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PII: S0955-2863(17)30216-4
DOI: doi: 10.1016/j.jnutbio.2017.09.005
Reference: JNB 7841

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 4 March 2017
Revised date: 25 August 2017
Accepted date: 5 September 2017

Please cite this article as: Rukavina Mikusic Natalia L., Kouyoumdzian Nicolás M., Del Mauro Julieta S., Cao Gabriel, Trida Verónica, Gironacci Mariela M., Puyó Ana M., Toblli Jorge E., Fernández Belisario E., Choi Marcelo R., Effects of chronic fructose overload on renal dopaminergic system: alteration of urinary L-dopa/dopamine index correlates to hypertension and precedes kidney structural damage, *The Journal of Nutritional Biochemistry* (2017), doi: 10.1016/j.jnutbio.2017.09.005

Effects of chronic fructose overload on renal dopaminergic system: alteration of urinary L-dopa/dopamine index correlates to hypertension and precedes kidney structural damage

Running title: Urinary L-dopa/dopamine index and fructose overload

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This work was supported by grants from the ANPCYT (PICT 2012-1775), Universidad de Buenos Aires (UBACYT 20020110200048 and 20020130200105BA) and Sociedad Argentina de Hipertensión Arterial (Stimulus Grant for Research on Hypertension 2014-2015).

Keywords: *dopamine, fructose, hypertension, insulin resistance, Na^+, K^+-ATPase, microalbuminuria*

Abstract

Insulin resistance induced by a high fructose diet has been associated to hypertension and renal damage. The aim of this work was to assess alterations in the urinary L-dopa/dopamine ratio over three time periods in rats with insulin resistance induced by fructose overload and its correlation with blood pressure levels and the presence of microalbuminuria and reduced nephrin expression as markers of renal structural damage. Male Sprague-Dawley rats were randomly divided in six groups: control (C) (C4, C8 and C12) with tap water to drink; and fructose-overloaded (FO) rats, (FO4, FO8 and FO12), with a fructose solution (10% w/v) to drink; for 4, 8 and 12 weeks. A significant increase of the urinary L-dopa/dopamine ratio was found in FO rats since week 4, which positively correlated to the development of hypertension and preceded in time the onset of microalbuminuria and reduced nephrin expression observed on week 12 of treatment. The alteration of this ratio was associated to an impairment of the renal dopaminergic system, evidenced by a reduction in renal dopamine transporters and dopamine D1 receptor expression, leading to an over-expression and over-activation of the enzyme Na^+, K^+- ATPase with sodium retention. In conclusion, urinary L-dopa/dopamine ratio alteration in rats with fructose overload positively correlated to the development of hypertension and preceded in time the onset of renal structural damage. This is the first study to propose the use of the urinary L-dopa/dopamine index as marker of renal dysfunction that temporarily precedes kidney structural damage induced by fructose overload.

Keywords: *dopamine, fructose, hypertension, insulin resistance, Na^+, K^+-ATPase, microalbuminuria*

1. Introduction

Fructose is a simple and highly lipogenic sugar present in added dietary sugars, honey and fruit [1]. Fructose consumption has been associated to many of the components of metabolic syndrome such as insulin resistance, dyslipidemia, and hypertension [2]. In fact, a well-known experimental model of metabolic syndrome is based on a diet characterized by high fructose consumption [2-4]. Many mechanisms link insulin resistance with hypertension, including an increase in sodium reabsorption and stimulation of sympathetic nervous and renin-angiotensin systems [5-7]. On the other hand, several studies have suggested a close relationship between metabolic syndrome and renal damage [8,9]. In this way, insulin resistance induced by long term exposure to a high fructose diet is associated to renal structural damage, evidenced by kidney inflammation, proteinuria, glomerular hypertrophy, sclerosis and tubulointerstitial injury [10,11].

Renal dopamine, synthesized in tubular cells, acts as a local, non-neural and natriuretic system that regulates renal function and blood pressure levels through inhibition of sodium transporters at tubular level, being the most important the enzyme Na^+, K^+- ATPase [12,13]. Several studies have established a relationship between insulin resistance and the impairment of renal dopaminergic system (RDS) in the pathogenesis of hypertension. In this way, alterations in dopamine receptor D1 (D1R) were associated to hyperinsulinemia-induced hypertension in different animal models of obesity and metabolic syndrome [14,15]. It has also been demonstrated a reduction in dopamine

production in proximal tubular cells in rats with insulin resistance induced by fructose overload [16].

The conversion of L-Dopa to dopamine in the kidneys accounts for all the free dopamine found in urine [17]. Therefore, the ratio between urinary L-dopa and dopamine could reflect RDS functionality. Considering these facts, it can be proposed that the urinary L-dopa/dopamine index could be altered in pathological conditions that lead to renal dysfunction such as insulin resistance. To date, there is no evidence regarding the use of urinary L-dopa/dopamine index as an early biochemical marker of renal dysfunction that could precede and anticipate renal established structural damage associated to insulin resistance in this model of metabolic syndrome.

The aim of the present study was to evaluate the alterations in the urinary L-dopa/dopamine index over three time periods in rats with metabolic syndrome induced by fructose overload and its correlation with blood pressure levels and the presence of microalbuminuria and reduced expression of nephrin as indicators of renal structural damage.

2. Materials and Methods

2.1 Animal protocol and diet

Male Sprague-Dawley rats weighing 180-200 g at the beginning of the study were used. The experiments were approved in advance by the local ethics committee on animal research (protocol #2100-15; 0035638/15) according to “International Ethical Guiding Principles for Biomedical Research on Animals” established by the CIOMS (Council for

International Organizations of Medical Sciences). Animals were housed in cages with a 12-hour light/dark cycle under conditions of controlled temperature (22±2ºC) and humidity. Until the day of the experiment all animals were given free access to liquid and fed with standard chow with the following composition (w/w): 20% proteins, 3% fat, 2% fiber, 6% minerals and 69% starch and vitamin supplements (Commercial Rodents Purina Chow; Cooperación SRL, Buenos Aires, Argentina). 48 rats were randomly divided in six groups and studied at 4, 8 and 12 weeks of treatment: Three control (C) groups (C4, C8 and C12), with tap water to drink, and three experimental fructose-overloaded (FO) groups, (FO4, FO8 and FO12), with fructose (Parafarm, Buenos Aires, Argentina) solution (10% w/v) to drink (n=8 for each group). The rats were weighed before dietary manipulation and at the end of each experimental period.

2.2 Systolic blood pressure measurement

The rats were trained to the procedure of blood pressure measurement at 10.00 AM, twice a week, for 2 weeks, after randomization (baseline blood pressure), and prior to be sacrificed. Indirect systolic blood pressure (SBP) was measured by means of a photoelectric tail-cuff connected to an amplifier (II TC model 47; Innovators in Instrumentation, Landing, NJ, USA) in series with an oscilloscope (type 532, Tektronic Inc., Portland, OR, USA).

2.3 Measurement of food, fluid and caloric intake

Three days before the end of each period time, the rats were placed in metabolic cages for 48 hours in order to allow adaptation to the new environment. Food consumption and water or fructose solution intake were measured for control and FO

rats, respectively. Caloric intake for control rats was calculated using the formula for standard rat chow [food intake (g) x 3.3 kcal/g], while caloric intake for FO rats was calculated using the formula [food intake (g) x 3.3 kcal/g] + fructose intake (mL) x 0.4 kcal/mL].

2.4 Blood samples recollection and biochemical measurements

After 4, 8 or 12 weeks, all groups of experimental and control animals were fasted for 5 hours. Under anesthesia with ketamine (80 mg/kg, PRO-SER SA) and xylazine (12 mg/kg, PRO-SER SA), blood samples were collected to measure plasma triglycerides, cholesterol, glucose and insulin levels. Plasma triglyceride and cholesterol levels were measured by means of commercial kits (TG Color GPO/PAP AA, Colestat Wiener Labs, Rosario, Santa Fé, Argentina) using a spectrophotometric method; plasma glucose was determined by a blood glucose meter (Accu-Chek, Roche Diagnostics GmbH, Mannheim, Germany) and insulin by ELISA (Millipore Corporation, Billerica, MA, USA). Homeostasis model of assessment – insulin resistance index (HOMA-IR) was calculated by using the following equation: HOMA = fasting glucose (mmol/L) x fasting insulin (mIU/L)/22.5, being 2.5 points or higher, the cut-off point to define insulin resistance [18].

2.5 Urine samples recollection and urinary measurements

Using the metabolic cages, 24 hour urinary samples from control and FO rats were collected at the end of each experimental period to determine diuresis and urinary excretion of L-dopa, dopamine, sodium and creatinine. To determine urinary L-dopa and dopamine concentration by HPLC, a fraction of urine was collected at the end of the experimental period into polyethylene tubes containing 100 µl of 6 N HCl.

2.5.1 Renal function parameters calculation

To evaluate renal functionality, the following parameters were determined: urine and plasma sodium and creatinine (by standard methods using an autoanalyzer), glomerular filtration rate (GFR) estimated by creatinine clearance (CrCl), fractional and urinary sodium excretion (FENa and UNa.UV, respectively) and daily diuresis. FENa, UNa.UV and CrCl were calculated according to standard formula. Daily diuresis is expressed as mL/day/kg, CrCl as mL/min/kg, UNa.UV as mEq/day/kg and FENa as the percentage (%) of filtered sodium.

2.5.2 Urinary L-dopa and dopamine assay

A 50-µl aliquots of urine were partially purified by batch alumina extraction, separated by reverse-phase high-pressure liquid chromatography (RP-HPLC) using a 4.6×250 mm Zorbax RxC18 column (DuPont Company, Delaware, USA) and quantified amperometrically by the current produced upon exposure of the column effluent to oxidizing and then reducing potentials in series using a triple-electrode system (ESA, Bedford, Mass., USA). Recovery through the alumina extraction step averaged 80–90%. L-dopa and dopamine concentrations in each sample were corrected for recovery of an internal standard. Levels of L-dopa and dopamine were further corrected for differences in recovery of the internal standard in a mixture of external standards. The limit of detection was about 20 pg/volume assayed.

2.6 Kidney dissection and processing

After urine and blood samples collection, the animals were sacrificed and both kidneys were dissected and then processed to perform biochemical and molecular analyses.

2.6.1 Western blot analyses

Semiquantitative Western blot analyses were carried out in renal cortex from all animals. Expression of L-dopa transporters (LAT-1, LAT-2), dopamine transporters (OCT-2, OCT-N1/2/3, OCT-N1), dopamine receptor (D1R) and nephrin was studied. The right kidney from all animals was removed and the renal cortex was immediately dissected and separated under refrigeration. Tissue samples were then homogenized on ice with a Tissue Tearor (Biospec Products Inc, Bartesville, Oklahoma, USA), 1:3 weight/volume, in a buffer mixture: 50 mmol/L Tris, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% Triton-X-100, 1 mmol/L PMSF, 1 µmol/L pepstatin, and 2 µmol/L leupeptin, 1x protease inhibitor cocktail (Roche Diagnostics, Buenos Aires, Argentina). Then, samples were centrifuged at 3400 g and 4°C during 15 minutes. Protein concentration in the Triton-soluble supernatant was determined by Lowry technique [19]. Samples of renal cortex, containing similar amounts of protein (100 µg protein/lane) were separated by electrophoresis in 10% SDS-polyacrylamide gels (Bio-Rad Labs Inc, Hercules, California, USA) and then transferred to a PVDF membrane (Bio-Rad Labs Inc) and incubated with goat polyclonal anti-LAT-1 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA, 1:800 dilution), goat polyclonal anti-LAT-2 (Santa Cruz Biotechnology, Inc. 1:2000 dilution), goat polyclonal anti- OCT-2 (Santa Cruz Biotechnology, Inc. 1:800 dilution), rabbit polyclonal anti- OCT-N1/2/3 (Santa Cruz Biotechnology, Inc. 1:2000 dilution), goat polyclonal anti- OCT-N1 (Santa Cruz

Biotechnology, Inc. 1:800 dilution), goat polyclonal anti-D1R (Santa Cruz Biotechnology, Inc. 1:800 dilution) or goat polyclonal anti- nephrin (Santa Cruz Biotechnology, Inc. 1:400 dilution). A secondary immunoreaction was performed with a biotinilated donkey antigoat IgG or antirabbit IgG respectively (Santa Cruz Biotechnology, Inc. 1:1500 dilution), followed by a third step using streptavidin conjugated with horseradish peroxidase (GE Healthcare Life Sciences; Buenos Aires, Argentina, dilution of 1:2000). The samples were revealed by chemiluminescence using ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1–5 min. The density of the respective bands was quantified by densitometric scanning using a Hewlett-Packard scanner and Image J analyzer software (RSB). To avoid inaccuracies in protein loading, GAPDH or beta-tubulin were measured as internal standard (Santa Cruz, anti-GAPDH rabbit polyclonal first antibody, dilution of 1:2000; Abcam, anti-beta-tubulin rabbit polyclonal first antibody, dilution of 1:2000, second biotinilated anti-rabbit antibody, 1:2000 dilution; third streptavidin conjugated with horseradish peroxidase, 1:2000 dilution) for each blot. Protein levels were calculated and expressed as the ratio between the optical densities of the bands corresponding to LAT-1, LAT-2, OCT-2, OCT-N1/2/3, OCT-N1, D1R and nephrin and the one corresponding to the respective internal standard.

2.6.2 Immunofluorescence

Immunofluorescence detection of Na^+, K^+-ATPase was performed on renal cortex from all animals. A sagittal cut of the left kidney from all animals was performed. Then, the tissues were fixed in a solution of 10% paraformaldehyde (pH 7.20) and then embedded with a 30% sucrose solution. Tissues were freezed at -80 °C and 5-15 µm thick

tissue sections were cut using a cryostat. Three micron sections were made and mounted on slides for immunofluorescence. After washing with cold Ringer buffer, the nonspecific binding sites were blocked by incubating at 22°C with Ringer's buffer containing 0.5% gelatin for 30 minutes. They were incubated at 4°C overnight with specific purified rabbit anti total Na^+, K^+-ATPase antibody (Abcam, Cambridge, UK, 1:250 dilution). Then, they were washed with phosphate saline buffer (PBS) and incubated for 45 minutes at dark with corresponding marked secondary antibody (donkey anti-rabbit IgG FITC, Santa Cruz Biotechnology, Inc., 1:500 dilution in PBS).

2.6.3 Specific Activity of Na^+, K^+-ATPase

Sample tissues from renal cortex weighing 50 mg were homogenized (1:10 weight/volume) in 25mM imidazole/1mM EDTA/0.25M sucrose solution and centrifuged at 4700 g at 4°C for 15 minutes. Na^+, K^+-ATPase activity was assayed in the supernatant using Fiske-Subbarrow method [20]. ATPase activity was measured by colorimetric determination of released orthophosphate and ouabain was used to inhibit specifically Na+, K+-ATPase activity [21]. Proteins were determined by the method of Lowry *et al* [19]. Results are expressed as percentage of Na^+, K^+-ATPase activity, considering control values as 100%.

2.7 Statistical analysis

All results are expressed as means ± SEM. Data was processed using Graph Pad InStat Software (San Diego, CA, USA). The Gaussian distribution was evaluated by the Kolmogorov Smirnov method and to compare between groups, ANOVA followed by Newman-Keuls test or Tukey was used as appropriate. Statistical analysis was performed

using Kruskal-Wallis test (Nonparametric ANOVA) and multiple comparison test of Dunn, for those non-Gaussian distribution parameters. $p<0.05$ was considered statistically significant.

3. Results

3.1 Physiological and metabolic parameters

As shown in table 1, FO rats had significant higher fluid intake and lower food intake compared to control rats from week 4 of treatment. Caloric intake did not differ between control and FO rats for each experimental period. Body weight did not differ between control and FO animals in any experimental period. SBP significantly increased from week 4 of treatment in FO rats compared to control groups, reaching higher levels at weeks 8 and 12. Plasma triglyceride levels significantly increased since week 8 of fructose overload, without significant changes in total cholesterol values in any experimental period. Fructose overload significantly increased plasma insulin levels from week 4 of treatment compared to control rats, while blood glucose levels were higher, but not significantly different, in FO rats at 4 , 8 and 12 weeks. HOMA-IR levels significantly increased in FO respect to control rats and were higher than 2.5 since week 4.

3.2 Renal function parameters

Table 2 shows that daily diuresis significantly increased in FO animals from week 4, compared to control groups. Sodium urinary excretion and fractional excretion of sodium were significantly reduced in FO rats throughout all treatment. Furthermore, creatinine clearance did not change along the entire treatment in FO compared to control groups.

3.3 Renal dopaminergic system

3.3.1 L-dopa and dopamine excretion

Urinary L-Dopa excretion significantly increased along all the treatment with fructose while urinary dopamine excretion simultaneously decreased, compared to control levels (figure 1). The urinary L-dopa/dopamine ratio significantly increased in FO rats from week 4, as regard to their respective controls (figure 2). Figure 3 shows a positive and significant correlation between the elevation of SBP and urinary L-dopa/dopamine ratio levels along the 12 weeks of fructose treatment.

3.3.2 *Renal expression of L-dopa and dopamine transporters and D1 receptor*

LAT-1 expression did not change in FO animals in any of the experimental periods, compared to control rats (figure 4a). Conversely, LAT-2 expression significantly increased since week 4 of treatment with fructose, suggesting a possible increase in L-dopa uptake process by tubular cells compared to controls (figure 4b). OCT-2 expression significantly diminished since week 8 in FO rats respect to controls (figure 5a). These changes were accompanied by a significant reduction of OCT-N1/2/3 and OCT-N1 expression since the same week (figures 5b and 5c). On the other hand, D1R expression was reduced in FO rats since week of 4 compared to control rats (figure 5d).

3.3.3 *Na^+, K^+-ATPase expression and specific activity*

Specific activity levels of Na^+, K^+-ATPase in renal cortex were significantly increased from week 4 of treatment with fructose respect to control groups (figure 6). Na^+, K^+-ATPase expression, determined by immunofluorescence, increased in renal cortex in FO rats, from the same time period (figure 7).

3.4 *Biomarkers of renal damage*

Microalbuminuria was defined as a urinary albumin/creatinine ratio between *30-300* mg/g. The presence of microalbuminuria was detected in week 12 of treatment with fructose (figure 8). A significant reduction of nephrin expression was observed at the same experimental period in FO rats compared to controls (figure 9).

4. Discussion

Experimental models of metabolic syndrome, such as the model of FO in the diet, are characterized by hemodynamic and metabolic changes including hyperinsulinemia and insulin resistance, dyslipidemia and hypertension, and are useful to investigate the pathophysiological processes that lead to kidney damage and their clinical implications [5,22]. In our study, plasma insulin levels were significantly increased in FO rats compared to controls since week 4. Plasma glucose levels were also higher in FO respect to control rats, although the difference was not significant. Rats with FO showed significantly higher levels of HOMA index compared to control rats since week 4 of treatment, reaching higher values at weeks 8 and 12, indicating the development of insulin resistance [18]. Regarding lipid metabolism, FO rats exhibited increased plasma triglycerides since week 8 of treatment without changes in total cholesterol in any experimental period compared to control rats. It is well established that fructose leads to an increase in plasma triglycerides, which in turn contributes to the development of insulin resistance [23].

Body weight values did not differ between control and FO animals in any experimental period. The absence of body weight gain in rats with a high fructose diet has been reported in several studies [22,24]. At the same time, caloric intake between FO and control rats was not different either. This could be explained by the fact that in FO animals, the increase in caloric intake given by fructose intake was compensated by a reduction in food intake, compared to control rats.

Concerning renal function, the 24 hour diuresis significantly increased in FO rats respect to controls. This increment is explained as a consequence of increased fluid intake in FO compared to control rats, which has been well documented [3]. One explanation could be the sweet taste of the solution that would enhance the palatability thereby increasing fluid intake [3]. On the other hand, urinary sodium excretion and urinary fractional excretion were significantly reduced in rats with FO since week 4 of treatment, indicating the existence of sodium retention. Many authors have suggested that increased renal sodium reabsorption induced by insulin is enhanced in insulin resistance states and possibly contributes in part to the development of hypertension [25].

In addition, RDS constitutes a local natriuretic system that regulates sodium excretion and blood pressure levels and several studies have established a relationship between insulin resistance and the RDS in the pathogenesis of hypertension [13,16]. Our results show an increase in the urinary L-dopa/dopamine ratio in FO rats from week 4 of treatment, indicating the existence of an impairment in renal dopamine synthesis in this model. The increase in L-dopa/dopamine ratio was due to a significant decrease in urinary dopamine excretion accompanied by a significant increase in urinary L-dopa excretion. L-

dopa uptake through the apical membrane of proximal tubular cells represents the rate-limiting step in dopamine synthesis, and it is mediated by at least, two major Na^+-independent amino-acid transporters, named LAT and $b^{o,+}$ systems [26]. Alterations in L-dopa uptake in states of insulin resistance have been described [27]. Although, we found no significant difference in LAT-1 expression in FO rats compared to controls in any experimental period, while LAT-2 expression was significantly increased in FO *versus* control rats since week 4. This increase would imply an increase in L-dopa uptake and a decrease in urinary L-dopa excretion. Nonetheless, the results obtained in our study are in accordance with previous findings that indicate a reduced L-dopa uptake in insulin resistance states [27,28]. One possibility that would explain high values of urinary L-dopa with an increased LAT-2 expression is that these transporters could show a defect in their activity, being unable to enter L-dopa into the tubular cells. Another possibility is that LAT-2 increased expression could be located on intracellular stores and not necessarily inserted in the plasma membrane. Further experiments would be needed to confirm or discard any of these hypotheses.

In order to achieve its actions, dopamine must leave the proximal tubular cells through the apical border to reach the tubular lumen, where it can bind and activate specific dopamine receptors located outside the apical border of tubular cells. Dopamine can be uptaken from plasma into proximal tubule by members of the SLC22A family (solute carrier superfamily) called organic cation transporters (OCT-1, OCT-2 and OCT-3) located at the basolateral membrane. Dopamine leaves the tubular cells by other members of SLC22A family named OCTN-1, OCTN-2 and OCTN-3, which are mainly located

at the apical membrane [29]. The lower urinary excretion of dopamine observed in our experimental model could be a consequence of an impairment in dopamine transportation by OCTs and OCTNs. In this sense, reduced expression of OCT-N1 from week 8 in FO rats compared to controls was consistent with reduced expression of total OCTNs from the same period. OCT-2 expression was also reduced since week 8 in FO rats, indicating a decrease in dopamine uptake from circulation. These results may help to explain the lower values of urinary dopamine excretion induced by FO and are in accordance with other studies in which a high fructose diet was associated with impaired OCTs expression and activity and renal dysfunction [30,31].

In our study, FO rats showed higher SBP levels respect to control since week 4 of treatment, reaching the highest levels at weeks 8 and 12 with values close to 160 mmHg. Although the precise mechanism by which hypertension develops in states of insulin resistance is still unknown, it has been reported both in patients with essential hypertension and in rats with insulin resistance by FO, a reduced urinary dopamine excretion together with reduced endogenous content of the amine, inversely proportional to the levels of blood pressure [16,32]. Supporting this evidence, a progressive increase of the urinary L-dopa/dopamine ratio, was found from week 4 of treatment, with a positive significant correlation with SBP levels since the same time period.

Dopamine exerts its actions by binding to receptors classified into D1-like (D1 and D5) and D2-like (D2, D3 and D4) subtypes based on their structure and pharmacological properties [13,26]. In conditions of normal sodium balance, more than 50% of renal sodium excretion is regulated by D1R [26,33]. In our experiments, D1R expression was

significantly reduced since week 4 of treatment in FO rats. These results are consistent with other studies showing alterations in D1R expression and functionality in experimental models of insulin resistance [14,16,34]. Na^+, K^+-ATPase, an enzymatic pump of basolateral localization in renal tubular cells, is inhibited by dopamine mainly through D1R [26]. It has been reported that Na^+, K^+-ATPase is stimulated in states of insulin resistance and chronic exposure of kidney cells to hyperinsulinemia, leading to increased reabsorption of sodium and water and therefore to hydrosaline retention [34,35]. In this way, a defective D1R in a context of insulin resistance could lead to stimulation and increased expression of Na^+, K^+-ATPase, as it was found in FO rats since week 4 in our study.

Several clinical studies have proposed the existence of a relationship between metabolic syndrome and renal disease [8]. Microalbuminuria is an early indicator of kidney disease and a predictor of ischemic heart disease in essential hypertension [36]. On the other hand, nephrin is a transmembrane protein located at the specialized podocyte cell–cell junction that constitutes the slit diaphragm, maintaining the integrity of the filtration barrier of glomerulus [37]. Several studies have demonstrated the onset of renal damage with microalbuminuria and nephrin expression alterations in FO rats [11,38,39]. In our study, microalbuminuria and a significant reduction in nephrin expression were observed at week 12 in FO rats, evidencing the existence of structural alterations in the filtration barrier.

In conclusion, this study demonstrates that insulin resistance induced by FO is associated with an impairment in RDS since week 4 of treatment, given by alterations in L-dopa and dopamine transport and dopamine actions through D1R. This impairment could

be proposed as one of the mechanisms leading to sodium retention and hypertension. FO is also associated to renal damage over time, evidenced by the presence of microalbuminuria and reduced nephrin expression at week 12 of treatment. Taking into consideration that alterations in the L-dopa/dopamine ratio were evidenced since week 4 of FO, the urinary L-dopa/dopamine index could be proposed as a marker of renal dysfunction that temporarily precedes renal structural damage evidenced by microalbuminuria and reduced nephrin expression. A suggested mechanism is illustrated in figure 10.

This is the first study to propose the use of the urinary L-dopa/dopamine index as marker of renal dysfunction in a model of metabolic syndrome induced by FO. Further studies are needed to confirm the relevance of the index in other experimental models of hypertension and/or metabolic syndrome. This would set the precedent in the proposal of the use of this biochemical parameter in future clinical studies in order to assess its usefulness as a diagnostic marker, as predictor of response to treatment or to monitor changes in blood pressure and kidney damage.

Acknowledgments

We thank María C. Kravetz, Marcela Pandolfo, Ana Uceda, Susana Gorzalczany and Barcelo Foundation for their collaboration to the study.

Conflict of interest

None

References

[1] Johnson RJ, Sanchez-Lozada LG, Nakagawa T. The effect of fructose on renal biology and disease. J Am Soc Nephrol 2010; 21:2036-39.

[2] Dekker MJ, Su Q, Baker C, Rutledge CA, Adeli K. Fructose: a highly lipogenic nutrient implicated in insulin resistance, hepatic steatosis, and the metabolic syndrome. Am J Physiol Endocrinol Metab 2010; 299:E685-94.

[3] Mamikutty N, Thent ZC, Sapri SR, Sahruddin NN, Yusof MR, Suhaimi FH. The establishment of metabolic syndrome model by induction of fructose drinking water in male wistar rats. Biomed Res Int 2014; 263897;doi:10.1155/2014/263897.

[4] Wong SK, Chin KY, Suhaimi FH, Fairus A, Ima-Nirwana S. Animal models of metabolic syndrome: a review. Nutr Metab (Lond) 2016; 13:65.

[5] DeFronzo RA, Ferrannini E. Insulin resistance—a multifactorial syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care 1991; 14:173-94.

[6] Defronzo RA, Cooke CR, Andres R, Faloona GR, Davis PJ. The effect of insulin on renal handling of sodium, potassium, calcium, and phosphate in men. J Clin Invest 1975; 55:845-55.

[7] Rowe JW, Young JB, Minaker KL, Stevens AL, Pallotta J, Landsberg L. Effect of insulin and glucose infusions on sympathetic nervous system activity in normal in man. Diabetes 1981; 30:219-25.

[8] Gluba A, Mikhailidis DP, Lip GY, Hannam S, Rysz J, Banach M. Metabolic syndrome and renal disease. Int J Cardio 2013; 164(2):141-50.

[9] Locatelli F, Pozzoni P, Del Vecchio L. Renal Manifestations in the Metabolic Syndrome. J Am Soc Nephrol 2006; 17:S81-5.

[10] Sánchez-Lozada LG, Tapia E, Jimenez A, Bautista P, Cristobal M, Nepomuceno T, et al. Fructose-induced metabolic syndrome is associated with glomerular hypertension and renal microvascular damage in rats. Am J Physiol Renal Physiol 2007; 292:F423-9.

[11] Gersch MS, Mu W, Cirillo P, Reungjui S, Zhang L, Roncal C, et al. Fructose, but not dextrose, accelerates the progression of chronic kidney disease. Am J Physiol Renal Physiol 2007; 293:F1256-61.

[12] Aperia A. Dopamine action and metabolism in the kidney. Curr Opin Nephrol Hypertens 1994; 3:39-45.

[13] Carey RM. Theodore Cooper Lecture: Renal DA system: paracrine regulator of sodium homeostasis and blood pressure. Hypertension 2001; 38:297.

[14] Moreira-Rodrigues M, Quelhas-Santos J, Serrão P, Fernandes-Cerqueira C, Sampaio-Maia B, Pestana M. Glycemic control with insulin prevents the reduced renal dopamine D1 receptor expression and function in streptozotocin-induced diabetes. Nephrol Dial Transplant 2010; 25(9):2945-53.

[15] Muhammad AB, Lokhandwala MF, Banday AA. Exercise reduces oxidative stress but does not alleviate hyperinsulinemia or renal dopamine D1 receptor dysfunction in obese rats. Am J Physiol Renal Physiol 2011; 300(1):F98-104.

[16] Hasegawa K, Yoshida H, Ura N, Murakami H, Hagiwara M, Shimamoto K. The role of renal natriuretic and depressor systems in insulin-resistant hypertensive rats. Hypertens Res 2004; 27:501-8.

[17] Eisenhofer G, Kopin I, Goldstein D. Catecholamine metabolism: A contemporary view with implications for physiology and medicine. Pharmacol Rev 2004; 56:331-49.

[18] Matthews DR, Hosker JR, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28:412-41.

[19] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1):265-75.

[20] Fernandes-Cerqueira C, Sampaio-Maia B, Quelhas-Santos J, Moreira-Rodrigues M, Simões-Silva L, Blazquez-Medela AM, et al. Concerted action of ANP and dopamine D1-receptor to regulate sodium homeostasis in nephrotic syndrome. Biomed Res Int 2013; 397391 doi: 10.1155/2013/397391.

[21] Lowry OH, López JA. Determination of inorganic phosphate in presence of labile P esters. J Biol Chem 1946; 162:421-8.

[22] Abdulla MH, Sattar MA, Johns EJ. The relation between fructose induced metabolic syndrome and altered renal haemodynamic and excretory function in the rat. Int J Neph 2011; 934659 doi: 10.4061/2011/934659.

[23] Nseir W, Nassar F, Assy N. Soft drinks consumption and nonalcoholic fatty liver disease. World J Gastroenterol 2010; 16(21):2579-88.

[24] Bezerra RMN, Ueno M, Silva MS, Tavares DQ, Carvalho CRO, Saad MJA. A high fructose diet affects the early steps of insulin action in muscle and liver of rats. J Nutr 2000; 130(6):1531-5.

[25] Salyer S, Lesousky N, Weinman EJ, Clark BJ, Lederer ED, Khundmiri SJ. Dopamine regulation of Na+-K+-ATPase requires the PDZ-2 domain of sodium hydrogen regulatory factor-1 (NHERF-1) in opossum kidney cells. Am J Physiol Cell Physiol 2011; 300:C425-44.

[26] Armando I, Villar VA, Jose PA. Dopamine and renal function and blood pressure regulation. Compr Physiol 2011; 1(3):1075-117.

[27] Carranza A, Karabatas L, Barontini M, Armando I. Decreased tubular uptake of L-3,4-dihydroxyphenylalanine in streptozotocin-induced diabetic rats. Horm Res 2001; 55:282-7.

[28] Carranza A, Mendez CF, Barontini M, Nowicki S. Insulin enhances L-dopa renal proximal tubule uptake: a regulatory mechanism impaired in insulin resistance. Pflugers Arch 2004; 448(1):85-92.

[29] Koepsell H; Lips K; Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm Res 2007; 24:1227-51.

[30] Hu QH, Wang C, Li JM, Zhang DM, Kong LD. Allopurinol, rutin, and quercetin attenuate hyperuricemia and renal dysfunction in rats induced by fructose intake: renal organic ion transporter involvement. Am J Physiol Renal Physiol 2009; 297(4):F1080-91.

[31] Grover B, Buckley D, Buckley AR, Cacini W. Reduced expression of organic cation transporters rOCT1 and rOCT2 in experimental diabetes. J Pharmacol Exp Ther 2004; 308: 949-56.

[32] Iimura O, Shimamoto K, Ura N. The pathophysiological role of renal dopamine, kallikrein-kinin and prostaglandin systems in essential hypertension. Agents Actions Suppl 1987; 22:247-56.

[33] Hussain T; Lokhandwala MF. Renal dopamine receptors and hypertension. Exp Biol Med (Maywood) 2003; 228:134-42.

[34] Banday AA, Fazili FR, Marwaha A, Lokhandwala MF. Mitogen-activated protein kinase upregulation reduces renal D1 receptor affinity and G-protein coupling in obese rats. Kidney Int 2007; 71(5):397-406.

[35] Iannello S, Milazzo P, Belfiore F. Animal and human tissue Na,K-ATPase in normal and insulin-resistant states: regulation, behaviour and interpretative hypothesis on NEFA effects. Obes Rev 2007; 8(3):231-51.

[36] Gerstein HC, Mann JF, Yi Q, Zinman B, Dinneen SF, Hoogwerf B, et al. Albuminuria and risk of cardiovascular events, death and hearth failure in diabetic and nondiabetic individuals. JAMA 2001; 286:421-6.

[37] Glassock RJ. Is the Presence of Microalbuminuria a Relevant Marker of Kidney Disease? Curr Hypertens Rep 2010; 12(5):364-8.

[38] Zhai L, Gu J, Yang D, Hu W, Wang W, Ye S. Metformin ameliorates podocyte damage by restoring renal tissue nephrin expression in type 2 diabetic rats. J Diabetes 2016; doi: 10.1111/1753-0407.12437.

[39] Prince PD, Lanzi CR, Toblli JE, Elesgaray R, Oteiza PI, Fraga CG, Galleano M. Dietary (-)-epicatechin mitigates oxidative stress, NO metabolism alterations, and inflammation in renal cortex from fructose-fed rats. Free Radic Biol Med 2016; 90:35-46.

Figure Legends

Figure 1: Renal excretion of L-dopa and dopamine from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; *p<0.05; #p<0.01; &p<0.001; *versus* respective control**.**

Figure 2: Urinary L-dopa/dopamine ratio from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; &p<0.001 *versus* respective control

Figure 3: Correlation between SBP and urinary L-dopa/dopamine ratio from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; SBP: systolic blood pressure

Figure 4: Renal cortex protein expression of LAT-1 (a) and LAT-2 (b) from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; AU: arbitrary units; *p<0.05 *versus* respective control.

Figure 5: Renal cortex protein expression of OCT-2 (a), OCT-N1/2/3 (b), OCT-N1 (c) and D1-R (d) from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; AU: arbitrary units; *p<0.05; $^{\#}$p<0.01 *versus* respective control.

Figure 6: Values of specific activity of the Na^{+}, K^{+}-ATPase pump in renal cortex from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; S.A.: specific activity; *p<0.05; $^{\#}$p<0.01 *versus* respective control.

Figure 7: Immunofluorescence of Na^+, K^+-ATPase in renal cortex from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; a) C 4 weeks; b) FO 4 weeks; c) C 8 weeks; d) FO 8 weeks; e) C 12 weeks; f) FO 12 weeks.

Figure 8: Urinary albumin/creatinine ratio from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; Alb: albumin; Cr: creatinine; $^{\&}p<0.001$ *versus* respective control**.**

Figure 9: Nephrin expression in renal cortex from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

C: control group; FO: 10% fructose overload group; AU: arbitrary units; *p<0.05 *versus* respective control**.**

Figure 10: A proposed mechanism by which insulin resistance leads to hypertension and renal damage.

List of non-standard abbreviations

COMT: catechol-O-methyltransferase

D1R: dopamine receptor D1

FO: fructose-overloaded

LAT: L-aminoacid transporter

MAO: monoamine oxidase

NHE3: sodium–hydrogen exchanger 3

OCT/OCTN: organic cation transporter

RDS: renal dopaminergic system

SBP: systolic blood pressure

w/v: weight/volume

Table 1: Physiological and metabolic parameters from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

	4 weeks		8 weeks		12 weeks	
	C	**FO**	**C**	**FO**	**C**	**FO**
Liquid intake (mL/day)	43±3	94±9$^{\#}$	43±3	109±11$^{\#}$	41±3	95±12$^{\#}$
Food intake (g/day)	26±1	18±1$^{\#}$	24±2	15±1$^{\#}$	30±2	22±1$^{\#}$
Caloric intake (kCal/day)	85±5	96±7	80±6	92±7	99±6	110±9
Body Weight (g)	305±23	325±30	410±23	416±16	417±34	437±14
SBP (mmHg)	121±8	145±1*	130±4	161±10$^{\$}$	133±5	163±4$^{\$}$
Colesterol (mg/dL)	50±7	61±10	43±7	37±7	63±11	83±14
Triglycerides (mg/dL)	61±9	49±16	44±8	116±10$^{\#}$	68±30	153±12$^{\#}$
Glycemia (mg/dL)	127±6	145±7	128±4	150±14	122±4	147±19
Insulinaemia (ng/mL)	0.22±0.02	0.38±0.06*	0.35±0.04	0.58±0.08*	0.34±0.02	0.589±0.09*
HOMA Index	1.52±0.01	2.99±0.02$^{\#}$	2.41±0.01	4.69±0.06$^{\#}$	2.28±0.01	4.70±0.09$^{\#}$

C: control group; FO: 10% fructose overload group; *p<0.05; $^{\#}$p<0.01; $^{\$}$p<0.005 *versus* respective control.

Table 2: Renal functional parameters from control and fructose overloaded rats at weeks 4, 8 and 12 of treatment.

	4 weeks		8 weeks		12 weeks	
	C	**FO**	**C**	**FO**	**C**	**FO**
Diuresis (mL/day/kg)	38.69±4.66	75.45±6.30*	38.12±4.41	74.35±6.97*	35.20±3.84	70.10±6.11*
CrCl (mL/min/kg)	4.43±0.56	4.58±0.40	4.02±0.61	4.06±0.29	3.82±0.36	3.64±0.27
FENa (%)	0.41±0.03	0.28±0.03*	0.32±0.03	0.22±0.01*	0.35±0.04	0.2±0.02$^{\#}$
uNa.V (mEq/day/kg)	3.31±0.16	2.55±0.12*	2.49±0.20	1.73±0.12$^{\#}$	2.50±0.19	1.46±0.09$^{\#}$

C: control group; FO: 10% fructose overload group; ClCr: creatinine clearance; FENa: fractional excretion of sodium, uNa.V: urinary sodium excretion; *p<0.05; $^{\#}$p<0.01; *versus* respective control**.**

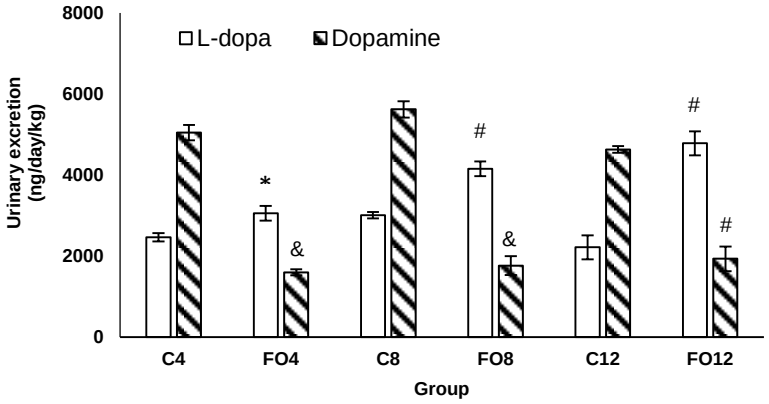
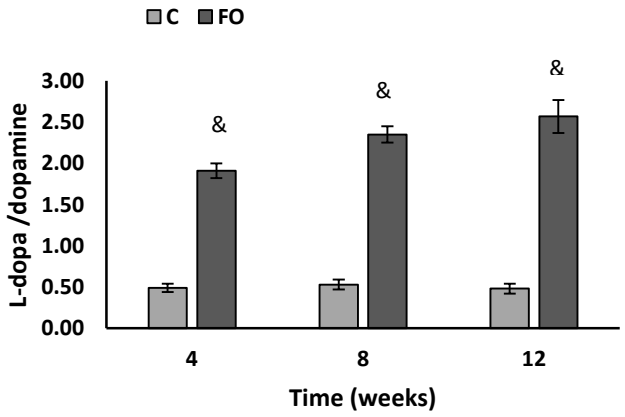


Figure 1



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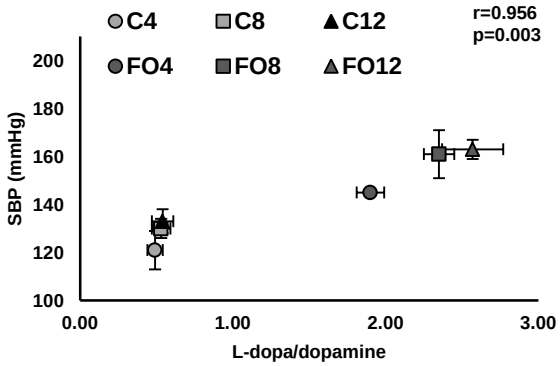

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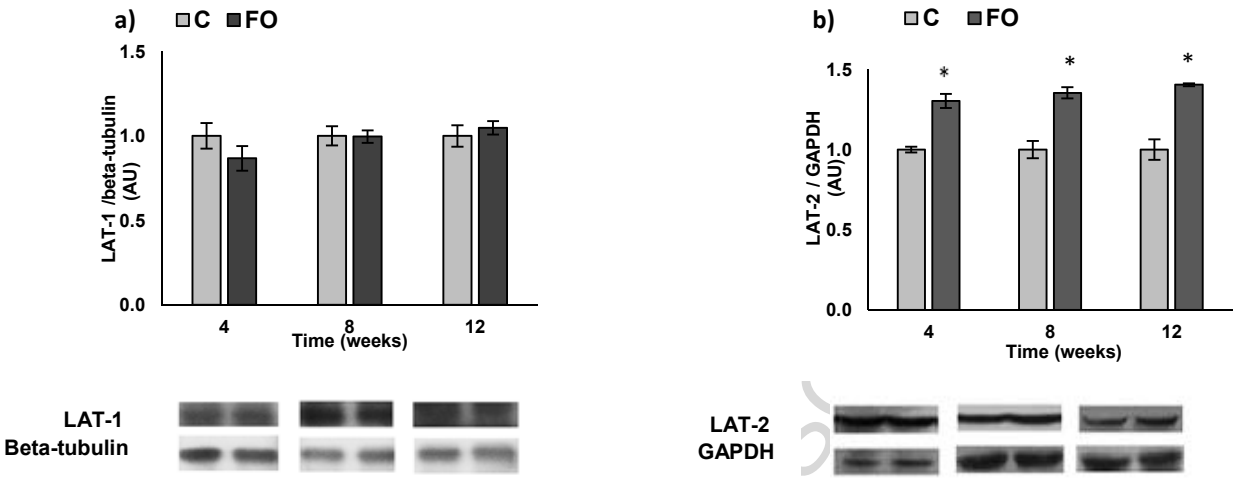

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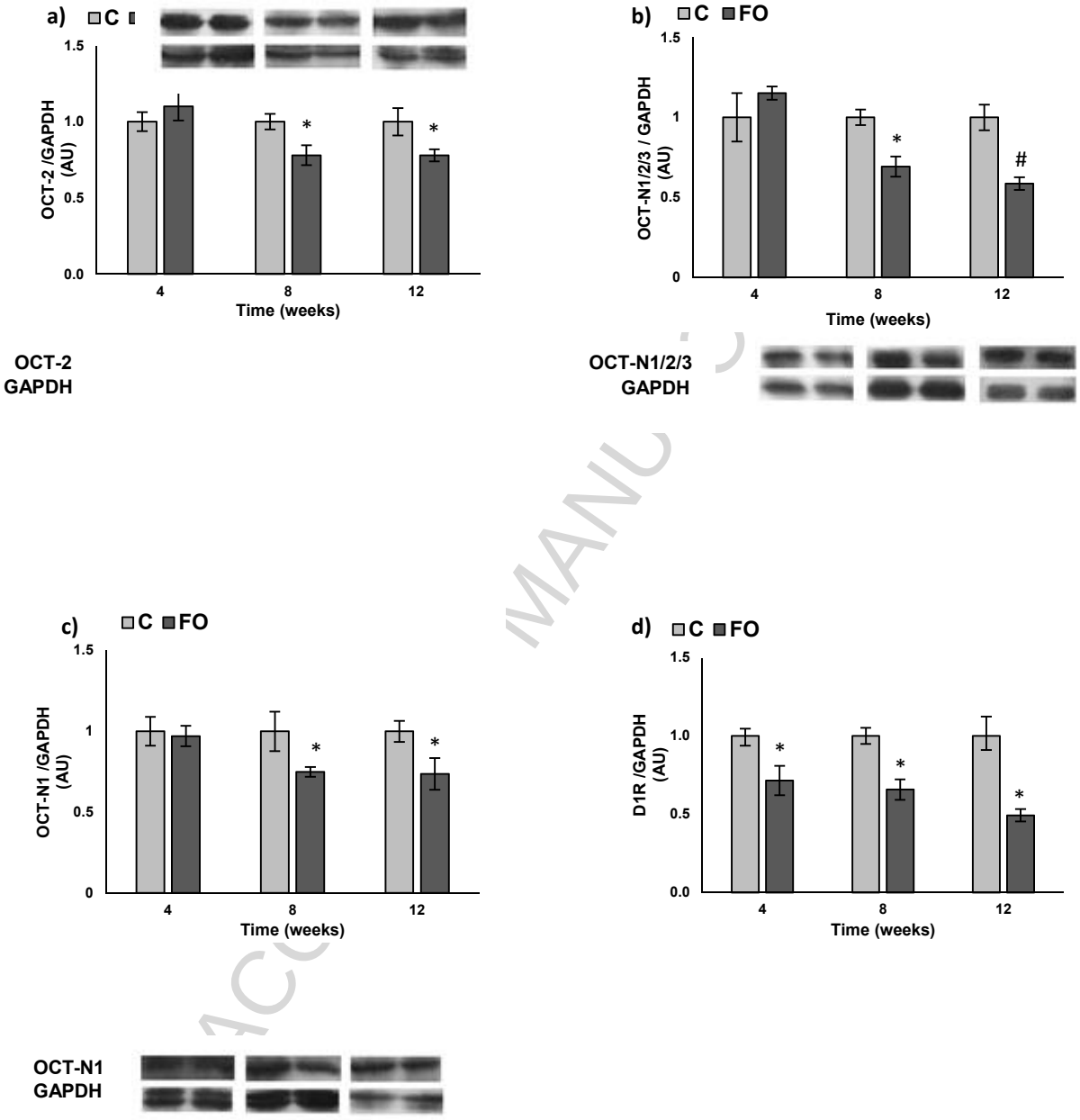
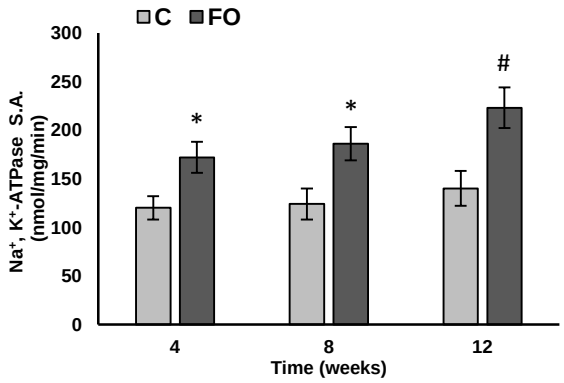


Figure 5



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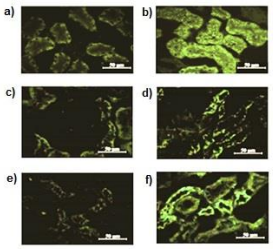

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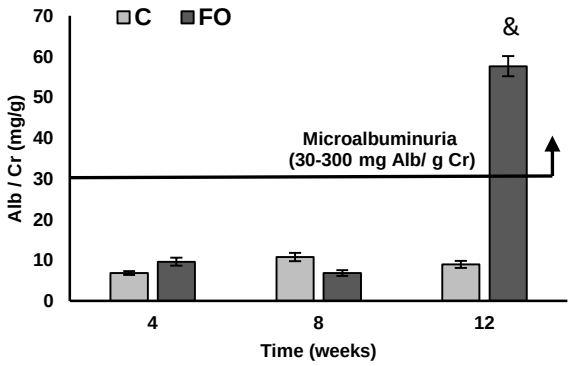

Figure 8

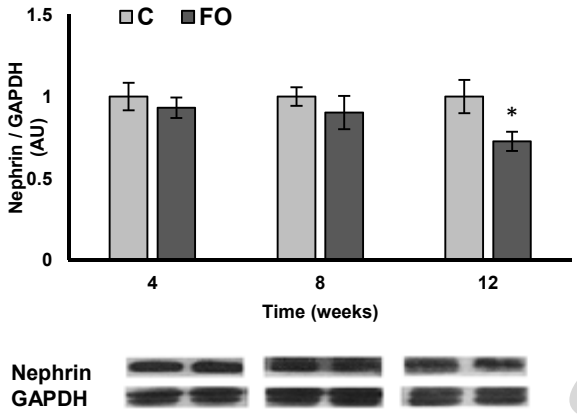

Figure **9**

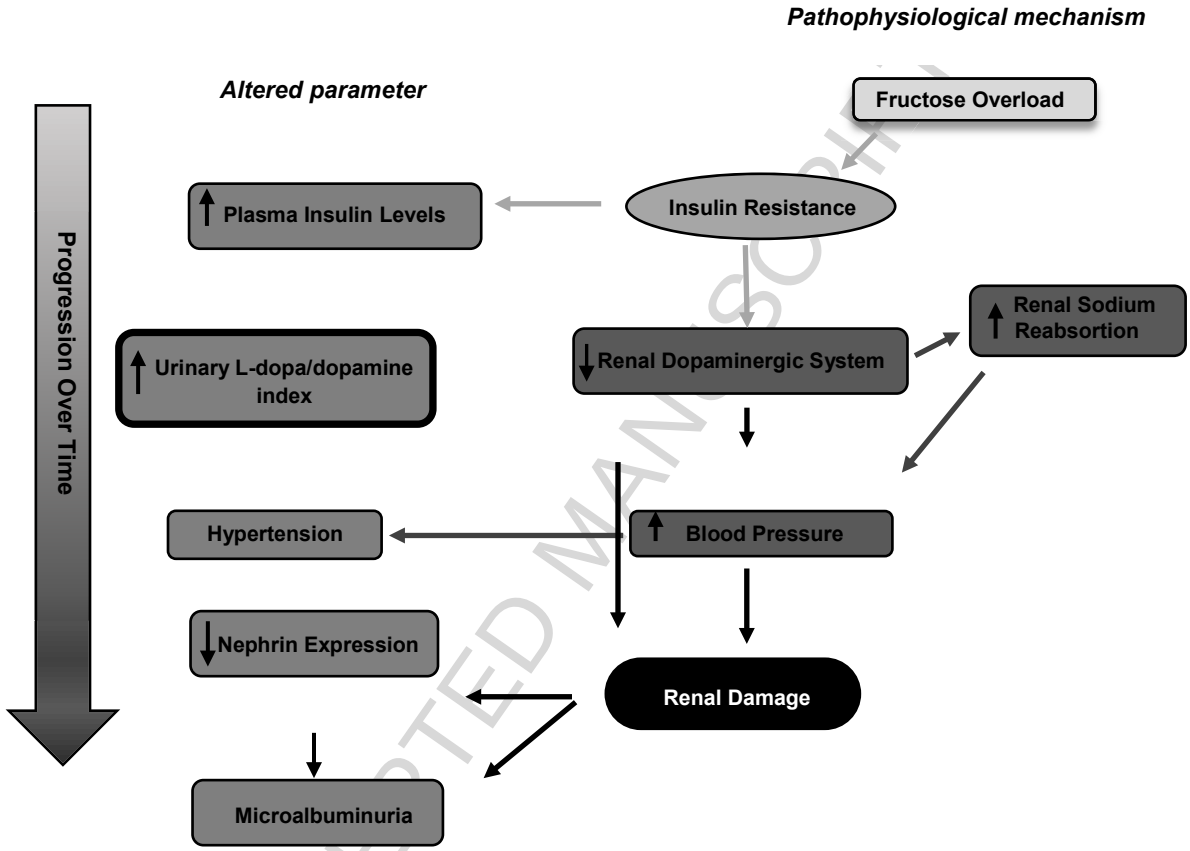

Figure 10