Full Length Research Paper

# Effects of *Agave salmiana* Otto ex Salm-Dick highfructose syrup on non-diabetic and streptozotocindiabetic rats

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Diabetes has become a serious public health problem worldwide. In Mexico, it has become the leading cause of death. Fructose in diabetic diet remains controversial because it's potential adverse effects on serum lipids. Forty eight rats were randomly assigned to one of the eight treatments resulting from a complete 2 X 4 factorial arrangement of treatments. In non-diabetic (ND) and streptozotocin-diabetic (D) rats we evaluate HFAS (high-fructose agave syrup) intake on plasma concentrations of diabetes related compounds. HFAS (0.0, 0.5, 2.0 and 5.0 g/kg body weight) was fed daily for six weeks to ND and D rats. Plasma glucose, cholesterol, triglycerides, blood glycosylated hemoglobin (HbA<sub>1c</sub>), urine albumin and creatinine and liver steatosis were evaluated. Intake decreased linearly in D rats and showed a quadratic trend in ND rats. The HFAS did not affect weight gain. Triglycerides in D rats increased linearly as HFAS doses increased. There were quadratic opposite trends of glucose and HbA<sub>1c</sub>, as HFAS doses increased; 0.5 g dose had the major impact on these variables. Streptozotocin-diabetic rats fed 2 and 5 g HFAS/kg body weight had lower liver steatosis than those non-supplemented with HFAS. Dietary HFAS did not show negative effects on food intake, weight gain and hiperglucemia in both ND and D rats. Apparently HFAS had a protective effect on steatosis in D rats.

Key words: High-fructose agave syrup, diabetes, glucose, triglycerides, liver steatosis, sweetener.

# INTRODUCTION

Diabetes has become the leading cause of death in Mexico and the third-leading cause of death among those living along the US side of the border (PAHO, 2007). Nearly 6.5 million Mexicans are diabetic and the prediction expects 11.7 million by 2025 (Aguilar-Salinas et al., 2003). Thus, diabetes would bankrupt the country's health system within the next decade if illness levels were not controlled. Glycaemic control is one of the most important goals in diabetic patients. Fructose induced lower blood glucose and insulin secretory reactions than other dietary carbohydrate (that is, sucrose). Thus, fructose has been extensively evaluated (Bell et al., 2000) and reviewed (Gaby, 2005). However, dietary fructose may induce hypertriglyceridemia (Johnson et al., 2007), obesity (Elliott et al., 2002), the accompanying abnormalities of insulin resistance syndrome (Basciano et al., 2005), and predisposition to develop cardiorenal disease (Johnson et al., 2007). In addition to diabetes, the prevalence of obesity has risen concurrently with an increased availability of other added sugars like highfructose corn syrup (HFCS), mainly in sugar sweetened beverages. Therefore, sweetened beverages with HFCS, versus sucrose, may play an important etiologic role in obesity (Bray et al., 2004). Nevertheless, energy balance consequences of HFCS sweetened soft drinks are not

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quite different from those produced by a sucrose-drink (Soenen and Westerterp-Plantenga, 2007). It was previously noted that neither sugar nor carbohydrate consumption has been clearly identified as a direct cause of obesity (Jenkins et al., 2004). Thus, based on the currently available evidence, an expert panel concluded that HFCS does not appear to contribute to overweight and obesity any differently than do other energy sources (Forshee et al., 2007).

In Mexico, high-fructose agave syrup (HFAS) is a sweetener commercially produced from some species of agave (that is, Agave salmiana Otto ex Salm-Dick). According to Michel et al. (2008), HFAS has 60% fructose, similar to that defined as HFCS-55 with 55% fructose (Hanover and White, 1993). Nonetheless there are chemical differences between HFCS-55 and HFAS. The HFCS-55 has 41% glucose (Hanover and White, 1993) and HFAS has only 22% glucose (Michel et al., 2008), mainly derived from fructans (Mancilla-Margalli and Lopez, 2006), which might have additional health benefits, as modulating lipid metabolism (Delzenne et al., 2002). Hence, the objective was to evaluate the HFAS intake on plasma concentrations of glucose, cholesterol, triglycerides, blood glycosylated hemoglobin (HbA<sub>1c</sub>), urine albumin and creatinine, as well as liver steatosis of non-diabetic (ND) and streptozotocin-diabetic (D) rats.

#### MATERIALS AND METHODS

#### Animals

Male Wistar rats (200 - 250 g) were installed in an appropriate room with inverted light-dark cycle (lights on at 1900 h, lights of at 0700 h), mean temperature  $25 \pm 3^{\circ}$ C and supplemented with food and water *ad libitum*. The experiments were performed in the dark phase (between 0900 and 1600 h) and according to the guidelines of the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOOO-1999) as well as in compliance with international rules on care and use of laboratory animals.

Forty eight white male Wistar rats 10 to 12 wk of age (200 - 250 g of body weight) were housed in polypropylene metabolic cages (one rat per cage) and maintained in a small animal house at  $25 \pm 3 \,^{\circ}$ C with a 12 h light/12 h dark cycle. Rats were acclimated to our animal quarters for at least 10 days prior to the experiment. During this time rats were allowed free access to tap water and to a commercial rodent feed (Rodent Laboratory Chow 5001, Purina, St Louis, MO, USA).

#### Induction of experimental diabetes

Diabetes mellitus was induced by single intraperitoneal injection of freshly prepared Streptozotocin (STZ) (40 mg/kg bw; Sigma SO130, St Louis, MO, USA) in 0.1 M citrate buffer (pH = 4.5) in a volume of 1 ml/kg bw. Diabetes was developed and stabilized in these STZ treated rats (D) over a period of three days (Sarkar et al., 1996). The control animals (non streptozotocin-diabetic rats) were treated with citrate buffer (pH = 4.5). Three days after STZ administration, plasma glucose levels of each rat were measured. Rats with a fast plasma glucose range of 300 - 350 mg/dl were considered diabetic and were included in the D rat treatments, which received immediate daily NPH human insulin (Eli Lilly, Indianapolis, IN, USA)

supplement. The insulin was injected subcutaneously and adjusted individually to control blood glucose concentration within 250 to 300 mg/dl range. Insulin supplement was initiated when diabetes was confirmed and continued daily at about the same time of the day ( $\sim$  1500 h) for a period of six wk. All ND rats received during the same period 100 µl saline hypodermically, but no insulin.

#### Blood collection and biochemical evaluation

Weekly, in the mornings, approximately 3 h into the dark cycle and 3 h after HFAS administration, whole blood samples were drawn from the tail vein. At the same day of blood samples collection, urine samples were also collected from individual rats in metabolic cages. Blood samples were transferred into micro-centrifuge tubes and centrifuged at 3000 rpm for 30 min at 4°C (Centra CL3-R, Thermo IEC, San Antonio Tx USA). The plasma was removed and stored at -18℃ until biochemical evaluation. The plasma glucose, cholesterol and triglycerides concentrations were measured by enzymatic methods (Bayer, Sées, France) using an UV-VIS spectrophotometer (Agilent 8453, Palo Alto, CA, USA). Also, blood glycosylated hemoglobin (HbA<sub>1c</sub>) was measured using an automatic analyzer (DCA 2000, Bayer Healthcare LLC, Elkhart, IN, USA). Urine samples were immediately analyzed for albumin and creatinine concentration using an automatic analyzer (Analyst 2000, Newbury, UK).

#### Liver histopathology examination

After six wk of experimental study, three rats per treatment were given an overdose of sodium pentobarbital (65 mg/kg body weight; Euthanyl, Maple Leaf Foods, Cambridge, Ontario, Canada) and perfused through the hearth with saline (50 ml) and 50 ml of phosphate buffered paraformaldehyde (2%). Liver was removed quickly from the carcass. Liver tissues were excised, rinsed, chilled (0.2 M phosphate buffer at pH 7.2), fixed (4% neutral pharaformaldehyde solution), embedded in paraffin and stained (haematoxylin eosin). Five cuts of 7  $\mu$ M of each liver were obtained. Preparations were observed through a light microscopy (Labophot-2, Nikon, Japan) for detailed independent evaluation by two pathologists.

#### Experimental design and statistical analysis

There were eight treatments that resulted from a complete factorial arrangement. The Factor A was the diabetic factor with two levels; ND and D. The Factor B was the HFAS with four levels; 0.0, 0.5, 2.0 and 5.0 g/kg body weight. Therefore, the evaluated treatments were ND-0.0, ND-0.5, ND-2.0, ND-5.0, D-0.0, D-0.5, D-2.0 and D-5.0. The data was analyzed according to a completely randomized experimental design with six repetitions and repeated measurements (1 to 6 weeks) using the "MIXED" option of SAS (1999).

Forty eight rats were randomly assigned to one of the eight treatments resulting from the combination of diabetic factor (ND and D levels) and HFAS factor (0.0, 0.5, 2.0 and 5.0 g/kg body weight levels). All rats were allowed free access to tap water and to the commercial rodent feed. We used commercial HFAS from *A. salmiana* (IIDEA, S.A de C.V., Guadalajara, Jal., Mexico) with 52% fructose and 17% glucose. The HFAS dose was orally administered twice a day by six wk via an esophageal tube. Sterile water was used to adjust at the same volume the syrup administered to the rats. Food intake (g/day) was recorded daily and body weight was measured once a week by 6 wk.

Initial body weight of rats was included in the model as a covariable (p < 0.0001) to analyse data of week gain and feed intake. Orthogonal polynomials were used to test effects of HFAS levels. As HFAS levels were not equally spaced, coefficients were calculated using the "IML" option of SAS to test linear and quadratic



**Figure 1.** Effect of high-fructose agave syrup (HFAS) on daily feed intake (A) and weight gain (B) of non-diabetic and streptozotocin-diabetic rats. Values at 3 h after HFAS intake.

effects. Significant differences were accepted when P  $\leq$  0.05. The covariance data structure was chosen according to Littell et al. (1998).

### **RESULTS AND DISCUSSION**

#### Feed intake and weight gain

Daily feed intake and weight gain (g body weight /week) are shown in Figure 1A and 1B. Weight gain was not affected by health status and HFAS level. Daily feed intake was affected ( $p \le 0.05$ ) by health status and HFAS; however the interaction of these main factors was not significant. As expected D rats had a higher food intake

than ND rats. There was a quadratic relation of feed intake in ND rats, with a diminishing trend toward the 5 g HFAS level. Feed intake decreased linearly as HFAS level increased in D rats. Weight gain was not affected by HFAS level in the ND and D rats. The relationship between HFAS intake and weight gain has been a controversial topic. High-fructose intake promotes weight gain due to its low effects on satiety as result of reduction of insulin and leptin concentrations (Elliott et al., 2002; Bray et al., 2004; Basciano et al., 2005). Thus, highfructose intake has been related with obesity and nonsatiety (Gaby, 2005; Johnson et al., 2007), but the currently available evidence is insufficient to implicate HFCS per se as a causal factor in the overweight and obesity problem (Forshee et al., 2007; Soenen and Westerterp-Plantenga, 2007). The reduction of fed intake trends as HFAS doses increased is according to Friedman (1990) who found an association between fructose consumption and a reduction in food intake. The present study shows that HFAS did not affect weight gain in ND or D rats, in agreement with Bell et al. (2000) evaluating high-fructose feeding on D rats, and Davail et al. (2005) studying the effects of dietary fructose on overfed mule ducks.

# Plasma glucose

Time did not affect any blood variable. Plasma glucose concentrations of ND and D rats are shown in Figure 2A. As expected, glucose concentrations of D rats were higher than those of ND. The interaction of health status and HFAS level ( $p \le 0.05$ ) suggest that in D, but not in ND rats, there was a quadratic decrease of plasma glucose concentrations as HFAS level increased, being the highest glucose concentration with 0.0 g HFAS and keeping constant with 0.5, 2.0 and 5.0 g. Similar results have been found in animals and humans; normally, a rapid and direct regulation of hepatic glucose fluxes by glucose per se helps maintain plasma glucose levels within a narrow range, but glucose effectiveness is decreased in type 2 diabetics due to both lack of glucose mediated suppression of endogenous glucose production and diminished stimulation of glucose uptake (Mevorach et al., 1998). In spite of it, the quadratic effect of HFAS doses on glucose concentrations in D rats suggests that fructose could activate hepatic glucokinase (Hawkins et al., 2002) and glucose re-captures by glycogen synthesis (Shiota et al., 1998) and thus avoids hyperglycemia (Moore et al., 2001; Wolf et al., 2002).

# Plasma triglycerides and cholesterol

No differences were detected for plasma triglycerides and cholesterol between ND and D rats (Figures 2B and 2C). Plasma cholesterol concentrations for D and ND rats were not affected by HFAS. Results after a similar short period (6 weeks) shown that fructose did not alter plasma cholesterol concentrations on ND (Bantle et al., 2000) and D subjects (Moore et al., 2001), but using longer periods (12 months) fructose could increase plasma cholesterol of healthy subjects (Levi and Werman, 1998).

Contrary to Bantle et al. (2000) results, which suggested that dietary fructose was associated with increased fatness and postprandial plasma triacylglycerol concentrations in healthy men, plasma triglycerides of ND rats were not impacted by HFAS levels. Conversely, the interaction ( $p \le 0.05$ ) of health status and HFAS level suggest that in D rat plasma triglycerides concentration linearly rose as HFAS dose increased. It has been summarized from a large number of works (Uusitupa, 1994; Basciano et al., 2005; Johnson et al., 2007) that elevations of serum triglycerides can occur after fructose ingestion, but our results and those summarized by Gaby (2005) suggests that triglycerides reaction to fructose ingestion appears to depend on whether or not the subject is carbohydrate sensitive or insulin resistant. Thus, the effect of the diet on triglyceride levels was more pronounced in D rats than in ND ones.

It is important to point out that different mechanisms have been proposed to explain the fructose effects on triglycerides metabolism, but the significantly enhanced rate of *de novo* lipogenesis and triglycerides synthesis, driven by the high flux of glycerol and acyl portions of triglycrides molecules coming from fructose catabolism, is the most acceptable (Basciano et al., 2005). Therefore, in insulin resistant states, fructose might induce a dyslipidemia, as was evidenced in this study.

# Blood HbA<sub>1c</sub>, urine creatinine and urine albumin

Blood HbA<sub>1c</sub> and urine albumin were affected ( $p \le 0.05$ ) by health status and HFAS level, while urine creatinine was only affected by HFAS level; there were not interactions of health status with HFAS level. Blood HbA<sub>1c</sub>, urine creatinine and urine albumin in ND rats nonsupplemented with HFAS were 3.3%, 5.6 and 0.9 mg/dL, respectively. Blood HbA1c percentage of ND rats was lower than that of D rats fed 0.5 g HFAS; urine creatinine was similar in both ND and D rats; urine albumin of ND rats was lower than those of D rats fed 0.0, 2.0 and 5.0 g HFAS, but similar to that of D rats fed 0.5 g HFAS. Regression analysis of blood HbA<sub>1c</sub> percentage and urine creatinine and urine albumin concentrations (mg/d) of D rats, are shown in Figure 3. There was a quadratic trend of blood HbA<sub>1c</sub> in D rats as HFAS dose increased, being 0.5 g the dose that induced the highest concentration. Urine creatinine concentrations linearly increased as HFAS dose increased. Urine albumin concentrations of D rats were quadratically affected as HFAS dose increased, being 0.5 g HFAS the dose that induced the lowest concentrations.

Glycosylated hemoglobin (HbA1c) is a marker to evaluate the glycemic level in diabetic patients and to predict risks of developing and/or progression of diabetic complications (Chun-Kuang et al., 2007), mainly in longterm circumstances. Even this study was a short-term one, HbA<sub>1c</sub> percentages were higher at 6 wk than starting the experiment. Fructose intake increases HbA<sub>1c</sub> of rats (Levi and Werman, 1998), such as it was observed in the rats fed 0.5 g HFAS in our study. Lingelbach et al. (2000) found that this effect was related to food restriction or low energy intake, but this is not according to HbA<sub>1c</sub> percentages of D rats fed 2.0 and 5.0 g HFAS. Hence, HbA<sub>1c</sub> percentages of D rats fed 0.5 g HFAS may suggest some diabetic complications, even those percentages are not corresponding with the low plasma glucose concentrations. In fact, HbA<sub>1c</sub> can continue to exert a pathogenic influence in vivo, even if normoglycemia is restored (Vaisman et al., 2006).



Figure 2. Effect of high fructose agave syrup (g/kg body weight) on plasma concentration (mg/dL) glucose, cholesterol and triglycerides of non-diabetic and streptozotocin-diabetic rats.



**Figure 3.** Effect of high fructose agave syrup (g/kg body weight) on plasma glycosylated hemoglobin (HbA<sub>1c</sub>, %), urine creatinine and albumin (mg/dL) of streptozoctocin-diabetic rats. Values for non-diabetic rats were 3.3%, 5.6 and 0.9 mg/dL for HbA<sub>1c</sub>, creatinine and albumin, respectively.

Urine albumin, as an indicator of glomerular hyperfiltration in diabetic subjects (Amin et al., 2005) was quadratically altered as HFAS dose increased. With a short-term fructose intake urinary albumin was not affected, but with a long-term (16 wk) fructose intake urine protein did increase in rats (Bell et al., 2000).



**Figure 4.** Liver photomicrographs (H-E 1000 x) of non-diabetic (ND) rats fed high-fructose agave syrup (HFAS): A) 0.0, B) 0.5, C) 2.0 and D) 5.0 g HFAS/kg body weight. Normal hepatocytes (long-arrow) with cell borders well defined, some of them binuclear (short-arrow).

Kizhner and Werman (2002) showed that fed fructose, as compared to fed glucose and sucrose did increase urine creatinine concentration in rats; however, urine creatinine concentrations of rats fed fructose versus water were similar. Even though urine creatinine concentrations in our experiment were linearly related to HFAS dose increments, their range (9.7 - 11.8 mg/dL) is inside the normal limits of healthy rats (Chinn, 1966).

#### Liver steatosis

Liver photomicrographs of ND and D rats are shown in Figures 4 and 5, respectively. Livers of ND rats fed 0.0, 0.5, 2.0 and 5.0 g HFAS were morphologically similar with no signs of macrovesicular steatosis; their hepatocytes showed a normal morphology, except some of them were binuclear, but with well defined borders.

These results differ from those of a study where fructose did induce hepatic macrovesicular and microvesicular



**Figure 5.** Liver photomicrographs (H-E 1000 x) of streptozotocin-diabetic (D) rats fed high-fructose agave syrup (HFAS): A) 0, B) 0.5, C) 2 and D) 5 g HFAS/kg body weight. A and B: hepatocytes with cell borders non-defined (long-arrows) and cytoplasm fat accumulation (shortarrows). C and D: hepatocytes without intracellular fat and cell borders well defined (long-arrows).

fat deposits in rats (Ackerman et al., 2005).

Livers of D rats fed 0.0 g HFAS had a marked development of steatosis as compared to livers from D rats fed 0.5 g HFAS. Nonetheless this effect was slightly diminished in areas coexisting with fatty deposits. Livers of D rats fed 0.0 and 0.5 g HFAS had marked development of hepatic macrovesicular and microvesicular fat deposits. However, livers of D rats fed 2.0 and 5.0 g HFAS had no signs of macrovesicular steatosis, with minimal microvesicular steatosis and lobular and portal inflammatory changes. Hepatic macrovesicular and microvesicular fat deposits are common in diabetic subjects as a reaction to hyperglycemia, increment of hepatic glucose production, novo lipogenesis and hipertriglyceridemia (Browning and Horton, 2004; Donnelly et al., 2005). These findings can explain why triglycerides in D rats increased and steatosis reduced as HFAS dose increased. There are no previous studies about the effects of HFAS on liver steatosis and most of the related studies have been developed with HFCS. The explanation about why HFAS relieved liver steatosis in D rats is not clear, but some residual non hydrolyzed inulin-type fructans detected in agave syrup (Mancilla-Margalli and Lopez, 2006) could be the responsible factor (Roberfroid, 2007), through its modulation of de novo lipogenesis, as a result of a reduced glucose level (Beylot, 2005). Hence, HFAS could act as a functional food with the ability to protect against toxic effects (that is, steatosis) possibly induced by other nutrients (that is, fructose) that are eaten concomitantly (Kok et al., 1996). This protective effect of HFAS on steatosis in rats would be interesting, if confirmed in humans, since steatosis is one of the most frequent liver disorders in overweight and diabetic subjects (Delzenne et al., 2002).

#### Conclusions

Dietary HFAS did not show negative effects on food intake, weight gain and hiperglucemia in both ND and D rats. Even though a linear increased in plasma triglyceride of D rats was observed as HFAS dose increased, it was not reflected on hepatic steatosis. Even more, apparently HFAS had a protective effect on hepatic steatosis in D rats. More studies with HFAS and a longer observational period are needed in order to verify that its beneficial effects on liver steatosis are long-standing before this syrup can be recommended to diabetic subjects.

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