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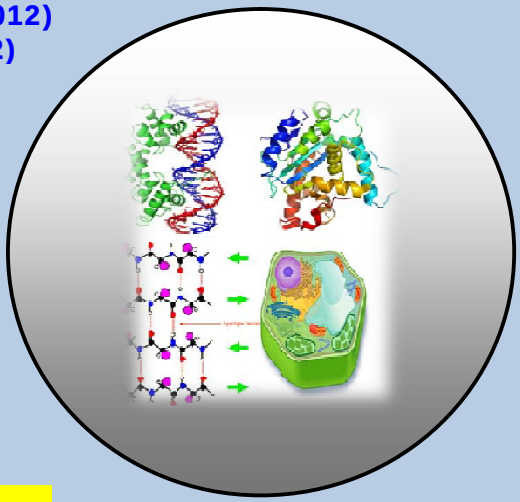

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RESEARCH PAPER

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A Long Term High Fat Diet/Low Dose Streptozotocin Develops a Type 2 Diabetic Rat Model Showing Clinical Presentation and Pathophysiology of Natural History of Diabetes

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ABSTRACT

Many animal models are representing and claiming as non-insulin dependent type 2 diabetic models but none of them showed clinical presentation and pathophysiology of natural history of diabetes in human. Our study presents a new progressive type 2 diabetic rat model that closely simulates with human type 2 diabetic condition. Wistar rats were fed with high fat diet (HFD) for an unspecified period of time to study the metabolic changes occurred. This was followed by another study with same HFD for 6 months followed by a low dose streptozotocin (30 mg/kg) injection. Four distinct stages were observed in the first experiment. Stage 1, covering first two months, was without any abnormality. Stage 2 comprising of 3rd month (FBG <100 mg/dl) with impaired glucose tolerance, and stage 3 comprising of 4-6 months (FBG >100 mg/dl) with impaired glucose tolerance, respectively, were the dynamic periods of metabolic abnormalities. Stage 4, from 7-9 months with no significant changes. Diabetic state (FBG >200 mg/dl) was obtained by injecting streptozotocin after 6 months of HFD feeding. Progressive changes in insulin resistance, adiponectin, glucose uptake and Glut4 gene expression were observed during stages 2 and 3 as well as after streptozotocin injection. Our model represents a new progressive type 2 diabetic rat model with distinct stages i.e. insulin resistance and pre-diabetes which are the pathophysiology of natural history of type 2 diabetes in human.

Running Title: HFD/Low Dose STZ Develops T2D in Rat Model

Keywords: Animal model, Type 2 diabetic rats, High fat diet, Streptozotocin and Glut4 mRNA.

INTRODUCTION

Diabetes is one of the most challenging public health concerns whose number of incidences is getting worse. People are still waiting for its remedy. A large number of drugs are being produced and screened for their efficacy. Screening of the anti-diabetic drugs is another concern of scientists. For this purpose they are producing and trying different animal models but no model, up till now, is claimed as perfect representing actual human pathological conditions. There are many animal models representing and claiming as non-insulin dependent type 2 diabetic models but none of them was given a status of a true model. That is why, different animal models are being represented till today and efforts to develop more realistic model is still under the way.

Type2 diabetes can be defined as a state of metabolic disorder occurred through the progressive decline in the insulin action for an indefinite period of time followed by the intoxication of β-cells resulting in the less production of insulin relative to the body demand [K. Srinivasan, B. Viswanad, A. Lydia, C.L. Kaul and P. Ramarao (2005)]. The increase in the prevalence of type 2 diabetes is closely linked to the upsurge in obesity. Obesity as a major factor is involved in the development of insulin resistance which ultimately converted into type 2 diabetes [J.M. Olefsky (2001)]. Now it is well accepted that insulin resistance as well as obesity, both has an outstanding role in the onset of type 2 diabetes [J. Wong, L. Molyneaux, D. Zhao, M. Constantino, R.S. Gray, S.M. Twigg, Z.R. Xu and D.K. Yue (2008)]. Obesity is one of the top most worldwide problems of today which, along with insulin resistance and type2 diabetes, is associated with hypertension, hyperlipidemia, atherosclerosis and other cardiovascular diseases. In the way of developing type 2 diabetes, insulin resistance is a characteristic intermediate stage which occurs and expend many years prior to the development of diabetes [J.H. Warram, B.C. Martin, A.S. Krolewski, J.S. Soeldner and C.R. Kahn (1990)]. Continuation of insulin resistance may lead to pancreatic β-cells dysfunction and their combination lead to the hyperglycemic type 2 diabetes [J.M. Olefsky (2001)]. On the basis of this linkage between obesity and type 2 diabetes, chronic high fat diet (HFD) feeding to the Wistar rats seems a realistic approach towards the development of type 2 diabetes through obesity and insulin resistance in the way of developing a better animal model of type 2 diabetes. Many researchers have worked on development of HFD-induced type 2 diabetic models and claimed for the close resemblance with human diabetic conditions [M.J. Reed, K. Meszaros, L.J. Entes, M.D. Claypool, J.G. Pinkett, T.M. Gadbois and G.M. Reaven (2000); K.V. Axen, A. Dikeakos, A. Sclafani (2003) and M.S. Islam, H. Choi (2007)], but they all have some shortcomings. A major issue is reproducibility in which other groups could not prepare the same model due to the standardization issues of HFD that is, percentage (%) of fat contents in the prepared feed rather than the added quantity, proportion of saturated to unsaturated fatty acids, type and proportion of individual fatty acids etc. Another issue is that focus of the many workers in the way of developing new models are on the physiological and pathological conditions of type 2 diabetes and in this way, they often bypass the critical progressive stages towards the development of diabetes.

The present study is, therefore, planned to investigate the metabolic changes occurred during the long term chronic HFD feeding in the way to develop a progressive rat model having distinct stages towards type 2 diabetes.

MATERIAL AND METHODS

Animals and experimental design

Male Wistar rats weighing (145-160 g) were used in this study. They were maintained at standard environmental conditions that is temperature 25 ± 2 °C, relative humidity 50-55% and 12/12 hours light & dark cycles. The rats were housed in plastic cages. After one week of acclimatization period, rats was fed with HFD enriched with 40% animal butter with the exception of control one that was fed on normal standardized diet without additional fat. Detail composition of HFD and its fatty acid composition are given in Tables 1a & b, respectively. The animals were fed HFD continuously for an unspecified period of time and periodic evaluation of their body weight, fasting blood glucose and glucose tolerance level were performed. In another group of experiment, the rats were fed HFD for 6 months followed by 30 mg/kg streptozotocin (Wako Pure Chemical, Osaka, Japan) injection intravenously through tail vein and monitored the metabolic changes occurred. All animals were treated according to the guidelines for care and use of laboratory animals with the approval of Institutional Ethics Committee of the ICCBS, University of Karachi, Pakistan. At the time of evaluation, the animals were fasted for 14-16 h in order to perform FBG and oral glucose tolerance test (OGTT). FBG was checked by hand held Glucometer (Accu Check, Roche) after pricking the tail vein. Then for OGTT, the rats were given glucose solution (2 g/kg) orally followed by the determination of blood glucose at 0.5, 1, 2 and 3 hours. Next day (day 2), insulin tolerance test (ITT) was performed. At day 3, after an overnight fast, blood was taken from the tail after which the animals were anesthesized with diethyl ether. Epididymal fat pads were rapidly isolated. After isolation, half the tissue was rapidly frozen in liquid nitrogen and kept at -80 °C until analysis. The other half of the tissue was also rapidly washed with warm phosphate buffer pH 7.4 and immediately put for the isolation of adipocytes. Serum was separated from the collected blood samples for the determination of biochemical parameters.

Insulin and adiponectin analysis

Serum insulin and adiponectin concentrations were determined by using ELISA kits. Rat insulin determination kit (DRG Instruments GmbH, Marburg, Germany) and rat adiponectin kit (Chemicon International, MA, USA) were used for this purpose.

Insulin tolerance test

In order to perform whole body insulin sensitivity, insulin tolerance test was performed [R. Buettner, K.G. Parhofer, M. Woenckhaus C.E. Wrede, L.A. Kunz-Schughart, J. Schölmerich and L.C. Bollheimer (2006)]. Briefly, after 14 h fasting, glucose was determined periodically till uniformity. After that, 0.15 U/kg insulin was injected intra-peritoneally followed by the monitoring of blood glucose level in 10 min interval till 30 min.

Adipocytes isolation

The epididymal fat pads were removed from the border of epididymis and put into sterile normal saline in a plastic petridish under sterile conditions.

The blood vessels in the fat pads were removed as much as possible and washed these fat pads several times with sterile normal saline at 37 °C to remove the remaining blood vessels and blood clots [P. Bjorntorp, M. Karlsson, H. Pertoft P. Pettersson, L. Sjostrom and U. Smith (1978)]. Isolated epididymal adipose tissues weighted 1 g from each animal were subjected to digestion in Erlenmeyer flask containing 10 ml Krebs-Ringer bicarbonate buffer consisting of 0.1M HEPES buffer, 1.5% w/v bovine serum albumin (BSA), 0.12 M NaCl, 0.05 M KCl, 0.001 M $CaCl_2$, 0.005 M glucose and 2 mg/ml collagenase (Sigma, St. Louis, MO, USA). This solution was incubated for 30 minutes at 37 °C in a shaking incubator. After incubation, the tissue remnants were removed by passing through a nylon screen with a mesh of 250 µm into a siliconized test tube. This suspension was briefly centrifuged for 1 minute and waited for few minutes to complete the floatation of the adipocytes on the surface. The infranatant was then aspirated through a siliconized pipette.

Deoxy D-glucose (2DG) uptake assay

The isolated adipocytes were subjected to glucose uptake assay by using radio labeled 2DG (MP Biomedicals, CA, USA) as described by X.Y. Park, Y.H. Choi, W. Lee (2008). Briefly, adipocytes suspensions were incubated in freshly prepared KRB buffer containing 1% BSA 30 minutes prior to the assay. Adipocytes were then stabilized for 1 hour in incubation vials at 37 °C with or without insulin. In insulin containing vials, the 100 nM insulin was added after 40 minutes during the stabilization period (60 min). 2DG uptake was measured by adding 2DG (2 µM) with the tracer amount of radio labeled 2DG to the cell suspension for 5 min at 37 °C. Phloretin (0.1 mM) was added to stop the further uptake of glucose. Then each vial was mixed with 0.1 ml silicon oil and centrifuged at 6,000 rpm for 1 minute to separate the adipocyte in the oil layer. The adipocytes were then placed in separate vials containing 3 ml scintillation fluid [R. Vettor, R. Fabris, R. Serra, A.M. Lombardi, C. Tonello, M. Granzotto, M.O. Marzolo, M.O. Carruba, D. Ricquier, G. Federspil and E. Nisoli E (2002)] and measured the radioactivity with a liquid scintillation counter (Beckman Coulter, Foster City, CA). The assay was also done after adding 50 µM cytochalasin B (MP Biomedical) to subtract that glucose which was uptaken non-specifically or present on extracellular region.

Semi-quantitative mRNA expression analysis

Total RNA was isolated from the frozen part of the adipose tissues by using TRIAzol reagent according to the manufacturer's instruction (Promega, Madison, WI 53711 USA). Total RNA from 5-7 rats in each group was pooled and aliquots were subjected to further analysis. The concentration and purity of RNA was determined spectrophotometrically by taking their absorbance at 260 nm and 260/280 ratio respectively.

RT-PCR analysis was performed as described by J.C. Hans, S.Y. Park, B.G. Hah, G.H. Choi, Y.K. Kim, T.H. Kwon, E.K. Kim, M. Lachaal, C.Y. Jung and W. Lee (2003). The PCR conditions were established by considering the scope of the analysis that is comparison between the expressions of transcript so that the numbers of cycles were kept at minimum possible number. One (1) µg total RNA was used as template in order to determine the relative expression level of Glut4 mRNA. Briefly, RNAs were treated at 37 °C for 1 h with RNAse-free DNAse in 100 mM Tris-HCl, pH 7.5, and 50 mM $MgCl_2$. Reverse-transcription was done with Moloney murine leukemia virus reverse transcriptase (Promega) in 20 ml of buffer containing 0.4 mM dNTPs, 2 units/ml RNAse inhibitor, 0.8 mg oligo (dT) 15 primer

(Promega). PCR was performed using Taq DNA polymerase (Promega) in 25 µl of standard buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$ and 200 mM dNTPs) and 1 nmol of each specific oligonucleotide primers. The primer sequences were: 5′-GCTTCTGTTGCCCTTCTGTC-3′ (sense) and 5′-AGTGGACGCTCTCTTTCCAA-3′ (anti-sense) manufactured by Gene link, USA. The gen was amplified using 30 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by 5 min final extension at 72 °C. Transcription level of β-actin was used as control with the same amount of RNA and thermal cycles. RT-PCR products were subjected to 2% agarose gel electrophoresis containing ethedium bromide. Images of the gel were pictured and recorded by using the computer aided gel imaging system (BioRad, Hercules, CA, USA). Bands of Glut4 product were normalized to that of β-actin followed by the calculation of relative band intensities of each sample.

HOMA-IR index and ß-cell functions derivation

HOMA-IR index was calculated from fasting glucose and fasting serum insulin by using homeostasis model assessment (HOMA) calculator (version 2), where HOMA-IR indicates insulin resistance index.

The correlation between insulin releases to that of insulin sensitivity was derived on a hypothetical scale described by S.E. Kahn, R.L. Hull, K.M. Utzschneider (2006).

Statistical analysis

All of the data are presented as means ± SEM from 7-11 rats/group. The statistical analyses were performed using SPSS for Windows (version 12, SPSS Inc., Chicago, IL, USA). Data were analyzed using one way ANOVA with post-hoc Bonferroni. Statistical significance is indicated by $P < 0.05$.

RESULTS *Changes of body weight, fasting blood glucose and 2h blood glucose of HFD-fed rats*

In persuasion of body weight gain of HFD fed rats, it was found that the body weights were increased gradually both in control and HFD fed rats from the very first week of experiment whereas the difference between the two groups also arose gradually as expected. The gradual increase lasted for 6 months followed by a stable period of 3 months in the control rats whereas in HFD fed rats, decline in the body weight occurs but very slowly and the difference was <50 g in 3 months period (Fig. 1a).

Table 1(a) Composition of experimental high fat diet (HFD)

Ingredients	Normal diet (g/kg)	HFD (g/kg)
Wheat flour	290	194
Oat flour	290	194
Gram flour	194	140
Corn gluten	145	115
Fish meal	50	50
Milk powder	6	6
Vitamin supplement	0.4	0.4
Edible vegetable oil	24	-
Butter oil	-	300

The body weight gain curve shows 4 distinct regions. First one comprising of 0-4 months with fastest rate of weight gain followed by the second region (4-6 month) were the rate of weight gain is relatively slow. This is followed by another 2 regions with no growth (6-7 month) and decline in body weight (7-9 month), respectively. In comparing, the HFD fed to that of control rats, the average body weight differences after 6, 7, 8 and 9 months were 169, 163, 144 and 128 g, respectively (Fig. 1a).

Table 1(b) Fatty acid profiles of HFD. The values represent the composition (g/100g); given are the maen±S.D. of three independent experiments

Fatty acid	Composition (g/100g)
Butyric acid (C4:0)	0.70 ± 0.19
Caproic acid (C6:0)	0.47 ± 0.22
Caprylic acid (C8:0)	0.29 ± 0.04
Capric acid (C10:0)	0.87 ± 0.10
Lauric acid (C12:0)	1.85 ± 0.13
Myristic acid (C14:0)	5.26 ± 0.24
Myristoleic acid (C14:1)	0.81 ± 0.04
Pentadecanoic acid (C15:0)	0.88 ± 0.02
cis-10- pentadecanoic acid (C15:1)	0.24 ± 0.01
Palmitic acid (C16:0)	26.16 ± 0.40
Palmitoleic acid (C16:1)	1.59 ± 0.06
Heptadecanoic acid (C17:0)	0.68 ± 0.03
cis-10-heptadecanoic acid (C17:1)	0.39 ± 0.01
Stearic acid (C18:0)	7.82 ± 0.13
Oleic acid (C18:1)	32.70 ± 0.51
Linoleic acid (C18:2)	14.72 ± 0.10
Linolenic acid (C18:3)	1.34 ± 0.04
Arachidic acid (C20:0)	0.27 ± 0.01
cis-11-eicisenoic acid (C20:1)	0.65 ± 0.07
cis-11,14-eicisenoic acid (C20:2)	0.22 ± 0.01
Behenic acid (C22:0)	0.19 ± 0.03
Others	1.90 ± 0.02
Total saturated	**45.44**
Total unsaturated	**54.66**

When comparing the fasting blood glucose (FBG) levels between the HFD fed and control groups, the picture can be divided into 3 distinct regions on the basis of curve shape (Fig. 1b). In the first region, the difference in the mean FBG level was very close to significant (P = 0.06 after 3 months) and increased with the rate of 1.51 mg/dl/month but remain less than 100 mg/dl after 3 month HFD feeding. After that the rate increased and kept increasing till 6th month with the average value and rate of 105.7 mg/dl and 3.13 mg/dl/month, respectively (Fig. 1b). In the next 3 months, the rate of increase in FBG reduced very much (0.5 mg/dl/month) with an average FBG level of 107.2 mg/dl after 9 months. The first significant difference (P <0.05) was appeared after 4 months in HFD fed group in comparison to the 0 hr reading of the same group.

The HFD fed group crossed the limit of 100 mg/dl after 3 months but the FBG values remain insignificant till 5 months as compared to 3 month's reading. After 6 months, the significant difference was observed as compared to the 3 month's value (Fig. 1b).

Periodic evaluation of impaired glucose tolerance via 2h blood glucose level during OGTT, showed somewhat different pattern (Fig. 1c). The picture can also be divided into 3 distinct regions comprising of 0-1, 1-6 and 6-7 months. In the first region, no significant change was occurred, but in the second region, a fast increase in the rate of change (3.7 mg/dl/week) in the blood glucose level occurred. After that the rate (0.75 mg/dl/week) became reduced very much till 9th month with no significant difference to that of 6th month value.

Changes of body weight, fasting blood glucose and 2h blood glucose of HFD-fed rats after STZ-induction

On the basis of the results of HFD feeding for a periods of 9 months, the similar experiments was done but this time a low dose of STZ was injected after 6 months of HFD feeding. After 15 days of STZ injection, body weights were reduced significantly ($P < 0.01$) as compared to the pre-injection state (Fig. 2a). On the other hand, FBG and 2h blood glucose levels increased very sharply and significantly ($P < 0.001$) (Fig. 2b & c).

Comparison of fasting serum insulin in the HFD-fed rats before and after STZ-induction

The concentration of insulin in the HFD fed rats also increased gradually (Fig. 3). After 3 months of HFD, the concentration of insulin was significantly ($P < 0.05$) different to that of same time control group. At 6th month, the difference between control and HFD groups increased very much with an approximately 3-fold higher concentration of insulin in HFD fed group. After STZ injection, the level of insulin decreased significantly ($P < 0.01$) to that of 6 month HFD group's level.

Comparison of HOMA-IR index and β-cell function in the HFD-fed rats before and after STZ-induction

In this HFD fed rats, it was found insulin resistance increased in a time dependent manner. Respective insulin resistance increased in 3 month HFD feeding and remain increases till 6 months of HFD feeding (Fig. 4a). However, after that when STZ was injected, a very little change of insulin resistance was observed.

Fig. 4b shows that 3 months HFD correlation data point lies in the normal region but with lesser insulin sensitivity. After 6 months of HFD feeding, this correlation data point at the edge of the normal limits and after STZ injection it is found at the edge of the type 2 diabetes limits.

Periodic comparison of fasting serum adiponectin in the HFD-fed rats before and after STZ-induction

No significant difference of adiponectin levels were found in the normal diet group during the 6.5 months of experimental periods (Fig. 5). However, serum adiponectin levels were decreased in 3 months HFD fed group compared with those in normal diet. Interestingly, there was a dramatic decreased in adiponectin levels in 6 month HFD group. Streptozotocin has a very little effect on adiponectin level in this 6 month HFD feeding group.

Comparison of insulin tolerance level in the HFD-fed rats before and after STZ-induction

The whole body insulin resistance was analyzed in different time intervals of HFD (0, 3, 6 months and after streptozotocin injection) through insulin tolerance test (Fig. 6).

After insulin injection, the blood glucose level dropped from 90.5 ± 5.1 mg/dl to 79.9 ± 5.0 mg/dl in control (0 month) rats, from 98.9 ± 5.7 mg/dl to 90.9 ± 6.7 mg/dl in HFD fed (3 month) rats, from 115.7 ± 1.91 mg/dl to 111.2 ± 3.4 mg/dl in HFD fed (6 month) rats and from 322.3 ± 16.2 mg/dl to 280.2 ± 17.7 mg/dl in HFD-STZ rats at 30 minutes. The insulin induced glucose disposals were 11.7%, 8.1%, 3.9% and 13.7% in normal, HFD 3 months, HFD 6 month and HFD-STZ rats, respectively.

Comparison of 2-deoxy-D-glucose (2DG) uptake in isolated adipocytes from HFD-fed rats before and after STZ-induction

The uptake of 2DG by the adipocytes isolated from normal diet fed, 3 months HFD fed, 6 months HFD fed, HFD-STZ groups were measured. The comparison was done in relative terms by taking the basal control as 100%. The basal and insulin stimulated 2DG uptake by the control rat's adipocytes was significantly higher than all other groups (Fig. 7). However, the insulin stimulated 2DG uptake was significantly decreased in HFD 3 ($P < 0.05$) and HFD 6 ($P < 0.01$) groups. The difference between HFD6 and HFD-STZ groups remained insignificant.

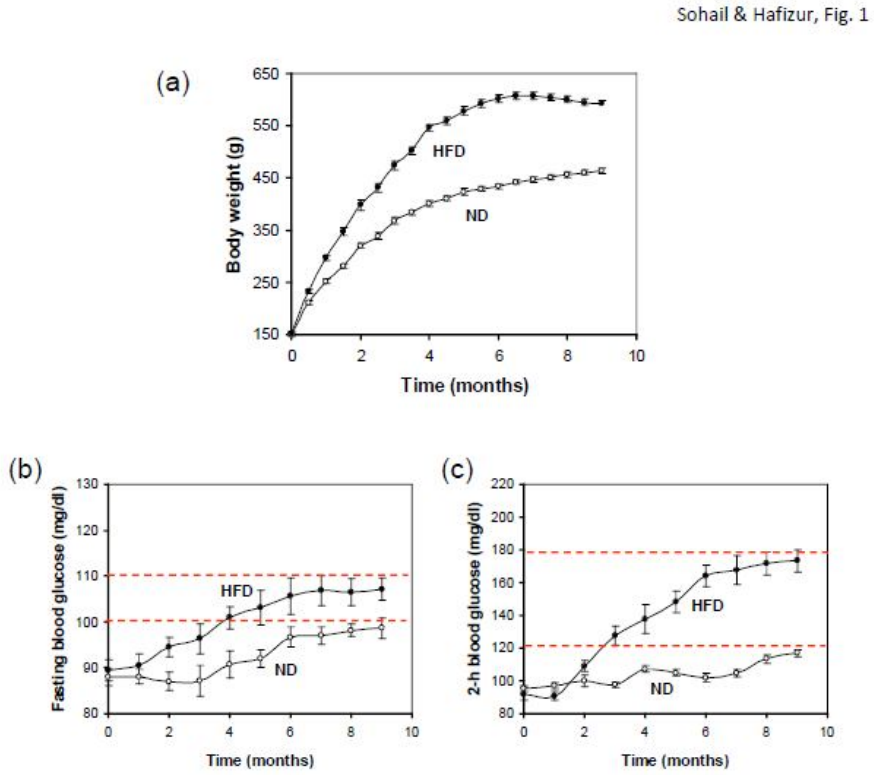

Fig. 1– Changes of (a) body weight (g), (b) fasting blood glucose (mg/dl), and (c) 2h blood glucose of HFD-fed rats. Results are expressed as means ± SE for 9-11 rats per group. Red dotted lines represent the lower and upper limit of pre-diabetes. ND, normal diet; HFD, high fat diet.

Glut4 expression in rat adipocytes

As shown in the Fig. 8, the total adipocytes content of Glut4 mRNA in all the HFD fed groups were decreased significantly ($P < 0.05$) compared to the mRNA isolated from the normal diet fed group. The adipocytes content of Glut4 mRNA in adipose tissue remains 78 ± 2.9 % in the 3 months HFD fed group to that of control. These Glut4 mRNA content further decreased drastically during the next 3 months HFD fed period. This was the same period when the rats develop hyperglycemic condition. In the next 3 months, the expression level of Glut4 gene decreased further with the higher rate as compared to the initial 3 months decrease. After the STZ injection, the Glut4 expression level decreased drastically and reached near 10% to that of normal rats.

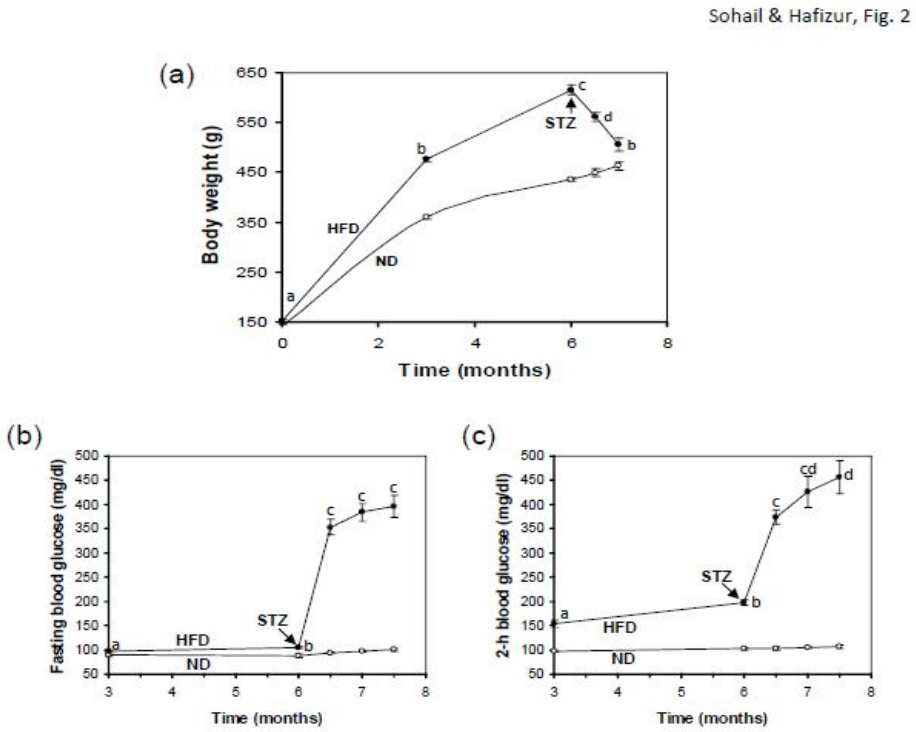

Fig. 2– Changes of (a) body weight (g), (b) fasting blood glucose (mg/dl), and (c) 2h blood glucose of HFD-fed rats after STZ-induction. Results are expressed as means ± SE for 9-11 rats per group. Data points that do not share a common notation are significantly different at $P < 0.05$ (one way ANOVA with post-hoc Bonferroni). ND, normal diet; HFD, high fat diet.

Sohail & Hafizur, Fig. 3

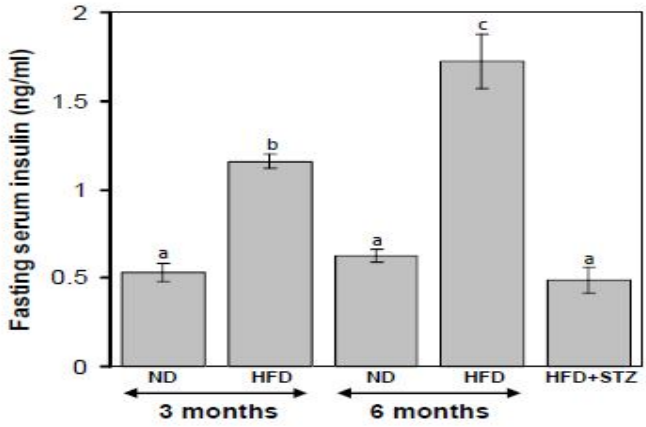

Fig. 3. Changes of fasting serum insulin (ng/ml) in the HFD-fed rats before and after STZ-induction. Results are expressed as means ± SE for 9-11 rats per group. Bars that do not share a common notation are significantly different at $P < 0.05$ (one way ANOVA with post-hoc Bonferroni). ND, normal diet; HFD, high fat diet.

Sohail & Hafizur, Fig. 4

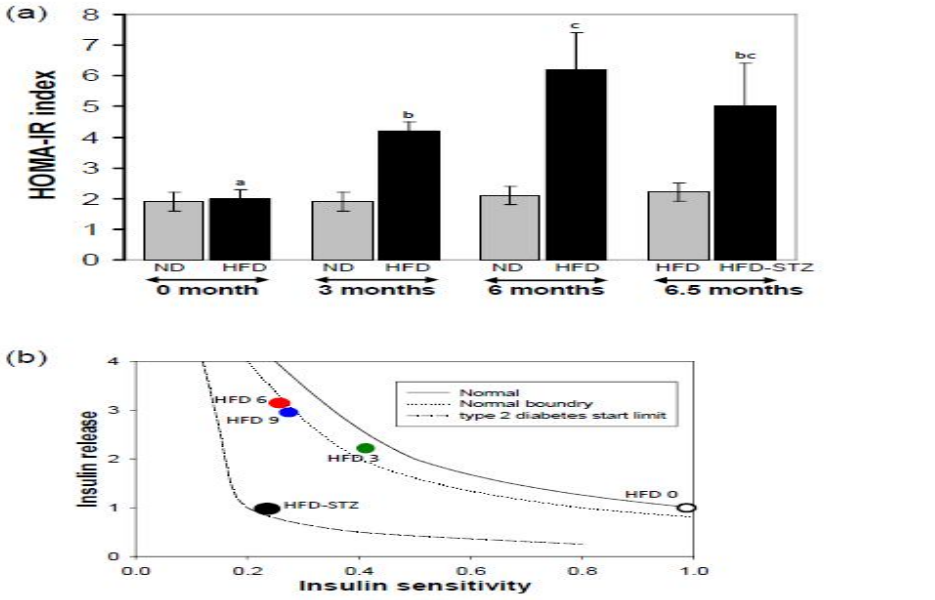

Fig. 4. Comparison of HOMA-IR index (a), and β-cell function (b) in the HFD-fed rats before and after STZ-induction. For β-cell function, the arbitrary units of insulin sensitivity and insulin release (the respective values of normal rats were taken as 1) were used to draw relative data points on a hypothetical scale representing the various regions from normal to type 2 diabetes. Results are expressed as means ± SE for 9-11 rats per group. Bars that do not share a common notation are significantly different at $P < 0.05$ (one way ANOVA with post-hoc Bonferroni). ND, normal diet; HFD, high fat diet.

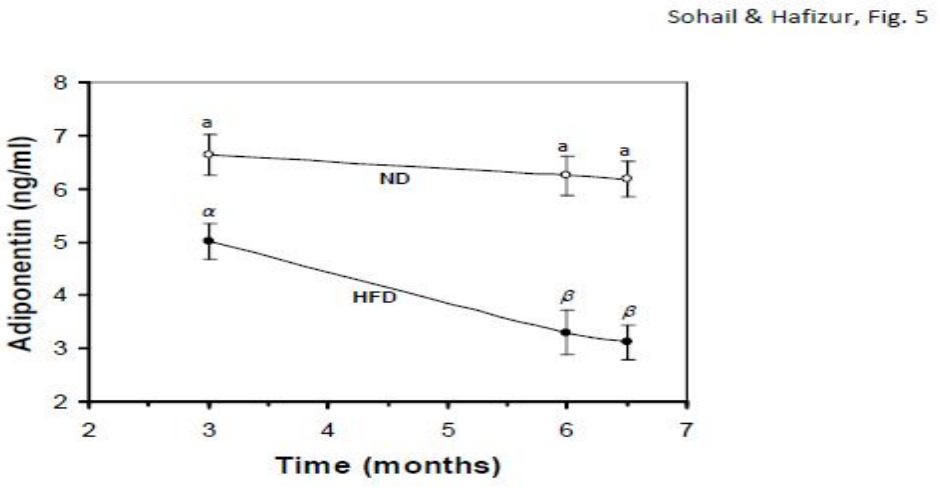

Fig. 5 Periodic comparison of fasting serum adiponectin (ng/ml) in the HFD-fed rats before and after STZ-induction. Results are expressed as means ± SE for 9-11 rats per group. Data points that do not share a common notation are significantly different at $P < 0.05$ (one way ANOVA with post-hoc Bonferroni). ND, normal diet; HFD, high fat diet.

DISCUSSION

This study was designed to investigate the effects of chronic HFD feeding on the metabolic and molecular changes occurred in Wistar rats. On the basis of this study results, our further goal was to develop a progressive rat model towards the type 2 diabetes which have distinctive stages towards the type 2 diabetes as well as reproducibility. It is now well accepted that HFD is more susceptible of having impaired glucose metabolism, tolerance and ultimately type 2 diabetes as supported by many studies [S.E. Kahn, R.L. Hull, K.M. Utzschneider (2006); A.H. Lichtenstein, U.S. Schwab (2000) and E.E. Blaak (2007)]. In the way of these pathological complications, obesity is an intermediate and a way to many other complications beside type 2 diabetes [J. Wong, L. Molyneaux, D. Zhao (2008)].

A major hurdle towards the reproducibility of this type of animal model is the characterization of the HFD. The first consideration is the selection of fatty medium for the preparation of HFD. Many studies have been done in this regard to comparatively evaluate different sources of fats in the way of developing obesity and type 2 diabetes in terms of quality as well as quantity [M.A. Alsaifa, M.M.S. Duwaihy (2004)]. As far as quality is concern, the fats having more amount of saturated fatty acid are associated with more severe insulin resistance [A.A. Rivellese, S. Lilli (2003) and G. Riccardi, R. Giacco, A.A. Rivellese (2008)]. On the other hand both saturated and mono-unsaturated fatty acids present in a single source are also reported to be associated with adipocity and diabetes whereas ω-3 and polyunsaturated fatty acids are inversely associated with obesity and insulin resistance [M.A. Alsaifa, M.M.S. Duwaihy (2004) and J.C. Lovejoy (2002)].

Sohail & Hafizur, Fig. 6

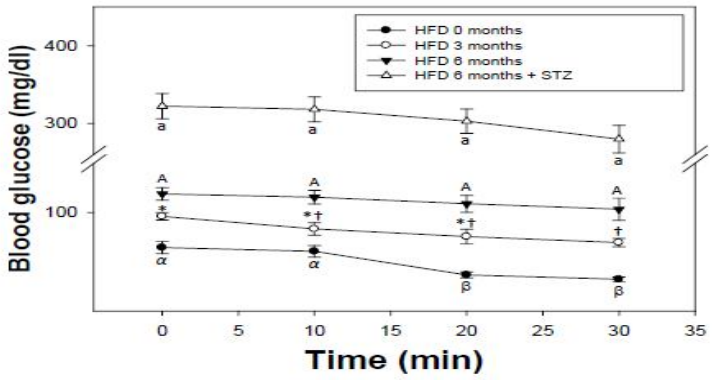

Fig. 6 Comparison of insulin tolerance level (mg/dl) in the HFD-fed rats before and after STZ-induction. Results are expressed as means ± SE for 9-11 rats per group. Data points do not share a common notation are significantly different at $P < 0.05$ (one way ANOVA with post-hoc Bonferroni). ND, normal diet; HFD, high fat diet.

Sohail & Hafizur, Fig. 7

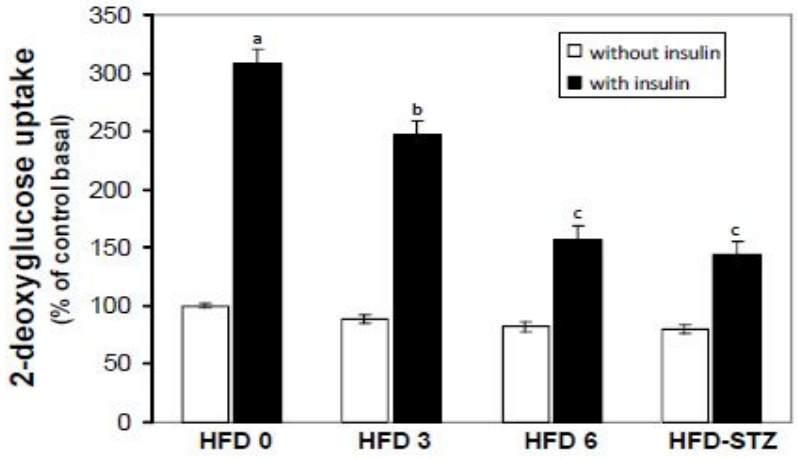

Fig. 7 Comparison of 2DG uptake in isolated adipocytes from HFD-fed rats before and after STZ-induction. Basal (white bars) and insulin stimulated (black bars) 2DG uptake in isolated adipocytes from the HFD-fed rats. The isolated adipocytes were incubated in the presence of [^{14}C] 2DG and then the cellular presence of ^{14}C were measured by liquid scintillation counter. Results are expressed as means ± SE for 9-11 rats per group. Bars that do not share a common notation are significantly different at $P < 0.05$ (one way ANOVA with post-hoc Bonferroni). ND, normal diet; HFD, high fat diet.

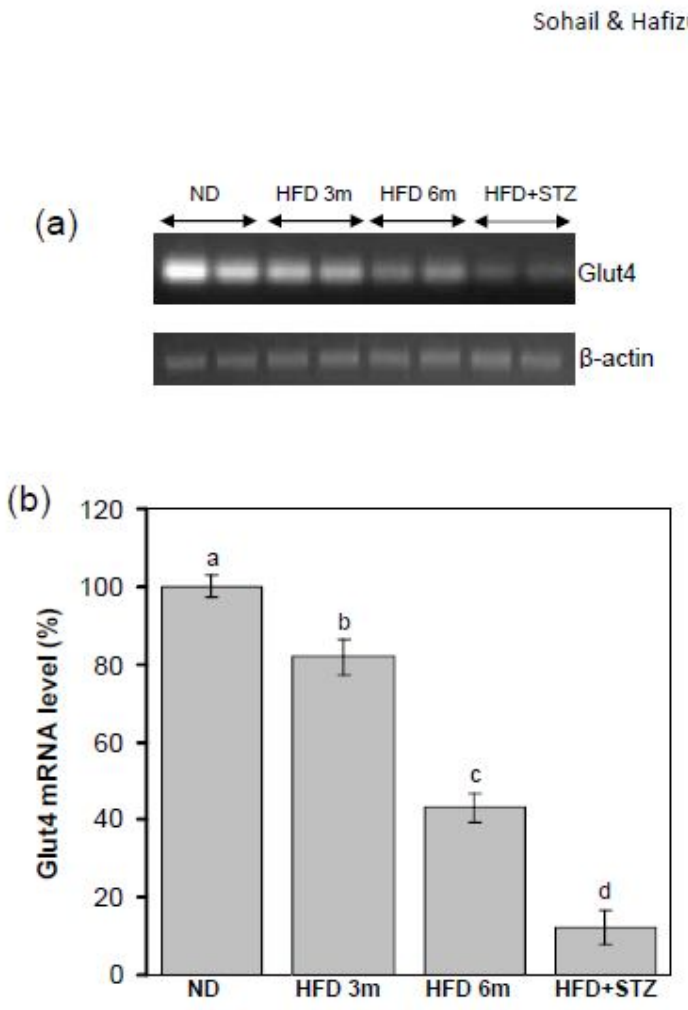

Fig. 8– Comparison of Glut4 mRNA expression levels in the HFD-fed rats before and after STZ-induction. (a) The RNA extraction was performed from the adipocytes followed by the semi-quantitative RT-PCR analysis. The PCR products was then separated by agarose gel electrophoresis and detected by ethidium bromide staining. The analysis of β-actin transcription levels under the identical conditions were used as control. (b) Percentage relative intensities of the bands were calculated after the normalization with the β-actin intensity where the intensity of the ND group was taken as 100%. Results are expressed as means ± SE for 9-11 rats per group. Bars that do not share a common notation are significantly different at $P < 0.05$ (one way ANOVA with post-hoc Bonferroni). ND, normal diet; HFD, high fat diet.

Butter from animal source was selected in this study because of: *i*) more than 80% of fatty acids are composed of saturated and mono-unsaturated fatty acids, *ii*) several studies have shown that butter has the amazing capacity to generate the state of insulin resistance [M.A. Alsaifa, M.M.S. Duwaihy (2004) and P. Hossain, B. Kawar, M. EL Nahas (2007)], *iii*) In Indo-Pak region, butter is among the major sources of fat.

The HFD was standardized by determining its fatty acid composition as well as the % of fat (29.6%) present. This characterized HFD made this model reproducible which is a major requirement and need for an animal model. This HFD fed continuously to the young rats for a period of 9 months and periodically evaluated the metabolic changes occurred in the context of type 2 diabetes. The focus was our pre-defined parameters for the determination of insulin resistance, pre-diabetes and diabetes stages. Pre-diabetes consensus conference at American College of Epidemiology highlighted the importance of impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and insulin concentration as a predictor of diabetes [T. Zachary, M.D. Bloomgarden (2008)]. Our initial focus was these parameters as well as body weight and adiponectin level as the parameters for the obesity. Our data showed that the metabolic changes occurred during the course of HFD can be divided into 4 distinct stages. First stage spans the initial 2 months with normal metabolic conditions. Second stage spans the month 3 where IFG remain below 100 mg/dl but the concentration of insulin increased slowly and gradually (Fig. 1b). Slight impairment was also observed in the glucose tolerance test but the reading resided below 150 mg/dl (Fig. 1c). Body weights and adiponectin -two predictors of obesity, also changed slightly but in the inverse manner (Figs. 1a & 5). Whereas Glut4 data shows that relatively less amount of glucose is passed through adipocytes (Fig. 8). Relatively high insulin concentration, less amount of Glut4 and mild IGT show that the animals are insulin resistant at this stage where normal fasting glucose concentration was maintained by the rise in the insulin concentration. Beyond that, the curves of body weight gain and fasting glucose uplift relatively and reached to their maximum at round about 6 months period (Fig. 1a,b). At that time, all the data fulfill the predefined conditions of severe insulin resistance or pre-diabetic stage. The quantity of insulin was approximately 3-fold high (Fig. 3) as well as IFG cross the limit of 100 mg/dl. Beside that serum adiponectin levels were decreased significantly in the HFD fed rats (Fig. 5). There was a gradual decrease of serum adiponectin levels during the HFD treatment period. The strong negative correlation ($r = -0.746$) of serum adiponectin and HOMA-IR index indicate that HFD feeding causes insulin resistance, partially through decrease in adiponectin (Figs. 3 & 4). IGT also observed relatively high as compared to the 3rd month condition where 2h blood glucose crossed the limit of 150 mg/dl. This data fulfill the conditions defined by K.G. Alberti (1998) and American Diabetes Association (1997) for pre-diabetes. Accordingly, pre-diabetes is defined by a FBG level of 100-125 mg/dl or a 2h glucose level of 140-200 mg/dl. It has also been stated that individuals having either IFG or IGT hold 3-fold increased chance of diabetes whereas as people having both of these conditions hold 5- fold rise in the risk of developing type 2 diabetes [Z.T. Bloomgarden (2008)].

Beyond 6 months HFD treatment, a steady metabolic state was observed in which FBG level did not vary significantly and this condition lasts till 9 months of HFD feeding. As per 1997's American Diabetes Association definition the people having >125 mg/dl fasting blood glucose and 2h post oral glucose tolerance level of more than 200 mg/dl are declared as diabetics. HFD feeding till 9 months did not fulfill these diabetic conditions and the chances of attaining diabetic state in the near future was not very hopeful as it may take 5-10 years for the development of type 2 diabetes after the pre-diabetic condition [G. Nijpels, W. Boorsma, J.M. Dekker, F. Hoeksema, P.J. Kostense, L.M. Bouter and R.J. Heine (2008)].

Another set of experiment, Wistar rats were fed HFD for 6 months followed by the administration of 30 mg/kg streptozotocin intravenous injection for the artificial semi-destruction of pancreatic β-cells as streptozotocin is used to destroy pancreatic β-cells partially in low dose (30-45 mg/kg) or completely in high dose (>60 mg/kg) [M.J. Reed, K. Meszaros, L.J. Entes (2000)]. Following streptozotocin injection, the diabetic conditions developed rapidly fulfilling all the definitions for type 2 diabetes.

The correlation between insulin release and blood glucose level was derived in the form of HOMA-IR which shows that insulin resistance is positively correlated with the extent of diet. On the other hand, the destruction of β-cells or lesser insulin has a little or no effect on the state of insulin resistance. Another correlation between insulin release and insulin sensitivity determines the β-cells functions [S.E. Kahn, R.L. Hull, K.M. Utzschneider (2006)]. It shows that in relation to the variation in the insulin sensitivity, the reciprocal decrease or increase of insulin release happened in order to keep the blood glucose concentration within normal limits. During the progression towards the type 2 diabetes, the intermediate steps are the insulin resistance where decreased insulin sensitivity is compensated by the relative increase in the insulin concentration thereby keeping the blood glucose in normal limits. Following that, beyond a certain stage, the compensatory release of insulin remains insufficient to cope with the decreasing insulin sensitivity and hyperglycemia occurred. After that the β-cell function remains decreasing continuously for an indefinite period of time and enters into the stage of type 2 diabetes.

Insulin resistance, pre-diabetic and diabetic conditions were evaluated at cellular and molecular level for further insights and standardization of the model. In previewing the fact that HFD cause insulin resistance in peripheral tissues [B.B. Kahn, O. Pedersen (1993)], we determined the insulin resistance state at cellular as well as whole body level. Adipocytes were used for the comparative evaluation of glucose transport rate via labeled 2DG and ITT was used to determine the whole body resistance (Fig. 7). Our results clearly indicate that insulin resistance was increased with HFD feeding. This resistance was mild during the first 3 months of HFD in which a lesser amount of glucose was transported across the adipocytes in the presence of a fixed insulin concentration. Beyond that insulin resistance increased and after 6 months of HFD, the glucose transport rate became very low. More or less same condition sustained after the streptozotocin injection. These results further strengthen the fact that HFD is associated with insulin resistance but conditions of insulin resistance vary upon the duration and composition of HFD.

Glut 4 is considered as major player of insulin stimulated glucose transport in those tissues that are sensitive to insulin where Glut1 is relatively insensitive and relatively less abundant [D.J. Dean, J.T. Brozinick, S.W. Cushman, and G.D. Cartee (1998)]. Insulin resistance is likely to be associated with the reduced transfer of Glut4 containing vesicles but beside this mechanism whether HFD also affecting the other mechanisms like the expression of Glut4 gene in adipocytes, we studied the Glut4 gene expression level in previously defined dietary conditions. The results showed that HFD also affected on the Glut4 gene expression in a time-dependent manner (Fig. 8). It has previously been reported that large scale reduction of adipocyte Glut4 contents results in the impairment of glucose homeostasis which make an individual a likely susceptible to impaired glucose tolerance as well as diabetes [X.Y. Park, Y.H. Choi, W. Lee (2008)].

It is believed that no animal model complies exactly with any human syndrome; none of the available animal models of type 2 diabetes exactly simulate the human type 2 diabetic condition. However, long term HFD followed by low dose streptozotocin injection rat model showed clinical presentation and pathophysiology of natural history of diabetes and have the potential to be one of the suitable experimental animal models of type 2 diabetes.

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