Synergistic Effects of Ad-Libitum Low-Dose Fructose Drinking and Low-Dose Streptozotocin Treatment in Wistar Rats: A Mild Model of Type 2 Diabetes

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Abstract- To develop a convenient animal model of T2D by pretreatment with low-dose 10% w/v fructose (FRC) solution followed by the injection of low doses of streptozotocin (STZ) in Wistar rats. For this 8-week experimental study; rats were first fed a standard chow ad-libitum diet and either tap water (n=40) or 10% w/v FRC solution (n=40) for 4 weeks. Next, rats in each category were randomly allocated to 4 subgroups (n=10 each) of low-dose STZ (25,35, and 45 mg/kg). The final mean fasting blood sugar (FBG) of FRC+STZ45 (197±55.87 mg/dl) were significantly higher than that of the STZ45 (P=0.015) and FRC (P=0.019) groups. FRC+STZ45 showed the highest insulin resistance demonstrated by insulin tolerance test [area under the curve (AUC) of insulin tolerance test; P<0.05]. AUC was not significantly different between the STZ45 and non-STZ groups and between FRC and non-FRC fed groups. Furthermore, FBG levels did not differ between FRC and non-FRC groups. Body weight measurement showed that the FRC+STZ45 group had the lowest body weight compared to all other groups. Our data provide the evidence that FRC and STZ45 synergistically could induce hyperglycemia and insulin resistance in Wistar rats. Here we presented a feasible model for initial forms of T2D by employing pretreatment with low-dose FRC solution and treatment with low-dose STZ.

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Keywords: Experimental animal model; Hyperglycemia; Type 2 diabetes; Insulin resistance; Streptozotocin; Fructose

Introduction

Type 2 diabetes (T2D) is a heterogeneous disorder recognized by progressive insulin resistance followed by the failure of β-cell mass compensation for insulin resistance. These events eventually lead to glucose intolerance, hyperglycemia and overt diabetes (1-3). It is believed that T2D could be the biggest epidemic in human history. A total number of people with diabetes was 171 million in 2000 and is estimated to rise to 366 million in 2030 (4). In spite of considerable efforts devoted during the last two decades for the prevention and treatment of T2D, the glycemic control still is a major problem in diabetic patients. In this regard, different animal models of diabetes have been introduced to study the pathophysiology and treatment strategies for diabetes (5).

Animal models of diabetes can be generated spontaneously (genetic models), or induced by chemicals agents, dietary modifications, surgical manipulations and/or by a combination of these factors. One of the extremely common methods for inducing diabetes is the use of streptozotocin (STZ), a chemical drug which selectively destroys the insulin-producing pancreatic β cells. Administration of STZ causes a drop in plasma insulin level leading to hyperglycemia in the experimental animals. Partial pancreatectomy which directly reduces β cell mass is another procedure for producing animals with mild/moderate hyperglycemia and insulin secretory defect. Both procedures produce animals that mimic human type 1 diabetes rather than T2D (5-7). The pattern of the disease initiation and development in most of these models do not seem to be similar to the clinical situation in humans (2).

The combination of a high-calorie diet and STZ is also used for inducing T2D. This model seems to be a

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better model for studying diabetes, as the induction of insulin resistance by high-calorie diet following the destruction of β cells by STZ injection can be observed in this model (2,5,6,8). At this context, the combination of a high-fat diet and low-dose STZ has been previously studied (2,5,6,9). These combined models are cheap, easy to develop and suitable for studying the pathophysiology of T2D (5). High-fat diet in these models can induce insulin resistance by increasing the fat depot in the body.

Studies also suggest that increased consumption of carbohydrate especially high in fructose (FRC) increases the risk of insulin resistance. Feeding normal rats with an FRC enriched diet induces adverse metabolic alterations including the hyperglycemia, hyperinsulinemia, and glucose intolerance (10-12). Although the diet-induced models can better reflect the pathophysiology of diabetes in humans; to date, most of these models produce inconsiderable hyperglycemia. In addition, the diabetogenic and fattening abilities of various STZ doses along with various doses and routes of FRC ingestion had been controversial (11-14). Therefore, in this study, we aimed to introduce a new mild model of T2D by combining FRC and STZ in rats. There are two major questions that have yet to be explored; first, whether pre-treatment with ad-libitum low-dose FRC drinking followed by low-dose STZ could induce insulin resistance and hyperglycemia, secondly whether this combined model (FRC+STZ) exerts its probable diabetogenic roles through inducing the obesity in animals. To address these questions we conducted an experimental animal study on Wistar rats subjecting to various combinations of low-dose STZ injections after 4 weeks pre-treatment with ad-libitum low-dose FRC drinking.

Materials and Methods

Materials

Chow diet was purchased from Behparvar (Tehran, Iran). Insulin was from Sigma Aldrich (Germany). Fructose was purchased from Merck (Germany). Accu-Check Active glucometer was purchased from Roche Diagnostic Corporation, Mannheim (German).

Rats

This research is an experimental study. Seven-week-old male Wistar rats weighing 70-120 g were obtained from (Pasteur Institute, Tehran, Iran) and housed in individual cages. The animals were maintained under controlled conditions of temperature (22±3° C) and light (12-h light/dark cycle). Prior to the intervention, rats were acclimated for one week, during this week rats had free access to tap water and chow food (Behparvar, Tehran, Iran). During the acclimation, the daily food intake was documented. According to the manufacturer protocols, the composition of the food was: energy, 3.84-3.86 kcal/g; carbohydrates, 51.5-52.5%; total protein, 19.5-20.5%; total lipid, 3.5-4.5%; cellulose, 4-4.5%; maximum ash, 10.0%; maximum moisture, 10.0%. All procedures and protocols were performed in accordance with the institutional guidelines for animal care and use. At the end of the study, rats were euthanized by cervical dislocation under the ketamine anesthesia.

FRC+STZ protocols

After acclimation period, rats were fed a standard chow ad-libitum diet and either tap water (n=40) or water supplemented 10% w/v FRC (n=40) for 4 weeks. After 4 weeks, each group was randomly divided into four subgroups (n=10 each). After 16 h fasting, rats were undergone intra-peritoneal injection of three single doses of STZ (25, 35, and 45 mg/kg), while the respective control rats received the vehicle citrate buffer. All animals were then followed 4 weeks. The nomination of groups in this study is as follows: 1, Control (ad-libitum food+tap water); 2, FRC (ad-libitum food+10% w/v FRC); 3, STZ25 (ad-libitum food+tap water+STZ 25 mg/kg); 4, FRC+STZ 25 (ad-libitum food+10% w/v FRC+STZ 25 mg/kg); 5, STZ 35 (ad-libitum food+tap water+STZ 35 mg/kg); 6, FRC+STZ 35 (ad-libitum food+10% w/v FRC+STZ 35 mg/kg); 7, STZ 45 (ad-libitum food+tap water+STZ 45 mg/kg); 8, FRC+STZ 45 (ad-libitum food+10% w/v FRC+STZ 45 mg/kg).

Rat weights and fasting blood glucose (FBG)

Rat weights were measured on the same day and time of each week. At the end of acclimation week, rats fasted for 16 h, and a tip of the tail was snipped with sharp scissors and gently squeezed for a drop of blood. Then plasma glucose concentrations were assessed by glucometer (Accu-Check Active; Roche Diagnostic Corporation, Mannheim, German). FBG was determined every 2 weeks on the same day and time during the FRC+STZ phase. Glucometer was calibrated with the help of calibrators provided by the manufacturer.

Chow food intake and fructose consumption

Chow food intake was measured and taken away at 16:00 h each day. Consumed volume of fructose

solution was freshly prepared and changed every two days. Unfortunately, we were unable to use metabolic cages to measure the exact amount of calorie intake of rats. We assessed the amount of consumed fructose solution by ordinary laboratory volume equipment.

Insulin tolerance test (ITT)

ITT was conducted at the end of study after overnight fasting (16 h). Insulin (0.8 U/kg in 0.9 salines) was injected intraperitoneally; and glucose concentrations were measured at the time points of 0, 15, 30, 60, 90, and 120 minutes.

Statistical analyses

Data are reported as means±SE and were analyzed using IBM™ SPSS 20. One-way ANOVA was used for exploring the differences between group means. The Shapiro-Wilk test was used for testing the normality of continuous variables. A Tukey's or Tamhane's T2 post hoc test was conducted to discover the significant differences between group means. Repeated measures ANOVA test was used to test the longitudinal measures of body weight, food intake, calorie intake, FRC intake, and biweekly FBG. In cases of significant Mauchly's test of sphericity, Greenhouse-Geisser epsilon was used for reporting the significance of within-subjects effects. $P<0.05$ was considered statistically significant.

Results

Glucose concentrations

Longitudinal FBG measures of rats are shown in Figure 1a. The final FBG levels at weeks 8 are also displayed in Figure 2a. Repeated measures ANOVA showed a significant time effect ($P=0.006$) and time×groups interaction ($P=0.003$). The significant time×groups interaction indicates hyperglycemia was developed in some FRC+STZ groups over time. The final mean FBG of groups were significantly different based on one-way ANOVA ($P=0.003$) so that only FRC+STZ45 rats showed significantly higher FBG compared to STZ45 ($P=0.015$) and FRC ($P=0.019$) rats. Both the STZ45 (135.86±40.19 mg/dl) and FRC+STZ45 (197±55.87 mg/dl) protocols could induce hyperglycemia.

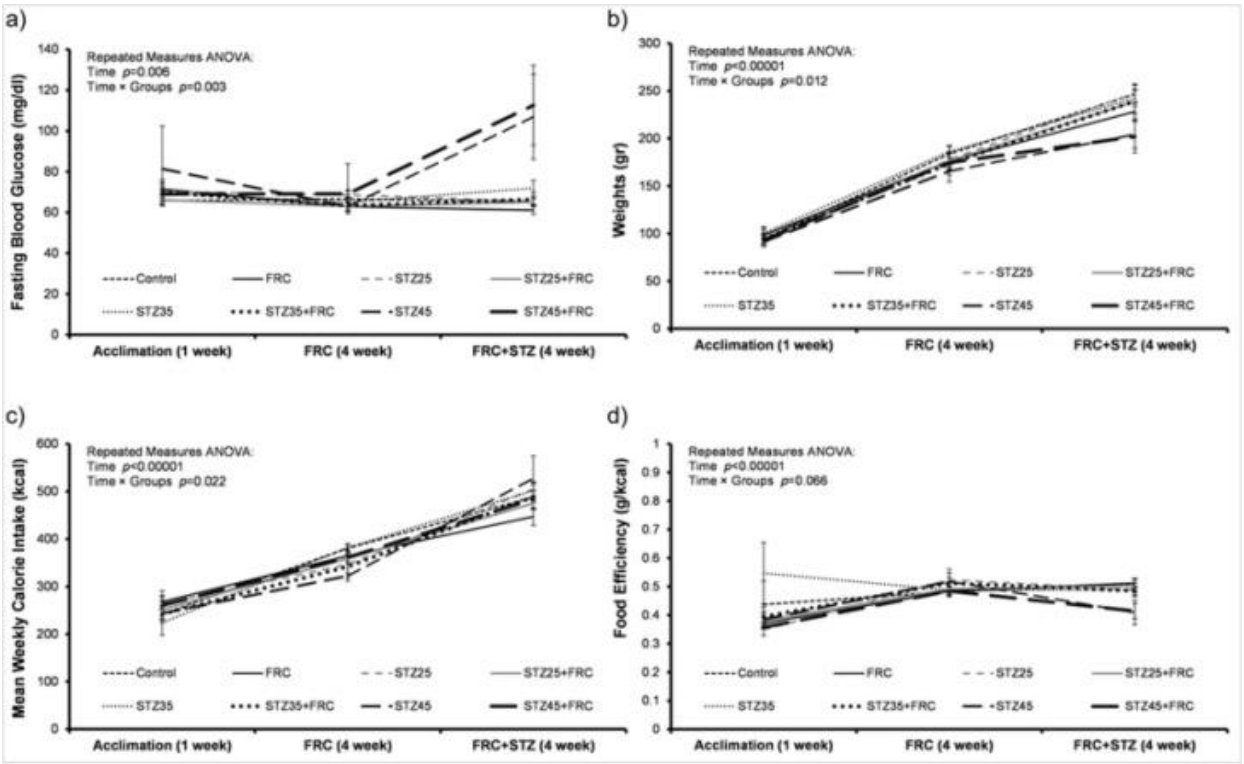

Figure 1. Longitudinal measures for a) fasting blood glucose; b) weight; c) mean weekly calorie intake; and d) food efficiency along with 9 weeks study (1-week acclimation, 4 weeks FRC, and 4-week FRC+STZ regimens). Values are expressed as means±SE. Repeated measures ANOVA with Tukey's *post hoc* test were conducted for between-group analyses. $P<0.05$ was considered statistically significant

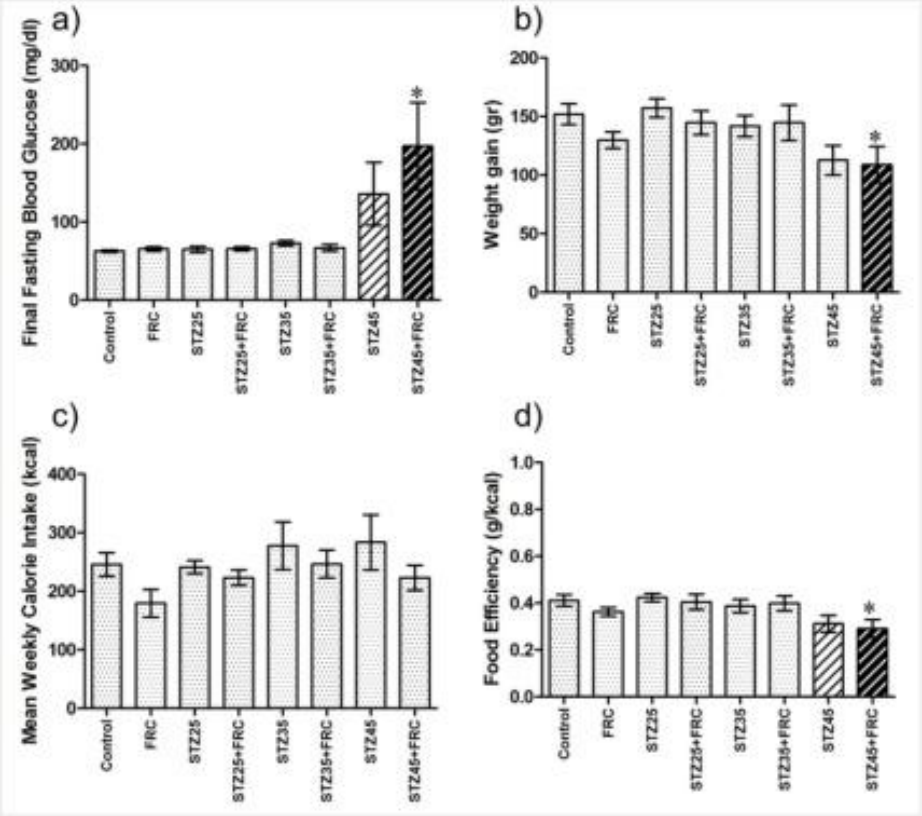

Figure 2. Mean fasting blood glucose (a), weight gain (b), weekly calorie intake (c), and food efficiency (d) of rats. One-way ANOVA followed by Tukey's *post hoc* were performed for detection of significant differences. Data are expressed as means±SE and $P<0.05$ was considered statistically significant (*).

Body weights

Repeated measures of body weights and weight gain through 8-weeks study are displayed in Figure 2b and Figure 2b, respectively. We observed a significant time effect ($P<0.00001$) indicating that all groups gained weight over time. In addition, significant time×groups interaction ($P=0.012$) explains a different pattern of fattening between different groups, as the FRC+STZ45 showed the significantly lowest weight gain ($P=0.022$).

Calorie intake

The consumed energies from both chow food and FRC solution were merged to clarify the energy intake of rats. Repeated measures of calorie intake and mean weekly ad-libitum calorie intake are shown in Figure 1c and Figure 2c, respectively. The significant time effect ($P<0.00001$) reflects that the calorie intake of all rats was increased during the study. Although mild, significant time×groups interaction ($P=0.022$) describes the different pattern of calorie intake among groups along the follow-up, the mean weekly ad-libitum calorie intake of rats was not significantly different between groups.

Food efficiency

We also estimated food efficiency [rats weight gain (g) per calories consumed (kcal)]. Longitudinal changes in food efficiency are displayed in Figure 1d. The significant time effect ($P<0.00001$) shows that the food efficiency of all rats changed during the study, while borderline significant time×groups interaction ($P=0.066$) reflects the significant lower ($P<0.05$) food efficiency of FRC+STZ45 rats compared to the others (Figure 2d).

Insulin tolerance test

Results of ITT are depicted in Figure 3a. There was a significant ($P=0.0035$) "Area Under the Curve (AUC)" between 8 groups, in which FRC+STZ45 group had the highest insulin resistance. The significant time effect ($P<0.00001$) in ITT showed a considerable decrease of FBG in response to insulin, and borderline significant time×groups interaction ($P=0.091$) could hint us significant differences of insulin resistance. Furthermore, AUC was not significantly different between the STZ45 and non-STZ groups and between the FRC and non-FRC fed groups.

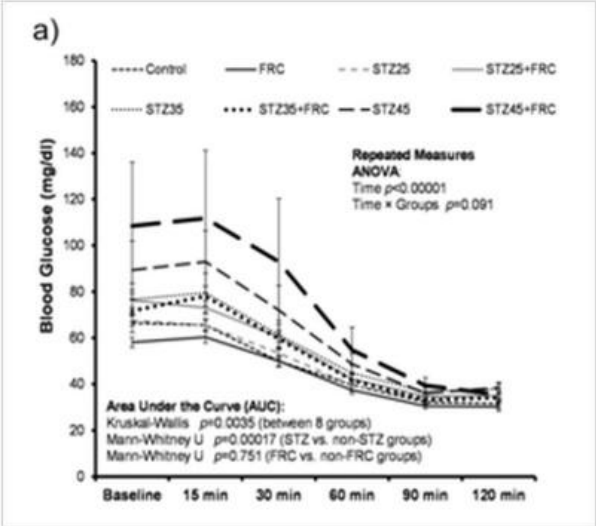

Figure 3. Insulin tolerance test (ITT), repeated measures ANOVA with Tukey's post hoc test was conducted for between-group analyses. Differences between areas under the curve (AUC) of groups were tested by Kruskal-Wallis and Mann-Whitney U tests. Values are expressed as means±SE. $P<0.05$ was considered statistically significant.

Discussion

The development of a model which closely reflects the metabolic characteristics and natural history of human diabetes have always been a challenge (5). This study aimed to develop an animal model of T2D by the possible interactions between STZ-induced β-cell destruction and FRC-induced insulin resistance. In this study, we introduced a mild model of T2D in Wistar rats, by a combination of low-dose FRC drinking and low-dose STZ.

In order to induce insulin resistance in the model, we used 10% w/v FRC solution, the lowest FRC concentration that has been previously used to induce insulin resistance in different animal models. We used this amount of FRC to avoid the severe insulin resistance that may cause by high FRC concentrations. In the present study, we observed that FRC intake leads to weight gain at the end of the 4th and 8th weeks of the study (Figure 1b), and this result is in line with a previous report (15). However, 10% FRC had no an obvious influence on FBG of FRC and FRC+STZ (doses lower than 45 mg/kg) groups, In addition, 10% FRC solution could not induce insulin resistance per se, as demonstrated by AUC of ITT (Figure 3a). In line with these findings, several reports have shown that this amount of drinking fructose is unable to induce insulin resistance in different animal models (15,16). Moura *et al.*, reported that the administration of fructose (10 %) to adult (90 d) male Wistar rats did not change the metabolic parameters and could not induce T2D (14). Anvari *et al*., have also suggested that the consumption of FRC (20%), but not FRC (10%) for 14 weeks could induce insulin resistance and hyperglycemia in male Sprague-Dawley rats (17). In addition, glucose level did not alter in mice which were treated with 15% fructose solution for 16 weeks and in Wistar rats administrated with fructose solution (10%) for 8 weeks (18,19). However, a positive effect of FRC solution (10%) on insulin resistance and glucose metabolism has been reported in other studies. Sanchez-Lozada *et al*., (19) compared the metabolic effects of fructose (10 %) in drinking water and a high-fructose (60 %) diet in Sprague-Dawley rats. Both FRC concentrations could induce hyperuricemia and hypertriacylglycerolaemia. However, the 60% fructose diet was directly associated with worsening the metabolic syndrome parameters (20). Moraes-Silva *et al*., found that Wistar rats receiving fructose in drinking water (10%) for 10 weeks were developed the features of the metabolic syndrome (21). Therefore, the metabolic alterations in fructose-fed rats are quite divergent among the studies, and these differences can be attributed to the strain of rat used, such as Wistar and Sprague-Dawley; the amount and route of fructose administration, diet (60 %), oral administration (8 g/kg) or drinking water (10 %); the age of the animals used, young or adults, and the period of fructose administration from 4 weeks to several months (14).

The next step of the study was to induce β-cell destruction by low doses of STZ on FRC fed rats. These doses appear to destruct β-cells without much

circulating insulin deficiency. Based on previous studies, an STZ dose of <50 mg/kg is low-dose and sub-optimal to induce insulin insufficiency per se (22,23). In this study, we used single low doses of STZ (25, 35, or 45 mg/kg) at the end of the 4th week of 10% FRC consumption. Only FRC+STZ45 regimens could induce hyperglycemia (Figure 2a). Our FRC regimen, as stated earlier, could not induce insulin resistance and hyperglycemia, however, the combination of FRC with STZ45 led to a higher FBG (197.5 vs. 135.8 mg/dl) and AUC of ITT levels, compared to the STZ45 group, indicating the synergy of FRC and STZ45 in inducing hyperglycemia and insulin resistance in Wistar rats. We believe that the evolution of insulin resistance in insulin resistant FRC rats rests upon its synergism with low-dose of STZ (45 mg/kg). This synergy produced frank hyperglycemia in the presence of circulating insulin concentration which seems to be approximately similar to normal rats, as the STZ45 and lower STZ doses could not significantly decrease the β cells insulin secretion enough to cause hyperglycemia in non-FRC groups. We were not able to observe an inducing effect of FRC on body weight in the STZ45 group. The fact that FRC does not exert a synergism on fattening of the rats with STZmight suggest that FRC amplifies its adverse effects only on peripheral tissues leading to insulin resistance rather than the fattening in STZ rats.

The development of a rat model possessing insulin resistance by substituting the water by FRC has been described (10,12,13,15,16,24). More exactly, high-FRC diets could increase weight gain and plasma triglyceride levels and hyperinsulinemia accompanied with insulin resistance/glucose intolerance in animals (24,25), a condition similar to prediabetic, insulin resistant state in humans (2). Reasons for developing T2D by chronic FRC overnutrition might be elucidated by the hexosamine hypothesis, where hexosamine flux is believed to regulate glucose and satiety-sensing pathways (10). Overexpression of the glutamine:fructose-6-phosphate amidotransferase, the pivotal regulatory enzyme in hexosamine synthesis, leads to a higher hepatic triglyceride-rich VLDL production leading to muscle insulin resistance and hyperinsulinemia in mice (10,11,26).

In this study, we had some limitations, as we could not use the metabolic cages to measure the exact metabolic profile of rats. In addition, we explored limited aspects of insulin-resistance, however, measuring FBG and ITT along with comprehensive information regarding daily calorie and FRC intake could result in valuable findings enabling us to introduce an innovative model for developing initial steps of T2D.

In conclusion, we elucidated for the first time that pretreatment with low-dose FRC solution in addition to treatment with low-dose STZ could be effectively used to produce a rat model mimicking the natural history and metabolic characteristics of the insulin resistance and mild initial forms of T2D. This model is cheap, easy to conduct and suitable for studying the initiation of T2D. This model is also useful for investigation of therapeutic agents for the early treatment of T2D. It is highly suggested to conduct more studies investigating the exact molecular and pathophysiological details of this animal model.

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