Sex differences in renal and metabolic responses to a high-fructose diet in mice

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Running Title: Sex differences in fructose metabolism
Abstract

High-fructose intake has been associated with increased incidence of renal disease and hypertension, amongst other pathologies. Most fructose is cleared by the portal system, and metabolized in the liver; however systemic levels of fructose can rise with increased consumption. We tested whether there were sex differences in the renal responses to a high fructose diet in mice. Two-month-old male and female C57Bl6/129/SV mice (n = 6/sex/treatment) were randomized to receive control or high-fructose (65% by weight) diet as pelleted chow ad libitum for 3 months. Fructose feeding did not significantly affect body weight but led to a 19 and 10% increase in kidney weight in males and females, respectively. In males, fructose increased the expression (~50%) of renal cortical proteins involved in metabolism including GLUT5 (facilitative fructose transporter), ketohexokinase (KHK), and the insulin receptor (β-subunit). Females had lower basal levels of GLUT5, which were unresponsive to fructose. However, female mice had increased urine volume and plasma potassium (K+), and decreased plasma sodium (Na+) with fructose, while males were less affected. Likewise, females showed a 2-3 fold reduction in the expression of the thick ascending limb, Na+K+2Cl cotransporter (NKCC2), and the collecting duct, aquaporin-2 (AQP2) with fructose, relative to control females; while males had no change. Overall, our results support greater proximal metabolism of fructose in male animals, and greater distal tubule/collecting duct (electrolyte homeostasis) alterations in females. These sex differences may be important determinants of the specific nature of pathologies that develop in association with high fructose consumption.
Introduction

Fructose is a 6-carbon monosaccharide that naturally exists in honey, fruits, and some vegetables. However, the development of high-fructose corn syrup, as a lower-cost sweetener, has led to a stark rise in the rate of its consumption. One estimate suggests that the average daily intake of fructose in the United States increased 32% between 1978 and 2004 (43). Fructose is about 1.4X sweeter than table sugar which is some respects makes it a reasonable substitute, as the caloric content (4 kcal/g) is the same as that of glucose. Although fructose, at first pass, would seem a safe and natural sweetener, cellular metabolism and regulation by satiety hormones such as insulin are known to be substantially different from that of glucose. These metabolic differences, along with its high consumption level, make it a highly critical and timely research topic.

While most dietary absorbed fructose is cleared by the liver, thus portal levels of fructose can be quite high (~1 mM), peripheral levels are elevated much less dramatically (~0.1 mM) (21). Even so, renal damage has been reported in response to high-fructose diets (36). However, whether these damaging changes are manifested as a direct effect of fructose metabolism within these cells or are indirectly caused by the elevated insulin, uric acid, or triglycerides in the circulation is not clear. The kidney expresses at least two major classes of proteins that transport fructose: 1) the glucose transporters (GLUTs) or gene family name Slc2a, and 2) the sodium-coupled transporters, Slc5a, super-families (7, 38), each with a number of different isoforms. In this study, we focus on the regulation of GLUT5. GLUT5 may be considered “insulin-dependent” in that insulin is thought to elicit its trafficking from intracellular domains to the cell surface (29). Barone, et al. demonstrated that global KO of GLUT5 abolished the hypertensive effects of dietary fructose (2).

In this respect, there are several studies in humans (16, 42, 60) and animals (34) demonstrating sex differences in response to fructose feeding. Nearly all studies show males are more sensitive. High-fructose feeding has been demonstrated to increase insulin resistance,
blood pressure, and *de novo* triacylglycerol production to a greater extent in males. Unfortunately few studies have tried to determine the mechanisms underlying differences in sensitivity between the sexes, nor have they focused on specific aspects of renal metabolism.

The first step in fructose metabolism is phosphorylation by fructokinase, also known as, ketohexokinase (KHK), an enzyme that has been associated with rapid ATP depletion (14). Fructose-1-phosphate is cleaved by aldolase B to dihydroxyacetone phosphate and glyceraldehyde, substrates for triglyceride synthesis or gluconeogenesis, depending upon the cell type. Rapid ATP depletion has been demonstrated to increase circulating levels of uric acid, a bi-product of ATP metabolism, in some individuals (5, 10, 62). Hyperuricemia has been associated with several of the pathological effects of fructose (10, 14, 62).

At the whole-body level, high consumption of high levels of dietary fructose has been linked to the development of the metabolic syndrome, i.e., a constellation of disorders consisting of hyperinsulinemia, hypertension, visceral adiposity, and dyslipidemia (9). Some of these effects are simply a manifestation of increased calorie consumption, and in this respect, any sugar will do. However, in this regard, fructose does not stimulate the release of pancreatic insulin, a major satiety hormone, which then can lead to over-eating. While sex differences in these responses have not been extensively studied, one study did show fructose increased blood pressure and led to metabolic perturbations in male, but not female rats (26)

Sex differences exist in a number of renal activities including, but not limited to, the regulation of transport including salt, water, and organic anion and cation reabsorption (40, 46, 52), metabolic activities including propensity toward oxidative stress (50), endocrine systems, such as the renin-angiotensin-aldosterone system (64) and endothelin (37), and drug disposition and clearance (52). Moreover, there are a number of metabolic differences between the sexes with regard to nutrient storage and utilization in a different tissue beds. For example, men have higher rates of gluconeogenesis and women higher rates of fatty acid oxidation. Men generally have a higher respiratory quotient (RQ) and rely on carbohydrates preferentially as an energy
source (51). Therefore, it would not be unexpected if the renal responses to dietary fructose would vary. Indeed most studies examining the effects of fructose on the kidney have been done in male animals, in general, because they are thought, and in a few situations, found to be more sensitive, at least to the major cardio-metabolic effects of fructose. Therefore, the aim of this study was to characterize sex differences in basic renal responses to chronic dietary fructose that might affect and explain sex-differential susceptibility to renal pathology, hypertension, and/or other water and electrolyte disturbances.

**Materials and Methods**

*Experimental animals*—Mice (C57Bl6/129/SV mixed genetic background) were bred and raised at Georgetown University under approved protocols within the established guidelines of the Institutional Animal Care and Use Committee (IACUC) of Georgetown University. Two-month-old male and female mice (n = 6/ sex/treatment) were randomized to receive control (C) (Teklad, TD01457) or high-fructose (Fr, 65% by dry weight, Teklad, TD01458) diet as pelleted chow. In the control diet, cornstarch primarily replaced fructose as the carbohydrate source. Both diets were formulated to contain the same amount of sodium (Na+), potassium (K+) and chloride (Cl-) per gram dry weight. Urine (24-hour) was collected near the end of the study in mouse metabolic cages (Hatteras Instruments, NC; MMC100) with the addition of a small volume (50 µl) of an antibiotic cocktail to inhibit bacterial growth (49). Mice had *ad-libitum* access to food and water and weekly body weights were recorded. After 3-months, mice were fully anesthetized with inactin (Inactin hydrate, Sigma @100 mg/kg-bw), and heparinized blood was collected by cardiac puncture. Next, the kidneys were perfused via the heart with phosphate-buffered saline, removed, and weighed. The right kidney was bisected and immersed in 4% paraformaldehyde for 24-hour fixation. The left kidney was placed in ice-cold isolation buffer prior to preparing for western blotting (22)
Glucose tolerance testing - Near the end of the 3 months, glucose tolerance was measured on the mice. Mice were fasted for 5 hours and then injected intraperitoneally with a filter-sterilized 20% dextrose solution (2 g/kg·bw). Tail vein blood glucose was measured at 0, 15, 30, 45, 60, and 90 min. Area-under-the-curve for glucose tolerance was calculated based on Tai’s Model (56).

Histochemical Analyses - The right kidney was embedded in paraffin blocks and sectioned by the Histology Core (Lombardi Cancer Center, Georgetown University). Sections from 3 mice in each group were stained with Masson’s Trichrome and analyzed for histological evidence of pathology. Sections were imaged with a Zeiss Axiovert 410 inverted microscope. Sections from 4 mice in each group were semi-quantitatively analyzed for GLUT5 subcellular localization using immunoperoxidase-based staining. Heat-induced target retrieval was performed on slides using a citrate buffer pH 6 (DakoCytomation, Carpinteria, CA) to unmask antigenic sites. Endogenous peroxidase activity was removed by incubation with 3% H$_2$O$_2$ for 10 minutes and blocking with avidin/biotin (Vector) and 10% normal goat serum. Sections were incubated with the GLUT5 primary antibody (Boster Immunoleader PA1737, 1:2000), overnight at 4°C. The secondary antibody was biotinylated goat anti-rabbit antibody (1:1000, BA-1000, Vector Labs, Burlingame, CA). 3–3’-diaminobenzidine tetrachloride dihydrate (DAB) was applied for 35 seconds. A positive reaction was identified as a dark brown stain. Pictures were taken with a Photometrics Cool Snap® camera (Scanalytics, Fairfax, VA) mounted to a Nikon Eclipse E600 microscope. The pixel densities (relative to background, i.e., luminal pixel density) of the apical and basolateral regions of 10 sampled proximal tubules per mouse were evaluated and averaged using Adobe Photoshop CS5 software. Values were obtained from tubule cross sections by outlining areas approximating the inner 1/3rd (“apical”) versus the outer 2/3rd (“basolateral”) regions of the tubule cross-section. Average density of each region and the ratio of the apical-to-basolateral staining were calculated for each tubule sampled.
Plasma and urine analysis: On 24-hour urine, urine osmolality was measured using a freezing-point osmometer (Model 3900; Advanced Instruments, Inc., Norwood, MA). Urine fructose levels were measured by an enzyme-based chromogenic assay (BioAssay Systems, CA; EFRU-100). Plasma creatinine was determined by an enzymatic assay kit (Mouse Creatinine Assay Kit #80350, Crystal Chem Inc. Downers Grove, IL). Urine creatinine was determined by a Jaffe reaction assay kit (Cayman Chemical Company, MI, #500701). Plasma and urine electrolytes (Na+, K+, Cl-) were measured using the Medica EasyLyte Analyzer. Plasma and urine uric acid was measured by a colorimetric kit (Cayman Chemical Company, MI; 700320). Plasma insulin and aldosterone were determined by ELISA assays (Mercodia, Uppsala, Sweden; 10-1247-01; Cayman Chemical Company, MI; 10004377, respectively).

Western blot analysis of proteins: The kidney cortex (dissected free from medulla) was prepared for western blotting. Briefly samples were homogenized in isolation buffer containing protease inhibitors (22). Protein concentration was determined by a bicinchoninic acid (BCA) assay kit (Pierce), and then protein solubilized in Lammeli buffer. Protein estimations were confirmed by Coomassie dye-stained loading gels. For immunoblotting, 10-25 μg of total protein was loaded from each sample onto individual gels of 10 or 12% polyacrylamide (precast, BioRad). Blots were probed with the following primary antibodies at the respective dilutions: 1) GLUT5 (H200):sc-30109, 1:1000, Santa Cruz Biotechnology, (Santa Cruz, CA); 2) insulin receptor beta subunit, 1 μg/μl, MAB1139, Bioscience Research Reagents, (Temecula, CA); 3) ketohexokinase, sc-366024, 1:1000, Santa Cruz Biotechnology; 4) NKCC2 and 5) AQP2 antibodies (1:1000) polyclonal rabbit from our lab (48, 58); 6) β actin, 1:5000, A5441, Sigma-Aldrich, (St. Louis, MO); 7) GAPDH 1:5000, SAB1405848, Sigma-Aldrich. Blotting and detection approach has been previously described (22). Western blot band density on film was analyzed by Image J (NIH) software (NIH, Bethesda, MD) and normalized to β-actin or GAPDH.
**Statistical Analysis** - In general, data were analyzed using SigmaPlot (Systat Software, version 10, Evanston, IL). Two-way analysis of variance (ANOVA) was used to determine differences due to the main factors, sex, diet, and their interactions for all variables. One-way ANOVA followed by a multiple comparisons test or unpaired t-tests were used to determine differences between individual pairs of means. P-values < 0.05 were considered to be significant.

**Results**

**Metabolic effects of fructose feeding** - Body weight and weight gain (Table 1) were not affected by fructose feeding; however, male mice of both treatments were about 35% heavier and gained twice as much weight. Male mice had a significantly reduced ability to rapidly clear a bolus of intraperitoneal glucose (glucose tolerance) as compared to females. Surprisingly, fructose feeding improved this ability in the males. Male mice also had significantly increased plasma insulin, and there was a significant interactive term in that females showed a slight increase in plasma insulin with fructose and males showed a decrease. Plasma aldosterone was not significantly affected by sex or treatment.

**Hypertrophy of the kidney and osmotic diuresis** - Fructose feeding resulted in significantly increased kidney wet weight (Figure 1A). The increase was about 19% and 10% in males and females, relative to sex-respective controls. Absolute weights of the kidneys were heavier in males (not shown), but no sex differences were observed when kidney weight was normalized for body weight. Urine volume (Figure 1B) was also significantly higher in fructose-fed mice of both sexes (increased 77 and 327%, in males and females, respectively). Urine osmolality, however, was not significantly lower in fructose-fed mice (Table 2). This resulted in a large and significant increase in total osmoles excreted in the fructose-fed mice (Figure 1C). Moreover, there was no sex difference in this parameter when normalized for body weight. Dietary fructose led to a large significant increase in urinary fructose (Table 2). Urinary fructose was
increased over 100-fold in males and 40-fold in females. Interestingly, male control mice had
significantly lower (less than half) urine fructose as compared to female controls. Gross
differences in collagen deposition in the cortex were evaluated by Masson’s trichrome staining
(Figure 2). In general, no obvious differences were observed as a result of diet or sex.

**Uric acid metabolism**—Uric acid metabolism was evaluated as a determinant of ATP
depletion. Urine uric acid was significantly increased by fructose feeding (over 100%) in both
males and females with no sex differences (Figure 3A). Surprisingly uric acid concentrations in
plasma were highest in male control mice and reduced significantly in both sexes by fructose
(Figure 3B). Uric acid clearance was highest in the female mice fed fructose, with an increase
of over 500% as compared to female controls (Figure 3C).

**Urine electrolytes**—Dietary fructose led to a significant increase in the excretion of Na+, K+
and Cl- ions in the urine (Table 2). There were no sex differences in this excretion (when
normalized by body weight). The ratio of the electrolytes in the urine was also evaluated as an
index of distal tubular electrolyte homeostasis. Fructose did not significantly affect any of these
ratios (as assessed by 2-way ANOVA); however, females had a lower ratio of K+ to Cl- in urine,
and this difference was attenuated in the fructose-fed mice. Fractional excretion (FE) of Na+, 
Cl- and K+ is shown in Figure 4. Mice are plotted individually to show increased variability in
fructose-fed animals. Fructose increased FE of all 3 electrolytes. This response was enhanced
in males (highly significant interactive term). Differences in the FE of electrolytes between the
sexes were primarily driven by a fall in urine creatinine in MFr. Urine creatinine levels were
(μmol/d/25 g·bw): 3.5 ± 0.5 (MC), 1.6 ± 0.6 (MFr), 2.9 ± 0.5 (FC), and 5.4 ± 1.9 (FFr); p = 0.032
for unpaired t-test between MC and MFr. In contrast, plasma creatinine was not significantly
different between groups (not shown).

**Plasma electrolytes**—Plasma Na+, K+, and Cl- were all significantly affected by fructose
(Figure 5). Plasma Na+ (Figure 5A) and Cl- (Figure 5C) were reduced by fructose, but only in
females (significant interactive term). Plasma K+, on the other hand, was increased by fructose
in both sexes. The increase was larger in the females, as control females had the lowest plasma K+. The ratio of Na-to-Cl was not different between groups and indicated that these two electrolytes were being regulated in parallel. In contrast, the ratios of Na-to-K and K-to-Cl were highly affected by fructose in that fructose reduced the first and increased the second. There was also a significant interaction for both of these ratios in that the effects were strongest in females.

**Expression of proteins involved in water balance**- Due to evidence of sex differences in plasma electrolytes with fructose feeding (modest hyponatremia in females), we tested the cortical expression of two major vasopressin-regulated proteins involved in urine concentrating, i.e., aquaporin-2, in the collecting duct, and the sodium-potassium-2-chloride cotransporter (NKCC2) in the thick ascending limb (Figure 6). Both AQP2 and NKCC2 were more highly expressed in the females under the control diet. Fructose feeding reduced both proteins, and the degree of reduction was greater in females.

**Expression of proteins involved in fructose metabolism/transport**- In contrast, the expression of proteins involved in fructose transport/metabolism or energy homeostasis (Figure 7) appeared more sensitive to fructose feeding in males. Males had substantially greater cortical expression of GLUT5 (fructose transporter) as compared to females. GLUT5 expression was increased by about 50% in males by fructose, but females did not show sensitivity to fructose feeding with regarding to GLUT5 abundance. Similarly, ketohexokinase (KHK), the first enzyme in fructose metabolism, and the insulin receptor (β-subunit) were increased by fructose only in males (significant interaction).

**Subcellular Localization of GLUT5**- The transporter GLUT5 in the S3 segment of PT requires apical brush border localization in order for luminal-to-interstitial directional transport to occur (29). To determine whether sex or dietary fructose affect the relative amount of protein in the apical versus basolateral aspects of the cell, we performed semi-quantitative
immunohistochemistry (Figure 8). Surprisingly, fructose had no effect in males on the average density in the basolateral or apical aspects sampled; however, in females control mice had greater apical density (relative to background) as compared to fructose-fed mice (Figure 8B). The ratio of apical-to-basolateral staining was compared in Figure 8C. Here there was a clear sex difference in that females had a greater ratio regardless of dietary treatment.

Na\textsuperscript{+}K\textsuperscript{+}ATPase expression in regions of the kidney- To determine whether electrolyte differences might result from altered driving forces, we evaluated protein levels (Figure 9) of the $\alpha$-1 subunit of Na-K-ATPase pump in homogenates from cortex (CTXH), inner stripe of the outer medulla (OMH), and inner medulla (IMH). Females had modestly, but significantly higher levels of the $\alpha$-1 subunit in CTXH (primarily proximal tubule-associated) and inner medulla (collecting duct-associated) as compared to males (2-way ANOVA). In addition, fructose had a tendency to increase expression in females only in all 3 regions leading to a significant interactive term for OMH (thick ascending limb-associated) and IMH, and a strong trend ($p = 0.061$) in CTXH.

Discussion

The consumption of fructose, around the world, has increased substantially in the last 3-4 decades (8, 9, 53, 57). However, whether or not this represents an inherent health risk and whether certain populations may be more susceptible, is still uncertain. Our studies were aimed to determine whether there were sex differences primarily in the renal-specific responses to fructose feeding in the mouse. We utilized a “mixed genetic background” approximately 80% 129/SV and 20% C57Bl6/J. It is important to note that background strain may affect overall results. Our mixed background would be predicted to increase overall variability, and therefore any sex differences found might be considered more robust. Overall, we found a pattern of differences that we interpret as supportive of greater proximal tubule (PT) metabolism of
fructose by male mice, and a greater impact, i.e., sensitivity of the distal tubule (TAL and CD) in 
the female mice. We will discuss these findings and interpretation in greater detail below.

Like glucose, fructose has the capacity to be reabsorbed from the filtrate in the proximal 
tubule (PT). While Slc5a2, e.g., SGLT2 is responsible for the majority of glucose reabsorption 
in the PT (63), it does not transport fructose (63). In the Slc2 family, Slc2a5 (GLUT5) appears to 
be the major facilitator of fructose reabsorption across the apical membrane of the PT (2). Our 
Western blotting supports our conclusion that male mice have higher total PT cellular levels of 
GLUT5 protein under control and dietary fructose feeding (Figure 6). Furthermore, while the 
expression of GLUT5 increased 2-fold in the fructose-fed males, it was unresponsive in the 
females. An increase in renal GLUT5 with fructose feeding in the males confirmed the 
observation of other laboratories (1, 2).

Nevertheless, our immunohistochemical (IHC) analysis (although clearly not as 
quantitative) did not show increased density in the male animals or due to fructose in either sex. 
In fact, female mice (on both diets) had a higher ratio of apical-to-basolateral staining, 
potentially indicative of transport potential. The absence of finding an increase in GLUT5 due to 
fructose feeding with IHC in either sex might be explained by the hypertrophy of the PT (~10% 
females,~19% males) which may have diluted the signal with IHC, but would not confound 
western blotting. Nonetheless, in females fed fructose both apical and basolateral staining, as 
quantified, was significantly reduced (relative to same sex controls). Thus, it appears that 
females either down-regulate or at least have attenuated upregulation of GLUT5 expression 
(relative to males) in response to dietary fructose in agreement with western blotting and 
directional changes in ketohexokinase.

Based on the fairly high increase in fructose in the urine in the fructose-fed mice, it is 
likely that fructose uptake into PT cells is not nearly as efficient as that of glucose. In support of 
this, the Km of GLUT5 in rat has been reported to be 12.6 mM, similar to small intestine, and
rather high relative to the blood levels (0.1-0.3 mM in rats consuming fructose) (11). A more recent study looking at mouse GLUT5 in transfected oocytes found similar kinetics to rat (15). In comparison, SGLT2 has a Km about 1.6 mM for glucose, but blood levels are near 5.5 mM (35). However, the fact that males had more GLUT5 and this level was increased by fructose suggests that they had the capacity to adapt in this manner. This may be due to the fact that GLUT5 is an “sry-regulated” gene (15). Sry is a transcription factor produced from the Y-chromosome which will increase the expression of this transporter similar to other proteins involved in differentiation of the male sex (55). In a recent study by Aoyama et al. (1), increased outer cortical GLUT5 expression in response to dietary fructose corresponded temporally to increased tubulointerstitial fibrosis in the DBA/2N mouse strain, suggesting cellular uptake of the sugar was necessary to initiate some modes of renal pathology.

This difference in GLUT5 expression may have also precipitated the approximate 100% increase in expression of ketohepxokinase (KHK) in the male, fructose-fed mice. KHK and GLUT5 expression are thought to be limited exclusively to the PT in kidney (18, 19). Thus while other renal tubule cells may reabsorb filtered fructose, or transport it from the basolateral side, it is unclear whether they can metabolize it. PT cells have similarities to hepatic cells, the major metabolic site for absorbed fructose. In that regard, our results do contrast to what has been reported in rats fed fructose, where female rats were found to have a greater upregulation of fructokinase (ketohepxokinase) in liver, as compared to male rats (61). In addition, the female rats in their study, suffered additional consequences of the diet (impaired GTT and elevated plasma insulin) to a greater extent than the males (61). This did agree with our findings in the mice on the whole-body impact of dietary fructose, which, we found, were somewhat more apparent in the females, e.g., increased plasma insulin and a trend for reduced GTT.

We also found males had an approximate 4-fold increase in the expression of renal cortical insulin receptor (β-subunit) with fructose feeding, whereas females started out with
higher levels of expression which did not change with fructose. Whether this increase in receptor number led to greater signaling capacity in these cells, or was in response to insulin receptor “resistance” is not known. We have found a number of factors that influence expression of the renal IR including obesity and high-fat diets (decrease expression), and insulin infusion (increase its expression) (59). Impaired insulin signaling in the PT may be associated with enhanced gluconeogenesis at this site (28).

Non-reabsorbed fructose travels down the tubule lumen where it can act as an osmolyte, drawing in fluid, and leading to osmotic diuresis. We did not find any sex differences in the absolute or body-weight normalized amount of fructose in the urine, which was substantial in both sexes (430 μmol or about 77 mg/d for males) and represented about 0.3 kcal (4 kcal/g X 0.077 g fructose). Nonetheless, maintenance energy requirements for a lean mouse have been estimated at about 124 kcal/kg^{3/4}/d (39) or about 10 kcal/d for a 30 g mouse. Thus urinary fructose would represent only about 3% of daily energy requirements and shouldn’t have substantially affected nutritional status.

Although we did not measure food intake as it is difficult with pelleted diet in chronic studies, we did monitor urine excretion of Na+, K+, and Cl- as an index of food intake in the chronic state. The diets were formulated to contain the same percentage of these electrolytes. While there were no significant differences in body weight gain between fructose- and control-diet fed mice, urine electrolytes were increased by about 94, 129, and 104% for Na+, K+ and Cl, respectively in males, and by 94, 156, 85%, for females in the fructose-consuming animals. It is not entirely clear what driving this increase in urinary electrolytes. Possibilities include: 1) increased food consumption due to lower energy bioavailability of the fructose and/or increased basal metabolic rate; or 2) losses from other tissues beds, such as bone, muscle, and extracellular fluid. Calculation of the fractional excretion of electrolytes revealed a greater response in males; however, this sex effect was primarily driven by a fall in urine creatinine in
male fructose-fed mice rather than increased urine electrolytes. Why male and not female mice experienced this reduction in urine creatinine with fructose feeding is unclear. Plasma creatinine levels were not significantly different.

The osmotic diuresis produced by fructose may have been the cause of the slight, but significant, disruption in plasma electrolyte homeostasis. Overall, fructose increased the ratio of K+ to both Cl- and Na+ in the plasma, and this effect was significantly greater in the females. Females fed fructose had significantly lower plasma Na+ and Cl- than all other groups. Both sexes experienced some rise in serum K+. Although the fall in serum Na+ and Cl- in females was modest, it might be exacerbated with aging or co-morbidities. Verbalis and colleagues have demonstrated sex differences in hyponatremia with females more sensitive (40). The rise in plasma K+ could be the result of impaired excretion or transcellular shifts in K+(44). Our urine analysis did not indicate impaired K+ excretion. In fact K+ showed the greatest percentage increase in excretion with fructose. Similarly the rise in expression at least for the α-1 subunit of Na-K-ATPase would be predicted to increase K+ excretion in exchange for sodium resorption at least in the collecting duct. Thus, we can assume that the increase in this subunit (which has been shown to be aldosterone sensitive)(47) may represent an attempt to compensate for hyperkalemia rather than the cause of it.

Relative hyponatremia and hypochloremia in the female, fructose-fed mice was accompanied by a fall in the expression of two major proteins involved in water balance, i.e., aquaporin-2 and NKCC2. We also found a fall in the abundance of these two proteins in our study done in male rats in response to fructose (54). The cause-and-effect relationship between the fall in these proteins and the 237% increase in urine volume in the female mice is uncertain. A reduction in the level of these proteins would clearly facilitate water excretion both by affecting urine concentrating and diluting capacity of the TAL and affecting permeability of the CD. The fall in these proteins would have been predicted to protect the mice from more severe
Hyponatremia (23, 40). It is unclear why these proteins weren't down-regulated in the male mice as well with fructose; however control levels were lower in the males. Furthermore, this supports a more distal phenotype in the females. Other studies have suggested that fructose feeding may lead to dehydration and stimulate vasopressin release (33). Vasopressin has been shown to upregulate the abundance of both AQP2 (20) and NKCC2 (24). Therefore, we do not predict that vasopressin levels were elevated in our study.

High dietary fructose has been associated with the development of the constellation disorder known as “metabolic syndrome” in humans and animals models, i.e., dyslipidemia, hypertension, visceral adiposity, and hyperinsulinemia (9, 25, 57). One potential contributing mediator to various aspects of metabolic syndrome is hyperuricemia (4, 32, 41, 45). Uric acid is the end product of purine metabolism generated by the action of the enzyme xanthine oxidase (4). We found an interesting phenomenon with regard to uric acid homeostasis in our mice. While urine excretion of uric acid was significantly increased by high fructose diet, as we predicted, plasma levels were reduced. In fact, male control mice had the highest levels of plasma uric acid. Reduced levels of plasma uric acid have also been found in patients with type I diabetes (5, 6). One potential explanation provided is that poorly controlled diabetes resulting in high circulating glucose levels can impair proximal tubule function (Fanconi-like syndrome), resulting in reduced serum circulating levels of uric acid, as urate can be reabsorbed at this site. Another explanation could be that high luminal fructose is competitively inhibiting urate reabsorption through transporters such as GLUT9 (Slc2a9) (38). GLUT9 has recently been shown to have the capability to transport urate in addition to fructose and glucose and is expressed in renal proximal tubule, along with GLUT5.

In this study we did not elect to measure blood pressure. In a previous set of studies in rats, we did not find an effect of dietary fructose on blood pressure (54), similar to other published reports (3, 17, 27). In contrast, some laboratories have observed fairly substantial
increases (12, 13, 30, 31), and even a sex difference in this response (26). Because it is likely
that this response is very dose/strain/species specific, we did not elect to focus on this aspect in
the current report. Moreover, we did not find clear evidence of tubulointerstitial fibrosis of
glomerulopathy in our fructose-fed mice. Our mice were not prone to excessive weight gain as
a result of the fructose diet which may have ameliorated many of the metabolic effects. We feel
this is a strength of our study as we did not have to interpret these confounding influences.
Lack of severe renal pathology is also consistent with other studies in mice showing only
modest changes which may be strain specific (1).

In sum, fructose feeding resulted in differential responses, at the level of the kidney in
male versus female mice. Our findings are consistent with more proximal effects in male mice
that may arise from increased cellular uptake and metabolism, and a more distal phenotype in
females, with effects on serum electrolytes and the expression of transporters/channels that
regulate these parameters. Nonetheless, it is not clear whether the changes in females
represent adaptive, maladaptive, or neutral responses to fructose. Additional studies are
warranted to address differences in vulnerabilities to dietary fructose between the sexes and the
mechanisms underlying them.

Figure Legends

Figure 1. Fructose increased kidney weight and urine volume- A. kidney weight (average of
both kidneys); B. urine volume (24-hour); C. urine osmoles excreted (24-hour). All data were
normalized to 25 g body weight. “A” is significantly greater than “B”, but not “AB” (results of
multiple comparisons test following a significant, p < 0.05, one-way ANOVA); two-way ANOVA
(sex X diet) results are provided in the panels.

Figure 2. Histology revealed no clear pathology due to fructose- Masson’s trichrome staining to
evaluate pathological changes in the kidney (collagen deposition) revealed no overt effects of
fructose-feeding (3-months) or sex differences in male or female mice.
**Figure 3.** Males had greater uric acid in plasma- A. Urine uric acid excretion; B. plasma uric acid; C. uric acid clearance. Data were normalized to 25 g body weight. “A” is significantly greater than “B”, but not “AB” (results of multiple comparisons test following a significant, p < 0.05, one-way ANOVA); two-way ANOVA (sex X diet) results are provided in the panels.

Figure 4. Fractional excretion of A. sodium, B. chloride, and C. potassium in mice fed control or fructose diet. Each mouse is plotted separately. Fractional excretion of all 3 electrolytes was increased by fructose-feeding, but predominantly in male mice.

**Figure 5.** Changes in plasma electrolytes with fructose were greater in females- Plasma concentrations of A. Na+; B. K+, C. Cl-; ratio of D. Na-to-Cl; E. Na-to-K; and F. K-to-Cl in plasma.

**Figure 6.** AQP2 and NKCC2 abundance showed greater sensitivity to fructose in females- A. Western blots of cortex homogenates (each lane is a different mouse sample) loaded with equal amounts of protein and probed with antibodies against AQP2, NKCC2, and GAPDH. GAPDH (reprobe) was used to normalize loading. MC- male control, MFr- male fructose, FC- female control, FFr- female fructose; “A” is significantly greater than ”B”, but not “AB” (results of multiple comparisons test following a significant, p < 0.05, one-way ANOVA); two-way ANOVA (sex X diet) results are provided in the panel.

**Figure 7.** Proteins involved in energy metabolism are upregulated by fructose in males- A. Western blots of cortex homogenates (each lane is a different mouse sample) loaded with equal amounts of protein and probed with antibodies against GLUT5, KHK, IR-β, and GAPDH. GAPDH (reprobe) was used to normalize loading. MC- male control, MFr- male fructose, FC- female control, FFr- female fructose; “A” is significantly greater than “B”, but not “AB” etc. (results of multiple comparisons test following a significant, p < 0.05, one-way ANOVA); two-way ANOVA (sex X diet) results are provided in the panel.

**Figure 8.** GLUT5 immunohistochemistry- A. Representative stained sections from the deep cortical region of the kidney from male (M) control (C)- upper left, male fructose (Fr)- upper right,
female (F) control- lower left, and female fructose- lower right- 400X image; B. Summary of density semi-quantification of “apical” and “basolateral” aspects of tubules sampled (sampling 10 tubules per mouse, n = 4 mice/group); C. Summary of average mean ratios of apical-to-basolateral staining calculated for each tube. *indicates a significant (p < 0.05) difference between control- and fructose-fed mice within the female sex (unpaired t-test); “A” is significantly greater than “B” (results of multiple comparisons test following a significant, p < 0.05, one-way ANOVA); two-way ANOVA (sex X diet) results are provided in the panel.

Figure 9. Regulation of Na-K-ATPase (α-1 subunit) in kidney regions by fructose- Western blots of whole-cell homogenates prepared from A. cortex (CTXH); B. inner stripe of the outer medulla (OMH) and C. inner medulla (OMH). Each lane is loaded with the same amount of protein from a different mouse sample and probed with a commercial antibody against α-1 Na-K-ATPase. The lower portion of each blot was probed with β-actin to control for loading. D. Densitometry summary- MC- male control, MFr- male fructose, FC- female control, FFr- female fructose; “A” is significantly greater than “B”, but not “AB” etc. (results of multiple comparisons test following a significant, p < 0.05, one-way ANOVA); two-way ANOVA (sex X diet) results are provided in the panel.

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Disclosures
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**Author Contributions**

N.S., L-J. L. and C.M.E. were involved in conception and design of research; N.S. and L-J. L. performed experiments; C.M.E. and N.S. analyzed data and interpreted results of the experiments; L-J. L., N.S., and C.M.E. edited and revised the manuscript and approved final version of manuscript; N.S., L-J. L. and C.M.E. prepared figures and drafted the manuscript.
References


56. **Tai MM.** A mathematical model for the determination of total area under glucose tolerance and other metabolic curves. *Diabetes Care* 17: 152-154, 1994.


### Table 1: Metabolic and Plasma Parameters*

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Weight gain (g/3 months)</th>
<th>Glucose Tolerance (AUC)†</th>
<th>Plasma Insulin (μM)</th>
<th>Plasma Aldosterone (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>32.0 ± 2.0&quot;</td>
<td>9.1 ± 1.5&quot;</td>
<td>504 ± 33&quot;</td>
<td>1.14 ± 0.15</td>
<td>5.4 ± 2.1</td>
</tr>
<tr>
<td>MFr</td>
<td>30.7 ± 1.9A&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.1 ± 1.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>367 ± 74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.58 ± 0.13</td>
<td>6.9 ± 2.7</td>
</tr>
<tr>
<td>FC</td>
<td>22.0 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.4 ± 1.0&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>286 ± 36&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.40 ± 0.14</td>
<td>21.3 ± 13.8</td>
</tr>
<tr>
<td>FFr</td>
<td>23.8 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.0 ± 1.0&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>317 ± 23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.54 ± 0.13</td>
<td>18.0 ± 6.9</td>
</tr>
</tbody>
</table>

Results of 2-Way ANOVA (Diet X Sex) p-values

<table>
<thead>
<tr>
<th></th>
<th>Diet</th>
<th>Sex</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.87</td>
<td>&lt;0.001</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>0.002</td>
<td>0.56</td>
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<tr>
<td></td>
<td>0.26</td>
<td>0.008</td>
<td>0.083</td>
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<td>0.012</td>
<td>0.02</td>
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<tr>
<td></td>
<td>0.92</td>
<td>0.12</td>
<td>0.77</td>
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</table>

*mean ± SEM; n = 6/group; M- male, F- Female, C- control, Fr- fructose; †AUC- area-under- curve (mg·hr/dl); Letters indicate the results of multiple comparisons testing following a significant (p < 0.05) one-way ANOVA; "A" equal to "AB" but not "B" etc.; for two-way ANOVA- significant (<0.05) p-values are bolded.
Table 2: Urine Analysis*

<table>
<thead>
<tr>
<th>Group</th>
<th>Urine Osmolality (Osm/kg-H$_2$O)</th>
<th>Urine Fructose (µmol/d)$^\dagger$</th>
<th>Urine Na$^+$ (µmol/d)$^\dagger$</th>
<th>Urine K$^+$ (µmol/d)$^\dagger$</th>
<th>Urine Cl$^-$ (µmol/d)$^\dagger$</th>
<th>Ratio (Na:Cl)</th>
<th>Ratio (Na:K)</th>
<th>Ratio (K:Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>2.08 ± 0.32</td>
<td>3.14 ± 0.81</td>
<td>54 ± 11$^b$</td>
<td>105 ± 18$^{ns}$</td>
<td>71 ± 15$^{ns}$</td>
<td>0.95 ± 0.25</td>
<td>0.53 ± 0.06</td>
<td>1.72 ± 0.25$^{ns}$</td>
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<tr>
<td>MFr</td>
<td>2.54 ± 0.36</td>
<td>430 ± 132</td>
<td>105 ± 19$^h$</td>
<td>240 ± 45$^h$</td>
<td>145 ± 27$^h$</td>
<td>0.73 ± 0.03</td>
<td>0.44 ± 0.03</td>
<td>1.68 ± 0.10$^h$</td>
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<tr>
<td>FC</td>
<td>2.85 ± 0.18</td>
<td>8.19 ± 1.56$^h$</td>
<td>47 ± 7$^d$</td>
<td>62 ± 9$^d$</td>
<td>68 ± 15$^d$</td>
<td>0.77 ± 0.12</td>
<td>0.62 ± 0.05</td>
<td>1.26 ± 0.05$^d$</td>
</tr>
<tr>
<td>FFr</td>
<td>2.84 ± 0.40</td>
<td>334 ± 74</td>
<td>91 ± 12$^{ns}$</td>
<td>174 ± 23$^{ns}$</td>
<td>115 ± 14$^{ns}$</td>
<td>0.79 ± 0.05</td>
<td>0.53 ± 0.05</td>
<td>1.52 ± 0.10$^{ns}$</td>
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Results of 2-Way ANOVA (Diet X Sex) p-values

<table>
<thead>
<tr>
<th>Diet</th>
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<th>0.002</th>
<th>&lt;0.001</th>
<th>0.002</th>
<th>0.46</th>
<th>0.10</th>
<th>0.46</th>
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<tbody>
<tr>
<td>Sex</td>
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<td>0.29</td>
<td>0.44</td>
<td>0.081</td>
<td>0.28</td>
<td>0.66</td>
<td>0.086</td>
<td>0.048</td>
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<tr>
<td>Inter</td>
<td>0.48</td>
<td>0.27</td>
<td>0.76</td>
<td>0.61</td>
<td>0.55</td>
<td>0.36</td>
<td>0.96</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*mean ± SEM; n = 6/group; $^\dagger$daily excretion normalized to 25 g·bw; M- male, F- Female, C- control, Fr- fructose: Letters indicate the results of multiple comparisons testing following a significant (p < 0.05) one-way ANOVA; $^\dagger$significantly different from MC by unpaired t-test; “A” equal to “AB” but not “B” etc.; for two-way ANOVA- significant (<0.05) p-values are bolded
A. 2-way ANOVA
Diet- 0.001
Sex- <0.001
Interaction- <0.001

B. 2-way ANOVA
Diet- <0.001
Sex- <0.001
Interaction- <0.001

C. 2-way ANOVA
Diet- 0.011
Sex- <0.001
Interaction- 0.002
A. Cortex

B. Outer Medulla

C. Inner Medulla

D. 2-way ANOVA
   Sex- **0.003**
   Diet- 0.24
   Interact.- 0.061

2-way ANOVA
   Sex- 0.05
   Diet- 0.38
   Interact.- **0.038**

Band Density (% MC, normalized to β-actin)

CTXH    OMH    IMH