deteriorate towards liver fibrosis, cirrhosis and ultimately cancer.

Histopathological samples of liver revealed the presence of immense inflammation and macrophage infiltration. Presence of inflammation is key indicator of activation of NFκB. NFκB as a master regulator of inflammation and present in cytosol in inactivated form. This

inactivation is by masking of nuclear localization segment (NLS) by IκB. After the treatement with STZ in diabetic mice and DEN+CCl_4 in hepatic inflammation mice, this IκB was phosphorylated by IKKβ and release NFκB. IKKβ helped release in NFκB that is why NFκB was found in high level in diabetic+hepatic injured mice hence it is I concurrence with previous studies in which Kpuffer cells activated in steatosis induced inflammation and insulin resistance [31]. On the other hand IKKβ also involved with the inactivation of IRS and downstream components of PI3k/Akt[32]. This caused the insulin resistance in hepatic cells. That is how liver inflammation played a role in initiation of T2DM. And controlling the hepatic insult can be a potential therapeutic target for enhancing insulin sensitivity[33].

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