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Assessment of gut associated changes and adipose tissue inflammation in high fat diet fed Streptozotocin-induced diabetic rats

Satvinder Singh*, Raj Kumar Sharma, Shilpi Malhotra, Ramesh Pothuraju, Umesh Kumar Shandilya

Animal Biochemistry Division, National Dairy Research Institute, Karnal 132001, Haryana, India

Abstract: The present study evaluated gut related changes and adipose tissue inflammation in high fat diet (HFD) fed and streptozotocin (STZ) treated diabetic rats. Male albino Wistar rats were fed on HFD (60% kcal fat) and normal chow (control) for 3 weeks. After 3 weeks, HFD fed rats were injected with STZ (35 mg/kg body weight) intraperitoneally to induce type 2 diabetes. High fat diet fed STZ treated rats had reduction in the oral glucose tolerance, plasma insulin, HDL-C and raised fasting blood glucose, glycosylated hemoglobin and atherogenic index in comparison to HFD fed rats. On the other hand, feeding of high fat diet resulted in decrease in the total number of bacteria as well as other bacterial groups in comparison to control. Bifidobacteria were most significantly reduced. The propionate proportions (%) in caecal contents were increased in HFD fed rats in comparison to control while HFD fed diabetic animals had decreased propionate proportions. Expression analysis of proglucagon and PC1 in cecum and IL-6 in epididymal fat was higher in HFD fed group and lower expression of adiponectin in epididymal fat as compared to control. STZ treatment significantly lowered the expression of proglucagon and PC1 in cecum and adiponectin in epididymal fat and increased the expression of TNF-α and IL-6 in epididymal fat. These results suggest that improvement of gut related parameters by nutritional/therapeutic approaches may have beneficial effect on obesity and diabetes.

Keywords: High fat diet, Obesity, Diabetes, Gut microbiota, Inflammation, Streptozotocin

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Obesity and type 2 diabetes are the metabolic diseases tuned by diet have become major health concern for the world. Dietary habits and life style are very important factors in association with the genetics behind these diseases as evidenced by rapid emergence of obesity and diabetes within a generation in the last few decades in all parts of the world. Diet exerts its effect through gut; therefore, it makes logic to explore gut associated changes in these diseases. Recently, the significance of gut microbiota in the manifestation of metabolic disorders has been receiving extra attention worldwide (Shen et al. 2013; Tremaroli V and Backhed F 2012; Harris et al. 2012; Burcelin et al. 2011; Cani PD et al. 2008; Membrez et al. 2008; Backhed et al. 2004). Study of change in microbiota and subsequently host genes in high fat fed animal model of metabolic disorders will pave new insight to combat these life style diseases. Microbiota ferments the undigested food material and produce short chain fatty acids (SCFAs) which are suggested to be beneficial for gut health. SCFAs in total or their relative proportions is important in terms of good health is also a subject of research. Therefore, in diet induced obesity the alteration of SCFA content in gut is also a very important phenomenon. Glucagon like peptide-1 (GLP-1), synthesized in intestinal enteroendocrine L-cells, is an incretin hormone which is responsible for the majority of nutrient stimulated insulin secretion. This, incretin effect is reduced or absent in type 2 diabetes (Drucker, 2003a). GLP-1 reduces appetite and food

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Corresponding Author:
Singh S. (✉) Animal Biochemistry Division, National Dairy Research Institute, Karnal-132001, Haryana, India
Email: satvinderbiochem@gmail.com

intake in humans (Verdich et al. 2001). Post-translational modification of proglucagon by prohormone convertase 1 (PC1) generates GLP-1 in the intestine (Drucker, 2003b). We hypothesized that HFD feeding might influence the expression of proglucagon gene in intestine.

Clinical and epidemiological studies have shown an apparent link between low-grade inflammation and metabolic diseases, mainly obesity and type 2 diabetes. Obese individuals have higher levels of TNF- α and IL-6 in their adipose tissue and reduction in body weight of these individuals is associated with a decrease in TNF-α and IL-6 expression (Ziccardi et al. 2002). Adiponectin levels in the plasma and adipose tissue are decreased in obese individuals, and production of adiponectin by adipocytes is inhibited by pro-inflammatory factors, such as TNF-α and IL-6 (Ryo et al. 2004). HFD fed and low dose of STZ treated rats have been suggested as a novel animal model that mimics the natural history and metabolic characteristics of common type 2 diabetes in humans (Srinivasan et al. 2005; Reed et al. 2000). Reed et al. (2000) reported that increase in plasma glucose concentration in STZ treated rats reached a plateau within 3 days and was stable for at least 21 days. Diabetes is generally associated with increased triglyceride, total cholesterol, LDL-cholesterol and decreased concentrations of HDL-cholesterol (Kontush and Chapman, 2006). In the present study, we explored the effect of high fat feeding and STZ treatment on different gut associated parameters and expression of inflammation related genes in adipose tissue.

MATERIALS AND METHODS

Materials

Paraformaldehyde, DABCO [1,4Diazabicyclo(2,2,2) octane] antifade, DAPI (4,6-diamidino-2-phenylindole di-hydrochloride), lysozyme used in fluorescent in situ hybridization (FISH), Streptozotocin (STZ) and trizma base were purchased from Sigma-Aldrich Chem. Co., USA. Fluorescently labeled (Cy-3) probes were custom synthesized from Integrated DNA technologies (IDT), USA. Rat insulin ELISA kit (Mercodia, Sweden) was procured from Krishgen BioSystems, Mumbai, India. For gene expression analysis, Tri reagent from MRC (Molecular Research Center), cDNA synthesis kit and Maxima™ SYBR Green Master Mix (Fermentas) were procured from Genetix Asia Pvt. Ltd, New Delhi, India. Lipid profile analysis was performed by diagnostic kits from Span Diagnostics Pvt. Ltd., Surat, India. Vitamins were purchased from CDH, New Delhi, India. Casein was a product of M/s Cepham Milk Specialities, Dera Bassi, (Punjab) India. Branded refined soybean oil and lard were purchased from local market. All other chemicals used in the study were of analytical grade (AR) or equivalent grade.

Animals

Male albino Wistar rats were procured from Chaudhary Charan Singh Haryana Agricultural University, Hisar, India for the study. All animal procedures were conducted at Small Animal House, National Dairy Research Institute, Karnal, India, in accordance with Institutional Animal Ethics Committee (IAEC). Three animals were housed per cage under 12 h light/dark conditions at 22±2°C. Before the experiment, all animals were fed normal chow and acclimatized for 7 days.

Feeding and Induction of type 2 diabetes

Twelve male albino Wistar rats (230±15 g) were fed on HFD (60% kcal fat) and six animals on normal chow served as control for 3 weeks. The composition of diet is given in Table 1. After that, six animals from HFD fed group were injected with a low dose STZ (35 mg/kg body weight) via intraperitoneally to induce type 2 diabetes. A single intraperitoneal injection of freshly prepared STZ in 0.1 M citrate buffers (pH 4.5) at 1 ml/kg body weight was administered. Other animals were given vehicle (citrate buffer). STZ treated rats were kept on 5% glucose solution for first 24 h to overcome drug-induced hypoglycemia. Induction of diabetes was confirmed by the determination of fasting glucose concentration on the third day post administration of STZ. Animals having blood glucose levels >300 mg/dl were considered to be diabetic. At the end of experiment all animals were euthanized under diethyl ether condition. Body weights were recorded weekly during the study.

Table 1 Composition of diets

Sr. No.	Component	Normal Chow (%)	High Fat Diet (%)
1	Starch	53	25
2	Casein	20	20
3	Sucrose	10	10
4	Fat	7*	35**
5	Cellulose	5	5
6	Vitamin mixture	1	1
7	Mineral mixture	3.5	3.5
8	Choline Chloride	0.2	0.2
9	Methionine	0.3	0.3

*(soyabean oil) ** (7% soyabean oil and 28% Lard)
Vitamin and Mineral mixture as per AOAC, 1990

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed between 09:00-11:00 h after 3 days of STZ treatment. The rats were deprived of food for 12-14 h before the oral administration of glucose (2 g/kg body weight; 200 g/l solution) by intra-gastric gavage. Blood was drawn by puncturing the tail with a needle gun at 0 (before), 30, 60 and 120 min after glucose administration. Glucose levels were determined by taking one drop of blood sample by a GlucoDr blood glucose test meter (Germany).

Collection and processing of blood and tissue samples

Blood samples were collected by cardiac puncture and stored in vials containing EDTA (2 mg/ml blood). Cecum and epididymal fat were excised. Cecal content was collected for bacterial enumeration and

SCFAs analysis. A part of epididymal fat and cecal tissue was collected in RNA later for gene expression analysis. Small volumes of whole blood were used for the determination of glycosylated hemoglobin (GHb) and free fatty acids (FFAs) and the remaining part was centrifuged at 2000 x g for 10 min at 4°C to collect plasma.

Analysis of biochemical parameters

The plasma insulin was measured by sandwich ELISA method as per manufacturer's instructions (Mercodia, Sweden). GHb was estimated by weakly binding cation-exchange resin based kit method (Crest Biosystems, Goa, India). Total cholesterol, triglyceride and high density lipoprotein (HDL)-cholesterol in the plasma were estimated using kits (Span diagnostics Ltd. Surat, India). These lipid fractions were analyzed as per the instructions of manufacturer. The kits were based on enzymatic colorimetric methods. Low density lipoprotein (LDL)-cholesterol, very low density lipoprotein (VLDL)-cholesterol and atherogenic index (AI) were calculated using Friedewald's equation (Friedewald et al. 1972). FFAs were estimated according to the method of Itaya (1977).

Changes in caecal microbiota-Fluorescent in situ hybridization

Enumeration of bacteria in caecal contents was performed by FISH technique using Cy-3 labeled probes. The method adapted was as described by Rycroft et al. (2001). Probes used in the study are LAB158 (GGTATTAGCAYCTGTTTCCA), BIF164 (CATCC GGCATTACCACCC) EREC 482 (GCTTCTTAGTCAR GTACCG) and BAC 303 (CCAATGTGGGGGACCTT). Total bacteria were enumerated by DAPI stain. Caecal samples were fixed in 4% paraformaldehyde (PFA). Each diluted sample was mixed by vortexing and 20 µl of it was applied to the well of a poly-l-lysine-coated slide. The slide was dried for ~15 min at 46–50°C in an incubator. In case of LAB 158 probe, an additional step of permeabilization with lysozyme was performed. For all other probes (and LAB 158 after lysozyme treatment), slides were dipped into 50, 80 and 96 % (v/v) ethanol (3 min each). The dehydrating effect of ethanol concentration series disintegrates cytoplasmic membranes, which therefore becomes permeable to oligonucleotide probes. Slides were dried for 2 min at 46–50°C. Oligonucleotide probe solutions (50 ng/µl) were thawed on ice and 5 µl of each was added to 50 µl of hybridization buffer, mixed well and 50 µl of the mixture applied onto the well. The lid was placed on the slide tray to ensure no evaporation of the hybridization mixture and placed carefully into the hybridization chamber. The samples were incubated for 4 h. Fifty milliliters of the washing buffer was poured in falcon tubes and kept in water bath at 50°C. Twenty microlitres of DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride; 50 ng/µl) was added to each tube of wash buffer. Just before the slides were due to come out of the hybridization chamber, lids on the centrifuge tubes were loosened so that the slides could be placed in the wash buffer quickly. After incubation, the hybridization box was carefully removed from the chamber. Two slides placed back-to-back were transferred to the wash buffer and kept for 10–15 min. The slides were taken out using tweezers and dipped into ice-cold distilled water for 2–3 sec. The slides were dried as quickly as possible by using a stream of air from dryer. Five microliter of antifade solution (DABCO) was placed over each well on the slide and a cover slip was placed on the slide. Slides were viewed in oil immersion at 1000X magnification under fluorescence microscope in dark conditions. The slides were focused under UV filter for DAPI. Once focused, the filter was changed from UV to green. The fluorescent cells (orange coloured) were counted in 15 random fields per well. The number of bacteria per gram caecal contents was determined using following formula: 0.8 x 10x average number of cells per well x microscopic factor x 50 x dilution factor, Where,

Microscopic factor = area of well/area of field of view

The values are represented as $\log_{10}$ cells/g caecal contents.

Estimation of SCFAs in caecal contents

Short chain fatty acids in rat caecal contents were estimated by gas chromatography (NUCON 5700) by following the methodology of Juskiewicz et al. (2006). The samples of caecal digesta (of about 0.2 g) were weighed, mixed with 0.2 ml of formic acid, diluted with deionized water, and then centrifuged at 10,000 × g for 5 min. Supernatant was loaded onto the chromatography stainless steel column (1.82 m × 2 mm; 80/100 Chromosorb 101). The chromatograph was coupled to a flame ionization detector. Conditions were as follows: oven temperature of 200°C; detector temperature of 230°C; injector temperature of 210°C.

Gene expression analysis by Real-time PCR

Total RNA from epididymal fat and cecum was isolated using TRI REAGENT® (MRC) as per the manufacturer's instructions. The first strand cDNA was synthesized using Fermantas First strand cDNA synthesis kit (Fermantas, Germany). The reaction mixture contained 2 µg of total RNA, 1 µl of random hexamers (0.2 µg/µl) and DEPC treated water up to 11 µl. The contents of the tube were mixed gently, spun briefly and then incubated at 65°C for 10 min to denature secondary structures of RNA followed by incubation for 2 min at room temperature. The following reagents were further added to the tube to make the final volume up to 20 µl: 4 µl 5X reaction buffer (250 mM Tris-HCl, pH 8.3; 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 1 µl of RNase inhibitor (20 IU), 2 µl of dNTP mix (10 mM), 2 µl of M-MLV Reverse Transcriptase (200 IU). The contents were mixed gently, spun briefly and incubated in thermocycler at 25°C for 10 min, 42°C for 30 min and 95°C for 3 min. PCR amplification was performed in a reaction mixture containing 2 µl of cDNA, 0.2 µM primers and 5 µl of 2X SYBR mix. The reaction volume was made up to 12 µl with the help of nuclease free water. The primers for genes of interest and housekeeping gene (β actin) for quantative RT-PCR are mentioned in Table 2. For relative quantification, β actin was used as normalizer

Table 2 Primers sequences used for real-time quantitative PCR

Gene	Primer sequence	Annealing temperature (°C)	Size of amplification product (bp)	Reference
β actin	CGGTCAGGTCATCACTATCG TTCCATACCCAGGAAGGAAG	60	78	Thakur et al. 2006
adiponectin	GGAGACGCAGGTGTTCTTGG AGCCCTACGCTGAATGCTGA	60	152	Romero et al. 2009
IL-6	CTCCGCAAGAGACTTCCAG GGTCTGTTGTGGGTGGTATC	55	120	Maurer et al. 2009
TNF-a	GTCGTAGCAAACCACCAAG AGAGAACCTGGGAGTAGATAAG	55	145	Maurer et al. 2009
PRG	ACCGCCCTGAGATTACTTTTCTG AGTTCTCTTTCCAGGTTCACCAC	60	122	Maurer et al. 2009
PC1	GGTACCCAAAAACTCCAGCA GGCTTGTTGAGCTTTTCCAG	60	203	Cani et al. 2005

to nullify any possible experimental error. PCR reactions were performed by heating the contents at 94°C for 5 min (preincubation), followed by 40 cycles of 94°C for 20 sec (denaturation), 60°C or 55°C for 15 sec (annealing), 72°C for 20 sec (extension), each step was followed by a plate read for data acquisition. The quantitative PCR results were analyzed by $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2012) and presented as fold change in comparison to control.

Statistical analysis

All the values are quoted as mean ± SEM. The data was statistically analyzed using GraphPad Prism Software (GraphPad Software, San Diego, CA, USA). Two way ANOVA with Bonferroni post tests was used for analysis of OGTT, SCFAs, FISH and gene expression analysis. Other parameters like body weights, plasma insulin, FFAs, GHb, and lipid profile were analyzed by one way ANOVA with Tukey's post test to compare the groups.

RESULTS

Body weight changes

During the preinjection phase, the animals gained weight continuously in the control as well as HFD fed groups. Body weights of animals fed on HFD were apparently higher in comparison to normal chow fed animals. However, the change was not statistically significant (Fig. 1).

After 3 weeks feeding, the body weights were found to be 273.6±6.66 and 286.3±4.34 g (mean±SEM) for normal chow and HFD fed groups, respectively. The net gain in body weight after 3 weeks was found to be 42.24±4.35 and 54.94±2.98 g in normal and HFD fed animals respectively. STZ treatment reduced body weight drastically ~ 20 g within 3 days.

Standardization of dose

In preliminary experimentation, different doses of STZ viz., 25, 35, 45 and 50 mg/kg body weight were administered. Low dose of 25 mg/kg did not induce desirable hyperglycemia (i.e. >300 mg/dl) while higher doses (45 & 50 mg/kg) led to fast deterioration of condition of animals and increased mortality. At 35 mg/kg body weight, there was no mortality in rats and found frank hyperglycemia.

Oral glucose tolerance test

OGTT data was collected at the stage of 3 days post STZ treatment in control, HFD and HFD+STZ groups. The data for glucose concentration at different time intervals during glucose tolerance test is presented in Figure 2. The increase in blood glucose level was significantly higher, both in case of HFD and HFD+STZ groups in comparison to control group as indicated by

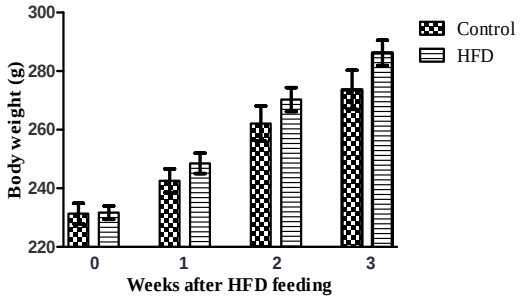

Figure 1 Effect of HFD feeding on body weights of rats. Values are expressed as mean±SEM

Area Under Curve (AUC):

Control	HFD	HFD+STZ
18713	25450	62728

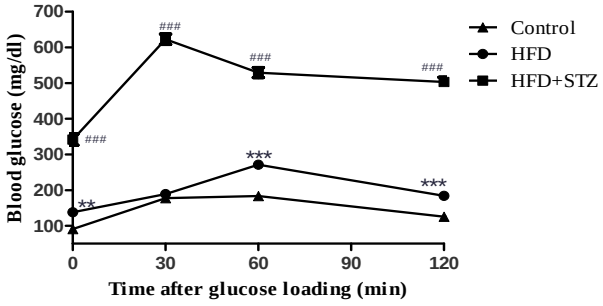

Figure 2 Effect of HFD feeding and STZ treatment on OGTT. Values are expressed as mean±SEM

the area under curve. The blood glucose level reached a maximum (623±14.29 mg/dl) at 30 min in case of HFD+STZ treated group. However, the maximum levels could reach to 277 and 183 mg/dl in HFD group and control group, respectively, in 60 min. In comparison to control, glucose tolerance was significantly (P<0.001) low in HFD group. Further in STZ treatment group, glucose tolerance decreased immensely (P<0.001).

Changes in biochemical parameters

The results for biochemical parameters after 3 days of STZ treatment are presented in Figure 3. Fasting blood glucose level was significantly higher in HFD as well as HFD+STZ group in comparison to control group. The values were 138.2±9.83, 341.5±15.44 and 91±4.96 mg/dl, respectively indicating a hyperglycemic effect in HFD and HFD+STZ treated animals. Plasma insulin level in

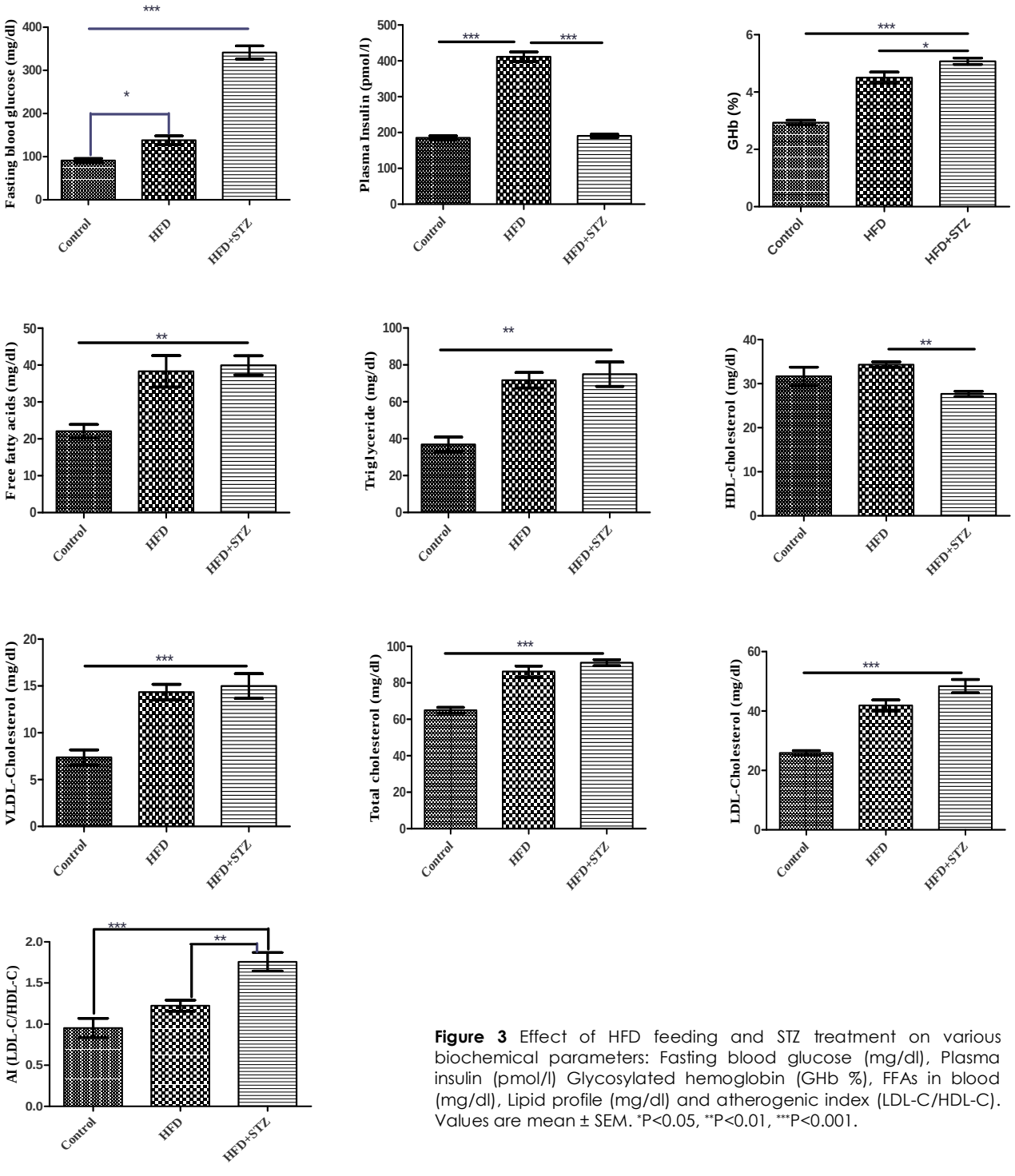

Figure 3 Effect of HFD feeding and STZ treatment on various biochemical parameters: Fasting blood glucose (mg/dl), Plasma insulin (pmol/l) Glycosylated hemoglobin (GHb %), FFAs in blood (mg/dl), Lipid profile (mg/dl) and atherogenic index (LDL-C/HDL-C). Values are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

HFD fed animals (411.5±13.37 pmol/l) were significantly higher (> 2 folds, P<0.001) than normal chow fed (185.2±5.53 pmol/l) control animals. However, STZ treatment was found to reduce insulin level (190.6±4.76 pmol/l) reaching almost near to the control. Glycosylated hemoglobin (%) was found to increase as a result of feeding the rats on HFD and it was significantly higher, being 4.50±0.19 (%) in comparison to animals fed on normal chow (2.93±0.08%). The level of GHb was also significantly higher in the STZ treated group (5.07±0.11%). The higher values of GHb could be correlated with hyperglycemia induced in HFD and HFD+STZ group. The feeding of animals on HFD for 3 weeks led to higher levels of FFAs in blood. These were found to be significantly higher in HFD and HFD+STZ group, being 38.30±4.24 and 39.93±2.60 mg/dl, respectively in comparison to control (22.06±1.83 mg/dl). However, there was no significant difference between HFD and HFD+STZ group.

Lipid profile was altered after 3 weeks of HFD feeding. Triglycerides, total cholesterol, LDL-cholesterol and VLDL-cholesterol were increased in HFD fed animals in comparison to control animals after 3 weeks of feeding. Interestingly, HDL-cholesterol levels were not significantly altered between HFD fed and control group animals. However, the STZ treatment was found to reduce the HDL-cholesterol levels, the values being 34.33±0.60 and 27.69±.061 mg/dl in HFD and HFD+STZ treated group. The atherogenic index (LDL-C/HDL-C) though apparently higher in HFD fed rats but was not statistically significant. However, a significantly higher AI (1.76±0.12) was found in HFD+STZ group.

Changes in caecal microbiota

FISH was carried out in ceacal contents to determine the changes in the major bacterial groups. The cy-3 labeled probes used were specific to *Enterococcus rectale-Clostridium coccoides* (EREC), bacteroides (BAC), lactobacilli (LAB) and bifidobacteria (BIF). Total bacteria were enumerated using DAPI stain. The data on different bacterial groups in caecal content (expressed as log_{10} cells/g) are presented in Figure 4.

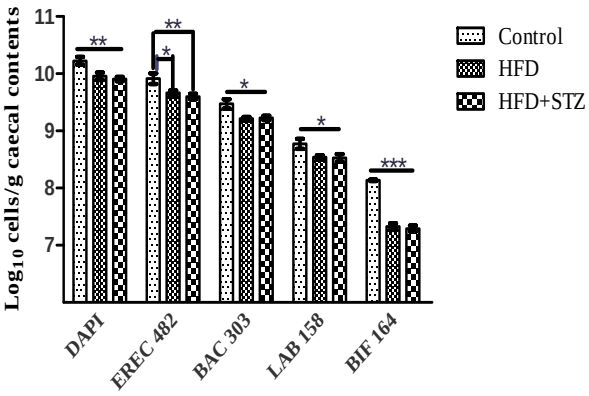

Figure 4 Effect of HFD feeding and STZ treatment on caecal microflora. Values are mean± SEM. *P<0.05, **P<0.01, ***P<0.001.

The total bacterial counts in HFD fed group were found to be significantly reduced (9.96±0.06 log_{10} cells/g) in comparison to the normal chow fed animals (10.22±0.07 log_{10} cells/g). However, there was no significant difference in HFD and HFD+STZ group. Similarly, the different bacterial groups were also found to decrease in HFD and HFD+STZ group. The bifidobacteria seemed to be most adversely affected as indicated by a decrease of >0.8 log_{10} cells/g in HFD fed group. However, there was no significant difference in bifidobacteria counts in caecal contents between HFD and HFD+STZ group. The bifidobacterial counts were 8.14±0.07, 7.33±0.06 and 7.29±0.05 log_{10} cells/g for control, HFD and HFD+STZ group, respectively.

SCFAs in caecal contents

The major SCFAs (acetate, propionate and butyrate) were determined in caecal contents and expressed as µmol/g caecal content, and their relative proportions were also calculated (Table 3). In HFD fed animals, acetate and propionate concentration were significantly higher in comparison to control, but no change was observed in butyrate concentration. Acetate concentration in control animals were 27.80±0.56 µmol/g caecal content that was raised to 35.60±1.2 µmol/g caecal content in HFD fed animals. No change could be observed in STZ treated group. Propionate concentration was measured to be 5.96±0.14 and 8.92±0.24 µmol/g caecal in control and HFD group, respectively. In HFD+STZ group, the propionate concentration was observed to decrease significantly. In terms of their relative proportions, HFD feeding altered propionate significantly. It was 29.78±0.24 and 33.57±0.45 % in control and HFD group. In case of STZ treated rats, propionate proportions were significantly reduced to 26.64±1.1%. Acetate and butyrate proportions were not affected in HFD group, but STZ treatment raised acetate proportions.

Table 3 Effect of HFD feeding and STZ treatment on SCFAs in caecal contents

Concentration (µmol /g caecal content)			
SCFA	**Control**	**HFD**	**HFD+STZ**
Acetate	27.80 ±0.56	35.60 ±1.2***	35.38 ±0.63***
Propionate	5.96 ±0.14	8.92 ±0.24***	6.45 ±0.31##
Butyrate	1.54 ±0.10	1.53 ±0.14	1.78 ±0.08
Proportion (%)			
Acetate	63.11 ±0.35	61.12 ±0.36	66.58 ±1.2***###
Propionate	29.78 ±0.24	33.57 ±0.45***	26.64 ±1.1**###
Butyrate	7.01±0.36	5.29±0.35	6.77 ±0.27

* Mean values were significantly different from those of the control. *P<0.05, **P<0.01, ***P<0.001. # Mean values were significantly different from those of the HFD fed group. #P<0.05, ##P<0.01, ###P<0.001

Gene expression in cecum and epididymal fat

Relative quantification of mRNA levels of different genes in HFD fed and HFD+STZ treated rats in comparison to control diet fed rats are illustrated in Figure 5 and 6. HFD feeding increased the expression

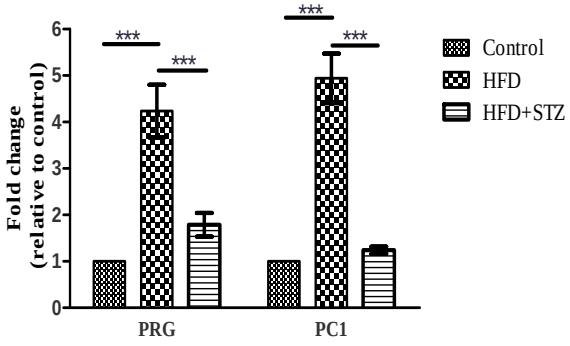

Figure 5 Effect of HFD feeding and STZ treatment on gene expression of proglucagon and PC1 in cecum. The amplification is compared by assuming the amplification in Control as 1. Values are mean ± SEM. ***P<0.001

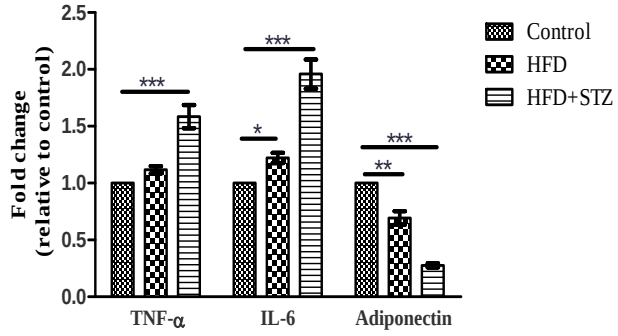

Figure 6 Effect of HFD feeding and STZ treatment on gene expression of TNF-α, IL-6 and adiponectin in epididymal fat. The amplification is compared by assuming the amplification in Control as 1. Values are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

of proglucagon and prohormone convertase 1 (PC1) genes in cecum, being 4.24 and 4.94 fold, respectively, in comparison to control animals. However, in case of HFD+STZ group, these were 1.8 and 1.2 fold for proglucagon and PC1, respectively. In epididymal fat, HFD fed rats had lower levels of adiponectin (P<0.01) mRNA and higher levels of IL-6 (P< 0.05) in comparison to control rats. However, there was no change in mRNA levels of TNF-α gene. In HFD+STZ treated group, the genes expression profile was significantly (P<0.001) altered in epididymal fat. The level of mRNA of adiponectin gene was lower while that of TNF-α and IL-6 was significantly higher than HFD fed animals.

DISCUSSION

Rats were first fed on HFD for three weeks to make them insulin resistant and then injected with low dose of STZ to develop type 2 diabetes. HFD fed and STZ treated (low dose) rats have been suggested as a novel animal model that mimics the natural history and metabolic characteristics of the common type 2 diabetes in humans, and is suitable for testing of antidiabetic compounds (Srinivasan et al. 2005; Reed at al. 2000). During initial 3 weeks of HFD feeding, body weight increased in comparison to normal chow fed animals but this change was not statistically significant. The additional gain in body weight on HFD feeding has been shown by numerous researchers (Zhang et al. 2008; Srinivasan et al. 2005). The additional gain in body weight in HFD fed rats might be due to the consumption of energy dense diet rich in saturated fat. After 3 weeks of HFD feeding, rats were made diabetic by injecting STZ intraperitoneally. STZ inhibits O-GlcNAcase of β-cell that accounts for its diabetogenic toxicity (Konrad et al. 2001). STZ treatment reduced the body weight drastically ~20 g within 3 days which is a characteristic feature of diabetes induced by STZ (Zhang et al. 2008; Ramadan et al. 2006; Srinivasan et al. 2005).

OGTT performed after 3 days of STZ treatment, showed massive decrease in glucose tolerance as insulin level decreased due to STZ induced destruction of pancreatic β cells. Therefore, great increase in $AUC_{glucose}$ was observed in diabetic rats. HFD feeding alone decreased the glucose tolerance in comparison to control rats while it decreased STZ treated rats many folds within 3 days of treatment. Reed et al. (2000) reported that increase in plasma glucose concentrations in STZ treated rats reached a plateau within 3 days. FBG was mildly elevated while plasma insulin levels were more than double in HFD fed rats in comparison to control rats which is a characteristic feature of insulin resistance (Srinivasan et al. 2005). Plasma insulin levels of STZ treated group was near to control group which clearly shows the effect of STZ on β- cells. Type 2 diabetic patients have comparable circulating day-long insulin concentrations in absolute terms to the values seen in non-diabetic individuals (Reaven, 1988). They differ only in their relative insulin efficiency. Another symptom of insulin resistance is abnormal blood lipid profile, mainly higher levels of TG, TC, LDL-C, VLDL-C and AI and lower level of HDL-C. These were also observed in HFD fed animals as compared to control except HDL-C. HDL-C level was low in STZ treated group resulting in higher AI in them. Due to hyperglycemia and hyperlipidemia, GHb and FFAs levels were increased in HFD fed animals as compared to control animals. GHb levels were further higher in STZ treated group. Srinivasan et al. (2005) reported that male Sprague–Dawley rats fed on HFD for 2 weeks exhibited significant increase in body weight, basal plasma glucose, plasma insulin, TG and TC levels as compared to normal pellet diet fed control rats. They also found frank hyperglycemia, reduction in plasma insulin, elevation in TG and TC after 7 days of STZ (35 mg/kg body weight) injection in HFD-fed rats. The difference in observations in our study could be due to difference in strain of rats and time of measurements after STZ injection. Many studies have suggested that impaired blood lipids, especially circulating FFAs are characteristic feature of insulin resistant subjects (DeFronzo and Tripathy, 2009; Hotamisligil, 2006). Our main focus was to explore the

mechanism of obesity and type 2 diabetes in terms of microbial modulation, SCFAs production and whether HFD feeding influences gene expression of proglucagon and PC1 genes in cecum, and expression of genes responsible for low grade inflammation in visceral adipose tissue.

In our study, HFD feeding exhibited decrease in total bacteria and other bacterial groups as compared to control diet fed animals. Most significant change was observed in bifidobacteria followed by *Eubacterium rectale-Clostridium coccoides* group. We did not find changes in caecal micrbiota in STZ treated group, the reason could be the short time (3 days) for diabetes induction in HFD fed rats. Dewulf et al. (2011) observed a drop in total bacterial counts and in most of the other analyzed bacterial groups in mice fed HFD for 4 weeks. Bifidobacteria have been reported to be low in HFD fed animals (Cani et al. 2007a,b). Waldram et al. (2009) reported lower number of bifidobacteria in the fa/fa obese rats thus proposing the protective role of bifidobacteria in obesogenesis. Brinkworth et al. (2009) compared the isoenergetic very low-carbohydrate, high-fat (LC) diet with a high-carbohydrate, high-fibre, low-fat (HC) in overweight and obese participants fed for 8 weeks and observed significant reductions in the LC group for counts of bifidobacteria. Schwiertz et al. (2010) also showed decrease in genus *bifidobacterium* in faecal samples of obese people. Cani et al. (2007a,b) also reported significantly lower numbers of the *Eubacterium rectale-Clostridium coccoides* group in mice fed the high-fat diet compared with control. In our study, bacteroides and lactobacilli were also reduced in HFD fed animals as compared to control. The decrease observed in these groups may be the result of decrease in total bacteria. Cani et al. (2007a, 2008) reported that HFD feeding increase harmful Gram negative bacteria and also increase in intestinal permeability that lead to higher concentrations of plasma lipoplysaccharide (LPS) which generate inflammation through TLR-4 and deteriorate the condition in obesity/diabetes.

Acetate, propionate and butyrate are the SCFAs produced by bacterial fermentation. In our study, HFD feeding increased the concentration of acetate and propionate while no change in butyrate concentration was observed. STZ treated animals had lower propionate concentration but no change in acetate concentration which suggests that propionate is an important metabolic modulator. It is further supported by data in terms of their relative proportions in which acetate proportion was increased while propionate proportion was decreased in STZ treated rats as compared to HFD fed rats Similar observations were made by Schwiertz et al. (2010) showing increase in faecal propionate levels in overweight people and total SCFAs in obese people. Recently, Payne et al. (2011) also reported significantly higher levels of propionate and total SCFAs in fecal sample of obese children in comparison to normal weight children. Contrary to these observations, Brinkworth et al. (2009) reported reduction in butyrate and total SCFA in very low-carbohydrate, high-fat (LC) diet fed overweight and obese participants in comparison to isoenergetic high-carbohydrate, high-fibre, low-fat (HC) diet fed for 8 weeks.

Post-translational modification of the proglucagon by prohormone convertase 1 (PC1) generates GLP-1 in the intestine (Drucker, 2003a). L-cells, producing GLP-1 increase in density along the length of the GIT, having highest numbers in the distal ileum and colon (Tolhurst et al. 2009). In our investigation, we found HFD feeding increase the expression of proglucagon and PC1 genes in cecum. HFD fed animals had more than 4 times expression of these genes in comparison to control animals. Along with numerous anti-diabetic effects, GLP-1 has been shown to have direct stimulatory effects on the pancreatic β cells (Tolhurst et al. 2009). In our study, increased expression of proglucagon and PC1 genes in cecum of HFD fed animals could be correlated with increase in plasma insulin in this group. STZ treatment produced sharp decline in their expression showing direct inverse correlation between hyperglycemia (due to lower plasma insulin) and GLP-1 expression.

Adipose tissue dysfunction dysregulate adipokine production and can have systemic effects on inflammatory responses, thereby contributing to the initiation and progression of obesity-induced metabolic and cardiovascular complications. Therapeutic strategies that counteract the imbalance of pro-inflammatory and anti-inflammatory adipokines could be an attractive and useful means for preventing and/or treating obesity-related metabolic and cardiovascular diseases (Ouchi et al. 2011). In our investigation, adiponectin gene expression was altered most significantly (decreased, $P<0.01$) followed by IL-6 (increased, $P<0.05$) in HFD fed group, no significant change was observed in case of TNF-α. STZ treatment altered gene expression profile in epididymal fat significantly ($P<0.001$). Expression of adiponectin was decreased while expression of TNF-α and IL-6 were increased. TNF-α gene has been reported to over express in the adipose tissues of various genetic models of insulin resistance (Hotamisligil et al. 1993). Yao et al. (2005) reported that serum adiponectin levels and mRNA levels of adiponectin in adipose tissue of type 2 diabetic rats (induced by HFD and STZ) were significantly decreased as compared to the normal control rats, and the mRNA levels of adiponectin in adipose tissue were inversely correlated with serum insulin, triglyceride, cholesterol, interleukin 6 and TNF-α. Brake et al. (2006) reported an increase in IL-6 mRNA levels but not in TNF-α in adipose tissue of C57Bl/6 mice in response to a high-fat diet for 3 weeks. Terra et al. (2009) also showed increased mRNA expression of IL-6 but no change in TNF-α and a decrease in adiponectin level in mesenteric adipose tissue of male Zucker Fa/fa rats fed on HFD (31.8% energy from fat) for 19 weeks. Lee et al. (2011) reported decreased levels of adiponectin, increased level of IL-6 and TNF-α in

plasma, and increased mRNA levels of inflammatory genes from epididymal adipose tissue at 3 days of HFD feeding in C57BL/6J mice.

Conclusion

Our study demonstrated that total bacteria, mainly bifidobacteria were reduced by HFD feeding. Propionate proportions, expression of proglucagon and PC1 genes in cecum, IL-6 gene in epidiymal fat were increased while expression of adionectin gene in epididymal fat was decreased in HFD fed rats. STZ treatment significantly reduced the propionate proportions, expression of proglucagon and PC1 genes in cecum and adiponectin in epididymal fat, and increased the expression of TNF-α and IL-6 genes in epididymal fat. These parameters could be addressed in research on obese and diabetic rats. Correction or improvement of these parameters by different nutritional/therapeutic approaches may have positive effect on obesity and diabetes. For this, probiotic, prebiotic, nutraceuticals and other functional foods can be explored which may improve these parameters and have positive effect on obesity and diabetes.

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