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Cardiovascular risk factors involved in Type 2 diabetic rat models: A long term preliminary screening

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Abstract

The study was to evaluate the cardiovascular risk factors involved in n-STZ (Split dose Streptozotocin in neonatal rats) and F-STZ (high fat diet and low dose STZ) rat models of type 2 diabetes mellitus (T2DM) on a long term. The n-STZ model was developed using a split dose regimen of STZ (50 mg/kg, i.p.) administered on 2nd and 3rd postnatal days. F-STZ model was developed by high fat diet for 2 weeks followed by low dose of STZ (L-STZ -35 mg/kg, i.p.). Cardiovascular risk factors were evaluated at regular intervals in both models along with fasting blood glucose (FBG) and oral glucose tolerance test (OGTT). The diabetic group in both the model was found to show elevated lipid levels, CK-MB and LDH. Elevated systolic blood pressure (SBP) and along with hypertrophy of heart was observed in n-STZ model which is in contrast to F-STZ model. Both the n-STZ and F-STZ models are appropriate to study the cardiovascular risk factors in T2DM, except SBP in F-STZ model. Obesity was not a characteristic feature in F-STZ model in long term basis.

Key-Words: Type 2 diabetic rat model, High fat diet, Streptozotocin, Blood pressure

Introduction

The prevalence of diabetes all over the world has increased over the recent past and this will continue for the next coming years. The major concerns related to diabetes are the development of micro and macro vascular complications which leads to morbidity and mortality associated with the disease [1]. The progression of the disease from pre diabetic state to overt diabetes and the development of complications take many years. To study both the pathogenesis and potential therapeutic agents, appropriate animal models of type 2 diabetes mellitus (T2DM) are required [1]. The animal models should mimic the human diseased conditions and the development of complications should be studied on long term basis. There is a need for such animal models because investigators were unsuccessful to recreate in experimental animals the complications of diabetes seen in humans [2]. In spite of emerging of genetic models of T2DM, the reproducibility of most of the phenotypes in animals was challenging and also not economical. The newer chemical entities screened for their anti-diabetic activity should also be screened for their effects on the complications of the disease.

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The screening of animal models will help in understanding the disease pathogenesis, prevention and the treatment as well. In the present study an attempt has been made to study the cardiovascular risk factors involved in the presently available T2DM rat models. Two appropriate rat models namely a split dose regimen of streptozotocin (STZ) to induce diabetes in a neonatal rat model (n-STZ model) [3] and combination of high fat diet and low dose STZ treated rats (F-STZ model) [4] were chosen for the study. The reason behind selection of these two models are as follows: Induction of diabetes in n-STZ model using a split dose on 2nd and 3rd day of post natal days was refinement of previous studies with advantages such as reduction in mortality, high success rate, economical and reliable hyperglycemic response [5]. n-STZ rat model is considered to be one of the suitable experimental animal models of T2DM [6]. On the other hand F-STZ model was unique and different from other combination rat models; the dose of STZ (35mg/kg) selected causes diabetes only in HFD-fed insulin resistant rats but it failed to induce the same in normal control rats resembling the situation in humans with risk factors of obesity and insulin resistance to be more prone to T2DM than others [4]. Thus, the study was started with the objective of evaluating some cardiovascular risk factors such as blood pressure, heart rate and markers for myocardial damage along with lipid profile and adiposity index.

Material and Methods

Materials

STZ was purchased from Sigma Aldrich, Germany. The feed ingredients such as casein (Casein India pvt. ltd), Ingredient required to prepare AIN[76] Vitamin and mineral mix (all from Himedia laboratories, Mumbai, India), Cholesterol and yeast powder (Loba Chemie, Mumbai, India), were procured from the commercial sources. Hydrogenated vegetable fat (HVF) **was** obtained from Ruchi gold, India,

Animals

Male Wistar rats (160–180 g) and neonatal rats procured from in-house animal facility were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature (22 ± 2 ^{o}C) and humidity (55 ± 5%) with 12:12 h light and dark cycle. All the rats were fed with commercially available rat normal pellet diet (NPD) procured from Amrut Diet, New Delhi and water ad libitum, prior to the dietary manipulation. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Govt. of India were followed and prior permission was sought from the institutional animal ethics committee for conducting the study.

Development of T2DM models

2.3.1 Development of n-STZ rat model

STZ was administered to neonatal rats on the 2nd and 3rd postnatal day in the dose of 50 mg/kg/day intraperitoneally (n-STZ group) [3], and vehicle (citrate buffer) was administered to control group. After 28 days, the pups were weaned and fasting blood glucose (FBG) and Oral glucose tolerance test (OGTT) were carried out at 8. The OGTT was carried out in response to 2 g/kg oral glucose using 50% w/v glucose solution. The animals having FBG ≤ 100mg/dl with impaired glucose tolerance (IGT) were considered to be in prediabetic state, which were likely to develop FBG ≥ 100 mg/dl in few days. The rats with FBG ≥ 100 mg/dl after 12 weeks of n-STZ administration were considered to be diabetic and included in the study.

2.3.2 Combination of High fat diet (HFD) and low dose STZ [4] (F-STZ rat model)

The adult rats were allocated into two dietary regimens consisting of 12 and 12 rats by feeding either NPD or HFD (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) ad libitum, respectively, for the initial period of 14 days. After the 14 days of dietary manipulation, a subset of rats (12 rats) fed with HFD was injected intraperitoneally with low dose of STZ (L-STZ) (35 mg/kg), while the rats fed with NPD (NPD group) were given vehicle i.e. citrate buffer (pH 4.5). The HFD used in the present study was slightly modified when compared to the earlier study [4] where lard was replaced with HVF and Vitamin and mineral mix (AIN[76]). The preparation of HFD was in line with earlier study [7]. At the end of 21 days i.e. 7 days after STZ and citrate buffer administration in HFD and NPD groups, respectively, the FBG and OGTT were carried out and were considered as 0 week of study period. The OGTT was carried out in response to 2 g/kg oral glucose. The rats with FBG ≥ 100 mg/dl were considered to be diabetic and were included for the further study. HFD and NPD diet for respective group were continued until the end of the study.

Collection of blood and biochemical analysis

Blood samples were collected from retro-orbital plexus of the rats under light ether anesthesia using capillary tubes into eppendorf tubes and were left aside to clot. The blood sample was centrifuged (5min, 8000 rpm) and the serum was analyzed for glucose (GOD-POD), triglycerides (GPO-POD) total cholesterol and HDL cholesterol (CHOD-POD) levels by using commercially available colorimetric diagnostic kits using Artos semi-auto analyzer (Swemed diagnostics, Bangalore, India). For OGTT estimation, blood samples were collected at 0, 15, 30, 60, 90 and 120 minutes.

Estimation of enzyme activity

Creatine Kinase-MB (CK-MB) and Lactate dehydrogenase (LDH) estimations were carried out by diluting the serum samples using potassium phosphate buffer pH 7.4 (100 mmol/L) using commercially available kits (Swemed diagnostics, Bangalore India).

Blood pressure and Heart rate

The rats were taken to the experimental room 4 hours before systolic blood pressure (SBP) recording. The rats were kept in a restrainer; tail cuff along with pulse detector connected to a data acquisition system (Power lab, Australia) was placed at the base of the tail. After confinement in a holder for 10-15 minutes at room temperature, tail pulsations were large enough for accurate estimation of SBP. Each recording was obtained by averaging 5-8 individual readings. The heart rate (HR) was calculated simultaneously.

Adiposity index

Body weight

The body weight of the animals was recorded every week throughout the study period. The total weight gain in the study period was calculated using the difference between the final body weight and the initial body weight observed.

Fat pad weights (FPW)

At the end of the study, perirenal, mesenteric, epididymal and pericardial fat pads were isolated from all the rats. The fat pads were weighed immediately to

obtain wet FPW and are expressed in g per 100 g of body weight. The sum of all FPW was taken as total fat pad weight (TFPW). TFPW value represents the overall adipose mass present in the rats.

The adiposity index (ADI) was calculated using the following formula [7]:

$$ADI = \frac{TFPW}{(bodyweight - TFPW)} x100$$

Statistical analysis

The results are expressed as mean ± S.E.M. The unpaired Student's t-test was used for analyzing the data between two groups, where as one-way ANOVA followed by multiple comparison test (Tukey's multiple comparison test) was employed when there were more than two groups. A value of $p<0.05$ was considered statistically significant.

Results and Discussion

Biochemical parameters in n-STZ model

At 8 weeks in n-STZ group, FBG did not increase significantly, while the AUCg significantly ($P<0.001$) increased when compared with the control group at 12 weeks. FBG, AUCg, TG and CK-MB significantly ($P<0.001$) increased, when compared with the control group and the levels were maintained up to 24 weeks after administration of STZ. LDH levels significantly increased ($P<0.05$) after 12 weeks and further significantly ($P<0.001$) increased after 16 weeks and the levels were maintained up to 24 weeks. TC levels elevated significantly ($P<0.05$) at 16 weeks and further significantly increased, $P<0.001$ and $P<0.01$ at 20 and 24 weeks respectively (Table 1).

Biochemical parameters in F-STZ model

Injection of STZ (35 mg/kg, i.p.) after two weeks of HFD, significant increase ($P<0.001$) was seen in FBG, AUCg, lipid profile (except TC showed significant increase up to $P<0.01$ at 0 weeks and further increased significantly up to $P<0.001$ throughout the study period) and LDH levels in F-STZ group, when compared to the NPD group; moreover the levels were maintained throughout the study period. CK-MB levels significantly increased ($P<0.05$) at 6 and 12 weeks, further significantly increased ($P<0.001$) at 18 weeks of the study period in F-STZ group when compared to the NPD group (Table 2).

Systolic Blood pressure and Heart rate in n-STZ and F-STZ model

SBP in n-STZ group was significantly ($P<0.05$) elevated only at 20 weeks after STZ administration and further increased significantly ($P<0.001$) at 24 weeks when compared the control group (Figure 1). In contrast, SBP in F-STZ group significantly ($P<0.001$) lowered at 6 to 18 weeks of the study period when compared with the NPD group (Figure 2). There was no significant change in heart rate in n-STZ group when compared with the control group. However, significant reduction ($P<0.001$) in heart rate was observed in the F-STZ group when compared with the NPD group (Figure 3).

Body weight gain in n-STZ and F-STZ rat model

Body weight gain calculated at different time intervals of the study period in the n-STZ group showed significant reduction ($P<0.01$ during 12-16 weeks and $P<0.001$ during 16-20 and 20-24 weeks) in body weight when compared with the control group (Figure 4). There was no significant change in the body weight at different time intervals of the study period in the F-STZ group when compared to the NPD group (Figure 5).

Fat pad weight and organ weight in n-STZ and F-STZ rat model

In n-STZ model, at the end of the study, there was no significant change in the FPW in the n-STZ group when compared with the control group except the pericardial fat pad showed significant ($P<0.05$) reduction in weight (Table 3). The ADI was significantly ($P<0.05$) decreased in the n-STZ group when compared with the control group. In the other model, the perirenal, mesenteric FPW and ADI decreased significantly ($P<0.001$) in the F-STZ group when compared to the NPD group. Heart weight significantly ($P<0.05$) increased and liver weight significantly ($P<0.01$) decreased in the n-STZ group when compared to the control group. Heart weight significantly reduced ($P<0.05$) in the F-STZ group when compared with the NPD group.

The n-STZ model and the F-STZ models of T2DM of the present study were found to show mild to moderate hyperglycemia. The elevated FBG and IGT in the n-STZ model at 12 weeks were maintained up to 24 weeks of the study period which indicates that the split dose administration of STZ on 2nd and 3rd postnatal days could induce stable moderate hyperglycemia. The FBG observed in the diabetic group of F-STZ model was found to be mild in the initial stages of the study period and gradually elevated towards the end of the study. The mild to moderate FBG may be due to the usage of HVF in the diet instead of animal fat [7]. IGT was observed in the diabetic group as well as in the HFD group but existed only for few weeks in the later. This observation from the HFD group creates an element of doubt whether insulin resistance existed throughout the study period in the F-STZ model.

The increase in the lipid levels observed in both the models can be considered as one of the major cardiovascular risk factor which is mostly seen in

T2DM patients. The AI was found to be elevated in both the models but only at later stages in the n-STZ model. This shows the importance of long term observation which would be unnoticed in short term studies.

The increase in SBP observed in the n-STZ model was found to be in concordance with previous report where in the neonatal T2DM rat model using 70mg/kg of STZ on day 5 showed elevation in SBP 12 weeks after n-STZ administration [8]. However, elevation of SBP, reported by Gokhale and co-workers [8] was studied for short span of time, whereas in the present work SBP was recorded from 8 to 24 weeks after n-STZ administration at every 4 weeks interval, to give a clear picture of induction of hypertension in the neonatal model. Thus, the supporting results in both the studies suggest that the SBP was elevated in n-STZ administered T2DM model, unlike the question of incidence of hypertension in STZ induced adult diabetic rat models. The elevation of SBP in the present study was found to be seen along with elevation of the TC levels, TC: HDL ratio and AI. The reason behind elevation of SBP in previous report was correlated with increase in insulin levels [8] (insulin resistance), however insulin administered in rats with hypertension was found to produce hypotension [9], and thus the mechanism of incidence of SBP in n-STZ administered T2DM rat model was unclear yet. It can be because of elevation in TC levels, TC: HDL ratio and AI which might be the causes for hypertension in the present study. The incidence of hypertension in the present study may be as a result of oxidative stress developed due to hyperglycemia existing in the rats. STZ administration causing increase in free radicals which in turn inducing oxidative stress and endothelial dysfunction was found to be reported in the earlier studies [10-12]. Nitric oxide (NO) is one of the most important factors causing vasodilatation. The increased free radicals quench the NO; thereby preventing release of it when required, thus leading to hypertension may be one of the reasons.

In spite of elevated FBG levels and elevated lipid levels, the F-STZ model did not show any significant elevation in SBP in the present study.

A slight decrease in the mean HR values was observed at 20 and 24 weeks after n-STZ administration as in Goto-Kakizaki rats (GK rats), one of the similar models of T2DM as n-STZ diabetic rat model [16]. Both the models were found to show elevated CK-MB and LDH levels which indicates myocardial damage in them.

Heart weight to body weight ratio observed in the present study was significantly increased in the n-STZ administered group of rats when compared with the control group. The increase in heart to body weight ratio shows the hypertrophied hearts in the diabetic rats which is in contrast with F-STZ model. The presence of insulin resistance in fat-fed/STZ model as reported by Srinivasan and co-workers [7] was well supported by other study where ADIPOR1 and ADIPOR2 the newly identified receptors for adiponectin were down regulated [47]. Both the receptors mentioned above were positively correlated with glucose disposal, thus down regulation of these was responsible for insulin resistance in peripheral tissues. This might be the strong reason for elevation of AUCg in the diabetic group, indicating IGT which may be due to insulin resistance.

Also the TFPW and ADI were found to be significantly increased in the F-STZ model in the study while the body weight gain in the F-STZ model was observed only in the initial stages of the study. This shows that adipogenesis and lipolysis were seen in fat-fed/STZ model as reported earlier where they demonstrated increase in mRNA levels of adiponectin and leptin genes in the same model [47].

Conclusion

The n-STZ T2DM model possesses adequate cardiovascular risk factors such as hypertension, abnormal lipid profile, increased AI and elevation in LDH and CK-MB activity. The ADI was less in this model; hence obesity is not a characteristic phenotype of this model. However, the split dose n-STZ induced T2DM matches most of the characteristic features of human T2DM and was found to be more appropriate model to study the cardiovascular effects of T2DM. Also, since the FBG levels were found to be maintained in the same range from 12 to 24 weeks, the model helps us to study the long term effects of drugs *in vivo*. On the other hand, F-STZ model showed all abnormalities like n-STZ model but did not show elevated SBP, hypertrophied hearts and decreased ADI.

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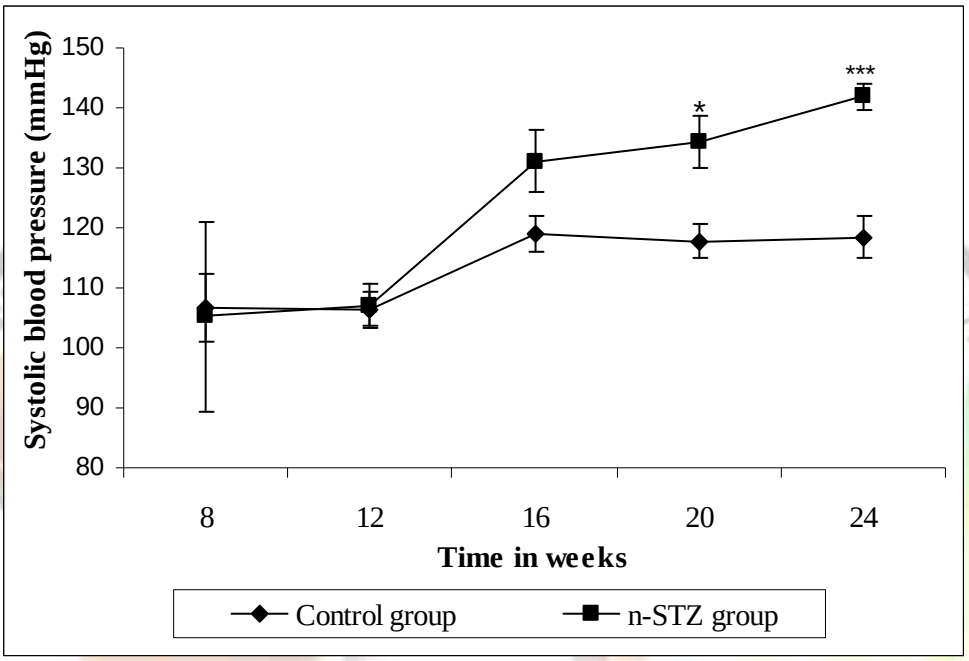

Fig. 1: Blood pressure recording in n-STZ model. Values expressed in mean ± SEM. * (P<0.001), ** (P<0.01) and * (P<0.05) Vs control group**

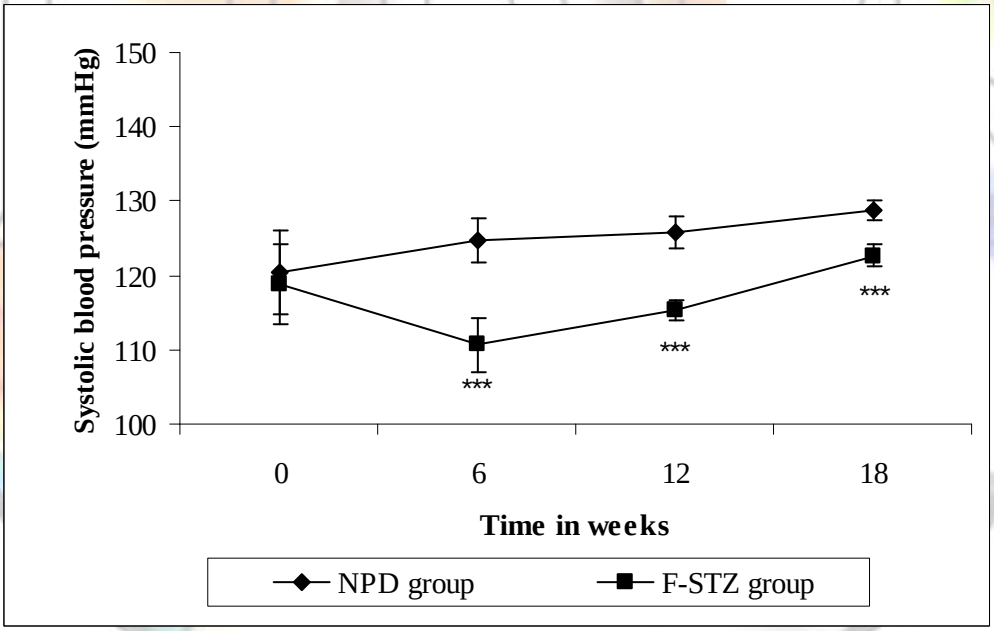

Fig. 2: Blood pressure recording in F-STZ model. Values expressed in mean ± SEM. * (P<0.001), ** (P<0.01) and * (P<0.05) Vs control group**

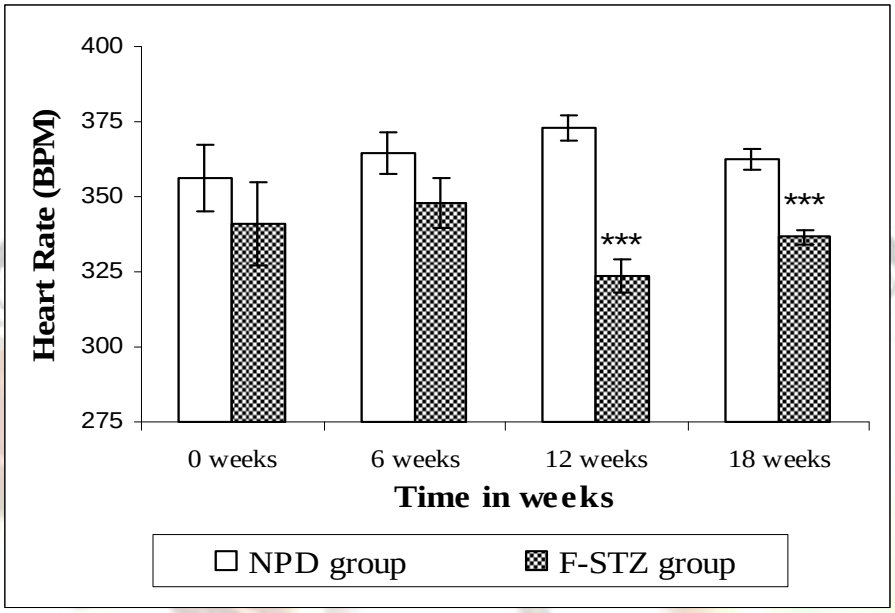

Fig. 3: Heart Rate recording in F-STZ model. Values expressed in mean ± SEM, * (P<0.001), ** (P<0.01) and * (P<0.05) Vs control group**

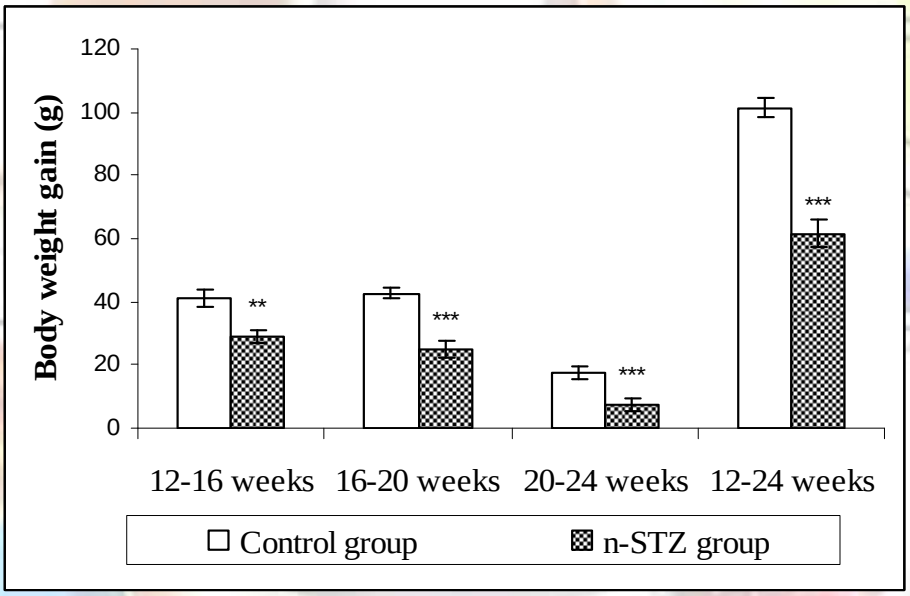

Fig. 4: Body weight gain in n-STZ model at different study period. Values expressed in Mean ± SEM. * (P<0.001),** (P<0.01) and * (P<0.05)**

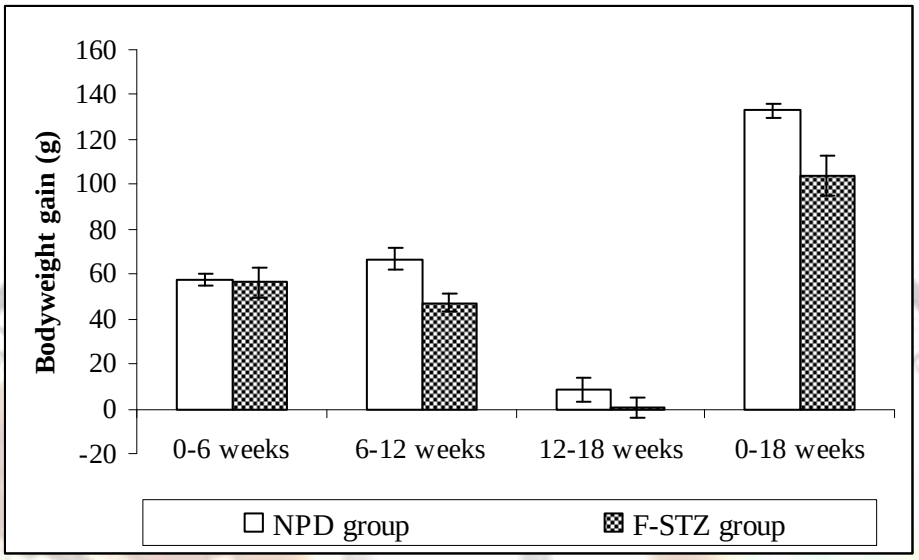

Fig. 5: Body weight gain in F-STZ model at different study period. Values expressed in Mean ± SEM. * (P<0.001), ** (P<0.01) and * (P<0.05)**

Table I: Serum biochemical parameters in n-STZ rat model

Study Period (Weeks)	Groups	FBG (mg/dl)	AUCg (g/dl*min)	TG (mg/dl)	TC (mg/dl)	AI	CK-MB activity U/L	LDH activity U/L
8	Control	65.62 ± 3.97	10.96 ± 0.43	ND #	ND	ND	ND	ND
	n-STZ	76.92 ± 2.6	22.24 ± 1.32***	ND	ND	ND	ND	ND
12	Control	66.28 ± 1.96	13.9 ± 0.12	84.24 ± 5.98	65.3 ± 4.46	0.63 ± 0.11	1292.5 ± 74.1	829.01 ± 91.63
	n-STZ	123.55 ± 5.28***	29.86 ± 1.46***	116.7 ± 6.78***	75.97 ± 1.44	0.61 ± 0.04	1822.7 ± 64.4***	1284.5 ± 84.33*
16	Control	70.98 ± 1.08	14 ± 0.1	83.58 ± 5.09	67.79 ± 2.6	0.47 ± 0.04	1406.9 ± 84.9	808.97 ± 33.81
	n-STZ	125.62 ± 6.60***	31 ± 2.43***	117.1 ± 4.77**	85.5 ± 5.32*	0.71 ± 0.07	2204.1 ± 97.4***	2209.6 ± 170.8***
20	Control	71.59 ± 1.54	14.32 ± 0.41	84.17 ± 4.99	70.38 ± 2.46	0.68 ± 0.03	1503.5 ± 74.3	699.65 ± 47.34
	n-STZ	131.69 ± 4.34***	32.72 ± 1.4***	123.2 ± 4.82***	88.56 ± 2.94***	0.98 ± 0.05***	2113.5 ± 64.7***	2527.3 ± 173.5***
24	Control	73.67 ± 1.10	15.07 ± 0.15	81.68 ± 3.82	74.75 ± 1.00	0.62 ± 0.02	1528.3 ± 41.3	949.26 ± 50.20
	n-STZ	130.79 ± 3.28***	33.36 ± 1.16***	117 ± 4.06***	93.19 ± 3.12**	1.23 ± 0.08***	2092 ± 73***	2134.4 ± 153.3***

Values are Mean ± SEM; #: not determined. The abbreviations denote FBG: Fasting blood glucose, AUCg: Area under the curve for glucose values from 0-120 minutes, TG: Triglycerides, TC: Total cholesterol, AI: Atherogenic index (AI = TC-HDL cholesterol divided by HDL-cholesterol). *** (P<0.001), ** (P<0.01) and *(P<0.05) Vs Control group.

Table II: Serum biochemical parameters in F-STZ rat model

Study Period (Weeks)	Groups	FBG (mg/dl)	AUCg (g/dl*min)	TG (mg/dl)	TC (mg/dl)	AI	CK-MB activity U/L	LDH activity U/L
0	NPD	70.95 ± 1.95	13.74 ± 0.19	78 ± 1.45	67.28 ± 1.11	0.37 ± 0.03	1264.9 ± 75.03	748.2 ± 50.55
	F-STZ	97.32 ± 2.27***	27.77 ± 0.62***	128.66 ± 6.44***	85.22 ± 2.1**	0.89 ± 0.08***	1073.02 ± 57.04	1995.1 ± 142.5***
6	NPD	78.21 ± 1.79	14.20 ± 0.26	81.6 ± 1.79	72.27 ± 1.42	0.46 ± 0.02	1066.8 ± 48.68	847.2 ± 55.19
	F-STZ	129.99 ± 4.71***	38.65 ± 1.56***	156.77 ± 7.69***	117.35 ± 4.3***	1.29 ± 0.13***	1465.09 ± 120.8*	2306.0 ± 142.01***
12	NPD	75.88 ± 2.01	13.38 ± 0.30	86.73 ± 2.91	73.25 ± 1.64	0.54 ± 0.04	1293.8 ± 66.94	843.8 ± 107.94
	F-STZ	152.55 ± 4.72***	37.55 ± 1.16***	176.63 ± 7.32***	127.09 ± 3.66***	1.38 ± 0.09***	1681.75 ± 109.03*	2343.5 ± 82.53***
18	NPD	73.49 ± 1.39	13.70 ± 0.47	84.62 ± 2.07	73.24 ± 1.21	0.51 ± 0.01	1145.24 ± 66.94	1089.8 ± 121.93
	F-STZ	156.83 ± 2.87***	41.10 ± 1.09***	176.13 ± 5.49***	127.69 ± 3.11***	1.43 ± 0.05***	1694.13 ± 63.61***	2483.6 ± 142.8***

Table III: Fat pad weights and organ weight in T2DM model

Parameters	Weight in gms / 100 gms of body weight			
	n-STZ model		F-STZ model	
	Control group	n-STZ group	NPD group	F-STZ group
FPW				
Perirenal	0.56 ± 0.01	0.46 ± 0.05	0.49 ± 0.02	0.88 ± 0.02***
Mesenteric	0.77 ± 0.02	0.63 ± 0.03	0.69 ± 0.01	0.59 ± 0.02***
Epididymal	1.01 ± 0.04	0.90 ± 0.06	0.97 ± 0.03	1 ± 0.02
Pericardial	0.07 ± 0	0.02 ± 0***	0.06 ± 0	0.05 ± 0
ADI	2.46 ± 0.03	2.09 ± 0.13*	2.26 ± 0.03	2.59 ± 0.06***
Organ weights				
Kidney	0.68 ± 0.01	0.72 ± 0.03	0.70 ± 0.01	0.67 ± 0.04
Heart	0.30 ± 0.00	0.38 ± 0.03*	0.30 ± 0	0.28 ± 0*
Liver	3.75 ± 0.08	3.04 ± 0.20**	3.67 ± 0.06	3.77 ± 0.07
Spleen	0.32 ± 0.01	0.30 ± 0.01	0.29 ± 0.03	0.24 ± 0.02

Values are Mean ± SEM; The abbreviations denote FPW : Fat pad weight, ADI: Adiposity index.***(P<0.001), **(P<0.01) and *(P<0.05) Vs Control