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# Development and characterization of late-stage diabetes mellitus and -associated vascular complications☆

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ABSTRACT

Preclinical investigation is the key mark of medical research, as the major breakthroughs including treatment of devastating diseases in biomedical research have been led by animal studies. Type 2 diabetes mellitus (T2DM) is a predominant metabolic disorder having high prevalence of morbidity worldwide which create an urgent need to understand the pathogenesis, complication and other possible influences by development of appropriate animal model. High-fat diet (HFD) fed animals (21 days) were treated with single cycle of repetitive dose (SCRD) of streptozotocin (STZ; 40, 30 and 20 mg/kg/per day in three respective group at 1st, 3rd, and 5th day) and double cycle of repetitive dose (DCRD) of streptozocin (STZ) (20, 10 and 5 mg/kg/per day in three respective group at 1st, 3rd, and 5th day in one cycle and 21st, 23rd, 25th day in second cycle of treatment) to induce late-stage diabetic complications. Induction of hyperglycemia was assessed by fasting and postprandial blood glucose, HbA1c, insulin, C-peptide, pancreatic β-cells and dyslipidaemia up to 12 weeks. Combined treatment of HFD and STZ (20 mg/kg) in the DCRD manner were significantly induced late-stage diabetic complication with sustained hyperglycaemia, no mortality, increased HbA1c and dyslipidaemia, reduced insulin, C-peptide and beta cells. Moreover, biochemical and histological assessment of micro and macrovascular tissues confirmed the significant cardio-renal injury, endothelial and hepatic damage. The study confirmed the development of chronic diabetic model in rat mimicked to clinical pathology with associated micro and macrovascular abnormalities which can further explore the molecular aspects of diseases.

## 1. Introduction

International Diabetes Federation reported the elevated burdens of type 2 diabetes mellitus (T2DM) over 400 million people. T2DM is characterized by low secretion of insulin or insulin resistance at target tissues [1–3]. Obesity is an associated risk factor of T2DM as an inappropriate elevation of non-esterified fatty acids in liver and muscle can cause lipotoxicity declining the glucose homeostasis by affecting insulin action (insulin resistance) [4]. Since the towering prevalence of T2DM urge to develop a novel therapeutic strategy; an appropriate animal model is needed that mimicked the clinical condition. Rodents have the high similarities in organ physiology, metabolism and blood bio-chemistry with human [5,6]. Consequently, these animals have been used to develop improved experimental T2DM model over the years with numerous manipulations including transgenic/knock-out alterations, spontaneous or planned genetic derivation, surgical treatments, chemical induction, dietary induction or combination of above. Conversely, some diabetic animal models have unavoidable limitation including high mortality rate, reversibility of hyperglycaemia and high cost. Certainly, the prior assessment of safety, toxicity, potency and therapeutic outcomes of any targeted treatment is based on laboratory animals. The advanced and validated method can propose the specified animal model for induction of T2DM based on the 3R scheme of animal model selection [7].

Mimicking the whole features of humans T2DM, a combination of high-fat diet (HFD) and streptozotocin (STZ) were used, as HFD associated obesity and low dose of STZ induced β cells (pancreas gland) destruction are the root cause of prediabetic followed by T2DM [8–10]. However, the use of combined application of HFD and STZ has been limited due to high mortality at the high dose of STZ and reversibility of hyperglycaemia at a low dose of STZ [9,10]. However, a unique combination of HFD and STZ is needed over several existing models to

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induce irreversible late-stage T2DM and associated complications with no mortality and in cost effective manner which may be more impersonator to human metabolic disorder. The present study was designed to develop a late-stage T2DM animal model -associated micro/macrovascular complications having the similar feature of human diabetic metabolic disorder and can use for targeted pharmacological screening of drugs.

## 2. Material and method

The experimental study for the present protocol was approved by 'Institutional Animal Ethics Committee' followed by 'Committee for the Purpose of Control and Supervision of Experiments on Animals' (with approval number: CPCSEA\AIP\2013\004) CPCSEA, Delhi, India. Young Wistar albino rats (24–25 weeks) of either sex (about 200 to 220 g) were used in the present study. Before initiating the protocol, all animals were acclimatized in 'Institutional animal house' and maintained on *ad libitum* access of HFD and water with normal day/night cycles (12 h/12 h), ambient humidity (60% ± 5%) and temperature (25 ± 5 °C).

The HFD was composed by consisting 45.5% standard chow, 22.7% lard, 22.7% vegetable shortening and 9% sucrose, whereas the standard diet (for control group) consisted of 100% chow. The standard chow diet provided 3.97 Kcal $g^{-1}$ whereas, the HFD provided 6.25 Kcal $g^{-1}$. The prepared HFD was stored up to 4 days in a refrigerator (temperature, 7 °C, ± 2).

STZ was purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. The enzymatic kit for lipid profile estimation (TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides), were purchased from Erba Diagnostics, Inc., USA. Glycated haemoglobin A1c (HbA1c) ELISA kit was purchased from Genxbio Health Sciences Pvt. Ltd. Delhi, India, rat C-Peptide ELISA kit and rat insulin ELISA kit were procured from RayBiotech, Norcross, GA. Estimation of cardiac cTnI and CK-MB, LDH were done by using a commercially available kit purchased from Logitech India Pvt. Ltd. (Delhi, India) and Transasia Bio-Medicals Ltd., Solan, India respectively. Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and serum creatinine, blood urea nitrogen (BUN) were measured for estimating the hepatic and renal damage respectively by using commercially available kits purchased from Sigma-Aldrich, US.

### 2.1. Experimental protocol

To induce experimental hyperglycaemia a chronic stress was given to islets of Langerhans by repeated dosing of STZ in HFD fed animals at different time interval with different dose as shown in Table 1. The animals were divided into three main groups including normal control and two STZ treated (a single cycle of repetitive dose (SCRD) and the double cycle of repetitive dose (DCRD)) animal groups which had *ad libitum* access of HFD.

1. The normal control group received vehicle injections (sodium citrate buffer, 2%), normal food pellets and water *ad libitum*.
2. SCRD of STZ treated group as detail in Table 1.
3. DCRD of STZ treated group as detail in Table 2.

The SCRD and DCRD of STZ treated groups were further divided into three subgroups respectively with different dose as shown in Table 3.

### 2.2. Development and standardization of animal model

To develop the STZ and HFD induced experimental diabetic model, we have revisited the reported technique with modified dose frequency and dose concentration [9,11,12]. The exposure frequency and dose concentrations were varied to achieve sustained late-stage metabolic disorder [11,12]. Different model with the varying mode of exposure and dose was screened and standardized on the basis of mortality rate, HbA1c level, insulin level, blood glucose level (fasting blood glucose, after oral glucose administration and random blood glucose level), C-peptide level and lipid profile.

Basically, HFD fed animals were treated by SCRD and DCRD of STZ with different dose to induce sustain hyperglycaemia for a longer duration at low mortality. In SCRD model, the concentration of STZ (as shown in Table 3) was administered on 1st, 3rd, and 5th day in HFD pre-treated animals. Whereas, in DCRD model, the concentration of STZ (as shown in Table 3) was administered on 1st, 3rd, and 5th day in the first cycle and similar treatment (with the same dose of STZ in respective animal group) were repeated after 15 days (on 21st, 23rd and 25th day) in the second cycle. All biochemical estimation for measuring the induction and confirmation of T2DM were evaluated after 3 days to 12 weeks of last dose administration of STZ as shown above in detail boxes.

#### 2.2.1. Measurement of body weight and blood glucose

Body weight of each animal was measured at the different time period after induction of DM (1 day, 1, 2, 4, 8 and 12 weeks). Blood glucose was measured by commercially available glucometers (Accu Chek Active Blood Glucose Meter Kit) in tail-tip blood samples from overnight fasted animals and oral glucose administered animals (postprandial blood glucose). Postprandial blood glucose was measured in animals having pre-treatment of glucose solution (2 g/kg/p.o) before 120 min of analysis [13].

#### 2.2.2. Measurement of mortality rate

Mortality rate was calculated by the measuring total death percentage during the complete study duration (12 weeks) after exposure of STZ in HFD animals as compared to normal control animals.

#### 2.2.3. Sustainability of blood glucose

To find prolonged sustainability of hyperglycaemia, blood glucose level was measured at 1, 2, 4, 8 and 12 weeks after the last dose of STZ. Sustainability of hyperglycaemia up to 12 weeks standardize the model for late-stage T2DM.

#### 2.2.4. Measurement of HbA1c, serum insulin, and C-peptide

HbA1c and serum insulin were measured by commercially available ELISA kit based on the Sandwich-based assay. Whereas, C-peptide was measured by commercially available ELISA kit based on competition-based assay as per the manual of respective kits.

**Table 1**
Detail of single cycle repetitive dose (SCRD) of STZ treated group and assessment of biomarkers in this group.

| Pre-treatment | Induction of diabetes mellitus | | | | Assessment of biomarkers | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 0–21 days | 1st day | 3rd day | 5th day | 6th to 8th day | 1st day | 7th day | 14th day | 28th day | 56th day | 84th day |
| HFD *ad libitum* | Repetitive dose of STZ | | | (3 days) | Confirmation and standardization assay for diabetic model | | | | | |
| HFD *ad libitum* | | | | | | | | | | |
| Total duration (3 weeks) | Total duration (1 weeks) | | | | 1 day | 1 week | 2 weeks | 4 weeks | 8 weeks | 12 weeks |
| | | | | | Total duration (12 weeks) | | | | | |

**Table 2**
Detail of double cycle repetitive dose (DCRD) of STZ treated group and assessment of biomarkers in this group.

| Pre-treatment | Induction of diabetes mellitus | | | | | | | Assessment of biomarkers | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0–21 days HFD *ad libitum* | 1st day 3rd day 5th day Repetitive dose of STZ | | | 6th to 20th day (15 days) | 21st day 23rd day 25th day Similar repetitive dose of STZ | | | 26th to 28th day (3 days) | 1st day 7th day 14th day 28th day 56th day 84th day Confirmation and standardization assay for diabetic model | | | | | |
| HFD *ad libitum* Total duration (3 weeks) | Total duration (4 weeks) | | | | | | | | 1 day 1 week 2 weeks 4 weeks 8 weeks 12 weeks Total duration (12 weeks) | | | | | |

**Table 3**
Detail of different groups with respective dose of treatment and frequency.

| Group | Category | Treatment | Day of treatment |
|---|---|---|---|
| **1** | Normal control | Standard diet *ad libitum*<br>3 vehicle injections (sodium citrate buffer, 2%) | Standard diet - throughout the study<br>Vehicle injection - 1st, 3rd, 5th and, 21st, 23rd, 25th day |
| **2** | SCRD | HFD *ad libitum*<br>3 dose of STZ (40 mg/kg/*i.p*) | HFD - throughout the study<br>STZ - 1st, 3rd, and 5th day |
| **3** | | HFD *ad libitum*<br>3 dose of STZ (30 mg/kg/*i.p*) | |
| **4** | | HFD *ad libitum*<br>3 dose of STZ (20 mg/kg/*i.p*) | |
| **5** | DCRD | HFD *ad libitum*<br>6 dose of STZ (20 mg/kg/*i.p*) | HFD - throughout the study<br>STZ - 1st, 3rd, 5th and 21st, 23rd, 25th day |
| **6** | | HFD *ad libitum*<br>6 dose of STZ (10 mg/kg/*i.p*) | |
| **7** | | HFD *ad libitum*<br>6 dose of STZ (5 mg/kg/*i.p*) | |

#### 2.2.5. Lipid profile (TC, TG, HDL, LDL)

Different marker for serum lipid profile including TC, HDL, LDL and TG were measured by using the commercially available kit as appraised by the previous report [14].

#### 2.2.6. Beta cell destruction

Destruction of beta cells presents in the pancreas was observed by evaluating the structural abnormalities of the pancreas. Pancreatic histological study of animal models was carried at the end of the study (12 weeks). In brief, animals of the selected model were sacrificed, the pancreas was immediately isolated in 10% formalin solution. Pancreatic tissues were fixed in paraffin wax and sections of about 5 μm thickness were cut. Haematoxylin-eosin (H&E) staining was applied to the sections and histological photomicrographs were evaluated under the microscope.

### 2.3. Selection of a best-fit model for chronic T2DM

The induction of late-stage T2DM animal model having associated micro and macrovascular complication were considered on the basis of no mortality rate, low dose of STZ consumption (economical) and high sustainability of blood glucose for longer duration in addition to dyslipidaemia, abnormal C-peptide, HbA1c, serum insulin level.

### 2.4. Optimization of an animal model for micro and macrovascular abnormalities

After induction and selection of animal model, the selected model has further optimized for late-stage T2DM-associated micro and macrovascular abnormalities including endothelial dysfunction, cardiovascular damage, hepatic damage, and renal abnormality.

Serum sample of the selected animal model of T2DM was used for estimation of various biomarkers to determine the functional state of the liver including SGOT, SGPT, and ALP using commercially available kits. Endothelial function was measured by estimation of serum nitrite/nitrate concentration as reported [15]. Moreover, the oxidative stress was measured by estimating serum TBARS. Cardiovascular damage was measured by estimation of serum biomarkers including cardiac cTnI and CK-MB, LDH by using commercially available kits. Renal damage was assessed in a selected model of late-stage T2DM animals by measuring serum creatinine and BUN by using commercially available kits. Furthermore, the structural changes in all associated micro and macrovascular organs were observed by measuring histological changes. Section of each tissue was stained by H&E staining and histological photomicrographs were evaluated under a microscope.

### 2.5. Statistical analysis

All values were expressed as mean ± SD. All biochemical data originating from each group of rats were analysed by one-way ANOVA followed by Tukey's multiple comparison tests using Sigma Plot version 11.0, from Systat Software, Inc., San Jose California USA. A '*p*' value of $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Assessment of body weight and mortality rate

Animals of each group were having the body weight in a range of 200–220 g at the initial stage but a significant reduction was observed in STZ-HFD treated animals. Concerning the effect of STZ in SCRD and DCRD fashion, all rats had significant ($p < 0.05$) reduction in mean body weight as compared to normal control group (Table 4). Additionally, the mortality rate is another critical factor for considering a novel animal model. Higher dose of STZ in SCRD groups (having treatment of STZ, 40 and 30 mg/kg) was shown significant mortality whereas, other groups of SCRD (having treatment of STZ, 20 mg/kg) and DCRD (having treatment of STZ, 20, 10 and 5 mg/kg respectively) did not induce any mortality (Table 4).

**Table 4**
Evaluation of selection parameters (Low dose, low mortality and hyperglycaemic sustainability for long duration) for different animal model. SCRD represents single cycle of repetitive dose, DCRD represents double cycle of repetitive dose, HFD represents high fat diet, STZ represent streptozotocin. All data are expressed as mean ± SD, $^{*}p < 0.05$ as compared with normal.

| S. N | Evaluating parameters | Normal control | SCRD | | | DCRD | | |
|---|---|---|---|---|---|---|---|---|
| | | | HFD + STZ (40 mg/kg/ once for 3 days) | HFD + STZ (30 mg/kg/ once for 3 days) | HFD + STZ (20 mg/kg/ once for 3 days) | HFD + STZ (20 mg/kg/once for 3 + 3 days) | HFD + STZ (10 mg/kg/once for 3 + 3 days) | HFD + STZ (5 mg/kg/once for 3 + 3 days) |
| **1** | Total dose of STZ given (mg/kg/i.p) | – | 120 | 90 | 60 | 120 | 60 | 30 |
| **2** | Total mortality (%, up to 12 weeks) | 0 | 33.34 | 16.6 | 0 | 0 | 0 | 0 |
| **3** | Body weight (gm) after last dose of STZ | | | | | | | |
| | 1 day | 205.45 ± 14.56 | 174.77 ± 15.85* | 172.23 ± 22.75* | 175.66 ± 14.82* | 176.44 ± 16.75* | 175.64 ± 19.75* | 179.47 ± 19.23* |
| | 1 week | 207.33 ± 12.36 | 172.76 ± 16.72* | 172.44 ± 22.34* | 176.84 ± 18.82* | 170.74 ± 15.65* | 176.54 ± 18.60* | 176.64 ± 18.44* |
| | 2 weeks | 201.12 ± 13.58 | 162.64 ± 14.75* | 163.76 ± 17.19* | 167.45 ± 16.65* | 165.37 ± 16.58* | 166.47 ± 15.65* | 163.94 ± 19.34* |
| | 4 weeks | 204.92 ± 11.18 | 154.76 ± 16.79* | 167.54 ± 21.75* | 166.82 ± 19.65* | 161.56 ± 18.39* | 164.84 ± 19.55* | 161.38 ± 15.15* |
| | 8 weeks | 210.34 ± 11.53 | 153.97 ± 16.97* | 154.84 ± 13.44* | 157.78 ± 21.97* | 155.49 ± 18.68* | 151.75 ± 16.96* | 159.09 ± 14.34* |
| | 12 weeks | 211.31 ± 12.86 | 132.87 ± 18.76* | 142.65 ± 14.67* | 142.33 ± 18.35* | 147.39 ± 19.65* | 139.77 ± 16.89* | 140.87 ± 15.78* |
| **4** | Hyperglycaemic sustainability after last dose of STZ | | | | | | | |
| | 1 day | 103.00 ± 5.18 | 491.67 ± 7.81* | 473.83 ± 7.25* | 436.00 ± 8.85* | 456.83 ± 7.25* | 404.17 ± 13.20* | 319.33 ± 8.14* |
| | 1 week | 104.50 ± 3.99 | 484.83 ± 5.67* | 478.72 ± 10.80* | 431.00 ± 5.93* | 456.17 ± 9.56* | 402.00 ± 13.56* | 291.50 ± 10.56* |
| | 2 weeks | 103.67 ± 4.55 | 484.00 ± 7.04* | 479.67 ± 8.87* | 400.17 ± 6.59* | 455.33 ± 8.69* | 366.33 ± 10.54* | 271.83 ± 8.73* |
| | 4 weeks | 103.83 ± 3.87 | 483.00 ± 9.36* | 477.67 ± 8.19* | 385.33 ± 8.50* | 455.00 ± 7.97* | 331.67 ± 11.55* | 242.50 ± 10.01* |
| | 8 weeks | 104.17 ± 4.22 | 481.33 ± 8.52* | 477.33 ± 7.94* | 364.17 ± 6.31* | 454.50 ± 8.04* | 327.83 ± 11.69* | 215.17 ± 8.77* |
| | 12 weeks | 105.33 ± 3.72 | 477.50 ± 9.59* | 470.67 ± 9.09* | 322.67 ± 7.20* | 451.17 ± 10.89* | 314.00 ± 5.55* | 163.50 ± 4.23* |

### 3.2. Blood glucose level

Measurement of blood glucose is mainly concern with fasting and postprandial blood glucose level. Level of fasting blood glucose in SCRD groups (having treatment of STZ 40, 30 and 20 mg/kg) were ranging from 333.17 to 324.50 mg/dl, 320.17 to 297.83 mg/dl and 230.67 to 251.83 mg/dl respectively during 12 weeks duration. Additionally, in DCRD groups (having treatment of STZ 20, 10 and 5 mg/kg), the level of fasting glucose was ranging from 263.83 to 250.67 mg/dl, 230.33 to 154.67 mg/dl and 225.17 to 119.33 mg/dl in respective groups throughout the 12-week duration. The level of fasting blood glucose throughout the whole duration was significantly higher in both groups (SCRD and DCRD) as compared to normal control rats (Supplementary Table 1). Moreover, the level of postprandial blood glucose for SCRD (having treatment of STZ 40, 30 and 20 mg/kg) and DCRD (having treatment of STZ 20, 10 and 5 mg/kg) were found to be raised throughout the 12-week duration as ranging from 549 to 503.33 mg/dl, 473.33 to 513.63 mg/dl, 449.67 to 454.33 mg/dl (for respective subgroups of SCRD) and 452 to 457.67 mg/dl, 431.17 to 326.83 mg/dl, 341.17 to 222.50 mg/dl (for respective subgroups of DCRD) as compared to normal control group (Supplementary Table 1).

### 3.3. Assessment of sustainability of hyperglycaemia

Even the increased level of fasting and postprandial blood glucose confirms the induction of chronic DM, but we further measured the random blood glucose to find the sustainability of hyperglycaemia up to 12 weeks. The level of random blood glucose in SCRD subgroups having treatment of 40, 30 and 20 mg/kg of STZ in HFD fed animals were ranging from 491.67 to 477.50 mg/dl, 473.83 to 470.67 and 436 to 322.67 mg/dl respectively. Whereas the level for DCRD subgroups having treatment of 20, 10 and 5 mg/kg of STZ in HFD fed animals were ranging from 456.83 to 454.17 mg/dl, 404.17 to 318 mg/dl and 319.33 to 163.50 mg/dl respectively in the duration of 12-weeks. Indeed, the level of glucose was moderately decreased with a time duration in all subgroups of SCRD and DCRD, except the subgroup of DCRD having treatment of 20 mg/kg STZ in HFD fed animals which have shown the sustainability in hyperglycaemia throughout the whole duration of the investigation.

### 3.4. Assessment of HbA1c

The presence of HbA1c represents the severity of hyperglycaemia as the level of %HbA1c was significantly raised in diabetic condition (Supplementary Table 1). Animals with %HbA1c level > 10 (almost double to normal level) were considered as diabetic. SCRD group has shown the considerable high level of %HbA1c as compared to the % HbA1c level in DCRD group up to 12 weeks. Similarly, the animal having exposure of DCRD of STZ at different dose (20, 10 and 5 mg/kg) has higher concentration of %HbA1c level as compared to normal control animals, but among them the low dose of STZ (10 and 5 mg/kg) did not give a sustain increased level of %HbA1c up to 12 weeks and thus these models have excluded for consideration.

### 3.5. Assessment of C-peptide and insulin

A sustained level of serum insulin and C-peptide was observed in normal control animals up to 12 weeks as compared to significantly decreased in diabetic groups. Animals with serum insulin and the C-peptide level below than 900 pg/ml and 600 pg/ml respectively up to 12 weeks has considered as diabetic. Exposure with different concentrations of STZ (40, 30 and 20 mg/kg) in SCRD and DCRD groups (20, 10 and 5 mg/kg) significantly reduced the level of serum insulin and C-peptide. Indeed, a small increase in insulin was observed in all groups of SCRD and DCRD, except in group having STZ treatment with 20 mg/kg in DCRD group, where a level of reduced insulin was

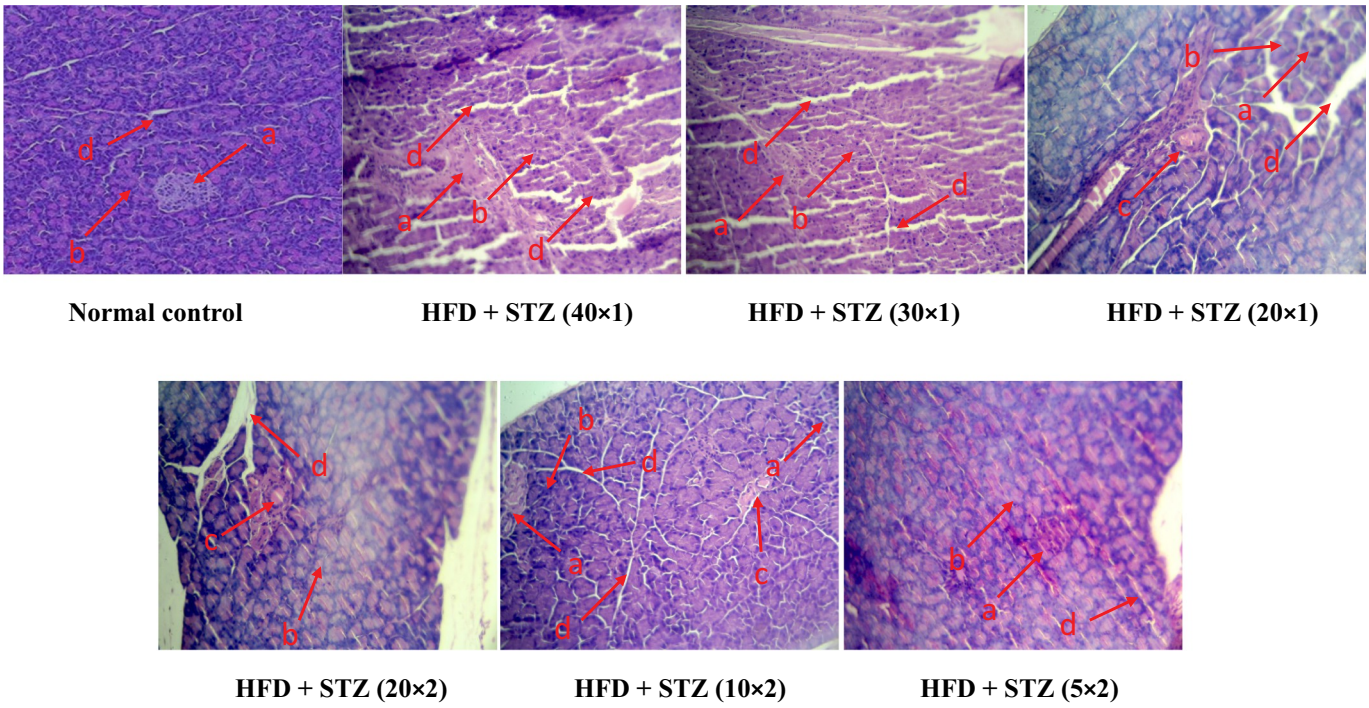


**Fig. 1.** Photomicrographs of rat pancreatic section of normal control and different experimental groups. Histological architecture of normal control rat shown the circular shape of islets of Langerhans 'a' packed in acinar cells 'b'. Pancreatic lobules seen in exocrine components are separated by intact intralobular 'c' and interlobular 'd' connective tissue septa. Whereas, histological section of diabetic pancreas has shown significant damage or entirely loss of islets of Langerhans 'a'. Moreover, the diabetic pancreas shown the swelled acinar cells 'b' with small vacuoles. Interlobular duct 'c' was completely flattened.

sustained or constant up to longer duration (Supplementary Table 1). Additionally, a significant reduction of C-peptide by all exposure of STZ in SCRD and DCRD was measured which was almost constant up to longer duration (12 weeks).

### 3.6. Assessment of lipid profile

A significant increase in TC, LDL, TG and reduced level of HDL was measured in all animal model of SCRD/DCRD groups having different exposure to STZ dose as compared to normal control group. The raising of TC, LDL and TG were constantly increased throughout the whole period of 12 weeks in all groups, whereas a substantial reduction in HDL concentration was observed in all groups as shown in supplementary Table 1.

### 3.7. Histological assessment of pancreas

Histological assessments of normal control group shown the normal architecture of islets of Langerhans which was tightly packed by surrounding acinar cells present in exocrine portion of pancreas [16,17]. Pancreatic structure has been divided by intact intralobular and interlobular connective tissue septa [16,17]. Whereas, in diabetic pancreas either of SCRD or DCRD groups shown the swelled acinar cells with small vacuoles in exocrine components, but the presence of islets of Langerhans was entirely lost or shrunk (Fig. 1). Moreover, the interlobular ducts were completely flattened with epithelium. These structural changes in diabetic pancreas shown the complete destruction of beta cells or islets of Langerhans which confirmed the induction of pancreatic damage by different dose of STZ in HFD pre-treated animals (Fig. 1).

### 3.8. Selection of best fit animal model

Preliminary, we have considered the prime factor including low mortality, low dose (STZ) and high sustainability of hyperglycaemia for longer duration. on the basis of lower mortality (0%) and higher sustainability (> 450 mg/dl up to 12 weeks) of blood glucose at minimum required dose of STZ (20 mg/kg); the animal group of DCRD was selected as ideal model for chronic diabetic mellitus. Furthermore, this animal models of diabetes were significantly altered the various biomarkers including dyslipidaemia, HbA1c, C-peptide and insulin concentration as in desired limit (Fig. 2).

### 3.9. Assessment of associated hepatic damage

The significant raised level of SGOT, SGPT, ALP and reduced level of albumin as compared to normal control animals revealed the presence of hepatic damage during late-stage diabetes mellitus (Fig. 3A, B, C and D respectively). Moreover, the histological assessment of normal control animals did not show any structural alteration but in selected diabetic animal, the swelling of hepatocytes, presence of inflammatory cells, karyomegaly, activated Kupffer cell, dysplasia of bile duct (hyperplasia and hyperactivation of epithelial lining of bile duct) were observed, which confirm the hepatic damage in selected animal model (Fig. 4).

### 3.10. Assessment of associated endothelial damage

The concentration of serum nitrite/nitrate was significantly reduced in selected animal model of chronic diabetes as compared to normal control (Fig. 3E). Additionally, the increased level of serum TBARS was measured to confirm the presence of oxidative stress in late-stage diabetic condition (Fig. 3F). Furthermore, the integrity of vascular endothelial layer was observed by histopathological study using H&E staining which revealed the disruption of (aorta) endothelial cell lining of diabetic rats as compared to the aorta isolated from normal control rats having uniform endothelial layer (Fig. 4).

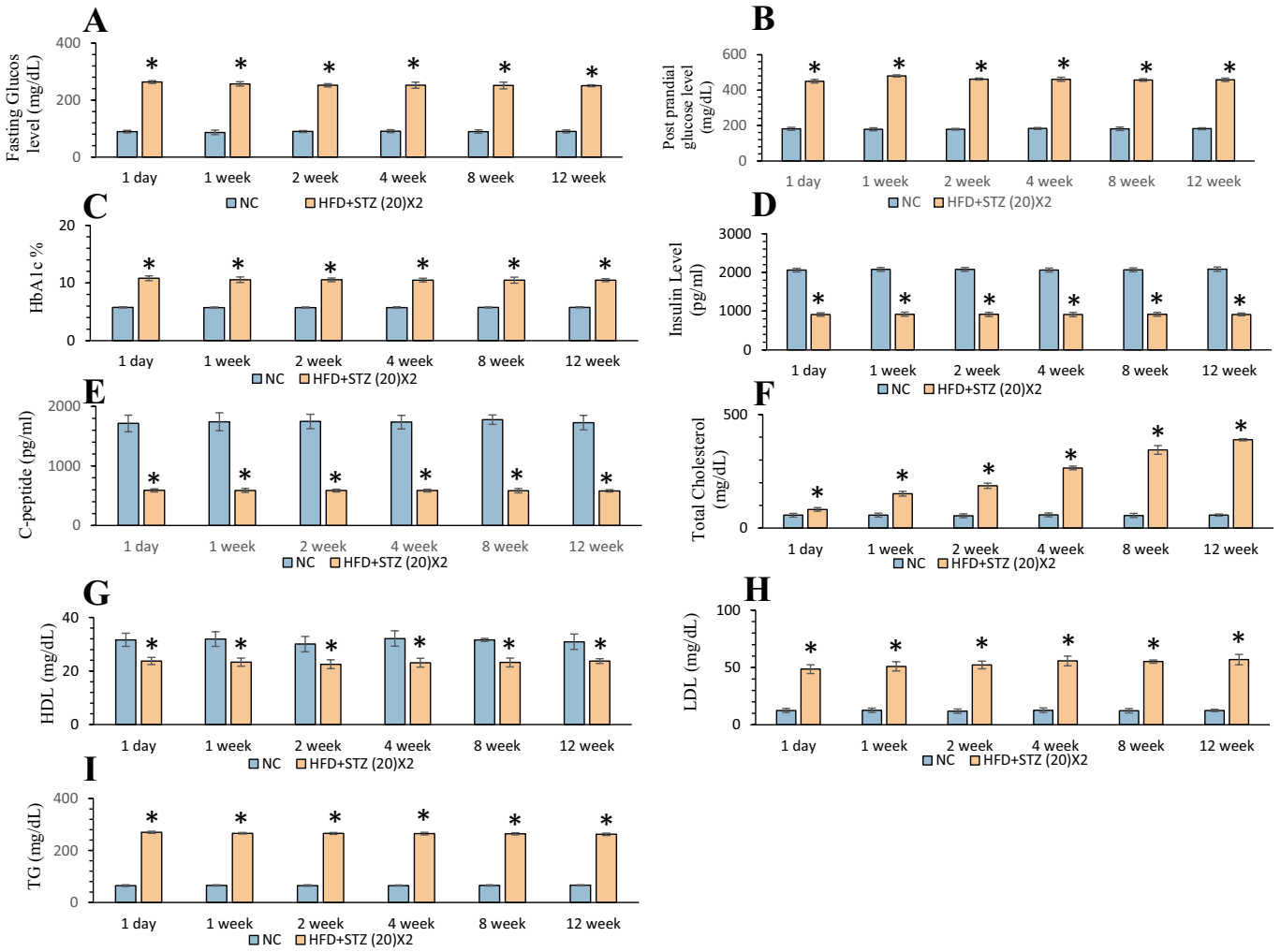


**Fig. 2.** Estimation of various biochemical markers including fasting blood glucose 'A', postprandial glucose level 'B', HbA1c 'C', serum insulin 'D', C-peptide 'E', total cholesterol 'F', high density lipoproteins 'G', low density lipoproteins 'H' and triglyceride level 'I' in selected animal model (HFD-STZ 20 mg/kg in DCRD group) verse normal control animals. All data are expressed as mean ± SD, value of significance has represented by $*p < 0.05$ as compared with normal.

### 3.11. Assessment of associated cardiovascular and renal damage

Substantial increase in cardiac biomarker including CKMB, LDH and cTnI were observed as compared to normal control which confirmed the presence of cardiac injury (Fig. 3G, H and I respectively). Moreover, the histopathological observation from treated animals further shown the presence of thrombus formation, contraction band necrosis, perivascular cuffing, increased intercalated and nuclei karyolysis, myofibrillar loss and increased intercalated space (widened intracellular spaces) as compared to no histological lesions in normal control animals heart (Fig. 4). Moreover, the increased levels of serum creatinine and BUN were observed in the selected animal model as compared to normal control group (Fig. 3J and K respectively). The associated complication was further confirmed by structural damages in glomerulus including vacuolation of endothelial lining glomerular tufts, atrophy of glomerular tuft, hypertrophy of glomerular tuft, congestion of renal blood vessels and tubular necrosis as compared to the renal histology of normal control animals shown normal glomerulus and normal renal tubules (Fig. 4).

## 4. Discussion

Chronic diabetes mellitus is the benchmark for several vascular disorders which would be fatal. To induce the unique animal model of late-stage diabetes mellitus, we have used several approaches with different dose and frequency of STZ administration in HFD fed animals. The reduction of body weight in diabetic animals represents the severe catabolic metabolism which may be due to compensatory hyperinsulinemia against hyperglycaemia and by a late reduction in insulin synthesis after the partial destruction and exhaustion of β-cell masses and this mimic the natural pathology of T2DM [18]. This study tends to develop an animal model of late-stage diabetes with a similar association of vascular complication as in human. The present investigational report clearly observed the significant reduction of mean body weight of STZ treated animals which indicate the induction of natural hyperglycaemia. Indeed, severe catabolic metabolism may lead to death, thus it is mandatory to use an optimum dose of STZ that can produce diabetes mellitus but not leads to mortality. Assessment of mortality in different animal's model was the prime factor of this study where the

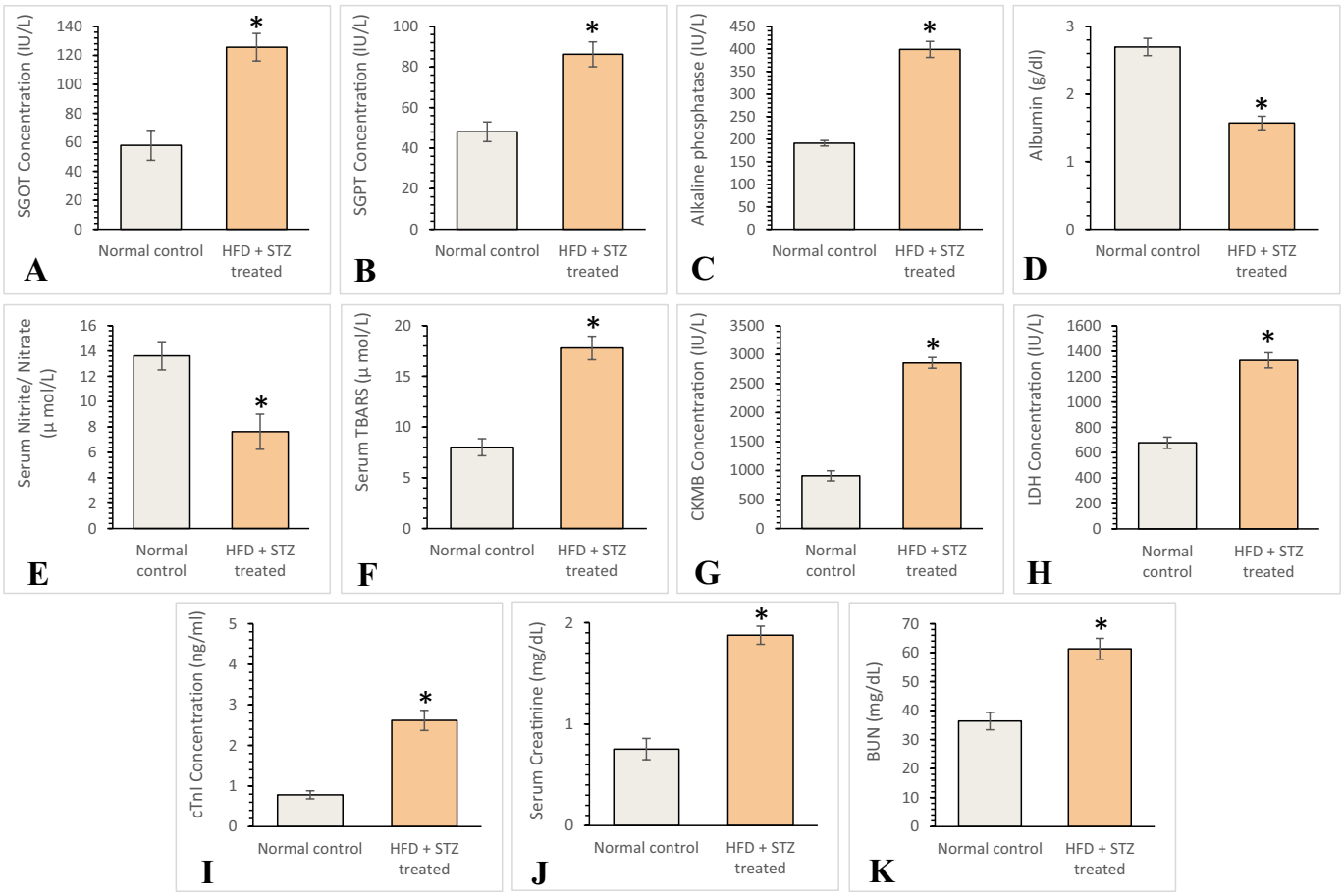


**Fig. 3.** Estimation of various biochemical markers for hepatic, endothelium, cardiac and renal damage in selected animal model exposed by HFD-STZ 20 mg/kg in DCRD verse normal control animals. All data are expressed as mean ± SD, value of significance has represented by $^{*}p < 0.05$ as compared with normal.

different dose of STZ was exposed to HFD treated animals to confirm the survival rate at higher, moderate and minimum dose of STZ in the different model of SCRD and DCRD. The mortality rate is always a critical factor for developing a new animal model [19]. STZ has a high rate of mortality due to extreme hyperglycaemia which limits the sustainability and meaningful outcomes of study [20,21]. Thus, to find the meaningful and significant difference of various biomarkers between the corresponding groups, the mortality rate must be minimal or zero, so that the outcome would be statistically significant. The mortality rate of SCRD subgroups was significantly more as a high concentration of STZ was given in these subgroups by a single administration. Whereas no mortality of animal was found in DCRD group. The results are more interesting for higher dose subgroups of both SCRD and DCRD group (having treatment of STZ 40 mg/kg and 20 mg/kg respectively), where DCRD subgroup induce no mortality as compared to SCRD groups even the total amount of STZ administration were same in the corresponding group as shown in Table 4. The consequences have supported by the published report which explained that, the high dose of STZ with less frequency or dosing cause infection, malnutrition, suffocation of lymphatic circulation and toxicity of STZ [21], whereas, the lesser dose with increased number of dosing or frequency minimize the chances of toxicity and mortality. Even minimum dose administration of STZ in DCRD group (10 and 5 mg/kg) also shown no mortality of animals up to 12 weeks but not able to sustain the hyperglycaemia for a longer duration.

The physiological and pathological consequence for diabetic patients can investigate by critical measurement of blood glucose level in experimental animal models. Certainly, the fasting and postprandial blood glucose is to be measured for estimating the overall hyperglycaemia. Recent evidence has clearly mentioned the strong association between increased postprandial hyperglycaemia and cardiovascular risk, oxidative stress and endothelial dysfunction [21,22]. For the present investigation, the animals with fasting and postprandial blood glucose level > 250 mg/dl and 450 mg/dl respectively up to 12 weeks were considered as diabetic. The consequences from different models signify the fact that low dose of STZ with repetitive frequency can effectively produce hyperglycaemia as compared to high dose with less frequency of STZ. These multiple low doses of STZ may induce inflammation-mediated destruction of the β-cells instead of the fast induction of the β-cell death induced by a single dose of STZ as reported in the previous report [23]. Moreover, the sustainability of blood glucose represents the constant level of hyperglycaemia up to 12 weeks after total administration of STZ dose. This increasing level of blood glucose for longer duration confirmed the existence of late-stage diabetes mellitus that can lead to associated micro and macrovascular disease [21,22].

Instead of body weight variation, mortality rate and blood glucose level, there is a number of other biomarkers which reflect and confirm the induction of chronic diabetes animal model. HbA1c is a unique biomarker to estimate the overall glucose, an excess amount of glucose level in blood react with haemoglobin and formed HbA1c which further reflect more harmful glycation sequelae, such as retinopathy and nephropathy [24,25]. Furthermore, this increased non-enzymatic glycosylation in diabetic patients is key marker that indicates the possible association between hyperglycaemia and vascular complications [24,25].

Moreover, C-peptide is the cornerstone to assist the interpretation of endogenous insulin secretion as both peptides are produced in

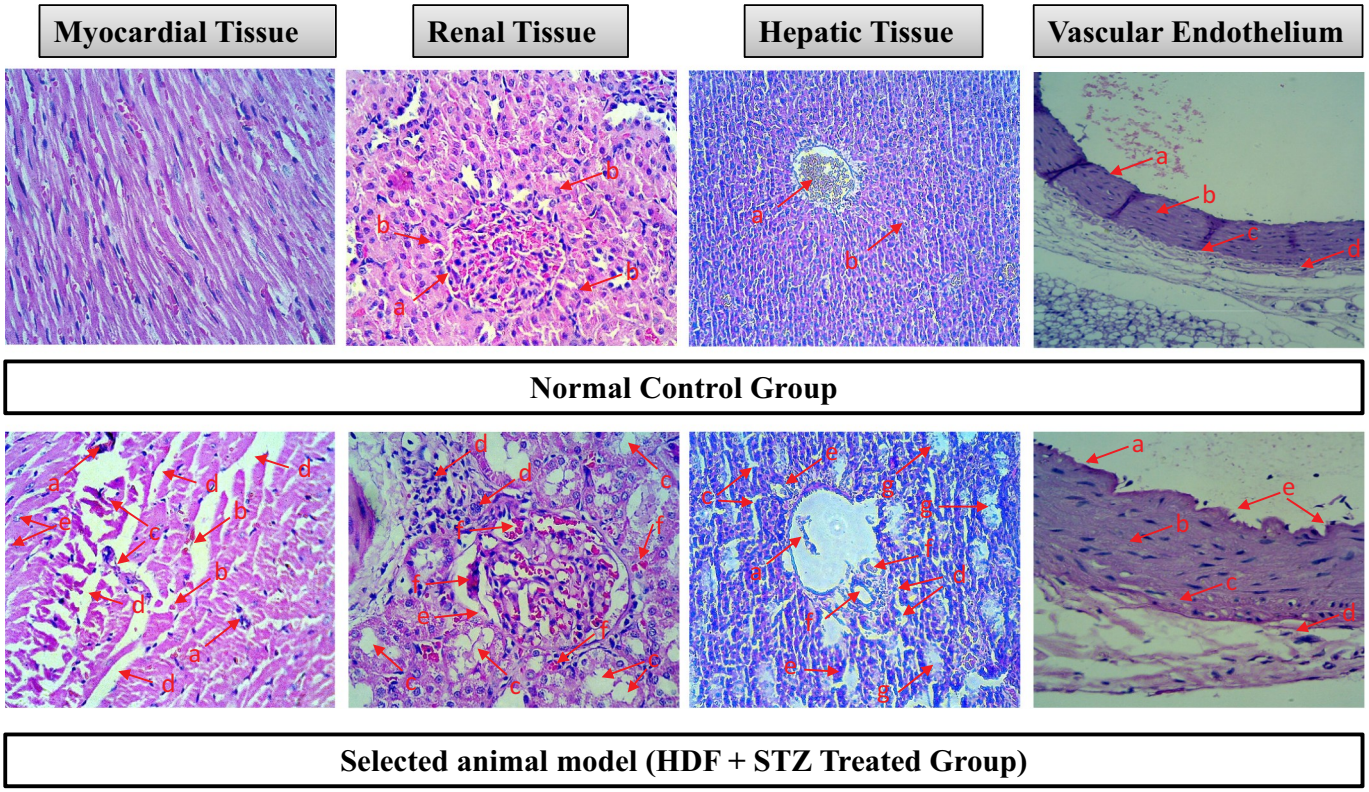


**Fig. 4.** Histological sections of myocardial tissue including normal control verse selected diabetic group, arrows highlight as: thrombus formation 'a', contraction band necrosis 'b', perivascular cuffing 'c', increased intercalated space (widened intracellular spaces) 'd' and nuclei karyolysis 'e', marked in different photomicrographs of different groups animal.
Renal histological sections arrow represents: normal glomerulus 'a', normal renal tubules 'b', vacuolation of endothelial lining glomerular tufts 'c', necrosis of epithelial lining renal tubules 'd', atrophy of glomerular tuft 'e', hypertrophy of glomerular tuft 'f', and congestion of renal blood vessels 'g'.
Histological sections of hepatic tissue including normal control verse selected diabetic group, arrows highlight as: central vein 'a', hepatic cords 'b', swelling of hepatocytes 'c', Kupffer cell activation 'd', karyomegaly 'e', dysplasia of bile duct 'f' and portal infiltration with inflammatory cell 'g'.
Histological sections of vascular endothelium including normal control verse selected diabetic group, arrows highlight as: intima 'a', media 'b', adventia 'c', elastic fibres 'd', damaged endothelium 'e'.

equimolar concentration from pancreatic β-cells during enzymatic cleavage of prohormone precursor proinsulin [26]. Assessment of C-peptide level as the measure of insulin secretion is more precise analysis as negligible extraction of c-peptide by the liver and longer half-life as compared to insulin [27]. However, recent meta-analysis report on the application of measurement of plasma insulin level has shown the practical importance to confirm the animal screening model for diabetes [28]. In spite of a considerable difference in plasma insulin after STZ administration, numerous researchers do not consider the practical importance of insulin level [26,29]. The release of insulin and c-peptide are interlinked as the source of the release is same for both peptides. Decreased level of serum insulin and C-peptide is the key biomarker of β-cell destruction [30,31]. The present investigation has shown a significant reduction in serum insulin and C-peptide in SCRD and DCRD groups with variant dose regimen of HFD-STZ treated animals that confirmed the chronic induction of diabetes mellitus. Indeed, a small increase in insulin was observed in all subgroups of SCRD and DCRD which may due to the overexpression of β-cell of pancreas under stress condition [32]. The consequence with the significant reduction of serum insulin and C-peptide reduced level-up to 12 weeks duration confirmed the constant T2DM with permanent damage of β-cell of the pancreas [33,34]. A chronic diabetes mellitus significantly leads to dyslipidaemia which is the root cause of major vascular complications including atherosclerosis, endothelial dysfunction, cardio and renal damage [35]. The abrupted lipid profile has significantly explored the presence of dyslipidaemia which is the most relevant metabolic disorder associated with diabetes mellitus [35]. The presence of dyslipidaemia was measured by the increased level of TC, LDL, TG, and decreased level of HDL as compared to normal control. All consequence from the present finding revealed the development of significant dyslipidemic condition for longer duration which has been considered a key marker for the development of T2DM and -associated vascular diseases [35].

Beside of biochemical markers, the structural alteration was measured to confirm the beta-cells destruction which is the common trigger for induction of diabetes mellitus. The portion of islets may disperse throughout the acinar lobule and interlobular ducts. Besides the presence of alpha-cells (glucagon), delta-cells (somatostatin) and other pancreatic polypeptides, the largest portion of islets cells are covered by β-cell which is responsible to secrete insulin [17]. The damage of β-cell sparks the down secretion of insulin. The similar observation was found in present investigation where the acinar cells were swelled and the islets of Langerhans was entirely lost or shrunk which confirm the loss of pancreatic function or complete destruction of β-cell as reported in previous reports [16,17]. On the basis of prime factors including low mortality, low dose (STZ), high sustainability of hyperglycaemia for longer duration and all other biochemical markers DCRD subgroup having treatment of 20 mg/kg was selected for further evaluation of micro and macrovascular complications.

Several clinical and preclinical reports revealed the altered biochemical parameters including increased level of SGOT, SGPT, ALP, and decreased serum albumin in hepatic toxicity [2,14,36]. Additionally, some recent report also revealed the liver damage due to over oxidative stress during chronic diabetes mellitus [36,37]. For the present study,

the effect of chronic diabetes on hepatic cells were assessed by SGOT, SGPT, ALP, albumin level and, the similar alteration has observed in present investigation that revealed the selected animal model of chronic diabetes mellitus has significantly induced experimental hepatic toxicity. Histological alteration also supports the existed report and confirms the structural hepatic damage [14,36,37]. Moreover, the persistent high level of blood glucose during T2DM can significantly increase the oxidative stress [16] which ultimately trigger various destructive signalling cascade including scavenging nitric oxide (NO) that has been considered a key factor for induction of endothelial dysfunction, cardiovascular and renal abnormalities [35,38–40]. The consequences of the present study confirmed the association of diabetes mellitus induced endothelial damage by increasing oxidative stress and reducing NO bioavailability as reported in previous studies [35,38]. The association of cardiovascular damage and the renal injury was confirmed by various biomarkers as suggested by a previous report [35]. Histopathological evaluation of cardio-renal tissue also confirmed late-stage diabetes associated cardio-renal damage in a selected animal model. Thus, altered biochemical marker and histological variation endorsed the late-stage diabetic animal model which can produce chronic micro and macrovascular alteration with a minimum dose of STZ and no sign of mortality for the longer duration. The proposed diabetic model also has some limitations as it has been shown that human T2DM is characterized with a long stage of insulin resistance and associated hyperinsulinemia leading to beta cells dysfunction while STZ treatment in animals leads to an increased insulin sensitivity rather than resistance. Under this context, the HFD intake and weight gain are suggested as the considerable factors for cytosolic ectopic accumulation of fatty acid metabolic (diacylglycerol and ceramides) which underlies the development of insulin resistance in skeletal muscles [41]. Moreover, the high dietary intake depresses electron transport activity, reduces gene expression (responsible for oxidative metabolism) and perturbed mitochondrial morphology in skeletal muscles that leads to metabolic imbalance, insulin resistance [41]. Thus, the combination of HFD intake and low dose STZ significantly upsurge the level of blood plasma glucose by disproportion in insulin release, beta cells destruction, increase oxidative stress and insulin resistance.

Conclusively, a unique and late-stage diabetic model in experimental animals opens the new window to explored new pathway for understanding the pathological events and treatment with advanced molecular approaches. As the preclinical model of late-stage diabetes mellitus to induce sustained hyperglycaemia for longer duration with no mortality rate and low dose of STZ has not introduce before, we have developed and characterized this new animal model which recapitulates the metabolic features of natural history of diabetes in human purely driven β-cell dysfunction (metabolic defect) or without specific gene invalidation or multiple congenic breeding strategies.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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