

A Comprehensive Study of Chronic Diabetes Complications in Streptozotocin-Induced Diabetic Rat

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Abstract

Background: The purpose of this study was to provide a reference of chronic diabetes complications by investigating the prolonged hyperglycemia effects on hematological, biochemical and histopathological changes (liver, kidney, spleen, cardiac muscle, adrenal gland, and endocrine pancreas) in diabetic rats induced by streptozotocin. **Methods:** Ten adult female Sprague-Dawley of uniform age were divided into two Groups. Group 1 was made diabetic by single intraperitoneal injection of streptozotocin (60 mg/kg/bw) whereas Group 2 served as control. After six months, the rats were anesthetized using pentobarbital. Cardiac puncture was performed to get 3 ml of the blood sample; following 12 hours of an overnight fast. Serum chemistry test and complete blood analysis for lipid profile and blood glucose test; liver and renal functions were performed. Tissue specimens of liver, kidney, spleen, cardiac muscle, adrenal gland, and endocrine pancreas were fixed in 10% formal saline and processed for histological study. **Results:** There were severe histopathological changes in the affected organs; and the presence of a significant abnormality of lipid profile, liver, and renal functions. **Conclusions:** The presence of histopathological changes with abnormal biochemical changes is related to the chronic absence of insulin production in the destroyed β -cells which reflect the diabetic complications in a human being.

Keywords: biochemical, histopathological, rats, streptozotocin

Introduction

Diabetes mellitus (DM) is the commonest metabolic diseases characterized by hyperglycemia resulting from deficiencies in insulin secretion or action or both; that affects carbohydrate, lipid, protein and nucleic acid metabolism. According to the international diabetes federation (IDF), more than 382 million adults throughout the world suffered from diabetes, and 5.1 million deaths occur yearly due to diabetes.¹ The frequency of diabetes has doubled in the last three decades, and it is predicted to continue rising to 592 million cases by 2035.¹ Experimental models of diabetes have been developed by using drugs such as Streptozotocin (STZ) to induce diabetes. These drugs inflate and ultimately destruct the β -cells of the pancreatic islets. STZ is an antimicrobial agent; initially developed as an antibiotic and antitumor agent.² It is now widely used to induce diabetes mellitus in many animal species resembling many features seen in human patients' condition including clinical, physiological and pathological features. STZ is the first choice for

diabetes induction in animals, mild to severe diabetes are generated depending on the animal strain, dose, period and route of drug administration.³ A single intraperitoneal injection of STZ (150 mg/Kg body weight) or multiple intraperitoneal injections of low dose (40 mg/Kg body weight) in mice produced a significant hyperglycemia similar to a type 1 diabetic. Mild diabetes, characterized by low glycemic intensity, was induced in rats received 100mg/kg STZ injection subcutaneously.^{4,5} Mild diabetes was also observed in rats injected intraperitoneally with a single dose of 100mg/kg of STZ.^{6,7} It was found that a single i.p. Injection of 100 mg/kg STZ in mice produced non-insulin-dependent DM characterized by impaired insulin response to glucose stimulation while 200 mg/kg STZ-induced insulin-dependent DM.⁸ The purpose of this study was to provide a reference of chronic diabetes complications by investigating the prolonged complications of untreated diabetes on hematological, biochemical and histopathological changes (liver, kidney, spleen, cardiac muscle, adrenal gland, and endocrine pancreas) in untreated diabetic rats after six months of treatment with STZ.

Methods

Animals and experimental design. Ten adult female Sprague-Dawley rats (weighing 200-225 g, 12 weeks old) were used. All experimental rats were kept in cages under the standard laboratory conditions (adequate cross ventilation; temperature: 22 ± 1 °C; 12:12 hrs light: dark cycle; relative humidity: 60-70%) and were allowed one week period to acclimatize before the experiment. The rats were maintained on standard commercial dry pellet diet and water ad libitum. The rats were randomly divided into two groups of 5 animals, control group and STZ treated group. STZ (Merck) was dissolved in 0.1M citrate buffer PH 4.5 immediately before i.p. Injection. Following an overnight fast, diabetes was induced under light anesthesia (Sodium pentobarbital) by a single intraperitoneal injection of 60 mg/kg of STZ. Rats were supplied with 5% glucose solution for 24 hrs. immediately after STZ injection to prevent the severe acute hypoglycemic effect. The rats of the control group received an equivalent volume of i.p injection of Citrate Buffer only. Three days post induction, fasting blood glucose level was measured, only rats with fasting blood sugar (FBS) level ≥ 15 mmol/L were considered as diabetic rats.

Collection of blood samples and biochemical assays. All rats were anesthetized with pentobarbital (90 mg/kg). The blood samples (3 ml) of each animal were withdrawn by cardiac puncture; following an overnight fast for 12 hrs. All blood samples preserved with suitable blood tube with specific agents. Serum chemistry test and complete blood analysis were performed in Biochemistry and Hematology Unit (Faculty of Medicine, International Islamic University Malaysia).

Histological studies. After six months, fasting blood glucose level was measured, animals from control and treated groups (STZ) were sacrificed by using mild anesthesia (Sodium pentobarbital 90 mg/kg); dissected and small pieces of different organ (Pancreas, liver, kidney, spleen, cardiac muscle and adrenal gland) were quickly removed, fixed in 10% formal saline for 72 hrs., dehydrated through graded alcohols and processed for light microscopy. Sections of (4-5 μ thickness) were stained with Ehrlich Haematoxylin and Eosin.

Statistical analysis. Data analyses were performed using statistic software IBM SPSS Statistics 20 (IBM Corporation, NY, USA). All data in this study are presented as mean \pm SEM. Data were analyzed by Mann-Whitney U test, the value of $p < 0.05$ was considered significant.

All experimental protocols involving animals and their care were approved by the institutional animal care and used committee of International Islamic University

Malaysia (Ref. no. 2015-6-34-IACUC-IIUM/) and the experimental protocols followed the "Guide for the care and use of laboratory animals".⁹

Results

Liver function tests. The changes in the liver function profile of diabetic and Non-diabetic rat are illustrated in Table 1. The result shows a significant reduction in total protein and albumin level in the serum of diabetic rats. The serum level of ALP, ALT, AST, as well as total bilirubin, was significantly higher in the diabetic rats group compared with control group.

Lipid profile and blood glucose test. Table 2 showed the lipid profile and blood glucose of diabetic and Non-diabetic rat. Lipid profile of diabetic rats was characterized by an extremely significant elevation ($p < 0.01$) in the levels of low-density lipoprotein (LDL) and triglyceride (TG), compared with the normal animals. The high-density lipoprotein (HDL) level is slightly decreased in the diabetic group, but it was not statistically significant ($p > 0.05$). Fasting glucose sugar (FBS) level increased significantly in the diabetic group ($p < 0.01$), compared with control group.

Kidney function tests. The renal function parameters of diabetic and Non-diabetic rat are illustrated in Table 3. Renal profile of diabetic rats was characterized by a significant decrease in calcium, chloride and sodium serum level compared with control group ($p < 0.05$). While serum urea concentration showed a significant increase ($p < 0.05$) in STZ-diabetic rats.

Hematological tests. The hematological profile of diabetic and normal rats are illustrated in Table 4. Red blood cell (RBC) count of the diabetic group showed a significant decrease ($p < 0.05$) compared with control group. The significant decline ($p < 0.05$) in the levels of mean corpuscular haemoglobin concentration (MCHC) and corpuscular Hb concentration mean (CHCM) were noted in diabetic rats. In contrast, there is a significant increase ($p < 0.05$) in the levels of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and cellular haemoglobin (CH) was observed in the diabetic animals. About White blood cell (WBC), the present data showed a significant decrease ($p < 0.05$) in STZ treated group when compared with control group. On the other hand, there was a substantial increase ($p < 0.05$) in Neutrophils (NEUT) and Monocytes (MONO). On the contrary, Lymphocytes (LYMP) and Leucocytes (LUC) showed a significant decrease ($p < 0.05$) when compared with normal control group.

Histological observation. The normal histological structure of the pancreas is observed in (Figure. 1A); the pancreatic islet cells are embedded within the acinar cells and surrounded by a fine capsule. The pancreatic

islets of diabetic rats revealed severe pathological changes. The STZ pancreas showed destructed pancreatic islets with severe atrophy and massive loss of islet cells leading to large cytoplasmic vacuoles; some vacuoles are filled with a semifluid-like material and dilated sinusoids with eosinophilic material deposition. The pathological changes also extended into surrounding exocrine tissue. The size of the exocrine cells was shrunken with the occurrence of peripheral widening between pancreatic acinar and islet cells

(Figure 1B). Microscopical examination of liver sections from the control rats showed normal architectures of the hepatocytes with distinctive central veins (Figure 1C). On the other hand, liver sections of the STZ treated group showed dilated and congested sinusoid with mild free (hemorrhage) RBC in the interstitial tissue, centrilobular necrosis, infiltration of inflammatory cells, deposition of amyloid material and fibrosis surrounding the portal triad (Figure 1C).

Table 1. Liver Function Tests of the Experimental Groups

Parameters	None- diabetic group (Control)	Diabetic group (STZ group)
Total protein (g/L)	74.00 ± 1.52	65.80 ± 2.08**
Albumin (g/L)	40.45 ± 1.53	25.61 ± 2.59**
ALP (U/L)	73.35 ± 3.78	606.02 ± 261.37**
ALT (U/L)	51.44 ± 4.65	127.80 ± 10.44**
AST (U/L)	144.84 ± 8.80	193.53 ± 12.20*
Total bilirubin (umol/L)	0.94 ± 0.12	2.23 ± 0.70*
Direct bilirubin (umol/L)	0.68 ± 0.10	1.36 ± 0.40
Indirect bilirubin (umol/L)	0.28 ± 0.07	0.86 ± 0.35

Data are presented as mean ± SEM and Mann-Whitney U test is used, $p < 0.05$ is taken as statistically significant at a 95% confidence interval. *significant ($p < 0.05$), **high significant ($p < 0.01$)

Table 2. Lipid profile & Fasting Blood Sugar (FBS) Tests of the Experimental Groups

Parameters (mmol/L)	Non- diabetic group (Control)	Diabetic group (STZ group)
LDL	0.22 ± 0.04	0.90 ± 0.42**
HDL	1.73 ± 0.09	1.36 ± 0.14
TG	0.51 ± 0.02	1.31 ± 0.32**
TC	2.02 ± 0.10	1.78 ± 0.15
FBS	3.90 ± 0.08	29.62 ± 1.06**

Data are presented as mean ± SEM and Mann-Whitney U test is used, $p < 0.05$ is taken as statistically significant at a 95% confidence interval. **high significant ($p < 0.01$)

Table 3. Kidney Function Tests of the Experimental Groups

Parameters	Non- diabetic group (Control)	Diabetic group (STZ group)
Creatinine (umol/L)	26.94 ± 2.12	25.23 ± 1.71
Uric acid (umol/L)	82.80 ± 12.08	79.11 ± 9.49
Urea (mmol/L)	5.45 ± 0.71	13.18 ± 2.85*
Calcium (mmol/L)	2.42 ± 0.10	2.12 ± 0.065*
Chloride (mmol/L)	102.53 ± 0.55	91.02 ± 1.53*
Potassium (mmol/L)	4.81 ± 0.21	5.25 ± 0.39
Sodium (mmol/L)	138.88 ± 0.24	133.48 ± 1.52*
Phosphate (mmol/L)	2.10 ± 0.07	1.87 ± 0.19

Data are presented as mean ± SEM and Mann-Whitney U test is used, $p < 0.05$ is taken as statistically significant at a 95% confidence interval. *significant ($p < 0.05$).

Table 4. Hematological Tests of the Experimental Groups

Parameters	None- diabetic group (Control)	Diabetic group
RBC (10 ¹² /L)	8.39 ± 0.21	7.17 ± 0.47*
HGB (g/d L)	14.45 ± 0.41	13.23 ± 1.04
HCT (%)	44.62 ± 0.97	42.45 ± 3.40
MCV (f L)	53.25 ± 0.25	59.00 ± 1.41*
MCH (pg)	17.28 ± 0.10	18.38 ± 0.43*
MCHC (g/d L)	32.43 ± 0.30	31.18 ± 0.21*
CHCM (g/d L)	33.48 ± 0.43	32.03 ± 0.38*
CH (pg)	17.78 ± 0.17	18.78 ± 0.50*
RDM (%)	12.70 ± 0.12	15.60 ± 1.17
HDW g/d L)	2.32 ± 0.02	2.52 ± 0.28
PLT (10 ⁹ /L)	1389.50 ± 45.98	594.00 ± 66.68*
MPV (f L)	7.40 ± 0.07	7.43 ± 0.25
WBC (10 ⁹ /L)	16.40 ± 2.07	4.54 ± 0.65*
NEUT (%)	4.35 ± 0.48	20.75 ± 3.47*
LYMP (%)	65.38 ± 2.96	31.10 ± 8.35*
MONO (%)	14.50 ± 1.11	39.83 ± 4.76*
EOS (%)	4.08 ± 1.87	0.85 ± 0.56
BASO (%)	0.28 ± 0.06	0.55 ± 0.45
LUC (%)	11.43 ± 0.33	6.98 ± 1.03*

Data are presented as mean ± SEM and Mann-Whitney U test is used, $p < 0.05$ is taken as statistically significant at a 95% confidence interval. *significant ($p < 0.05$).

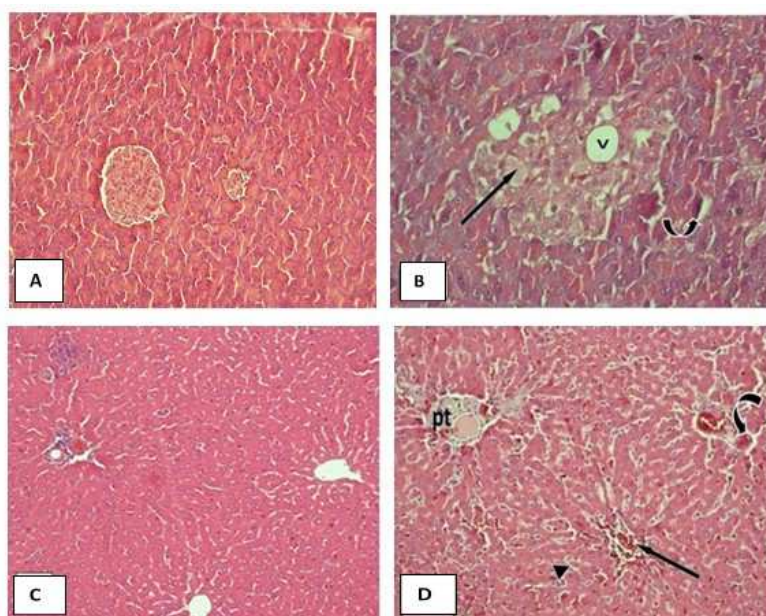


Figure 1. Photomicrographs of Sections of: A). Pancreas of Control Group, Showing Normal Histological Structure. B). Pancreas of STZ Treated Rats, Showing Severe Atrophy and Massive Loss of Islet Cells, Large Cytoplasmic Vacuoles (v), Dilated Sinusoids with Eosinophilic Material Deposition (Arrow), and Degeneration Extends to Exocrine Acinar Cells (Curved Arrow). C). Liver of Control Group, Showing Normal Architecture. D). Liver of STZ Treated Rats, Showing Centrilobular Necrosis (Arrow), Kupffer Cells Infiltration (Head Arrow), Lymphocytic Infiltration and Fibrosis Near the Portal Triad (pt), Amyloid Material and Fibrosis Near the Portal Triad (Curved Arrow) (H&E x 200)

The kidney of control rats showed normal histological structures of the glomeruli, and renal tubules (Figure. 2A). The kidney sections of diabetic rat exhibited tubular damage with fatty degeneration, dilated tubular lumen lined with degenerated epithelium and apoptotic cells, congested blood vessels, shrunken glomeruli, widening of Bowman space, thickening of the arterial wall, and periarterial mononuclear infiltration (Figure. 2 B&C). Sections of the wall of the heart of the of control rats showed normal histological appearance. The myocardium showed branching, anastomosing cylinders myocardium with centrally located oval basophilic nuclei (Figure. 2D). The myocardium of STZ rat revealed histological changes in the form of disorganization, distortion, degeneration and hyalinization of the myocardial fibers, increased interstitial space with chronic inflammatory infiltration, thickened vascular wall, dilated and congested blood vessels with eosinophilic material deposition (Figure.

2E). The spleen of control rats showed normal white and red pulp and with distinctive marginal zone (Figure. 3A). Examination of STZ diabetic rat's spleen sections revealed degeneration of the marginal zone of the white pulp. In addition to apoptosis and necrosis of lymphocytes in the red and white pulps, a decrease in the lymphocytes and macrophages in the germinal centre, and thickening of the central arteriolar wall, congested and distended venous sinuses and amyloid-like material deposition and fibrosis in red and white pulps (Figure. 3 B&C). The adrenal glands of control rats revealed normal appearance and were seen surrounded by thin connective tissue capsule (Figure. 3D). Sections of STZ diabetic rat's adrenal glands showed severe hydropic degenerative and pycnotic changes in zona glomerulosa and zona fasciculata, dilated and congested sinusoids in the cortex, thickened capsule and blood vessel wall and lobulation and fibrosis in the cortex (Figure. 3E).

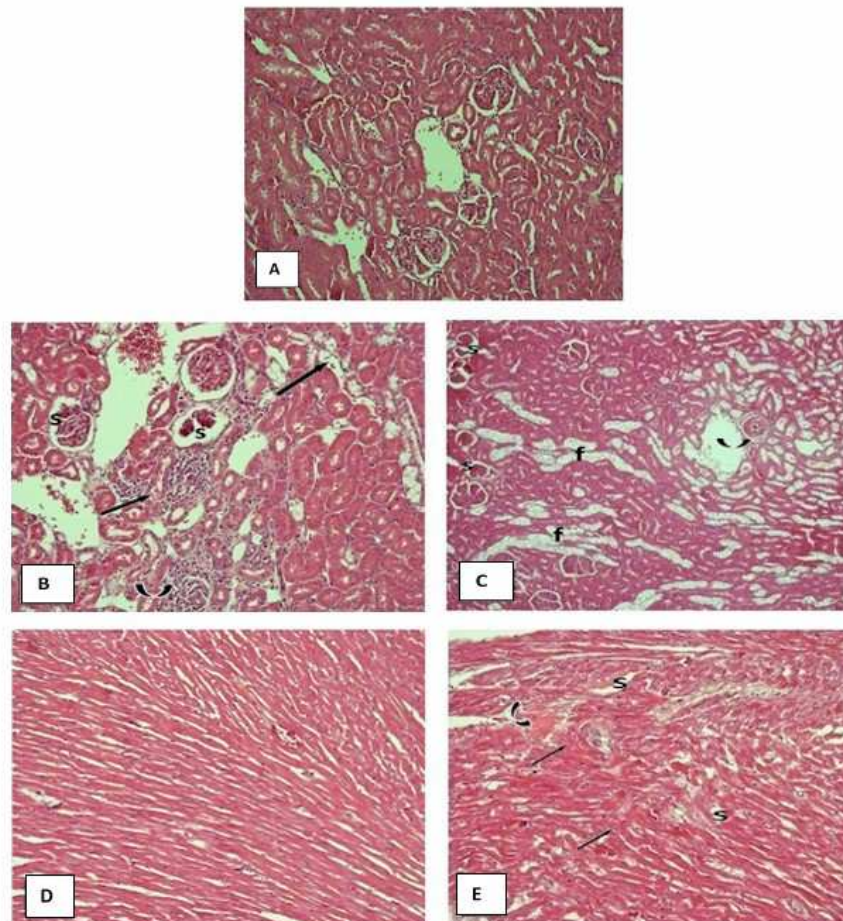


Figure 2. Photomicrographs of Sections of: A; Kidney of Control Group, Showing Normal Histological Structure. B and C; Kidney of STZ Treated Rats, Showing Shrunken Glomeruli, Dilated Bowman Space (s), Dilated Lumen of PCTs Lined with Degenerated Epithelium with Apoptotic Cells (Arrow), Abnormal Renal Tubules with Fatty Degeneration (f) and Mononuclear Infiltration(Curved Arrow). D; Myocardium of Control Group, Showing Normal Architecture. E; Myocardium of STZ Treated Rats, Showing Degeneration of the Myocardial Fibers (Arrow), Increased Interstitial Space(s), Dilated and Congested Blood Vessels with Eosinophilic Material Deposition (Curved Arrow). (H&E x A, B, C &D; 200, E; 400)

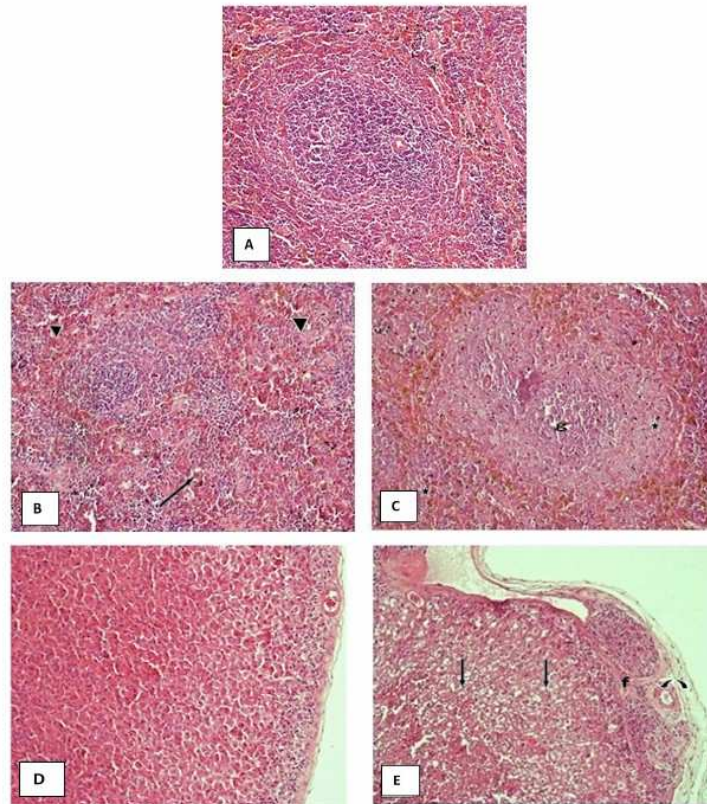


Figure 3. Photomicrographs of Sections of: A; Spleen of Control Group, Showing Normal Histological Structure. B and C; Spleen of STZ Treated Rats, Showing Congestion of Splenic Tissues with Markedly Distended Venous Sinuses (Arrows). Amyloid-like Material Deposition (Head Arrows), Degeneration of the Marginal Zone, Apoptosis and Necrosis of Lymphocytes in the Red and White Pulp (Stars). Decline in the Number of Macrophages and Lymphocytes in the Germinal Center (g), and Thickening of the Central Arteriolar Wall. D; Adrenal Gland of Control Group, Showing Normal Architecture. E; Adrenal Gland of STZ Treated Rats, Showing Severe Hydropic Degenerative Changes in Zona Glomerulosa and Zona Fasciculata (Arrows), Thickened Capsule and Blood Vessel Wall (Curved Arrow) and Lobulation and Fibrosis in the Cortex. (H&E x A, B, &D; 200, C&E; 400)

Discussion

Diabetes mellitus is a metabolic disease distinguished by numerous groups of disorders that disturb the metabolism of carbohydrates, fat, and protein.¹⁰ In the present study, diabetes induced by STZ was associated with many complications. These complications include dyslipidemia, electrolyte imbalance, hepatic enzymes disturbance and hematological abnormalities. Diabetes induced by STZ has been related to abnormal lipid profile. The increases in plasma triacylglycerol and low-density lipoprotein (LDL), and the decrease in high-density lipoprotein (HDL) indicated significant dyslipidemia in diabetic rats; similar results were obtained by several studies in human and experimental diabetes.^{11,12} Some biochemical mechanisms have been suggested as responsible for the hypertriglyceridemia in diabetes. These include an increase in the activity of hormone-sensitive lipase, which catalyses the mobilization of fatty acids from triacylglycerol stored in adipocytes.^{13,14} Therefore, the larger amounts of fatty

acids returning to the liver are reassembled into triacylglycerols and secreted in VLDL. It has additionally been reported that the activity of lipoprotein lipase is reduced in diabetes, and this explained the diabetic hypertriglyceridemia.¹²

Hepatic enzymes level of AST, ALT, and ALP were higher in the STZ diabetic rats. STZ has been noticed to have a significant role in the alteration of liver function.¹⁵ Associated with the progress of diabetes, a state of decreased total protein concentration is evidenced, which may have resulted from either hyperfiltration induced diabetic nephropathy and/or increased protein catabolism.¹⁶

In the present study, diabetes was associated with electrolyte imbalance, where a decrease in serum Na, Ca, and Cl, with increased serum K were recorded, this might be due to the osmotic diuresis produced by the state of hyperglycemia that causes marked urinary loss of water and electrolytes.¹⁷ Concerning sodium,

additionally, there is a translocation of Na⁺, K⁺-ATPase pumps from the basolateral membrane of proximal convoluted tubules to the cytosol, which leads to a decrease in sodium pumping from renal tubules to the blood.¹⁸ As for serum calcium, previous studies demonstrated lower concentration level associated with a reduction in the mineral content of bone and increased urinary excretion of calcium and phosphate in STZ diabetic rats. The decrement of calcium was related to several factors such as metabolic changes including chronic acidosis; insulin deficiency, impaired parathyroid hormone action and changed vitamin D metabolism have been implicated.¹⁹ The urea level, which has been considered as a significant marker of diabetic nephropathy was increased in the STZ-induced diabetic group, and this result is consistent with the other study.²⁰ It has been proved that the metabolic defects observed in uncontrolled diabetes lead to gluconeogenesis and consequently urea production.¹² This previous result is consistent with the reduction in the total serum protein level of diabetic rats in the present study.

Hematological evaluations revealed significant ($p < 0.05$) decline in the red blood cell (RBC) values in diabetic rats. These observations agree with existing literature that anemia is a common pathophysiology associated with diabetes mellitus.²¹ Recurrent or persistent hyperglycemia during diabetes causes non-enzymatic glycosylation of body proteins. Some biomolecules, such as Hb and RBC membrane proteins, are modified by glycation. RBC membrane hemolysis is due to the glycosylation of membrane proteins and lipid peroxidation, and this may lead to anemia.²² The reduced levels of WBC, PLT, and LYMP in diabetic rats indicate suppression of the immune system. These cells have an important role in identifying and eliminating pathogens, either by attacking larger pathogens through contact or by phagocytosis. They have the essential part of the innate immune system, which is also an important mediator of the activation of the adaptive immune system.²³ In addition to the previously mentioned, there are many abnormalities regarding MCV, MCH, CH, MCHC, CHCM, NEUT, MONO, and LUC. Diabetes mellitus is responsible for the abnormalities in the function, metabolism, and morphology of blood cells as well as the coagulation system. Numerous abnormalities are the consequence of hyperglycemia and protein glycosylation. Even though they are not frequent manifestations of diabetes, hematologic complications can result in anemia, infection, and hypercoagulability. Most hematologic deviations are reversible with effective metabolic control.²⁴

Histologically, STZ is well known for its selective pancreatic islet β -cell cytotoxicity and has been extensively used to induce diabetes mellitus in animals. It interferes with cellular metabolic oxidative

mechanisms. The decrease in cellularity within islets of Langerhans observed in the present study reflects the cytotoxicity of STZ.²⁵ STZ treatment in rats exhibited shrunken islets of Langerhans with cytoplasmic vacuolation of their cells associated with negative insulin immunoreaction; ultrastructurally the β -cells showed darkly stained nuclei with marked loss of granules.²⁶ The pathoanatomical changes in liver of diabetic animals have been previously reported.^{27,28} Liver, an insulin-dependent tissue, playing a pivotal role in glucose and lipid homeostasis, is severely affected by diabetes.²⁹ Previous studies have detected moderate to severe degeneration of the hepatocytes.⁶ The structural changes in kidneys could be attributed to altered metabolism in diabetes and the subsequent effects on the increased renal threshold for hyperglycemia.³⁰ Previous studies on the long-term effects of diabetes in experimental animals show glomerular nephropathy along with tubular and interstitial abnormalities.³¹ Massive inflammatory infiltrates in the interstitial tissue as well as vacuolar degeneration of tubular epithelial cells and renal glomeruli.^{30,32} The anomaly in the cardiac muscle in the present study is consistent with the earlier animal study, which demonstrated severe necrotic cardiac muscle in STZ diabetic rats. Previous observation by an electron microscope showed the disappearance of the myofilaments organization and pyknic mitochondria in the myocardium of diabetic rats.³³ The histopathological changes in heart could be attributed to the subsequent effects of hyperglycemia which induces degenerative changes in the tissues along with cardiomyopathy and nephropathy by oxygen free radicals.³⁴ The abnormalities in the zona glomerulosa and zona fasciculata in addition to the cortical fibrosis and thickened blood vessel wall of the adrenal gland in this study could be attributed to the hormonal changes as mentioned by previous investigation.³⁵ In the present study, the diabetic rats exhibited numerous abnormal histopathological features of the spleen characterized by apoptosis of the lymphocyte and necrosis in the white, and red pulps are consistent with the previous observation that demonstrated extensive parenchymal fibrosis involving the marginal zone and follicular areas of the white pulp.³⁶

Conclusions

This research regards as a comprehensive study of diabetes complications in an animal model. After six months, STZ-diabetic rats showed severe pathological changes in the Islets of Langerhans, liver, kidney, spleen, cardiac muscle and adrenal gland. These pathological changes associated with the deterioration of the liver and renal functions, lipid profile, and haematological parameters. On the other hand, this study can be used as a reference for many biochemical and histopathological abnormalities of diabetes complications in an animal model for other researchers. The results of this research

can be used in studies which looking at the treatment of diabetes complications for comparison purposes. Researchers recommend to follow the method of diabetes induction which has been used because they showed excellent results.

Acknowledgments

This research was sponsored by International Islamic University Malaysia (IIUM EDW B: 13-021-0906). The authors would like to appreciate all members of the pharmacology and physiology unit in the Faculty of Pharmacy for their support.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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