Evaluation of the chronic complications of diabetes in a high fructose diet in rats

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The increasing prevalence of type 2 diabetes is associated with increasing health costs, especially for the treatment of cardiovascular disease. The development of new treatment modalities requires animal models that mimic the range of pathophysiological changes seen in diabetic humans. Dietary fructose intake has been linked to the increase in insulin resistance as part of the metabolic syndrome; fructose-fed rats develop type 2 diabetes. This study has characterized the cardiovascular changes in young adult male Wistar rats fed a 61% fructose diet for 16 weeks. Our results extend the reported changes of hypertension, lipid abnormalities, impaired glucose tolerance and impaired oxidative defense to include ventricular dilatation with hypertrophy and decreased contractile function, together with increased inflammatory cell infiltration into the ventricular myocardium, resulting in excessive collagen deposition and an increased stiffness of the left ventricle. However, endothelial dysfunction, tactile allodynia as a symptom of peripheral neuropathy and retinopathy are not present in these rats, in contrast to the streptozotocin-induced model of type 1 diabetes. Thus, fructose feeding mimics many, but not all, of the symptoms of type 2 diabetes in humans.

Keywords: Type 2 diabetes, High fructose diet

The rising prevalence of type 2 diabetes in India has been described as the epidemic of the 21st century. The estimated burden of diabetes in India has increased from 22 million in 1990 and 33 million in 2000 to 40.9 million in 2006 with a prediction of 69.5 million in 2025. Data from the Chennai Urban Rural Epidemiology Study (CURES) suggest an overall prevalence of 15.5% for diabetes and 10.6% for impaired glucose tolerance. This study also assessed the complications related to diabetes with 21.4% of diabetic patients having coronary artery disease compared to 9.1% in subjects with normal glucose tolerance; the values for peripheral vascular disease were 6.3% in diabetic subjects and 2.7% in non-diabetic patients. These patients had an overall prevalence of diabetic neuropathy of 26.1% with these patients having an incidence of 24.1% of diabetic retinopathy and 51.1% of hypertension. The overall prevalence of diabetic retinopathy in these diabetic patients of 17.6% was lower than in earlier studies in Caucasians.

In a separate study based in Mumbai, the prevalence of insulin resistance was similar in both urban and rural populations at around 42% of the adult population, a very high value compared to a prevalence of around 15% reported in European populations such as Australia. The epidemic has been ascribed to both an increasing affluence and genetic predisposition. Analysis of the economic burden of diabetes in India has shown that the cost of providing routine care is only a fraction of the overall costs but without this routine care, costs escalate dramatically. Lifestyle interventions may help postpone the onset of diabetes; these interventions should be tailor-made to suit the needs of Indian patients with diabetes.

Rat models of human diseases such as diabetes have been widely used to investigate the progression of disease symptoms as well as possible treatment options. The most-used rat model of the complications of diabetes is that induced by streptozotocin. However, streptozotocin induces type 1 diabetes and so results from this model may be relevant only to a small proportion of diabetic patients. Type 2 diabetes is associated with complications such as hypertension, endothelial damage, cardiac hypertrophy, inflammation, atherosclerosis, ventricular contractile dysfunction, fibrosis, retinopathy, neuropathy and nephropathy. Diet-induced models of type 2 diabetes, rather than the streptozotocin-induced model of type 1 diabetes, should serve as a better vehicle to investigate possible interventions for these complications.
Both human and animal studies have shown that fructose is a highly lipogenic nutrient that contributes to insulin resistance, metabolic defects and the development of a prediabetic or diabetic state\textsuperscript{11}. The intake of processed sugars, in particular fructose, has been proposed to play a critical role in the epidemic of cardiovascular and renal disease\textsuperscript{12}. High fructose diets in rats (≥60% of the diet) have been used to induce cardiovascular symptoms such as hypertension, hypertriglyceridaemia, increased collagen deposition in the heart and kidneys associated with increased oxidant concentrations and decreased antioxidant defenses\textsuperscript{13,15}. Although these studies have defined the biochemical changes, the functional and structural changes, especially in the cardiovascular system, have not been thoroughly addressed. Other functional changes in fructose rats, especially common diabetic complications such as neuropathy and retinopathy have not been reported in this model of type 2 diabetes.

This study has characterized the common human diabetic complications in fructose-fed rats. Structural changes in the heart have been characterized by histology and echocardiography, whereas heart function has been measured \textit{in vivo} using echocardiography and \textit{ex vivo} in isolated perfused hearts. Isolated thoracic rings were used to measure vascular reactivity. Electroretinopathy was performed on these rats to determine the extent of retinopathy, while tactile allodynia as a measure of peripheral neuropathy was characterized by von Frey testing.

**Materials and Methods**

**Drugs and chemicals**

Heparin, noradrenaline, acetylcholine and sodium nitroprusside were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.) Noradrenaline, acetylcholine and sodium nitroprusside were dissolved in distilled water. Fructose and corn starch were obtained through The University of Queensland Chemical Store.

**Animals and physiological assessments**

Male Wistar rats were bred at The University of Queensland animal housing facility. All experimental protocols were approved by the Animal Experimentation Ethics Committee of The University of Queensland, under the guidelines of the National Health and Medical Research Council of Australia. Rats were given \textit{ad libitum} access to food and water and were housed in 12-h light/dark conditions. Body weight, food and water intakes were measured daily.

Two experimental groups of 8-9 week old Wistar rats were treated for 16 weeks with either a corn starch (CS) (n = 12) or fructose-fed (FF) (n = 12) protocol\textsuperscript{12,13}. The fructose diet consisted of fructose (610 g), skim milk powder (casein, 200 g), wheat bran (96 g), peanut oil (50 g), Hubble, Mendel and Wakeman salt mixture (35 g), L-methionine (7 g) and a vitamin diet fortification mixture (2 g) per kg of food. For the control diet, fructose was replaced with corn starch (610 g). Caloric intake was calculated from the following values (kJ/g): fructose, 15.4; corn starch, 15.94; skim milk powder, 15.12; wheat bran, 10.8; peanut oil, 34.0.

Systolic blood pressure was measured in rats after 0, 8 and 16 weeks under light sedation via i.p. injection of Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg), using an MLT1010 Piezo-Electric pulse transducer (ADInstruments) and inflatable tail-cuff connected to a MLT844 physiological pressure transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments, Sydney, Australia).

Fasting blood glucose concentrations were measured with blood taken from the tail vein. The rats were given 40% glucose solution in distilled water (2 g/kg body weight) via oral gavage. Tail vein blood samples were taken at 0, 30, 60, 90 and 120 min, following glucose administration. Medisense Precision Q I.D glucose meter (Abbott Laboratories, Bedford, U.S.A) was used to measure blood glucose concentrations.

**Assessment of tactile alldynia**

Calibrated von Frey filaments were used fortnightly to assess the development and maintenance of tactile alllodynia in the hindpaws of corn starch and fructose-fed rats. Von Frey filaments of varying tensile strength (2-20 g) were applied to the plantar surface of the hindpaw of the rat in ascending order of force, until there was a brisk paw withdrawal response. Higher forces were not used to prevent tissue damage to the footpad of the rat.

**Echocardiography**

Echocardiography was performed at The Prince Charles Hospital, Brisbane, small animal theatre by trained sonographers. Rats were anaesthetized via i.p. injection with Zoletil (tiletamine 15 mg/kg, zolazepam 15 mg/kg) and Ilium Xylazil (xyazine 10 mg/kg). A Hewlett-Packard Sonos 5500 echocardiography machine using a 12-MHz neonatal transducer with an image depth of 3 cm using two focal zones was used to
obtain images. Measurements of left ventricular posterior wall thickness and internal diameter were made using two-dimensional M-mode taken at midpapillary level.

Isolated heart preparation
The left ventricular function of the rats in all treatment groups was assessed using the Langendorff heart preparation. Terminal anaesthesia was induced via i.p. injection of pentobarbitone sodium 100 mg/kg (Lethabarb ®). Once anaesthesia was achieved, heparin (1000 IU) was injected into the right femoral vein. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a Maclab system. All left ventricular end-diastolic pressure values were measured by pacing the heart at 250 beats per min using an electrical stimulator. End-diastolic pressures were obtained starting from 0 mm Hg up to 30 mm Hg. The right and left ventricles were separated and weighed. Diastolic stiffness constant ($\kappa$, dimensionless) was calculated as in previous studies. Rate of contraction and rate of relaxation were obtained at an interpolated diastolic pressure of 10 mm Hg.

Organ bath studies
Thoracic aortic rings (4 mm in length) were suspended in an organ bath chamber with a resting tension of 10 mN. Cumulative concentration-response (contraction) curves were measured for noradrenaline; response (relaxation) curves were measured for acetylcholine and sodium nitroprusside in the presence of a submaximal (70%) contraction to noradrenaline.

Organ weights
Following euthanasia, the heart, liver, kidneys and spleen were removed and blotted dry for weighing. Organ weights were normalized relative to the body weight at the time of their removal (in mg/g).

Histology
Collagen distribution was measured in the left ventricle, following staining with picrosirius red and analysed by laser confocal microscopy. Tissues were initially fixed for 3 days in Telly’s fixative (100 ml of 70% ethanol, 5 ml of glacial acetic acid and 10 ml of 40% formaldehyde) for 2 days. The samples were then dehydrated and embedded in paraffin wax. Thick sections (15 µm) were cut and placed on glass slides coated with Mayer’s albumin solution (1 g powdered egg albumin, 50 ml glycerol, 50 ml distilled water), left to air dry for 2 days and then heated in an oven at 56°C for 1 h. Phosphomolybdic acid (0.2% in distilled water, 5 min) was applied to reduce non-specific binding of the stain to the section and then washed in distilled water. The collagen-selective picrosirius red stain (0.1% sirius red F3BA in saturated picric acid) was then used and allowed to incubate for 90 min. The sections were washed, dehydrated and mounted in Depex with a coverslip. Image analysis of the stained sections took place on the laser scanning confocal microscope (BioRad MRC-1024 – Rhodamine/Texas red filter, 568 nm, emission 609 DF 32 by green excitation). Randomly assigned slides and sections were scanned representing the areas of the left ventricle. The images were taken with an objective lens of X40 magnification and analysed for pixel intensity in a specified area of the section. The data were compiled by a software image-rendering program (IA-IP-Lab, Scanalytics Inc., Australia). Immunostaining for ED1-positive monocyte/macrophage infiltration into the left ventricle was performed as previously described.

Electroretinography
Electroretinography was performed after 16 weeks to quantify whether this high fructose diet induced retinal damage. Briefly, animals were dark-adapted for at least 6 h, anaesthetized as described previously and the pupils dilated with 1% tropicamide and 2.5% phenylephrine. The animals were placed in a custom-designed dome, the interior of which was completely covered with aluminium foil to obtain a Ganzfeld effect. Two cotton-wick electrodes, containing Ag-AgCl cells immersed in saline were used to obtain signals from both eyes simultaneously. The reference electrode was placed in the ear, while the ground electrode was positioned subcutaneously on the back. A Neuropack-MEB 7102 Evolved Potential Measuring system (Nihon-Kohden America, Foothill Ranch, CA, USA) was used to deliver a triggered output to the flash stimulator and collect signals from both eyes.

Statistical analysis
All data sets were represented as mean ± standard error of mean (SEM). Comparisons of findings
between groups were made via statistical analysis of data sets using either an unpaired t-test or one-way/two-way analysis of variance, followed by the Duncan test to determine differences between treatment groups. A $P$-value of <0.05 was considered as statistically significant.

**Results**

Young male Wistar rats fed a high carbohydrate diet containing fructose (61%) showed no differences in weight gain, food or water intake when compared with corn starch-fed rats (Fig. 1). Caloric intake was similar in both diets: corn starch-fed and fructose-fed rats averaged 408 ± 38 kJ/day and 391 ± 32 kJ/day, respectively. Fasting blood glucose concentrations were increased after 8 and 16 weeks of the diet (Table 1). Measurement of blood glucose concentrations after administration of a loading dose of glucose showed that fructose-fed rats were glucose-intolerant as the blood glucose concentrations reduced more slowly than in the corn starch-fed rats (Fig. 2). Abdominal fat pads did not differ between corn starch and fructose-fed rats, while plasma concentrations of hepatic enzymes increased in fructose-fed rats (Table 1). Measurement of tactile allodynia as paw withdrawal by the von Frey method and retinal function by electroretinography showed no differences between corn starch and fructose-fed rats (Table 1, Fig. 3).

Systolic blood pressure increased consistently in fructose-fed rats over the 16 weeks treatment with mean values of 153 ± 6 mm Hg at 8 weeks and 166 ± 6 mm Hg at 16 weeks; in contrast, the systolic blood pressure in corn starch-fed rats was 130 ± 9 mm Hg at 8 weeks and 133 ± 5 mm Hg at 16 weeks (n = 4-7) (Fig. 4). *In vivo* cardiac function as measured by echocardiography showed that fructose feeding produced ventricular dilatation (increased internal diameter in diastole) with increased estimated ventricular mass, aortic blood velocity and cardiac output, but with decreased contractility shown as a decreased fractional shortening and ejection fraction (Table 1). *Ex vivo* cardiac function, as measured in the
Langendorff isolated heart showed decreased contractility as ± dP/dt with a markedly increased cardiac stiffness (Table 1).

Post-mortem organ weights showed a selective increase in left ventricular wet weight, compared to the right ventricle and other major organs (Table 1).
Further, histological analysis showed that both interstitial and perivascular collagen deposition increased markedly in the left ventricle of fructose-fed rats (Table 1). ED1-positive monocyte/macrophages were found in low numbers in the left ventricle of corn starch-fed rats and in increased numbers in fructose-fed rats. Plasma malondialdehyde concentration (as a marker of oxidative stress) increased in both corn starch and fructose-fed rats (Table 1).

Vascular responses to noradrenaline, sodium nitroprusside (endothelium-independent relaxation) and acetylcholine (endothelium-dependent relaxation) were unchanged by chronic fructose feeding (Fig. 5).

Discussion

Type 2 diabetes is a complex disease with many hormonal changes contributing to the spectrum of pathophysiological changes\(^{21}\). Understanding the human disease state and proposing suitable interventions for use in patients requires investigations using the controlled situation available in animal models. To fulfill these requirements, these animal models should mimic the chronic changes observed in the human diabetic patient. The inability of animal models to mimic all of the changes in human diabetes has been emphasized, with rodent models suggested as the best alternatives\(^{10}\). This study has investigated the chronic changes, following a high fructose diet in rats, known to produce hypertension, lipid abnormalities, impaired glucose tolerance and impaired oxidative defense\(^{13,14}\). Our results extend these changes to include ventricular dilatation with hypertrophy and decreased contractile function, increased inflammatory cell infiltration into the ventricular myocardium, resulting in excessive collagen deposition or fibrosis and an increased stiffness of the left ventricle. However, endothelial dysfunction as a decreased relaxation to acetylcholine and tactile allodynia as a symptom of peripheral neuropathy were not present in these rats, nor was retinopathy observed following 16 weeks of this diet.

These results with chronic fructose feeding are very different to the chronic changes observed in the streptozotocin-diabetic rat, a model of type 1 diabetes, up to 24 weeks after diabetes induction\(^{22}\). Streptozotocin-diabetic rats remained normotensive with slowly developing systolic and diastolic dysfunction, minimal ventricular hypertrophy, rapidly-developing but stable tactile allodynia and cataracts developing to presumed blindness around 16 weeks after streptozotocin injection. Ventricular fibrosis leading to increased ventricular stiffness was common to both models\(^{22}\).

Dietary fructose transported into cells by the GLUT5 carrier induces marked carbohydrate oxidation stimulating rapid lipogenesis leading to insulin resistance, dyslipidaemia and hypertension\(^{11,23,24}\). Other cardiovascular abnormalities in the chronic fructose-fed rat include an increased collagen deposition\(^{13,14}\). Further, fructose feeding in rats resulted in the development of renal hypertrophy, afferent arteriolar thickening, glomerular hypertension and cortical vasoconstriction\(^{25}\) as well as acceleration of the rate of renal disease in a rat remnant kidney model\(^{26}\). The liver is another major target in the fructose-fed rat since fructose uptake is very high; long-term fructose-enriched diets lead to fatty liver and treatments that reduce or prevent hepatic lipid accumulation improve insulin sensitivity in the liver\(^{27}\).

Fructose is now a major source of carbohydrate for humans and this increased consumption has been proposed as the cause of the increased incidence of the metabolic syndrome, especially cardiovascular and renal disease\(^{12}\). Many mechanisms have been suggested to account for the responses to fructose, including a decrease in type 1 (slow twitch/oxidative) muscle, increased plasma triglycerides, increased muscle lipid deposition, increased portal delivery of free fatty acids to the liver, increased adipocyte size, protein kinase C activation, increased TNF-\(\alpha\) production to suppress insulin receptor signal transduction, inflammation and hyperuricaemia as a link between inflammation, oxidative stress and insulin resistance\(^{11,28,29}\).

The high fructose diet in rats mimics many of the symptoms of type 2 diabetes in humans, especially insulin resistance or glucose intolerance, dyslipidaemia and renal impairment together with hypertension. However, patients with diabetes are at a greater risk of peripheral neuropathy and retinopathy.
than non-diabetic patients; these changes were not observed in our fructose-fed rats, even after 16 weeks. Further, there was no significant endothelial dysfunction, defined as a decreased response to acetylcholine. Lastly, type 2 diabetic patients show increased abdominal fat deposition, unlike the fructose-fed rat. Thus, this model mimics most, but not all, of the symptoms of chronic type 2 diabetic humans. Since insulin resistance is a key aspect of the metabolic syndrome that is associated with many other changes such as endothelial dysfunction and abdominal fat deposition, then the fructose-fed rat also defines many, but not all, of the symptoms of the metabolic syndrome. Further, the role of the multiple genetic influences in human type 2 diabetes cannot be reproduced in the diet-induced diabetic state.

Despite these limitations, the fructose-fed rat model may be useful to define potential treatments for type 2 diabetes or the metabolic syndrome. Testing natural antioxidant-containing preparations from Indian plants such as the polyphenolic compounds present in green tea or the metabolic syndrome. Further, the role of the multiple genetic influences in human type 2 diabetes cannot be reproduced in the diet-induced diabetic state following fructose feeding in rats.

References

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