Implications of gut microbiota modulation biology essay

Science, Biology

Prasant Kumar Jena, Shilpa Singh, Bhumika Prajapati, Sriram Seshadri*Institute of Science, Nirma University, Ahmedabad, Gujarat, India

Running Title: Effect of Gut Microbiota Modulation on high Fructose fed rats

Corresponding Author:*Dr. Sriram SeshadriInstitute of Science, Nirma UniversitySarkhej-Gandhinagar Highway, Chharodi, Ahmedabad-382481, Gujarat, IndiaPhone: +91 2717 241901-04 ext752, Fax: +91 2717 241916E-mail: sriram. seshadri@nirmauni. ac. in, sriramsjpr@gmail. com

Abstract

Purpose: High consumption of dietary fructose increases incidence of metabolic disorders by altering the microflora balance. Manipulation of gut microbiota can prevent the development of metabolic changes. The objective of investigation was to study the effect of altering the gut microbiota by oral administration of pH sensitive cefdinir microspheres to high-fructose fed rats. Methods: Cefdinir microspheres were formulated for animal experimentation. High-fructose diet and cefdinir microspheres were given simultaneously for 30 days. Biochemical, histopathological and microflora population studies were performed for both the groups. Results: HFD rats showed hyperglycemia, hyperinsulinemia, hypertriglyceridemia and impaired glucose tolerance. Cefdinir treatment prevented the elevation in metabolic disorder associated biochemical changes as compared to HFD group. The cholesterol, triglyceride and body fat were significantly increased in HFD group. The histopathological changes in liver, small and large intestine were more profound in HFD group as compared to cefdinir treated

and control group. Lactobacillus population increase and decrease in Enterobacteriaceae in cefdinir treated group, indicated restoration of commensal microflora. Conclusions: Cefdinir microspheres reduced the development of metabolic changes induced by high fructose diet and increased gram positive and decreased gram negative bacterial population. Intestine targeted antibiotic delivery needs to be further explored for its therapeutic applications. Keywords: Cefdinir, Microspheres, Fructose, Gut microbiota, DiabetesAbbreviations: CEF, cefdinir; EL, eudragit L100-55; T2DM: type II diabetes mellitus; IGT, impaired glucose tolerance; DLS, dynamic light scattering; AIC, akaike information criterion; MSC, model selection criteria; MTCC, microbial type culture collection; OGTT, oral glucose tolerance test, LDLC, low density lipoprotein cholesterol; HDLC, high density lipoprotein cholesterol; FTIR, Fourier transform infrared spectra, SEM, scanning electron microscopy; ALT, alanine transaminase; AST, aspartate transaminase; SGOT, Serum glutamate oxaloacetate transaminase; SGPT, Glutamate pyruvate transaminase; HC, hepatic cells; CV, central vein; KC, kupffer cell; IDF, International Diabetes Federation.

INTRODUCTION

Diabetes is the most common endocrine disorder and number of people with diabetes in 2011 has reached a staggering 371 million, 4. 8 million deaths are due to diabetes and 300 million will subsequently added by 2025 (1). Obesity, type II diabetes and hyperlipidemia frequently coexist and are associated with significantly increased morbidity and mortality (2). A significant increase in total refined carbohydrate intake, particularly fructose

has paralled recent increased incidence of obesity and diabetes. Metabolism of sugars, particularly fructose occurs primarily in the liver and high fructose flux leads to enhanced hepatic triglyceride accumulation resulting in impaired glucose and lipid metabolism and increased proinflammatory cytokine expression (3). Sustained fructose consumption has been shown to decrease insulin sensitivity, increase inflammation, oxidative stress and pancreatic islet dysfunction promotes dyslipidemia which may increase the risk for development of Type 2 diabetes mellitus (4). T2DM is preceded by impaired glucose tolerance (IGT), where the metabolic and endocrine changes takes place which can be effectively prevented or even delayed through lifestyle changes or drug treatment (5). Because of the increasing prevalence of T2DM, there is a need to identify new effective strategies for diabetes prevention, including the potential use of nutritional supplements. In an attempt to answer this question, the investigation currently studied the effect of a fructose overload leading pathological changes and activity levels of the liver defense system as well as gut microbiota population. This would help both to better understand the role of gut microbiota involved in fructose-induced metabolic changes and to develop appropriate strategies for the prevention and treatment of obesity and T2DM triggered by unhealthy diets. Antibiotic therapy can affect not only the target pathogen but also commensal inhabitants of the human host. The extent of the impact on non-target microbial populations depends on the specific antibiotic used, its mode of action and the degree of resistance in the community (6). Besides immune and inflammatory mechanisms, other pathways may be involved which are the link between gut microbiota and metabolic syndrome.

The microbiota produces enzymes that degrade ingested polysaccharides, thereby promoting the absorption of nutrients (especially carbohydrates), resulting in increased liver lipogenesis, hepatic insulin resistance, and hyperinsulinemia. It has been demonstrated that high intake of cereal fiber is associated with reduced risk for T2DM (7). The mammalian host is colonized by trillions of microbes which inhabit the gastrointestinal (GI) tract, predominantly in symbiotic relationship with their host (8). Several lines of evidence suggest that dietary factors might profoundly influence gut microbiota composition. Switching to a high-fat diet resulted in a decrease in Bacteroidetes, whereas the numbers of Firmicutes and Proteobacteria increased (9). Importantly, this was observed in both the presence and absence of obesity, clearly suggesting that diet must be considered as a confounding factor affecting microbial composition. A change in the diet (i. e., from a low-fat, plant polysaccharide to a high-fat, high-sugar diet) shifted the structure of the microbiota within a single day, along with changes in metabolic pathways in the microbiome. Community population and function of the microbiota can change due to various ways including antibiotic treatment, inflammation, or changes in diet (10). Extended loss of the distinctive composition of gut microbiota has been linked with several disorders including inflammatory bowel diseases (11). Changes in microbial composition have been associated with obesity and weight loss; however, factors that cause these changes are not well defined (12). The alterations in community population, whether chronic or short-term, are accompanied by changes in the microbiota’s collective genome, or microbiome, and the patterns and specific metabolic capabilities (13). Recently it was observed

that feeding mice a high-fat diet also caused an increased Firmicutes and reduced Bacteroidetes type microbiota, as described previously (14). Therefore, the relevant variables, such as changes in diet, to changes in the microbiome, are important to understanding how environmental factors and behavior influence physiology of individuals. Many pharmaceutical dosage forms irritate the stomach due to their chemical properties. Others undergo chemical changes in gastric acid and through the action of enzymes. Specific eudragit acrylic polymers have been developed for peroral dosage forms with step-wise release of active ingredients in the digestive tract. The pharmaceutical principle of eudragit coating is to solubilize in a specific environmental pH value (15). Eudragit L100-55, composed of methacrylic acid and methyl methacrylate (1: 2, Mw= approx. 135, 000) (Scheme 1) was chosen as a pH sensitive polymer owing to its unique dissolution behavior above pH 5. 5. Eudragit have been used as pH-sensitive polymers in various applications including enteric coating materials and drug delivery vehicles and exhibited plastic deformation and significant speed sensitivity (16). Eudragit in combinations with other polymers, such as hydroxypropyl methyl cellulose and talc, stabilized loaded drugs and provided a controlled release of them (17). Microparticles made from eudragit polymers have been utilized for protein drug delivery to the lower intestine after oral administrations are based on the change of pH during the gastrointestinal passage (18). Cefdinir (8-[2-(2-amino-1, 3-thiazol-4-yl)-1-hydroxy-2-nitroso-ethenyl]amino-4-ethenyl-7-oxo-2-thia-6-azabicyclo[4. 2. 0]oct-4-ene-5-carboxylic acid; Fig. 1) is a semi-synthetic third-generation broad-spectrum oral cephalosporin active against both gram positive and negative bacteria, is widely used to

treat acute chronic bronchitis, rhinosinusitis and pharyngitis. It has only 21–25% of oral bioavailability (19), which is probably due to low aqueous solubility. The objective of the present work was to study the effects of gut microbiota alteration by using eudragit L100-55 coated cefdinir microspheres which used to disrupt the microbiota in small intestine of male wistar rat fed with fructose rich diet and evaluated their effect on health and physiological condition.

MATERIALS AND METHODS

Materials

Eudragit L100-55 (average molecular weight approximately 320, 000 g/mol) was supplied by Rohm GmbH and Co. KG (Germany). Cefdinir (Cefdinir is an extended spectrum oral third generation antimicrobial agent with a broad spectrum of activity against enteric gram-negative rods. it has low permeability) was received as a gift from Macleods Pharmaceuticals Limited, Mumbai, India. Polyvinyl alcohol (Mw 20, 000 Da, 80% hydrolyzed) was purchased from Sigma Aldrich. All other reagents and solvents were of analytical grade.

Methods

Preparation of microspheres

The preparation of microspheres was either based on an oil/water emulsification—solvent evaporation or solvent extraction method. The usually employed oil/oil emulsification process is given as a standard in the preparation of cefdinir microspheres. For all different techniques a fixed amount of polymer (100 mg) and drug (10 mg) were used. Accurately

weighed antibiotic cefdinir was taken in different drug polymer ratio (1: 2) and was added in an 5ml acetone organic phase (internal aqueous phase) having 10%w/v eudragit L100-55 (EL) and sonicated in an ultrasonicator (JY92-11DN, Syclon, Japan) for 10min . This solution was slowly injected (0. 33 ml/min) into an external aqueous phase containing paraffin light liquid (100 ml) and was emulsified containing the emulsifier, span 80 (2% v/v). The system was stirred continuously using a mechanical stirrer at 1000 rpm and 37° ± 0. 5° C for 5 h to form a uniform emulsion and allowed complete evaporation of the solvent to form microsperes. The paraffin was decanted off; the microspheres were washed 3-4 times with petroleum ether (40-60° C), collected by filtration and finally dried at room temperature for 3 h. (20).

Process yield

The process yield was calculated according to Eq. (1): Y% = (MMP/MT) ×100Where Y is the process yield, MMP is the mass of cefdinir microspheres recovered after freeze-drying step, and MT is the initial mass of eudragit L100-55 plus the mass of cefdinir.

Entrapment efficiency

30 mg cefdinir microspheres were dissolved in 50 ml of PBS (pH 7. 4) by shaking with magnetic stirrer for 24 h. The solution was filtered through Whatman no. 41 filter paper. An aliquot was assayed spectrophotometrically (UV-2450 Shimadzu Corporation, Japan) at 287nm. Drug entrapment efficiency was determined by using the following relationship.% EE: = (AS/ASt) × (MSt/DSt) × (DS/MS) × 100Where EE is the entrapment efficiency, AS is the medium area of sample peaks, ASt is the medium area of standard

peaks, MSt is the mass of standard, DSt is the dilution of standard, DS is the dilution of sample, and MS is the theoretical mass of drug in the sample.

In vitro release profile study

The transit time of a drug through the absorptive area of the gastrointestinal (GI) tract is between 9 and 12 hours, whereas γ scintigraphy studies confirm a short GI transit time from mouth to cecum of 4 to 6 hours Thus, assuming a maximum GI tract transit time of 12 hours, a formulation in the cecum is expected to release its drug load within 6 hours. Considering the same, in vitro drug release from all the batches of microspheres was studied for duration of 6 hours. The release profile of cefdinir from eudragit (L100-55) microspheres was evaluated in phosphate buffer (pH 5. 5) and which represents pH of small intestine. Microspheres equivalent to 1. 5 mg drug/ml were transferred to the dissolution media (20 ml) which maintained at 37º C ± 0. 5º C under stirring at 75 rpm. 0. 5 ml of samples were withdrawn every regular time interval up to 6 hours, and the withdrawn volume was replenished immediately by same volume of fresh phosphate buffer. Amount of drug released in the withdrawn sample was estimated by measuring absorbance in a UV-spectrophotometer at 287nm for cefdinir against a phosphate buffer (pH 7. 4 / pH 5. 5) as control blank.

Release Kinetics

In order to understand the drug release mechanisms, the results obtained were fitted in Weibull and Higuchi kinetic models. The rate constants were also calculated for the respective model. The release kinetic was determined

by considering correlation coefficient (R2) value, akaike information criterion (AIC) value and model selection criteria (MSC) value (21).

Enteric Nature of Microspheres

This test was performed to determine whether the drug would be released in the acidic environment of the stomach (i. e., pH between 1 and 3). Cefdinir microspheres equivalent to 1. 5mg drug was transferred to 20 ml of 0. 1 N HCl that was maintained at 37°C ± 0. 5°C under stirring at 75 rpm. 0. 5 ml of samples were withdrawn every regular time interval up to 3 hours, and the withdrawn volume was replenished immediately by same volume of 0. 1N HCl. Amount of drug released in the withdrawn sample was estimated by measuring absorbance in a UV-spectrophotometer at 287nm against a 0. 1N HCl as blank.

Physicochemical characterization of cefdinir eudragit microparticles

Scanning electron microscopy

The external and internal morphology of cefdinir microspheres was analyzed by scanning electron microscopy (SEM). The microspheres were fixed on supports with carbon-glue, and coated with gold using a gold sputter module in a high vacuum evaporator. Samples were then observed with the SEM (LEO-1430VP, UK.) at 10 kV.

Particle size analysis

The particles was analyzed basis on the dynamic light scattering technique (DLS) by using a Mastersizer (Malvern Instrument, Malvern, UK), Particle

sizes are expressed as the weighed mean of the volume distribution. Each value resulted from a triplicate determination.

FTIR analysis

Fourier transform infrared (FT-IR) spectra were obtained on a Perkin-Elmer, GX-FT-IR spectrometer. Eudragit L100-55 containing microspheres (with or without cefdinir) were prepared in KBr disks (2mg sample in 200mg KBr). The scanning range was 400–4000cm−1 and the resolution was 1cm−1.

In vitro antimicrobial profile study

In vitro antimicrobial studies of cefdinir microsphere were performed as described earlier (22) with modification. Cefdinir was dissolved in small amount of dimethyl formamide and suitably diluted with distilled water to obtain the final concentration. Similarly Cef EL and polymer was dissolved separately in distilled water and suitably diluted with the same to obtain the concentration of 5µg/ml. 1 mL overnight grown culture of E. coli MTCC 443 and L. casei MTCC1423 was inoculated in freshly prepared Luria bertani and MRS broth respectively. Both the broth was adjusted to pH 5. 5 before addition of inoculum. After 2 hr of incubation at 37°C at shaking 150rpm, prepared microsphere Cef EL (Cefdinir concentration: 5µg/mL (MIC 0. 1–0. 5µg/ml).) was added to the culture then incubated at 37°C with shaking at 150rpm. Changes in optical density (at 600 nm) were recorded every hour for 7 h (Fig. 2). Control cells were treated microsphere without cefdinir and eudragit L100-55 alone. The activities were compared with pure cefdinir and eudragit polymer.

In vivo experiments

Animals and Dosing

8 to 10 week-old healthy female wistar rats weighing 150–200 g were procured from the Animal Research Facility, Torrent Research Center, Ahmedabad (India) under the approval of Committee for the Purpose of Control and Supervision of Experiments on Animals, India, and were maintained at the animal house of Institute of Pharmacy, Nirma University, India. The animal caring, handling and the protocols were approved by the Institutional Animal Ethics Committee (IAEC), protocol no. IS/BT/PhD11-12/1004. The animals were acclimatized at temperature of 25±2°C and relative humidity of 50–60% under 12 h/12 h light/dark conditions for one week before experiments. Animals were assigned to a normal control group (CD; n= 6) that consumed a standard diet and normal drinking water, a high fructose-fed control group (HFD; n = 6) that consumed a standard diet having 65% fructose with normal drinking water and cefdinir microsphere treated group (HFD-cefdinir EL, n= 6), that consumed high fructose diet for 30 days except for the days before oral glucose tolerance tests (OGTTs) and blood collection. The composition of experimental diets is presented in Table 3. Animals had free access to food and water with or without fructose. The food and water intakes were recorded daily by correction of spillage and body weight was measured twice a week.

Oral glucose tolerance test

Oral glucose tolerance tests were performed between 8. 0 and 10. 0 h at weekly intervals. The food and fructose were removed from animal cages for

12 h before the administration of an oral glucose load (2 g/kg of body weight) by orogastric gavage. Blood samples were collected from the tail vein at 0, 15, 30, 60, 90 and 120 min after glucose administration. Glucose concentration was determined with an Accu-Check Advantage Blood Glucose Monitor (Roche Group, Indianapolis, IN, USA). Area under the curve for glucose (AUCglucose) was determined using the trapezoidal rule.

Blood and tissue sample collection

All animals were observed for their general condition, clinical signs and mortality. The blood samples (approximately 1 ml) were collected from the retro orbital plexus under mild anesthesia into the micro-centrifuge tubes for collection of serum on day 0 (for baseline values) and the last day of the experiment. The plasma was used to determine levels of blood glucose, total cholesterol, triacylglycerol, high-density lipoprotein cholesterol (HDL-C), Total protein and urea. The pathophysiology evaluations were carried out by biomarkers, (1) ALT (Glutamate pyruvate transaminase, SGPT) and (2) AST (Serum glutamate oxaloacetate transaminase, SGOT) was estimated from rat serum by Accucare diagnostic kit (India). The animals were sacrificed on 0 day and the end of the study by deep dose of anaesthesia. The liver, distal ileum and proximal colon were collected. Tissue samples were blotted with paper towel to remove blood, rinsed in saline, blotted to remove excess fluid for and stored at -70°C. A small portion of liver, distal ileum and proximal colon was excised from animals of each group fixed in 10% v/v formalin saline and processed for standard histopathological procedures. Paraffin embedded specimens were cut into 5 µm sections (Yorco Sales Pvt. Ltd.,

New Delhi) and stained with hematoxylin and eosin (H&E) for histopathological evaluations. The histopathological tissues sections were viewed and digitally photographed using a Cat-Cam 3. 0 MP Trinocular microscope with an attached digital 3XM picture camera (Catalyst Biotech, Mumbai, India).

Liver glycogen content

The liver glycogen was determined as described earlier (23). In brief Liver tissue (200 mg) was finely ground with 20 mL of 5% trichloroacetic acid in a homogenizer. The protein precipitate was filtered and clear filtrate was taken for analysis. Liver filtrate (2 mL) was pipetted into a 20-mL calibrated test tube and then 2 mL of 10 N KOH was added before placing it in a boiling water bath for 1 h. After cooling, 1 mL of acetic acid was added to neutralize the excess of alkali and fluid was brought up to the mark with water. Slowly, 2 mL of solution from the previous step was added to 4 ml of anthrone reagent in a separate test tube, which was placed in cold water to prevent excessive heating. After thorough shaking, the test tube was placed in a boiling water bath for exactly 10 min for the development of color and cooled with running tap water. The OD was read within 2 h at 650 nm.

Quantification of fecal bacteria

For quantitative determination of Enterobacteriaceae, lactobacilli and bifidobacteria, 1 gram cecal content 0. 9% NaCl solution and homogenized by vortexing for 10 minutes. Ten fold serial dilutions of each sample were performed to obtain 10-µL concentrations, which were plated with selective culture medium in triplicate. EMB agar was used for Enterobacteriaceae, MRS

agar was used for lactobacilli and BS agar was used for bifidobacteria. All media were obtained from Himedia (Mumbai, India). Results were reported as CFU/gm of cecal content. Microscopic characteristics of all of the colonies were investigated by Gram staining methods (24).

STATISTICAL ANALYSIS

All the values are expressed as mean ±S. E. M. Statistics was applied using graph pad prism software version 5. One way ANOVA followed by Tukey's multiple comparison tests was used to determine the statistical significance between various groups. Differences were considered to be statistically significant when P <0. 05.

RESULTS

Three different formulations containing cefdinir with eudragit L100-55 of various drug: polymer ratio were prepared. The properties of these formulations are listed in Table 1. The cefdinir microspheres were prepared by using emulsion solvent evaporation techniques by using eudragit L100-55. The production yield of microspheres determined by weighing the prepared microspheres by considering weight of all solid component used in the preparation of microsphere and results of entrapment yield are depicted in table (1). These yields were found to range from 60 to 82. 5% for the formulations (Table 1). From the results it was observed that increased polymer ratio with respect to drug leads to enhance drug entrapment efficiency, so as the amount of drug remaining and available for encapsulation increased as the theoretical drug loading increased. However the polymer with lower concentration, lowers the entrapment efficiency

because loss of product during the successive decantation, washing and drying procedure. Cefdinir loaded eudragit microsphere entrapment efficiency of cefdinir was found to be increased by changing drug to polymer ratio from lower to higher side and maximum entrapment efficiency was observed at drug to polymer ratio 1: 1 (Table 1). Fig. 2 shows the in vitro release profiles of cefdinir microspheres up to 6 h in simulated gastric fluid (pH 1. 2, 0. 1 N HCl) for 2 hour; in this duration, all the preparation showed negligible drug release (5-8%) under the said drug release media for drugs loaded with eudragit L100-55. As the concentration of polymer increases, release of drug was found to be much slower because more amount of drug entrapped inside the polymer matrix. Whereas more drug concentration, rapid release was observed for the absorption on the surface of microsphere. The values of correlation coefficient (r2) and rate constants (k) for Higuchi, Weibull, Hixson-Crowell, Makoid-Banakar and Korsmeyer-Peppas models are presented (Table 2). However, higher determination coefficients and lower residual mean square data were obtained from Weibull distribution with its parameters describing the types of dissolution profiles and dissolution time. The SEM images of cefdinir microsphere and eudragit L100-55 are presented in Figs. 6. The unprocessed cefdinir particles (Fig. 6a) have needle-like crystals, drastic change in the morphology and shape of particles was observed for both processed particles (Fig. 6c) and eudragit polymer (Fig. 6b). Fig. 5. exhibits the FT-IR spectra of intact cefdinir, eudragit L100-55 and eudragit L100-55 coated cefdinir (Cef EL). IR spectrum of cefdinir (Fig. 5A) is characterized by principal absorption peaks at 2928 cm−1 (O-H stretch COOH), 2849−1 (C-H stretch cyclic), 1761cm−1 (C= O), 1678cm−1 (C= C

alkene), 1620cm−1 (C = C aromatic), 1516cm−1 (N-H bending), 1391cm−1 (C- N stretch) and 656cm−1 (C-S). The spectrum of Eudragit L100-55 has a broad band characteristic of hydroxyl groups (O–H stretch vibration) in the range of 3476–2358cm−1, characteristic bands of methyl and methylene (C–H stretch vibration) at 2976cm−1, 2895cm−1, a strong band due to carbonyl groups (C-O stretch vibration) at 1733cm−1 and two bands due to ester linkages (C–O stretch vibration) at 1368cm−1 and 1266cm−1 (Fig. 5B). FTIR spectra of Cef EL polymer mixture shows prominent peaks at 3427cm−1 (O-H), 2922cm−1 and 2852cm−1 (C H), 1730 cm−1 (H -O- H bending). All the peaks of CEF completely disappeared with a shift of 1781–1767 cm−1. The FTIR spectra of Cef EL (Fig. 5C) complex shows complete disappearance of the CEF peaks at 3300, 2976, 2895 and at 652cm−1 with strong decrease in peak intensity. This suggested that, CEF could form inclusion complex with eudragit L100-55 in solid state. The microspheres of Cef EL did not show any new peaks, indicating no chemical bond formation in the complexes. The significant differences in the observed vibrational transitions and the bands in the spectrum of the crystalline form were clearer and sharper than the bands of the amorphous forms. In addition, in IR-spectra, it is already known that significant differences between hydrate and anhydrate form were observed around 2800–3800cm−1. Due to the O–H stretching vibration of water molecules, the unprocessed cefdinir, which is in monohydrate form, had characteristic peaks observed at 1164, 1118, 1620, 1761 3257, 3357cm−1, but not in processed particles. Under SEM examination, microspheres exhibited spherical morphology and smooth surfaces as well as a monodispersed size distribution shown in Fig. 3. The microsphere particle

size distribution of Cef EL (cefdinir and eudragit 100-55 drug polymer ratio 1: 1) was presented in Fig. 4. The antimicrobial activity of all Cef EL, cefdinir and eudragit L100-55 against Gram-positive (Lactobacillus casei MTCC1423) and gram-negative (E. coli MTCC443) species was checked and the results are summarized in Fig. 6. These studies revealed that Cef EL have shown similar antimicrobial activity like CEF alone against L. casei while eudragit L100-55 alone have no properties of microbial inhibition as it shows similar growth pattern like L. casei (Fig. 6A) However, Cef EL inclusion polymer complex has shown significant microbial inhibition against both the microorganisms as like pure CEF in pH 5. 5. However cefdinir known for its gram negative antimicrobial agents, the effects after eudragit polymer coating also not reduced much as shown in Fig. 6B. The antimicrobial activity of Cef EL polymer and pure CEF alone have identical inhibition properties against E. coli in pH 5. 5 where as eudragit alone had no inhibitory properties as grown with E. coli.

Body weight, food and water intake

As shown in Table 4, the rats fed a high-fructose diet (HFD) showed a slight increase in body weight, as compared to the normal control diet (CD) group at week 4. It was found that body weight was significantly reduced when administered with cefdinir microsphere (Cef EL) as compared to feeding HFD alone. The mean food consumption and water intake were not significantly different between the HF group and Cef EL group. Similar body weights were recorded in Cef EL and CD animals after the 4-week treatment period (198 ± 8. 3 vs. 195 ± 8. 5 g) but the HFD animals had higher gain in body weight.

The HFD animals drank a larger volume of water than CD (48 ± 5. 2 vs. 38 ± 4. 5 ml/ day) and increased rate in Cef EL group. On the other hand, the amount of food intake was significantly larger in CD (CD vs. HFD, 19. 93 ± 3. 2 vs. 16. 58 ± 2. 6 g/animal/ day). The weight of abdominal fat content was higher in the HFD group animals (12. 8 ± 0. 96) while CD (6. 32 ± 0. 43) and Cef EL (5. 17 ± 0. 26) group animals was almost similar.

Oral Glucose Tolerance Test

Figure 7. Shows that OGTTs value after 2nd week in the HFD group and the AUCglucose values were higher (46%) than that of the CD group while Cef EL group had 14% lower than that of HFD group (Fig. 7B). However, OGTTs were impaired after 4 week in the Cef EL animals and the AUCglucose values were lower (34%) than those of the HFD animals. After 4th week experimental period, the AUCglucose was 150% higher in the HFD than in the CD group, whereas in Cef EL animals it was 34% lower than that of the HFD animals yet 65% higher than that of the CD animals (Fig. 7D).

Blood and Tissue Biochemistry

Table 5 shows that the blood glucose levels in the HFD group were significantly higher (72% each) than those in the CD group. However, these variables were also higher (72%) in the Cef EL than in the CD group, but significantly lower (37%) than those of the HFD group. After 4 wk of fructose administration, liver triglycerides significantly increased (214%) while, liver cholesterol and hepatic glycogen decreased (32% and 21% respectively) in the HFD group as compared with normal values (Table 6). No significant differences were observed in liver glycogen between the CD and Cef EL

groups; however, plasma total cholesterol, triacylglycerol and LDL-C levels were significantly higher (i. e., 49%, 42% and 36% respectively) in the HFD than the CD group, whereas these variables in the Cef EL group were significantly lower (i. e., 27%, 26% and 8%, respectively) than those in the HFD group. However, HDL-C was significantly lower (20%) in the HFD group than in the CD group, whereas 6% increase differences were observed between the HFD and Cef EL groups. The activities of aspartate transaminase, alanine transaminase, total protein and total urea were shown in Table 5. The animals of the HFD group showed higher AST (42%) concentrations when compared with the CD group whereas Cef EL group were (17%) lower concentration than that of HFD group and little higher (17%) than CD group. The alanine transaminase concentration of animals of the CD and Cef EL groups had lower when compared to the HFD group. The urea concentration of Cef EL group was 21% higher than CD and 37% higher than HFD group. In addition, the HFD group showed low concentrations of liver total protein whereas no significant changes observed among CD and Cef EL group.

Histopathological analysis

Histopathological assessments were carried out to determine the possibility of pathogenicity induced by high fructose diet in the liver, small and large intestine rats. The histological examination of the liver showed a series of morphological alterations, notably hepatic steatosis in Fructose fed diet group. Reduced tissue damage and liver steatosis were observed in Cef EL + Fructose diet fed rats. In control group, liver sections, normal hepatic cells

(HC), central vein (CV) and kupffer cell (KC) were blurred observed due to staining (Fig. 8, series-IA). Fructose-fed rats showed macro- and micro-vesicular steatosis and necrosis around the portal triad as well as focal areas of the inflammatory cell infiltrate around the portal triad (Fig. 8, Series IB) while fig . 8, series IC shows the liver section of rats fed with HFD and treated with Cef EL. The liver section was shown ambiguous pathological symptoms in hepatic cells and vein systems. Liver sections of CD group rats and Cef EL were showed relatively less pathological symptoms as compared to HFD groups. Histological examination of the intestinal segments revealed villi edema, lymphocytes infiltration and goblet cells hyperplasia in rats of HFD group as compared to Cef EL and CD group animals. Control group small intestine appears normal villus and mucosal architecture while HFD group exhibited severe damage (i. e. villus denudation, loss of villus tissue, and even crypt infarction) but Cef EL group shows recovery of mucosa, villus, cryptal and repair of injury (Fig. 8, series II). HFD groups animal crypts of colon containing many goblet cells (Fig. 8, series III).

Enumeration of Fecal microbiota

Quantification of fecal bacteria of experimental animal was showed in Fig. 9. After oral administration of Cef EL into HFD group for 30 days, the Enterobacteriaceae population was increased 45% higher in HFD than CD group but Cef EL treated group had 58% lower than that of HFD group alone. The Lactobacillus content is higher in CD group and it reduced to 37% (HFD) and 27% (Cef EL) than CD group animals. In addition Bifidobacterium

numbers also dropped from HFD and Cef EL group than that of CD group by 6% and 14% respectively while Cef EL group is 8% lower from HFD group.

DISCUSSION

Present studies have focused on different strategy to avert and/or delay the onset of type 2 diabetes and its complications. In the present study, cefdinir was selected for its ability to target the Gram-negative microorganisms and its limited systemic impact. The pH sensitive polymer eudragit L100-55 was used to coat the cefdinir for the small intestinal drug release. The cefdinir microsphere (Cef EL) treatment on high fructose diet-induced rat resulted in a major alteration in the composition of the gut microbiota. The respective proportions of the three dominant genuses were altered dramatically with respect to each other, with a large reduction in gram negative and in particular the Enterobacteriaceae and a dramatic increase in lactobacilli. In the present study, we demonstrated that modification of gut microbiota by cefdinir microspheres ameliorated oral glucose tolerance and reduced hepatic steatosis in HFD rats. Earlier study reported that oxytetracycline (broad spectrum antibiotic) treatment reduced diabetic symptoms in BB rats (25). However, the favorable effects of the oxytetracycline treatment did not only depend on modulation of the gut microbiota, as tetracycline also regulates insulin secretion (26). Consequently, the antibiotics may directly influence the position of insulin sensitivity via unpredicted pleiotropic effects instead of gut microbiota modulation. The small intestine is the part of the GI tract where most of the host enzymatic digestion of the food occurs. The products of these digestive activities are absorbed in more distal parts of the

small intestine, the jejunum and especially the ileum. The conditions in the ileum are more favorable for microbial growth compared with the proximal part of the small intestine. The number of microorganisms in the ileum can be higher compared with the duodenum due to less acidic pH is and bile acids reabsorbtion (27). It is generally accepted that the commensal microflora of the small intestine is far more limited than that of the mouth or the large intestine. The microbial density increases from 101–104 microbial cells in the stomach and duodenum, 104–108 cells in the jejunum and ileum, to 1010–1012 cells in the colon and faeces (27). The gut microbiota also has important metabolic functions, breaking down dietary toxins and carcinogens, synthesizing micronutrients, fermenting indigestible food substances, assisting in the absorption of certain electrolytes and trace minerals, and affecting the growth and differentiation of enterocytes and colonocytes through the production of short-chain fatty acids (28). Finally, the normal gut microbiota helps to prevent luminal colonization of pathogenic bacteria, such as Escherichia coli and Clostridia, Salmonella, and Shigella species (29). Other studies have shown that modulation of the microbiota by broad-spectrum antibiotics results in a reduction in metabolic endotoxaemia in both high-fat-fed and ob/ob mice, and is associated with improvements in inflammation, glucose tolerance and hepatic steatosis, possibly through a mechanism involving Toll-like receptors (30, 31). However, whether the effect on weight gain is sustained or overcome by microbial compensatory adjustments is unclear, and more long term studies in animal models and humans are required. These data suggest that the ability of the gut microbiota to regulate inflammatory responses in diet-

induced obesity is important in the interaction between gut microbes and obesity-related metabolic dysfunction (32). Especially, the microbiota of the upper small bowel consists mainly of gram positive bacteria, and the numbers of gram-negative bacteria and anaerobes are low, suggests that the majority of the PAMPs present in the small intestine are likely to be derived from swallowed products of the oral microbiota, rather than from products of bacteria endogenous to the small intestine (33). The gut microbial alterations are associated with a reduction in body weight gain and an improvement in inflammatory and metabolic health of the host. Many earlier reports suggesting that an increase in body weight, glycemia, and insulinemia with the consumption of high-fructose diets in both humans and animal models (34). As expected, in our study HFD produced substantial weight gain and abdominal fat pad beginning at 4 weeks, with a continued increase in body weight throughout the experiment. Our results are consistent with previous studies which found that consumption of high-fructose diets markedly increase (160%) in body weight of HFD group than control group but Cef EL group has 49% lower weight gain that of HFD group. The abdominal fat content of HFD group was 102% higher than control animals but Cef EL group was 59% lower than HFD group. Diet-induced diabetes is largely caused by disorders of fat metabolism, resulting in a larger accumulation of fat in various tissues. Fructose metabolism occurs in the liver, which can produce energy in the oxidative chain or provide carbon skeletons for lipid synthesis that enter into the blood stream (3). Fructose is metabolically broken down before it reaches the rate-limiting enzyme (phosphofructokinase), thereby supplying the body with an unregulated

source of three-carbon molecules. These molecules are transformed into glycerol and fatty acids, which are eventually taken up by the adipose tissue, leading to additional adiposity. Because of its lipogenic properties, excess fructose in the diet can cause glucose malabsorption, and greater elevations in TG and cholesterol compared to other carbohydrates (35). Feeding of HFD to rat resulted in the elevation of various parameters of lipid profile. The repeated administration of Cef EL orally for a period of 30 days resulted in a significant decrease in the lipid profile in plasma when compared to the dyslipidaemic HFD control. The present investigation clearly demonstrates the cholesterol lowering effects of Cef EL in dyslipidaemic rats. In this context, the dominant presence of gram positive bacteria in the gut could be important for cholesterol elimination. Elevated levels of plasma TG have been correlated with the development of various metabolic diseases including type II diabetes and insulin resistance. While the HFD control groups exhibited significantly higher TG levels, Cef EL treated group registered a significant decline of TG in plasma. An increase in the plasma glucose level after glucose loading during OGTT in HFD fed rats, indicate an inability of insulin to stimulate glucose disposal in peripheral tissues associated with insulin resistance. From the obtained datum, in the OGTT, it is clear that in untreated (HFD) diabetic rats the blood glucose levels remained high even after 2 h. In contrast, in Cef EL treated diabetic rats the blood glucose levels reached a peak and returned again to fasting levels after 2 h. An increase in the plasma glucose level after glucose loading during OGTT in HFD fed rats, indicate an inability of insulin to stimulate glucose disposal in peripheral tissues associated with insulin resistance. Liver

glycogen is often lower in patients with type 2 diabetes, and restoration of liver glycogen storage is associated with increased hepatic insulin sensitivity (Magnusson et al. 1992, Membrez et al. 2008). Liver glycogen concentration was initially lower in the HFD group as compared to the control subjects. This finding does not appear to be related to the dietary conditions, since all subjects fed a standardized diet for three days before the study. It is possible that the diabetic subjects have a defect in liver glycogen synthesis as well as in muscle glycogen synthesis which has previously been shown (36). Liver glycogen synthesis has not been assessed directly in type II diabetic patients; however, splanchnic glucose uptake is decreased after glucose administration (37). Improvement of hepatic insulin sensitivity leads to the suppression of hepatic glucose output and an increase in liver glycogen storage. In the antibiotics-treated ob/ob mice, the level of liver glycogen was markedly increased (31). In the present study, the hepatic glycogen level of Cef EL group was marked increased 21% higher as compared to the HFD group but 3% lower than that of Control group (Table 6). Type II diabetes is related with several complications, such as hypertension, endothelial damage, cardiac hypertrophy, inflammation, atherosclerosis, ventricular contractile dysfunction, fibrosis, neuropathy and nephropathy (35). Rat models of human diseases, such as diabetes have been broadly used to examine the development of the disease symptoms as well as promising treatment options. The high fructose fed/induced rat diabetes model has an added advantage as it mimics many of symptoms of Type II diabetes in humans, especially insulin resistance or glucose intolerance, dyslipidemia and renal impairment together with hypertension (38). Fructose induced

insulin resistant states are commonly characterized by a profound metabolic dyslipidemia, which appears to result from hepatic and intestinal over production of atherogenic lipoprotein particles (3). Another factor common to the Western diet that is receiving attention in terms of metabolic disease is the sweetening of products, particularly soft drinks, with fructose. It has been found that fructose may be poorly absorbed by some subjects, leading to an expansion of bacteria in the small intestine. Accordingly, increased portal endotoxin levels and hepatic steatosis are observed in mice consuming water sweetened with fructose, but not glucose (39). The increase in both fructose consumption and high calorie intake, as well as the decrease in physical activity, have been identified as the main factors contributing to the growing numbers of obese and overweight individuals in many countries around the world (40) and experimental models have been widely used to analyze the metabolic effects of fructose-rich diets and exercise (41). The global epidemic of obesity and obesity-associated disorders, including diabetes, metabolic syndrome and cardiovascular disease, continues apace despite widespread public health education. Among the more stimulating developments in medicine in recent years, the evidence linking indigenous microbiota with obesity and obesity-associated complications has been significant. Discovery of a microbial-dependent pathway for metabolism of dietary phospholipids, which generates metabolites have pro-atherosclerotic effects after absorption and hepatic metabolism (42). Even more fascinating is the microbial contribution to the metabolic processes supporting obesity. This involves multidirectional signalling among the microbiota, host metabolism and immunity, with

dietary intake influencing each component of this triangular network. Several lines of evidence have linked the microbiota with these processes (32, 43). Obesity is associated with reduced microbial diversity and an increase in the ratio of Firmicutes: Bacteroidetes, the two dominant phyla or families of microbes in the human gut (43). Manipulation of the microbiota with antibiotics or pre- and probiotics improves the metabolic welfare of the host, in terms of glucose tolerance and cytokine profile, although exposure to antibiotics in infancy may actually predispose to obesity and metabolic disease in later life. Since inflammatory and metabolic pathways are inter-linked, the microbial signals acting on either or both may offer adjunctive therapeutic or preventive strategies for obesity (43). High fructose fed rats have been used regularly as a model for studying the metabolic factors associated with metabolic syndrome, a complex metabolic disorder linked to increased risk for cardiovascular disease (44), diabetes (45), and even different forms of cancer (46) and nonalcoholic fatty liver disease (47). Inclusion of 58% fructose in the purified diet in the current study did not produce a strong response in some of the metabolic parameters associated with metabolic syndrome. The prepared microspheres were found to be discrete, spherical and uniform in size with a range of 230-280 µm (Fig. 4). The SEM photograph indicates that the microspheres were smooth, spherical and free flowing (Fig. 3). From the result of FT-IR, it was clear that there was no interaction between drug and polymer used in the formulation (Fig. 5). The drug release from eudragit L100-55 polymer showed slow release at gastric pH but higher release obtained at intestinal pH. The Cefdinir microspheres with a coat consisting of eudragit L100-55 exhibited desired

antimicrobial properties in the ex vivo release study against Lactobacillus (Fig. 6a) and E. coli (Fig. 6b) with a pH of 5. 5. The microsphere started inhibiting the growth of L. casei and E. coli in different proportion after 2 hr of drug loading. But as per spectrum of the activity, higher gram negative microorganism E. coli inhibition was observed as compared to gram positive Lactobacillus. Earlier report of ranitidine hydrochloride microspheres coated eudragit E100 size was ranging from 247-286µm (48) and size of Eudragit RS 100 microparticles containing 2-hydroxypropyl β-cyclodextrin and glutathione was ranging from 150-350 µm for oral administration (49). Above results are comparable with the particle size of prepared cefdinir microspheres in the present investigation. There are no reports available till date for cefdinir coating with eudragit L100-55 polymer. As per our results, the size of cefdinir microspheres was suitable for oral administration following efficient targeting to the intestinal microflora within the pH range of 5. 5 for various therapeutic and preventive approaches.

CONCLUSION

In summary, gut microbiota modulation with cefdinir microsphere reversed the insulin resistance characteristic of male wistar rat. It is possible that the presence of certain bacteria in the gut might exacerbate the low-grade systemic inflammation, which further causes whole body insulin resistance. In the present study, rat treated with targeted intestinal delivery of antibiotics showed improved glycemic control, suggesting that gut microbiota influences whole body glucose homeostasis. Our results support the idea that modulating gut microbiota could be beneficial for improving

glycemic control. In conclusion, the data demonstrate that cefdinir microsphere administration produced distinctive modifications in the gut microbiota in fructose rich diet-induced diabetic rats. This further provides a confirmation on the role of the microbiota in metabolic dysregulation, and a supporting rationale for altering the microbiota as a prophylactic strategy using antimicrobial agents specifically targeting to intestinal delivery but specificity of action will be crucial. However, more work has to be done in order to prove that gut microbiota modulation is a safe and effective therapeutic strategy in treating or managing type II diabetes in humans.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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