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Losartan prevents the imbalance between renal dopaminergic and renin angiotensin systems induced by fructose overload. L-Dopa/dopamine index as new potential biomarker of renal dysfunction

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ABSTRACT

Background: The renin angiotensin system (RAS) and the renal dopaminergic system (RDS) act as autocrine and paracrine systems to regulate renal sodium management and inflammation and their alterations have been associated to hypertension and renal damage. Nearly 30–50% of hypertensive patients have insulin resistance (IR), with a strong correlation between hyperinsulinemia and microalbuminuria.
Objective: The aim of this study was to demonstrate the existence of an imbalance between RAS and RDS associated to IR, hypertension and kidney damage induced by fructose overload (FO), as well as to establish their prevention, by pharmacological inhibition of RAS with losartan.
Materials/Methods: Ninety-six male Sprague-Dawley rats were randomly divided into four groups and studied at 4, 8 and 12 weeks: control group (C4, C8 and C12; tap water to drink); fructose-overloaded group (F4, F8 and F12; 10% *w/v* fructose solution to drink); losartan-treated control (L) group (L4, L8 and L12; losartan 30 mg/kg/day, in drinking water); and fructose-overloaded plus losartan group (F + L4, F + L8 and F + L12, in fructose solution).
Results: FO induced metabolic and hemodynamic alterations as well as an imbalance between RAS and RDS, characterized by increased renal angiotensin II levels and AT_1R overexpression, reduced urinary excretion of dopamine, increased excretion of L-dopa (increased L-dopa/dopamine index) and down-regulation of D_1R and tubular dopamine transporters OCT-2, OCT-N1 and total OCTNs. This imbalance was accompanied by an overexpression of renal tubular Na^+, K^+-ATPase, pro-inflammatory (NF-kB, TNF-α, IL-6) and pro-fibrotic (TGF-β1 and collagen) markers and by renal damage (microalbuminuria and reduced nephrin expression). Losartan prevented the metabolic and hemodynamic alterations induced by FO from week 4. Increased urinary L-dopa/dopamine index and decreased D_1R renal expression associated to FO were also prevented by losartan since week 4. The same pattern was observed for renal expression of OCTs/OCTNs, Na^+, K^+-ATPase, pro-inflammatory and pro-fibrotic markers from week 8. The appearance of microalbuminuria and reduced nephrin expression was prevented by losartan at week 12.
Conclusion: The results of this study provide new insight regarding the mechanisms by which a pro-hypertensive and pro-inflammatory system, such as RAS, downregulates another anti-hypertensive and anti-inflammatory system such as RDS. Additionally, we propose the use of L-dopa/dopamine index as a biochemical marker of renal dysfunction in conditions characterized by sodium retention, IR and/or hypertension, and as a predictor of response to treatment and follow-up of these processes.

Abbreviations: Ang II, angiotensin II; AT_1R, angiotensin II type 1 receptor; CrCl, creatinine clearance; D_1R, dopamine sub-type 1 receptor; DA, dopamine; FENa, fractional sodium excretion; FO, fructose overload; GFR, glomerular filtration rate; IR, Insulin resistance; NF-kB, nuclear transcription factor kappa B; OCT/OCT-N, organic cation transporter; RAS, renin angiotensin system; RDS, renal dopaminergic system; SBP, systolic blood pressure; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α; UNa.UV, urinary sodium excretion.

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1. Introduction

Essential hypertension is the chronic pathology with the highest prevalence and incidence worldwide, and constitutes one of the main causes of cardiovascular and kidney disease [1]. The renal management of sodium is a determining factor in the regulation of blood pressure levels and is controlled by various endocrine, autocrine and neurogenic factors, which can be divided into two groups: one group causes sodium retention and vasoconstriction while the other causes natriuresis and vasodilation [2,3]. For many years, the existence of an over-activation of the intrarenal renin angiotensin system (RAS) as the primary mechanism of renal vasoconstriction, sodium reabsorption and development of hypertension has been established [4]. However, many studies have highlighted the participation of another local factor, renal dopamine (DA), formed mainly in proximal tubular cells from filtered L-dopa, as a counter-regulator of the hypertensive effects of angiotensin II (Ang II), arguing that a normal functioning of the renal dopaminergic system (RDS) is essential for the maintenance of normal blood pressure [3]. Supporting this theory, a complex interaction between RAS and RDS has been proven, with opposing and counterregulatory effects that operate at different levels [3,5]. One of the main targets of both systems is renal pump Na^+, K^+-ATPase, an enzyme whose alteration has been associated to sodium retention and hypertension [6].

Many factors that regulate renal sodium transportation are also able to mediate the immune response, participating in processes of inflammation, fibrosis and kidney damage [7]. In this context, renal Ang II and DA can modulate inflammation, controlling functions whose alterations lead to hypertension and renal damage [8–10].

On the other hand, about 30 to 50% of patients with essential hypertension have insulin resistance (IR), with a strong correlation between hyperinsulinemia and microalbuminuria [11,12]. It has been described that IR is present in several models of hypertensive animals, including spontaneously hypertensive rats and those fed with high fructose [13]. In this latter model, IR leads to an acquired form of hypertension in which the increase in blood pressure is not determined genetically but is induced by the diet [13]. Although alterations of RAS and RDS have been described in rats with fructose overload (FO), the existence of an imbalance between these two systems in this model has not been studied, as well as its association to hypertension, inflammation and kidney damage [14,15].

The aim of the study was to demonstrate the existence of an imbalance between RAS and RDS associated to the development of IR, hypertension and inflammatory kidney damage induced by FO, as well as to establish the prevention of these harmful processes, by pharmacological inhibition of RAS using losartan. Finally, we studied the behavior of the urinary L-dopa/DA index with the purpose of postulating it as a potential biochemical marker of renal dysfunction in conditions characterized by sodium retention, IR and/or hypertension.

2. Methods

2.1. Animal Protocol and Diet

Male Sprague-Dawley rats weighing 150 to 180 g at the beginning of the study were used. All animal experiments were performed in accordance with the "International Ethical Guiding Principles for Biomedical Research on Animals" established by the Council for International Organizations of Medical Sciences and were approved in advance by the local ethics committee on animal research (protocol #2100-15; 0035638/15). Animals were housed in cages with a 12-h light/dark cycle under conditions of controlled temperature (22 °C ± 2 °C) 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). Ninety-six rats were acclimated to the environment for 5 days and then they were randomly divided into four groups and studied at three experimental periods: 4, 8 and 12 weeks. Rats were randomly assigned to three control (C) groups (C4, C8 and C12), which received tap water to drink; three experimental fructose-overloaded (F) groups, (F4, F8 and F12), which received a 10% *w*/*v* fructose solution to drink (Parafarm, Buenos Aires, Argentina); three losartan-treated control (L) groups, which received losartan (30 mg/kg/day) in the drinking water; and three fructose-overloaded treated with losartan (F + L) groups, which received losartan (30 mg/kg/day) in the 10% *w*/*v* fructose solution ($n = 8$ for each group).

2.2. Control of Blood Pressure

The animals were trained to the procedure of blood pressure measurement at 10:00 a.m., twice a week, for 2 weeks, after randomization 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, NJ, USA) in series with an oscilloscope (type 532, Tektronic Inc., OR, USA). The value of SBP was calculated as the average of 5 determinations per rat.

2.3. Collection and Processing of Urine Samples

At the end of each experimental period, 24-hour urine samples were collected using metabolic cages. The urinary volume was determined by gravimetry. The urine samples obtained were used to determine the 24-hour diuresis as well as urinary concentrations of sodium, creatinine, albumin, L-dopa and DA. Urinary sodium, creatinine and albumin were measured by spectrophotometric method using an autoanalyzer (analyzer Automated Spectrum CCX, Abbott Diagnostics, IL, USA). The presence of microalbuminuria was defined as a urinary albumin/creatinine ratio between 30 and 300 mg/g [16]. To determine L-dopa and DA concentrations by high-performance liquid chromatography (HPLC), a urine fraction was collected in tubes of polyethylene containing 100 μl of 6 N HCl.

2.4. Collection and Processing of Blood Samples

At the end of each experimental period, all groups of animals were fasted for 5 h. Under anesthesia with ketamine (80 mg/kg) and xylazine (12 mg/kg), blood samples were collected to measure plasma sodium, creatinine, triglycerides, cholesterol, glucose and insulin. Plasma sodium and creatinine were measured by spectrophotometric method using an autoanalyzer (analyzer Automated Spectrum CCX, Abbott Diagnostics, IL, USA). Plasma triglyceride and cholesterol levels were measured by means of commercial kits (Colestat Wiener Labs, Santa Fé, Argentina) using a spectrophotometric method; plasma glucose was determined by a blood glucose meter (Accu-Chek, Roche Diagnostics, Mannheim, Germany) and insulin by an enzyme-linked immunosorbent assay (Millipore Corporation, MA, USA). Homeostasis model of assessment-insulin resistance index or HOMA-IR was calculated by using the following equation: HOMA = fasting glucose (mmol/L) × fasting insulin (μIU/ml)/22.5, with the cutoff point to define IR being 2.5 points or higher [17]. The laboratory where the analytical determinations in plasma and urine were performed is accredited under the IRAM (Argentine Standardization and Certification Institute)/ISO (Organization for Standardization) rule number 15189:2014.

2.5. Renal Function Parameters Calculation

To evaluate renal functionality, we determined glomerular filtration rate estimated by creatinine clearance (CrCl), fractional sodium excretion (FENa), urinary sodium excretion ($UNa \cdot UV$), and daily diuresis, which 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.

2.6. Kidney Dissection and Processing

After urine and blood sample collection, the animals were sacrificed by decapitation, and both kidneys were dissected and then processed to perform biochemical and molecular analyses. Protein immunoexpression was analyzed by western blot, immunohistochemistry and immunofluorescence techniques. The presence of collagen was determined by staining with Sirius red.

2.7. Total Activity of Na^+, K^+-ATPase

Sample tissues from renal cortex weighing 50 mg were homogenized (1:10 weight/volume) in 25 mM imidazole/1 mM EDTA/0.25 M sucrose solution and centrifuged at 4700*g* at 4 °C for 15 min. Na^+, K^+-ATPase activity was assayed in the supernatant using Fiske–Subbarow method [18]. ATPase activity was measured by colorimetric determination of released orthophosphate, and ouabain was used to inhibit specifically Na^+, K^+-ATPase activity [19]. Proteins were determined by the method of Lowry et al. [20]. Results are expressed as percentage of Na^+, K^+-ATPase activity, considering control values as 100%.

2.8. Statistical Analysis

All results are expressed as means ± S.E.M. Data were processed using Graph Pad InStat Software (CA, USA). The Gaussian distribution was evaluated by the Kolmogorov–Smirnov method. Data with normal distribution (dosages in blood and urine samples, Na^+, K^+-ATPase activity, protein expression by Western blot) were analyzed by means of two-way analysis of variance (ANOVA) followed by Tukey, and data with Non-Gaussian distribution (protein expression by immunohistochemistry and immunofluorescence) were analyzed by means of Kruskal–Wallis test (nonparametric ANOVA) and multiple comparison test of Dunn. $p < 0.05$ was considered statistically significant.

Antibodies, reagents and all other methodology are described in the Supplementary Experimental Procedures (Appendix A).

3. Results

3.1. Nutritional and Metabolic Parameters

Nutritional and metabolic parameters are shown in Table 1. FO was associated to an increase of drink intake as well as a reduction of food intake in F rats with respect to C, since week 4. Caloric intake was not different between F and C rats in any experimental period. Losartan did not alter drink, food or total calories intake in L and F + L rats compared to C and F, respectively, in any experimental period. Body weight was not modified by FO or losartan in any experimental period. FO significantly increased plasma insulin levels in F rats with respect to C from week 4, while blood glucose values showed a non-significant trend to increase. FO was associated to the development of IR from the same week, since F rats showed increased HOMA-IR levels, greater than the cut-off point to define IR. Losartan treatment did not alter blood glucose values in any group or experimental period. Treatment with losartan prevented the increase in insulinemia and development of IR induced by FO from week 4, since HOMA-IR levels of F + L rats were below the cut-off point to define IR. Total cholesterol levels were not modified by FO or losartan, in any experimental period. At week 8 and 12, plasma triglycerides were significantly increased in F rats compared to C, and losartan only prevented this increase in F + L rats at week 12. Losartan showed no effect on nutritional and metabolic parameters in L rats compared to C in any experimental period. Time-course analysis showed that HOMA-IR and triglyceride levels increased at weeks 8 and 12 of FO, with respect to week 4.

Table 1
Nutritional and metabolic parameters at 4, 8 and 12 weeks of fructose and losartan treatment in the different experimental groups.

	Week 4				Week 8				Week 12			
	C	L	F	F + L	C	L	F	F + L	C	L	F	F + L
Fluid intake (ml/day)	45 ± 4	42 ± 6	93 ± 8$^{\#}$	82 ± 3$^{\#}$	44 ± 2	37 ± 4	102 ± 9$^{\#}$	89 ± 6$^{\#}$	42 ± 3	40 ± 1	99 ± 9$^{\#}$	87 ± 4$^{\#}$
Food intake (g/day)	29 ± 3	26 ± 1	17 ± 2$^{\#}$	19 ± 2$^{\#}$	27 ± 2	28 ± 4	16 ± 1$^{\#}$	18 ± 3$^{\#}$	32 ± 3	30 ± 1	21 ± 1$^{\#}$	20 ± 2$^{\#}$
Caloric intake (kcal/day)	96 ± 4	86 ± 7	93 ± 6	96 ± 5	89 ± 5	92 ± 5	94 ± 7	95 ± 6	106 ± 5	99 ± 3	109 ± 9	101 ± 7
Body weight (g)	317 ± 19	325 ± 13	327 ± 22	313 ± 25	413 ± 23	404 ± 5	415 ± 16	407 ± 24	436 ± 17	447 ± 14	456 ± 22	461 ± 26
Glycemia (mmol/L)	7.09 ± 0.33	7.00 ± 0.67	8.02 ± 0.39	7.67 ± 0.78	7.15 ± 0.22	6.94 ± 0.28	8.63 ± 0.78	7.89 ± 0.47	6.88 ± 0.22	6.89 ± 0.28	8.17 ± 1.05	7.56 ± 1.22
Insulinemia (uUI/ml)	4.88 ± 0.44	4.18 ± 0.66	8.65 ± 1.24*	4.84 ± 1.54$^{\$}$	7.65 ± 0.87	7.67 ± 1.32	12.58 ± 1.56*	7.04 ± 1.64$^{\$}$	7.58 ± 0.49	6.50 ± 0.88	12.63 ± 2.08*	6.97 ± 0.88$^{\$}$
HOMA-IR	1.54 ± 0.06	1.67 ± 0.08	3.08 ± 0.02*	1.65 ± 0.05$^{\$}$	2.43 ± 0.04	2.36 ± 0.09	4.83 ± 0.03$^{\$/\epsilon}$	2.47 ± 0.02$^{\$}$	2.32 ± 0.05	2.00 ± 0.09	4.59 ± 0.07$^{\$/\epsilon}$	2.35 ± 0.05$^{\$}$
Cholesterol (mg/dL)	58 ± 7	55 ± 5	64 ± 6	56 ± 3	49 ± 6	54 ± 5	61 ± 6	57 ± 3	65 ± 10	60 ± 4	84 ± 12	58 ± 3
Triglycerides (mg/dL)	65 ± 8	63 ± 8	54 ± 12	72 ± 16	49 ± 7	50 ± 7	112 ± 17$^{*/\epsilon}$	105 ± 14*	69 ± 12	78 ± 14	157 ± 11$^{*/\epsilon}$	72 ± 10$^{\$}$

Fructose was administered in the drinking water ad libitum (10% w/v). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM (n = 8 animals per group). C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan.
$^{\#}$ $p < 0.01$ vs C and L.
* $p < 0.05$ vs C and L.
$^{\$}$ $p < 0.05$ vs F.
$^{\epsilon}$ $p < 0.05$ vs F4.

3.2. Hemodynamic and Renal Function Parameters

As it is shown in Fig. 1, SBP levels increased in F rats with respect to C from week 4, reaching the highest values at weeks 8 and 12. Losartan treatment prevented the increase in SBP in F + L rats compared to F, from week 4. CrCl was not altered by FO or losartan in any group or experimental period. Diuresis significantly increased in F rats compared to C, from week 4. Losartan treatment did not modify diuresis in any experimental period. UNa·UV and FENa were significantly reduced in F rats with respect to C, in all experimental periods. Losartan treatment prevented this reduction in F + L rats with respect to F, from week 4. Treatment with losartan alone showed no effect on hemodynamic and renal function parameters compared to C in any experimental period. Temporal analysis showed that SBP levels increased at weeks 8 and 12 of FO, with respect to week 4.

3.3. Renal Renin Angiotensin System

Ang II immunostaining significantly increased in renal cortex in F rats with respect to C, at 8 and 12 weeks. Losartan treatment (F + L

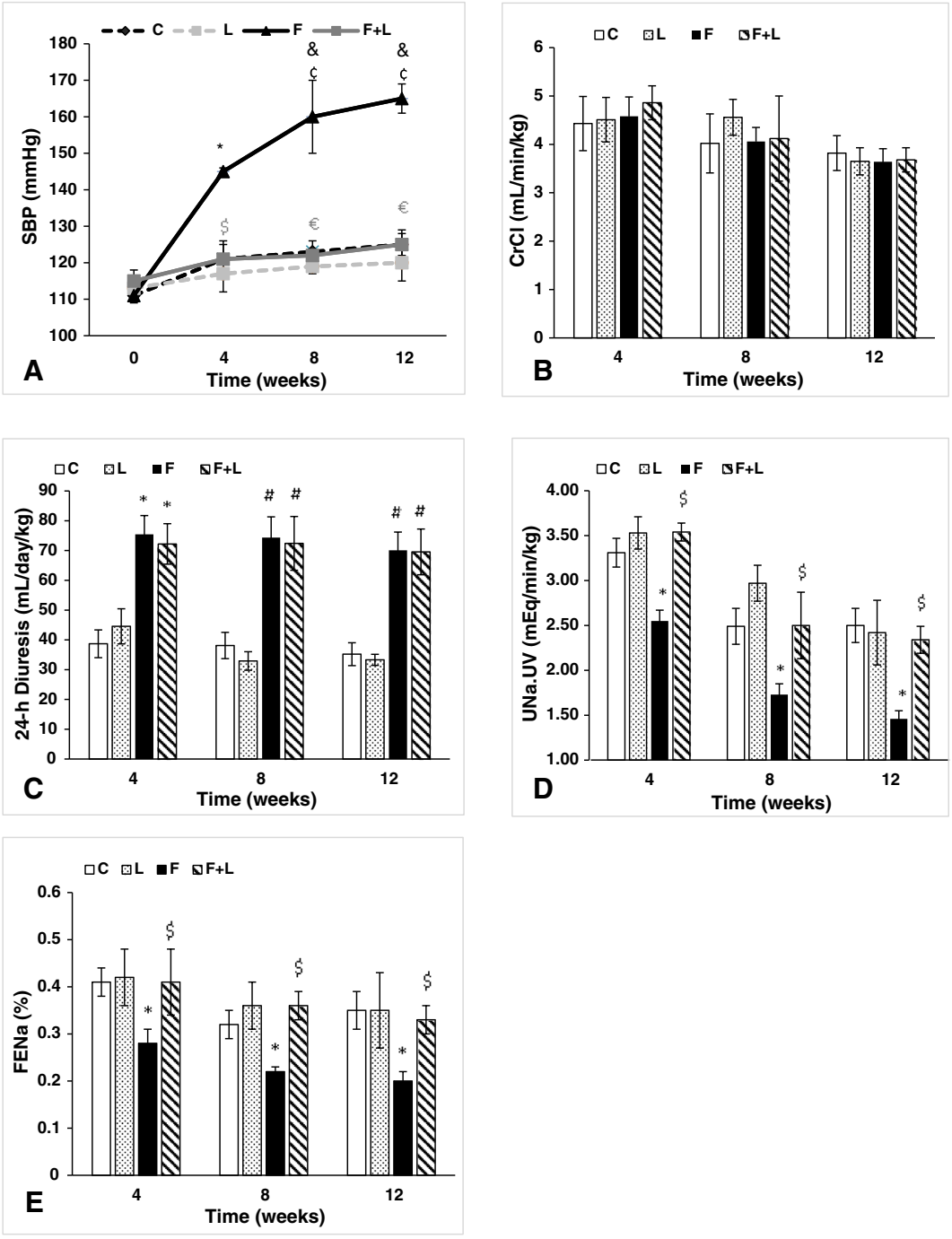

Fig. 1. Hemodynamic and renal function parameters at 4, 8 and 12 weeks of fructose and losartan treatment in the different experimental groups. A - Systolic Blood Pressure; B - Creatinine Clearance; C - 24-h Diuresis; D - Urinary Sodium Excretion; E- Fractional Sodium Excretion. Fructose was administered in the drinking water ad libitum (10% *w*/*v*). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM ($n = 8$ animals per group). C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; CrCl: Clearance of Creatinine; FENa: Fractional sodium excretion; UNa.UV: Urinary sodium excretion; $^{\#}p < 0.01$ vs C and L; $^{\&}p < 0.005$ vs C and L; $^{*}p < 0.05$ vs C and L; $^{€}p < 0{,}005$ vs F; $^{\$}p < 0.05$ vs F; $^{¢}p < 0.05$ vs F4.

group) lacked of effects in the prevention of this increase. Ang II type 1 receptor (AT_1R) expression was significantly increased in renal cortex of F rats compared to C, from week 4, effect prevented by losartan (F + L group). Losartan showed no effects on renal Ang II and AT_1R immunostaining in L rats compared to C in any experimental period (Fig. 2).

3.4. Renal Dopaminergic System

L-Dopa urinary excretion increased while DA urinary excretion decreased in F rats, compared to C, in all the experimental periods. Consequently, the urinary L-dopa/DA ratio significantly increased in F

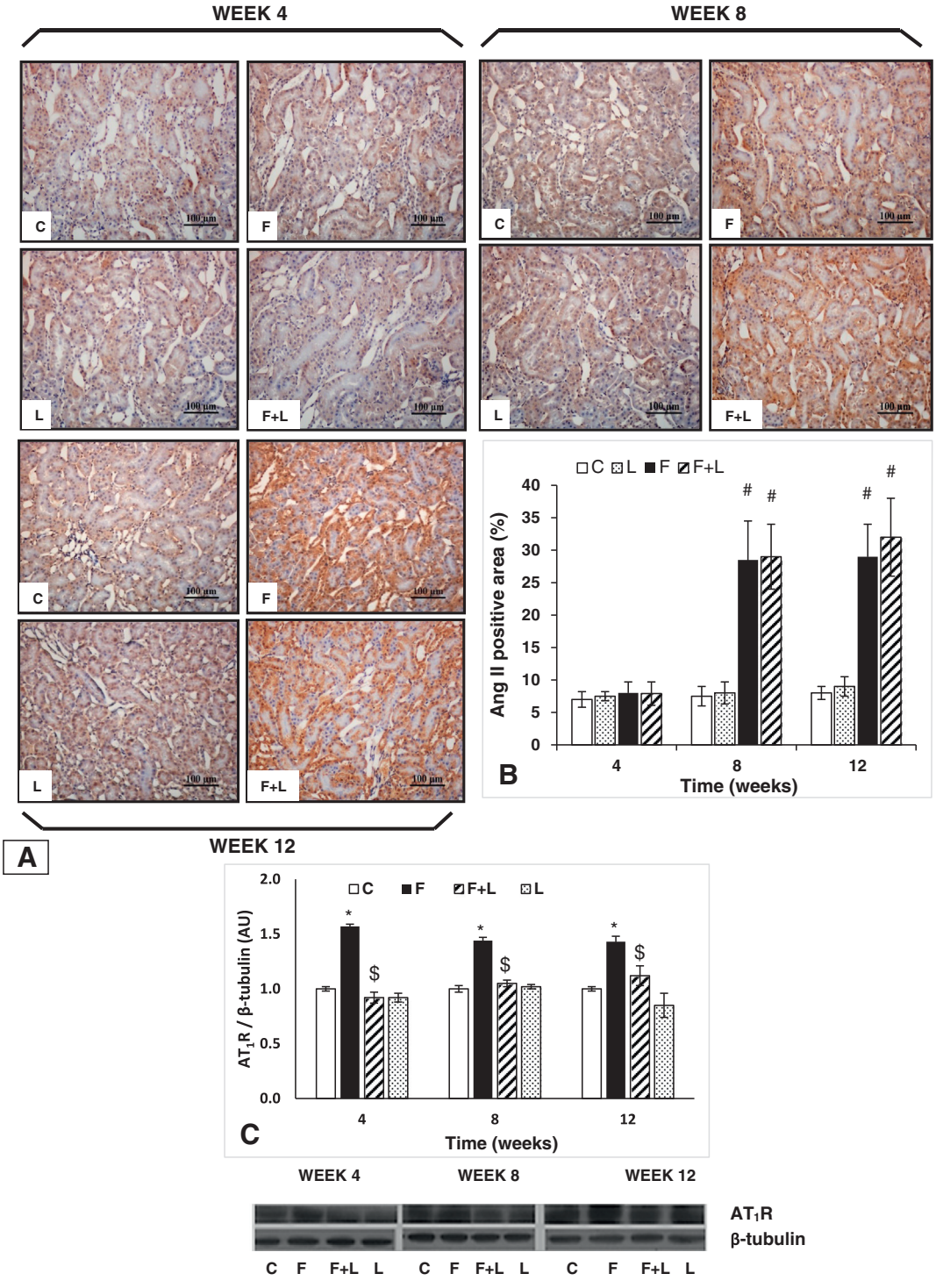

Fig. 2. Renal Renin Angiotensin System analysis at 4, 8 and 12 weeks of treatment with fructose and losartan in the different experimental groups. A - Representative microphotographs of Ang II immunostaining in renal cortex (200× magnification); B - Quantification of Ang II immunostaining in renal cortex; C - Protein expression values of AT_1 receptor in renal cortex. Fructose was administered in the drinking water ad libitum (10% *w/v*). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM ($n = 8$ animals per group). C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; Ang II: Angiotensin II; AT_1R: Angiotensin II Type 1 Receptor; $^{\#}p < 0.01$ vs C and L; $^{*}p < 0.05$ vs C and L; $^{\$}p < 0.05$ vs F.

rats compared to C, from week 4. Losartan treatment prevented the alteration of the urinary excretion of L-dopa and DA in F + L rats with respect to F, avoiding, in this way, the increase of the urinary L-dopa/DA ratio (Fig. 3A-C). The prevention of RDS alterations associated to losartan treatment was total at week 4, while it was partial at weeks 8 and 12. Time-course analysis showed that urinary L-dopa excretion increased at weeks 8 and 12 in F rats and also in F + L rats, with respect to week 4. Urinary DA levels were lower at weeks 8 and 12 in F + L rats with respect

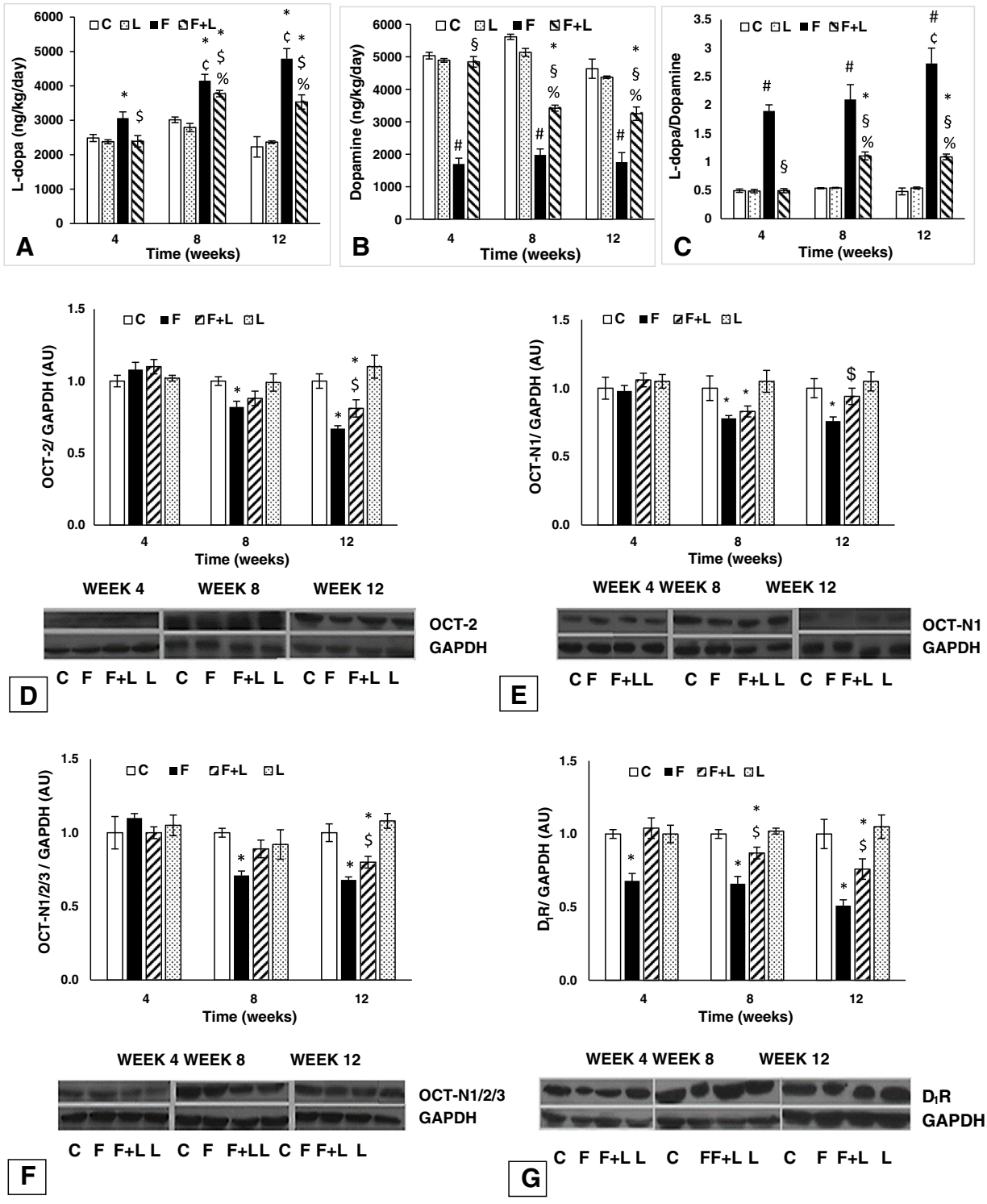

Fig. 3. Renal Dopaminergic System analysis at 4, 8 and 12 weeks of treatment with fructose and losartan in the different experimental groups. A - L-Dopa urinary excretion; B - DA urinary excretion; C - Urinary L-dopa/DA ratio; D - Protein expression values of OCT-2 in renal cortex; E - Protein expression values of OCT-N1 in renal cortex; F - Protein expression values of OCT-N1/2/3 in renal cortex; G - Protein expression values of D_1 receptor in renal cortex. Fructose was administered in the drinking water ad libitum (10% *w/v*). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM ($n = 8$ animals per group). C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; DA: Dopamine; D_1R: Dopamine Sub-type 1 Receptor; OCTs: Organic cation transporters; $^{\#}p < 0.01$ vs C and L; $^{*}p < 0.05$ vs C and L; $^{\S}p < 0.01$ vs F; $^{\$}p < 0.05$ vs F; $^{¢}p < 0.05$ vs F4; $^{\%}p < 0.05$ vs F + L4.

to week 4. Urinary L-dopa/DA ratio was higher at week 12 of FO with respect to week 4, while this ratio was higher at weeks 8 and 12 compared to week 4 in F + L rats.

Renal cortex expression of organic cation transporters (OCTs) OCT-2, OCT-N1 and total OCT-Ns (OCTN 1/2/3), which mediate tubular DA transportation, was significantly reduced from week 8 in F rats compared to C (Fig. 3D-F). Losartan showed a tendency to prevent the reduction of OCT-2 and OCTN 1/2/3 expression in F + L rats with respect to F at week 8, while at week 12, the prevention was partial but significant. Losartan treatment prevented the reduction of OCT-N1 expression in F + L rats compared to Fat week 12. DA receptor D_1 (D_1R) expression in renal cortex decreased in F rats compared to C from week 4, effect prevented by losartan. This prevention was total at week 4, while it was partial at weeks 8 and 12 (Fig. 3G). In any case, losartan showed no effects on RDS in L rats with respect to C.

3.5. Na^+, K^+-ATPase Expression and Total Activity

Renal cortex Na^+, K^+-ATPase total activity increased in F rats with respect to C from week 4. Losartan treatment prevented this increase in F + L rats compared to F, from the same experimental period (Fig. 4C). Na^+, K^+-ATPase expression in renal cortex was also increased in F rats with respect to C, at 8 and 12 weeks, and losartan prevented this

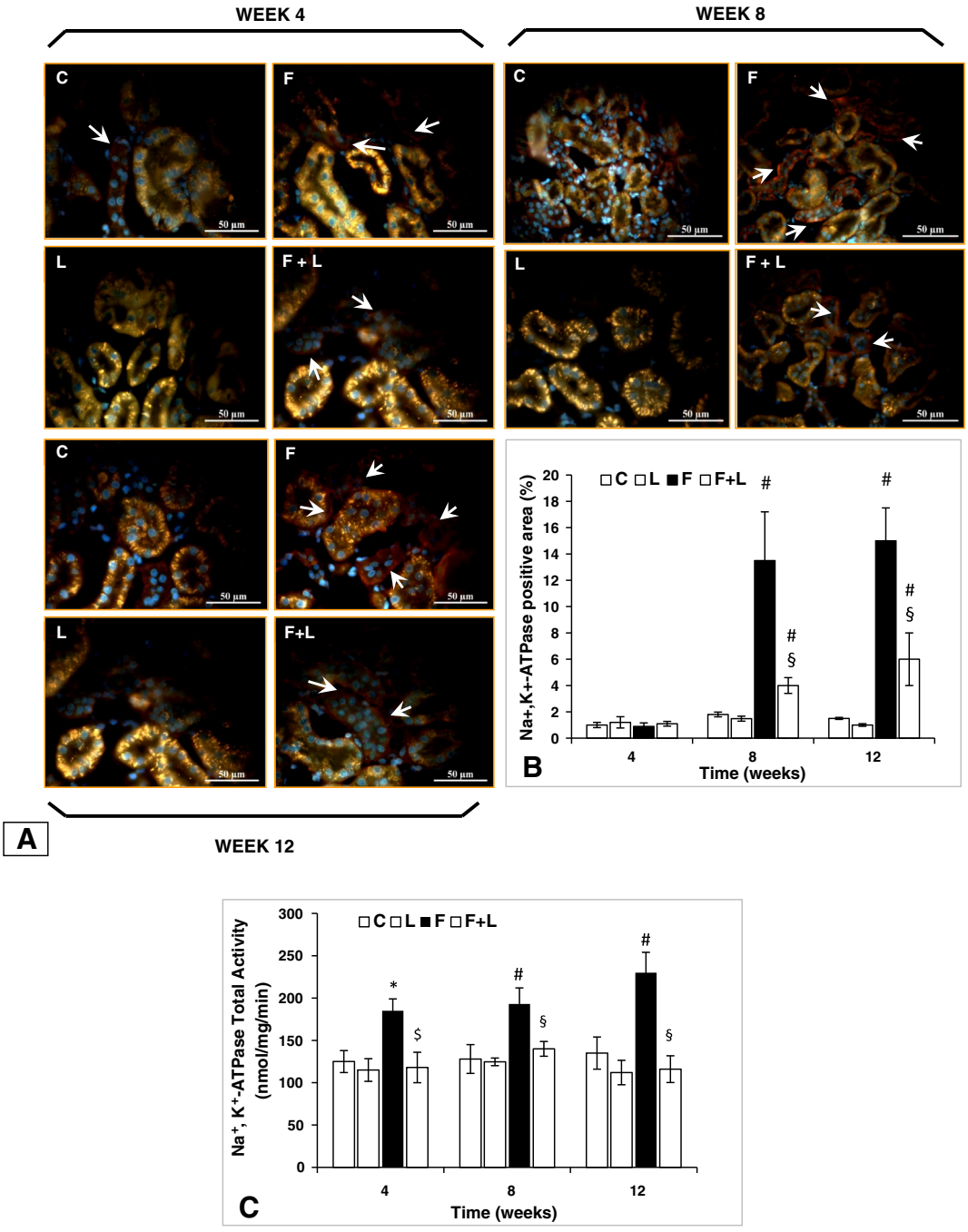

Fig. 4. Renal Na^+, K^+-ATPase analysis at 4, 8 and 12 weeks of treatment with fructose and losartan in the different experimental groups. A - Representative micrographs of the immunofluorescence of Na^+, K^+-ATPase in renal cortex (400× magnification); B - Quantification of the immunoexpression of the enzyme Na^+, K^+-ATPase in renal cortex; C - Total activity of Na^+, K^+-ATPase in renal cortex. Fructose was administered in the drinking water ad libitum (10% *w/v*). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM ($n = 8$ animals per group). The arrows indicate the areas of positive staining with rhodamine. C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; $^{\#}p < 0.01$ vs C and L; $^{*}p < 0.05$ vs C and L; $^{\S}p < 0,01$ vs F; $^{\$}p < 0.05$ vs F.

increase in F + L rats compared to F (Fig. 4A and B). Losartan treatment had no effect on Na^+, K^+-ATPase activity and expression in L rats with respect to C in any experimental period.

3.6. Biomarkers of Renal Damage

Renal cortex expression of the nuclear transcription factor kappa B (NF-kB) increased in F rats with respect to C, from week 4. Losartan partially prevented this increase in F + L rats with respect to F, in all experimental periods (Fig. 5A). Renal cortex expression of the cytokines tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), as well as fibrosis marker transforming growth factor β1 (TGF-β1) increased in F rats compared to C, at weeks 8 and 12, an effect that was partially prevented by losartan (F + L group) (Figs. 5B and C, 6 and 7). The positive staining area for Sirius Red increased in renal cortex of F rats with respect to C, at weeks 8 and 12, indicating the increase of interstitial

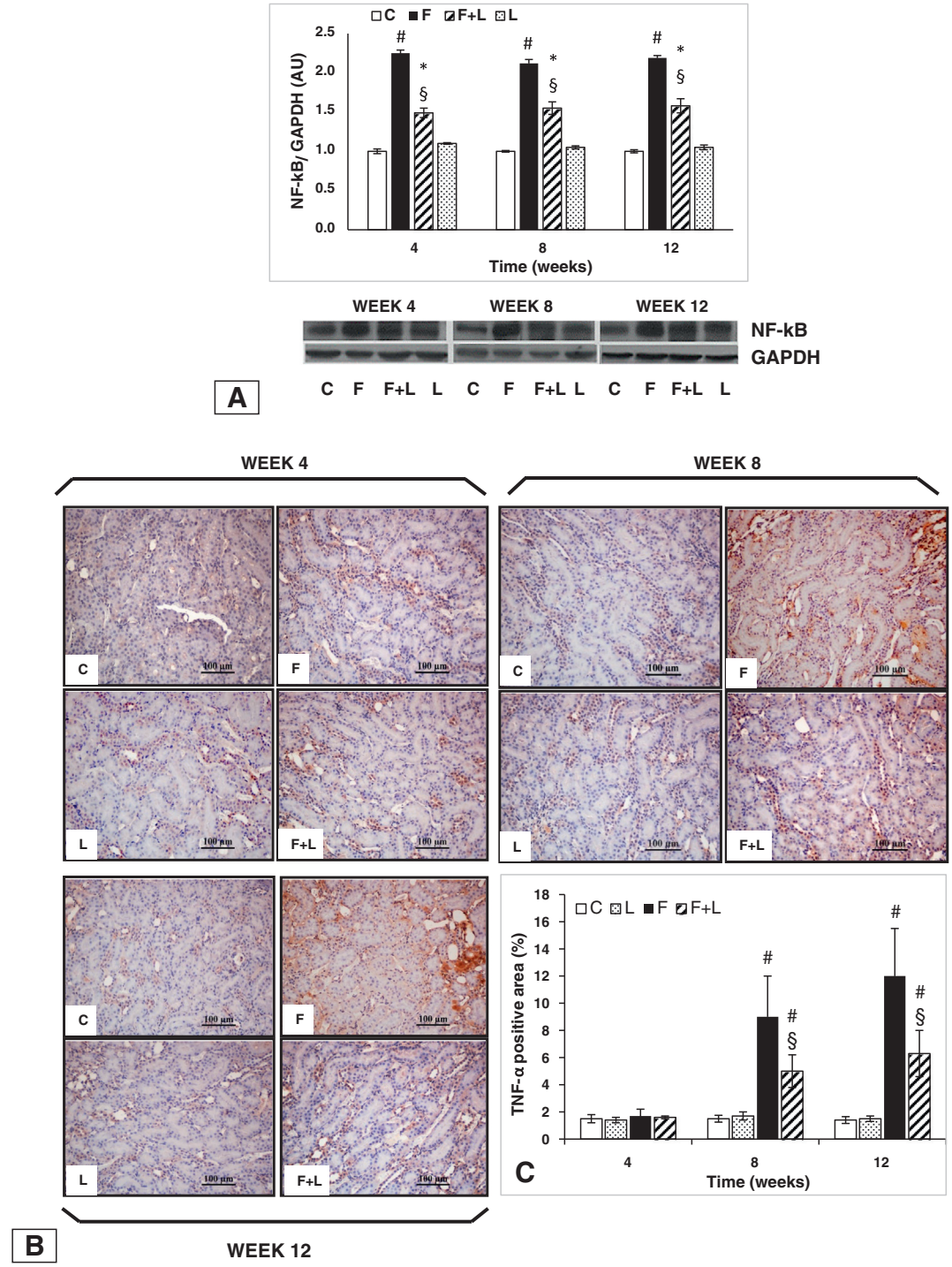

Fig. 5. Renal expression of proinflammatory markers: NF-kB and TNF-α at 4, 8 and 12 weeks of treatment with fructose and losartan in the different experimental groups. A - Protein expression values of NF-kB in renal cortex; B - Representative microphotographs of the immunoexpression of TNF-α in renal cortex (200× magnification); C - Quantification of the immunoexpression of TNF-α in renal cortex. Fructose was administered in the drinking water ad libitum (10% w/v). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM ($n = 8$ animals per group). C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; NF-kB: Nuclear transcription factor kappa B; TNF-α: Tumor necrosis factor α; $^{\#}p < 0.01$ vs C and L; $^{*}p < 0.05$ vs C and L; $^{\S}p < 0.01$ vs F.

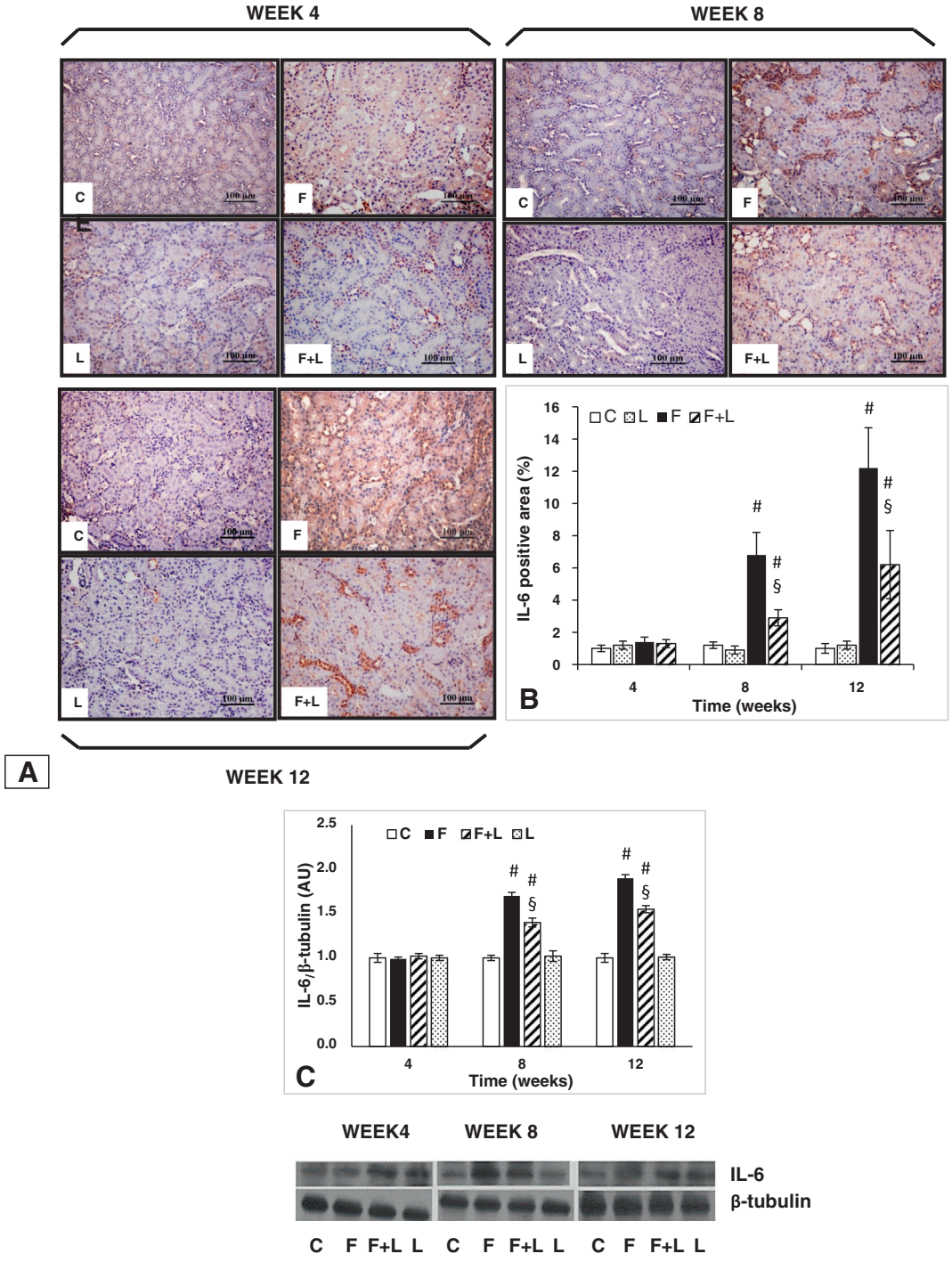

Fig. 6. Renal expression of proinflammatory marker IL-6 at 4, 8 and 12 weeks of treatment with fructose and losartan in the different experimental groups. A - Representative microphotographs of the immunoexpression of IL-6 in renal cortex (200× magnification); B - Quantification of the immunoexpression of IL-6 in renal cortex; C - Protein expression values of IL-6 in renal cortex. Fructose was administered in the drinking water ad libitum (10% *w/v*). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM ($n = 8$ animals per group). C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; IL-6: Interleukin 6; $^{\#}p < 0.01$ vs C and L; $^{\S}p < 0.01$ vs F.

and perivascular collagen content. Losartan partially prevented this increase in F + L rats compared to F in the same experimental periods (Fig. 8A and B). The presence of microalbuminuria was observed only in F rats at week 12. Losartan prevented the appearance of microalbuminuria in F + L rats in this experimental period (Fig. 8C). At cortical level, the protein expression of the glomerular structural damage marker, nephrin, was significantly reduced in F rats compared to C, at week 12. Losartan treatment prevented this reduction in F + L rats at week 12 (Fig. 8D). Losartan had no effect on renal damage markers expression in L rats with respect to C in any experimental period.

4. Discussion

The results of the study show the existence of an imbalance between renal RAS and RDS, in the context of IR and hypertension by FO. The existence of severe metabolic and hemodynamic alterations has been demonstrated in animal models treated with a high fructose diet, with the development of a metabolic profile similar to that observed in human metabolic syndrome [21]. To determine the beta-pancreatic cells functionality as well as the IR status, the model of evaluation of insulin homeostasis was used to calculate the HOMA index. F rats

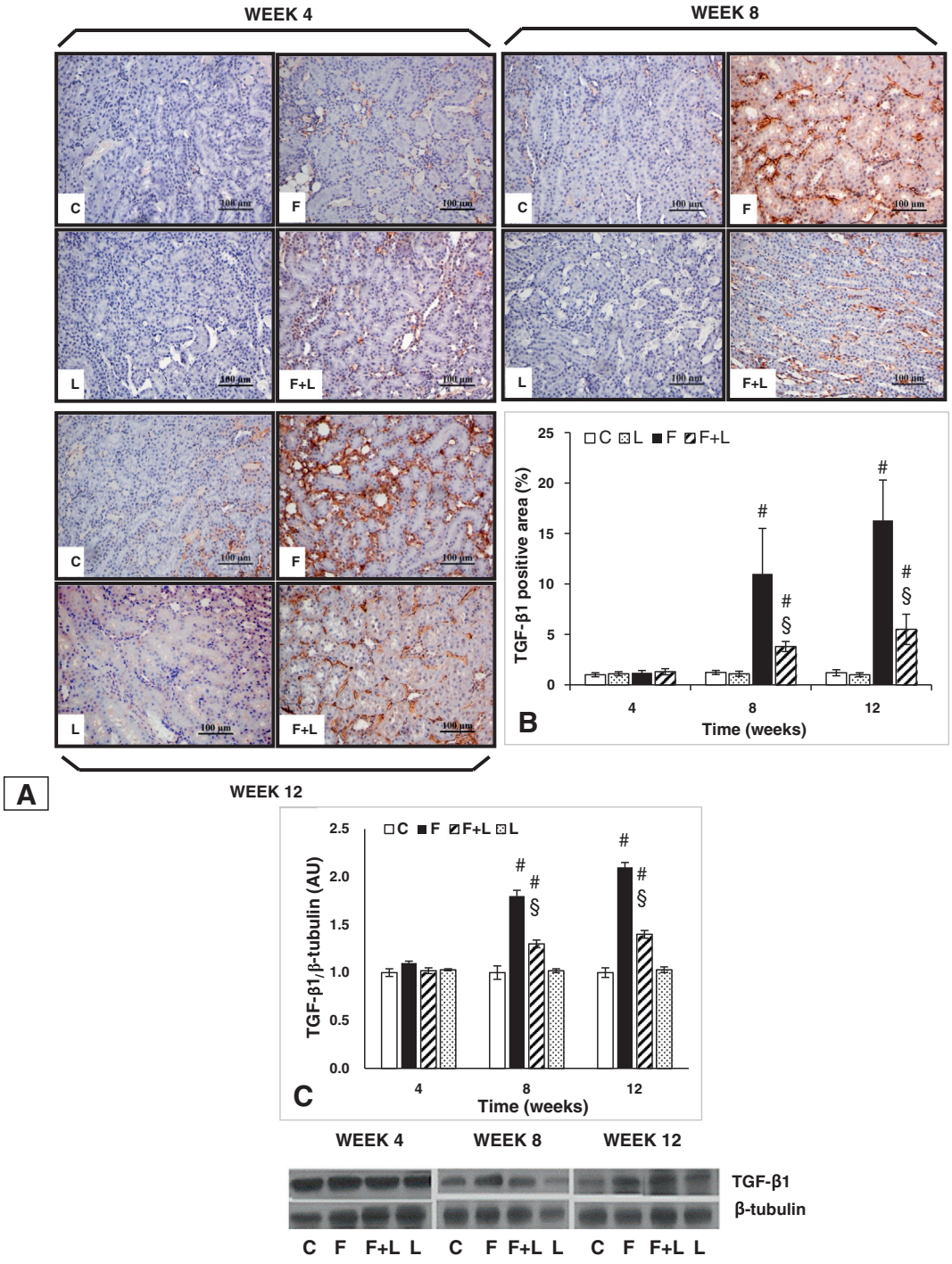

Fig. 7. Renal expression of profibrotic marker TGF-β1 at 4, 8 and 12 weeks of treatment with fructose and losartan in the different experimental groups. A - Representative microphotographs of the immunoexpression of TGF-β1 in renal cortex (200× magnification); B - Quantification of the immunoexpression of TGF-β1 in renal cortex; C - Protein expression values of TGF-β1 in renal cortex. Fructose was administered in the drinking water ad libitum (10% w/v). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM (n = 8 animals per group). C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; TGF-β1: Transforming growth factor β1; $^{\#}p < 0.01$ vs C and L; $^{\S}p < 0.01$ vs F.

showed higher HOMA-IR levels than C rats, greater than the cut-off to define IR, indicating the development of IR from week 4 of FO. These results coincide with those obtained by several studies in which a diet high in fructose is postulated as an IR model, with increased levels of insulinemia [21,22]. Regarding lipid metabolism, F rats showed a significant increase in plasma triglycerides compared to C, from week 8, while they developed hypertriglyceridemia (plasma triglycerides greater than or equal to 150 mg/dL) at week 12, with no changes in total cholesterol values in any experimental period. These results are coincident with those obtained by various experimental studies in which a FO in the diet was associated to hyperlipidemia in rodents [23]. On the other hand, F rats presented higher SBP levels compared to C, from week 4. In this sense, it has been reported that FO in the diet represents an animal model of acquired systolic hypertension [24].

Several studies showed that FO is able to induce the activation of various local RAS, with increase of AT_1R expression, renin/pro-renin index, and Ang II levels locally [14,15]. In our study, AT_1R expression increased in renal cortex of F rats from week 4, while Ang II

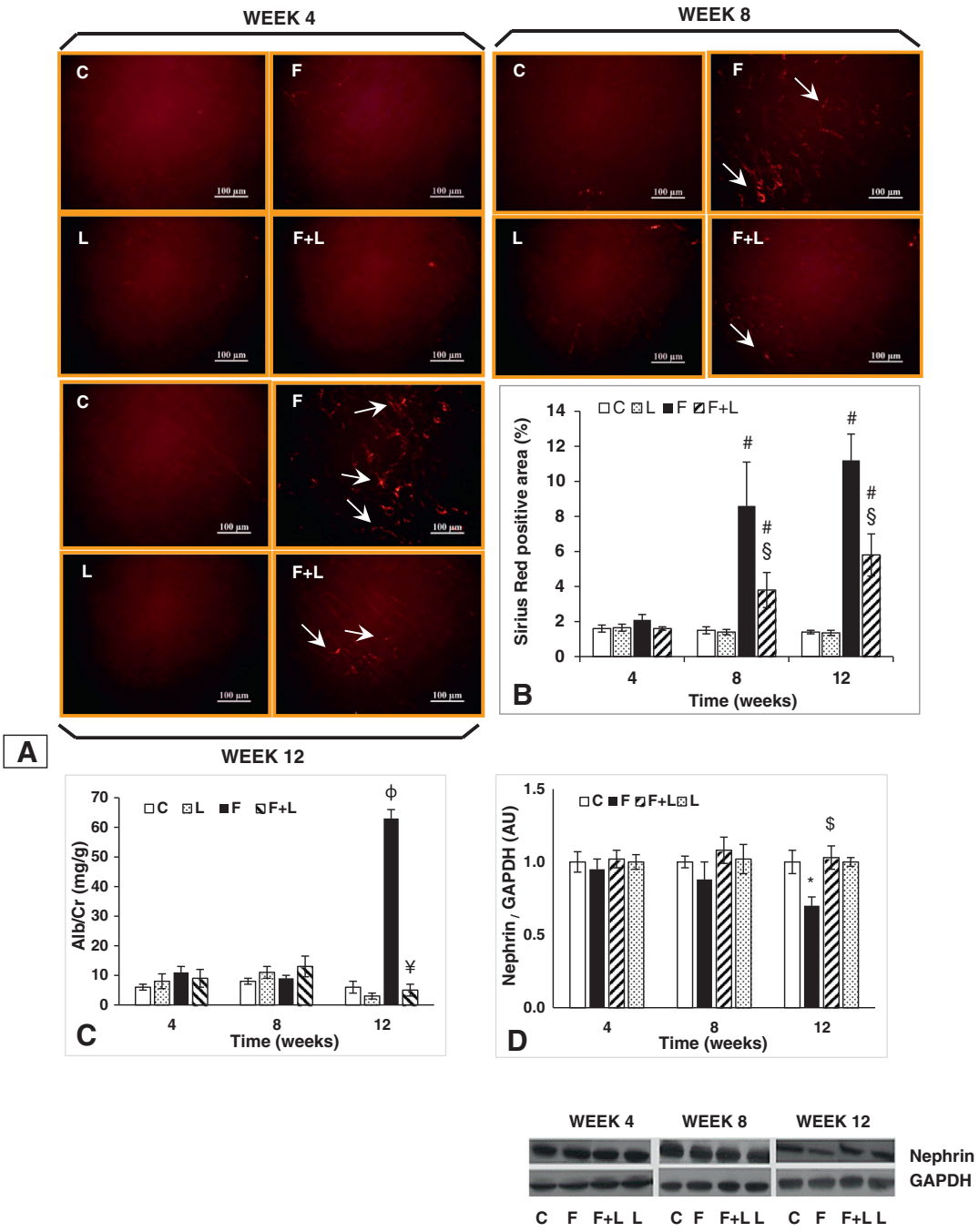

Fig. 8. Sirius Red Staining and biomarkers of structural kidney damage at 4, 8 and 12 weeks of treatment with fructose and losartan in the different experimental groups. A - Representative micrographs of the positive staining areas with Sirius Red in renal cortex (200× magnification); B - Quantification of positive staining areas with Sirius Red in renal cortex; C– Urinary albumin/creatinine ratio values; D - Protein expression values of nephrin in renal cortex. Fructose was administered in the drinking water ad libitum (10% w/v). Losartan was administered in the drinking water (30 mg/kg/day). The values are indicated as mean ± SEM (n = 8 animals per group). The arrows indicate the areas with different degrees of fibrosis. C: Control, L: Losartan; F: Fructose; F + L: Fructose plus losartan; Alb: Albumin; Cr: Creatinine; $^{\Phi}p < 0.001$ vs C and L; $^{\#}p < 0.01$ vs C and L; $^{*}p < 0.05$ vs C and L; $^{\S}p < 0.01$ vs F; $^{\$}p < 0.05$ vs F; $^{¥}p < 0.001$ vs F. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunostaining increased from week 8 of FO. On the other hand, many studies have established the existence of RDS alterations in states of IR and hypertension, such as the one induced by FO [25,26]. Taking into account the complex opposing and counter-regulatory interaction between RAS and RDS at renal level, it can be proposed that one of the effects of AT_1R over-expression and Ang II increased levels would be a reduction of DA production. Supporting this hypothesis, F rats showed an increased urinary L-dopa/DA ratio since week 4, due to a reduction in DA urinary excretion accompanied by an increase in L-dopa urinary excretion, as we have previously reported [27]. In vitro studies from our group have proven that Ang II is able to reduce DA tubular uptake, a process mediated by members of the SLC22A family called OCTs [28]. We hypothesize that urinary DA excretion reduction by FO could be associated to alterations in renal DA uptake from the circulation, as well as to a possible reduction in its transport from the intracellular compartment to the tubular lumen, process mediated by another members of the SLC22A family called OCTNs. In our study, OCT-2, OCT-N1 and total OCTNs expression was reduced in renal cortex of F rats since week 8. These results are consistent with those obtained by us and also by other groups, who demonstrated a reduction of kidney mRNA and protein levels of OCT-1, OCT-2 and OCT-N2 in Sprague-Dawley rats with FO for 8 weeks [27,29,30]. On the other hand, increased AT_1R

expression due to FO may contribute to the decrease of D_1R expression observed in F rats from week 4. Supporting this hypothesis, the existence of a heterodimeric complex between renal AT_1 and D_1 receptors, with opposing actions, has been demonstrated [9,31]. Additionally, the failure of the RDS to act as a counter-regulatory mechanism of RAS may, in turn, contribute to the over-expression of the latter, resulting in a feedback mechanism associated to the development of sodium retention and hypertension in this model.

It has been proven that RAS and RDS regulate the state of expression and activity of the enzyme Na^+, K^+-ATPase in an antagonistic and bidirectional way [32]. Increased Na^+, K^+-ATPase total activity since week 4, as well as increased expression from week 8, would indicate that, as result of RAS overexpression and RDS inhibition, Ang II action on Na^+, K^+-ATPase would prevail over DA actions. Likewise, these results could explain the reduction in natriuresis observed in F rats from week 4, and are coincident with those obtained by other groups [33,34]. 24-h diuresis increased in F rats compared to C, in all the experimental periods. This increase can be explained by taking into account the increase in drink intake in rats with FO. In fact, the existence of polydipsia and polyuria due to FO administered in the drinking water is well documented [35]. A possible explanation is based on the fact that the sweet taste of fructose would stimulate the palatability in rats, increasing its intake [35].

FO was also associated to an increase of pro-inflammatory markers expression in renal cortex of F rats from week 4. NF-kB expression increased from week 4, while TNF-α and IL-6 expression increased from week 8. Several experimental studies demonstrated the existence of an inflammatory response in kidneys of rodents treated with a high fructose diet [30,36]. It has also been proven that the increase of pro-inflammatory cytokines is associated to the appearance of renal fibrosis [37]. In this way, F rats showed increased fibrosis markers (TGF-β1 and collagen by staining with Sirius Red) from week 8. In this context, the imbalance given by AT_1R overexpression and increased Ang II levels, with pro-inflammatory actions, and by RDS inhibition, with anti-inflammatory actions that oppose those of Ang II, may be proposed as one of the mechanisms involved in the development of a pro-inflammatory environment at renal level [7,8]. As a result of the pro-inflammatory and pro-fibrotic processes associated to FO, the existence of structural renal damage was evidenced by the appearance of microalbuminuria and the reduction in nephrin expression at week 12. These results reveal the existence of structural alterations at the glomerular filtration barrier level. Microalbuminuria is an early indicator of kidney disease and a predictor of ischemic heart disease in essential hypertension [16]. Nephrin is a transmembrane protein located at the specialized cell junction of podocytes, constituting a fundamental part of the filtration diaphragm and maintaining the integrity of the glomerular filtration barrier [38]. Supporting our results, several studies have demonstrated the existence of kidney damage in rats with FO [39–41].

On the other hand, losartan treatment prevented the development of metabolic syndrome characteristics induced by FO. There are numerous evidences that indicate that RAAS inhibitors are capable of offering additional benefits, beyond the control of blood pressure, to patients with metabolic syndrome [42]. In our study, losartan treatment prevented the increase of SBP and the development of IR in F + L rats since week 4, as well as the development of hypertriglyceridemia at week 12. These results are consistent with bibliographic evidence and demonstrate the importance of Ang II actions in the pathogenesis of hypertension, dyslipidemia and IR in the model of FO [43–45]. On the other hand, treatment with losartan did not produce any change in drink, food and total caloric intake, as well as in body weight, in any group or experimental period. The absence of losartan effects on the increase in drink intake induced by FO in F + L rats could explain why losartan was not effective to prevent the increase in diuresis in this group, a fact supported by a previous report [46].

Consistent with bibliographic evidence, losartan prevented the increase in renal protein expression levels of AT_1R in F + L rats compared to F rats, from week 4 [47]. Treatment with losartan (F + L rats) had no effect on the increased renal Ang II immunostaining induced by FO at weeks 8 and 12. Considering the complex interaction between RAS and RDS, a fundamental objective of this study was to demonstrate that RAS blockade could affect RDS state. In this sense, losartan prevented the increase of urinary L-dopa/DA ratio, by preventing the reduction of DA excretion as well as the increase of L-dopa excretion. Losartan treatment showed preventive effects on the reduction of DA tubular transporters OCT-2, OCT-N1 and total OCTNs expression induced by FO, which could contribute to prevent the reduction of DA urinary excretion. Losartan treatment was also associated to a prevention of the decrease of renal D_1R expression, result in accordance with previous reports indicating that losartan is capable of acting as an allosteric effector of D_1R in the heterodimeric complex formed between AT_1 and D_1 receptors [48].

With respect to Na^+, K^+-ATPase, treatment with losartan avoided the increase of renal total activity and expression in F + L rats with respect to F rats. Taking into account Na^+, K^+-ATPase actions on the regulation of sodium excretion and blood pressure levels, the results obtained are consistent with the prevention of the reduction of natriuresis and increased SBP observed in F + L rats with respect to F. Losartan effects on Na^+, K^+-ATPase have been well documented [49]. In addition to AT_1R blockade, the prevention of D_1R expression downregulation by treatment with losartan may contribute to the prevention of increased expression and activity of the enzyme as well as decreased natriuresis and hypertension associated to dietary FO.

On the other hand, losartan treatment showed a renoprotective effect in rats with FO, by preventing the onset of microalbuminuria and reduction of nephrin expression observed at week 12. The renoprotective effects of losartan have been evidenced by several clinical and experimental studies [46,50]. In our model, these effects were associated to a prevention of the increase of pro-inflammatory and pro-fibrotic markers expression, which has also been documented [51,52]. In this regard, it should be noted that prevention of increased expression of pro-inflammatory and pro-fibrotic markers by losartan was not complete. Taking into account that losartan treatment fully prevented the development of IR, hypertension and hypertriglyceridemia, it is important to highlight that the increase of pro-inflammatory and pro-fibrotic markers expression in F + L rats with respect to C are probably due to the effects of renal metabolism of fructose per se, which are associated to oxidative stress and inflammation and have been widely reported in the literature [39,53].

To investigate whether losartan was capable of producing effects by itself in control animals, the drug was administered to rats fed a standard balanced diet, using the same dose as used in animals with a high fructose diet. The results obtained show that losartan per se did not alter any of the nutritional, metabolic, hemodynamic and renal function parameters, nor RDS and renal RAS, renal Na^+, K^+-ATPase and biomarkers of inflammation, fibrosis and kidney damage in control animals.

The results so far discussed allow us to establish the existence of a temporary association between alterations in RAS and RDS, with the development of hypertension, IR and kidney damage. Regarding the interaction between RAS and RDS, the reduction of DA excretion and D_1R expression was observed at week 4 of FO, together with AT_1R overexpression, while Ang II immunostaining was increased from week 8 of FO. On the other hand, AT_1R blockade by losartan led to a prevention of RDS alterations since week 4 of treatment. These results allow us to hypothesize that the AT_1R overexpression would have a fundamental role in RDS downregulation, more important than increased Ang II levels, fact supported by evidence demonstrating the intimate role between AT_1 and D_1 receptors at renal level [48].

Taking into account that alterations in the urinary L-dopa/DA ratio were observed from week 4 of FO, we can propose L-dopa/DA index as a marker of renal dysfunction in conditions characterized by sodium retention, insulin resistance and/or hypertension, which temporarily precedes the increase of pro-inflammatory cytokines and fibrosis

markers expression at week 8, as well as of renal structural damage evidenced by microalbuminuria and reduced nephrin expression at week 12. These results would complete the studies initiated by our group, in which we showed the existence of an alteration of the L-dopa/DA ratio associated to FO in the diet [27]. Also, urinary L-dopa/DA ratio was shown to respond to losartan, taking into account the prevention of the increase of this ratio in F + L rats. The prevention of L-dopa/DA ratio increase was total at week 4 of treatment, while it was partial at weeks 8 and 12. In this way, urinary L-dopa/DA index showed a behavior similar to that performed by pro-inflammatory and pro-fibrotic markers. The partial reversal of this parameter could be explained by considering experimental evidence that shows that DA production as well as regulation of D_1R expression and signaling, are highly sensitive to the development of oxidative stress and inflammation at renal level [54]. In this way, the development of oxidative stress and the increase of pro-inflammatory cytokines due to renal fructose metabolism, would affect RDS, independently of RAS blockade with losartan. In Fig. 9, we suggest a mechanism involved in the development of IR, hypertension and kidney damage in the model of FO.

The results of this study allow us to establish the time course of RAS and RDS alterations in our experimental model of metabolic syndrome and to establish the temporary association (along three experimental times) between these alterations and the development of hypertension, IR and kidney inflammation. In addition, we demonstrate the beneficial effects of AT_1R blockade by losartan to prevent hypertension, IR and kidney damage. Finally, the results provide new evidence supporting the potential use of urinary L-Dopa/DA index as biomarker of renal alterations related to sodium retention in this experimental model.

The main limitations of the study are: a) we did not determine the state of expression and activity of dopamine synthesizing and catabolizing enzymes (dopa decarboxylase, and monoamine oxidase and catechol *O*-methyl transferase, respectively); b) we did not evaluate the state of L-dopa transporters; and c) we did not compare the effects of losartan with other blood pressure-lowering drugs with different mechanism of action, on the imbalance between RDS and RAS and kidney inflammation. We have in mind to carry out further experiments to complete the assessment of the functional state of RDS as well as to determine the effects of other blood pressure-lowering drugs like calcium channel blockers in this model.

In summary, the results of this study bring new evidence about the mechanisms by which a pro-hypertensive and pro-inflammatory system such as RAS can downregulate another anti-hypertensive and anti-inflammatory system such as RDS, establishing a positive feedback loop for the development of hypertension and renal inflammation in the context of metabolic syndrome induced by FO. Finally, the results of this work allow us to postulate the urinary L-Dopa/DA index as a possible

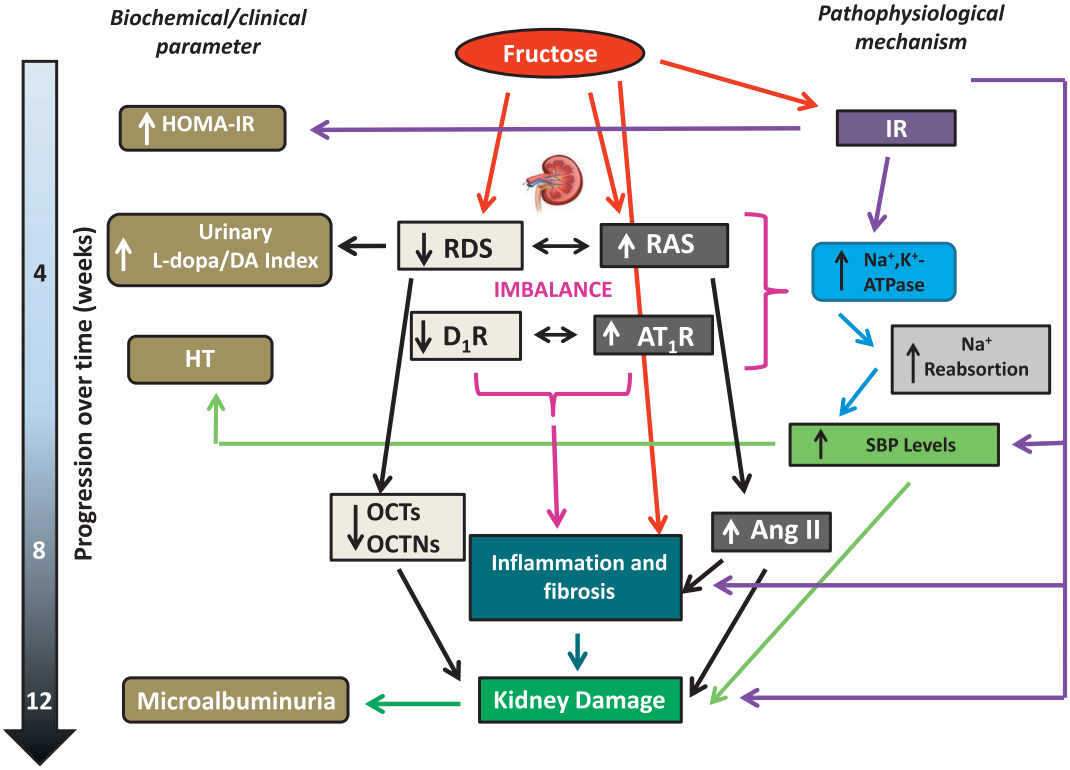

Fig. 9. Pathophysiological mechanism involved in the development of hypertension and kidney damage due to fructose overload in the diet. FO in the diet was associated to the development of IR from week 4, evidenced by increased HOMA-IR values in F rats. At renal level, FO was associated to an imbalance between RAS and RDS, consisting of increased AT_1R expression, simultaneously with the reduction of DA urinary excretion and D_1R expression. RDS alteration was verified by increased urinary L-dopa/DA index, from week 4. Taking into account the effects of both systems on the regulation of renal sodium homeostasis, the afore mentioned imbalance led to an increase in tubular sodium reabsorption, associated to the increase of Na^+, K^+-ATPase expression and activity, which contributed to the increase in SBP and development of HT. On the other hand, the IR present in the model, as well as fructose itself acting on the renal tubular cells, also contributed to the increase of sodium reabsorption and the development of HT. At week 8, the imbalance between both systems was magnified, taking into account the increase of Ang II renal levels as well as the reduction of DA tubular transporters (OCT-2, OCT-N1 and OCTN1/2/3) expression. Considering that Ang II and DA are capable of mediating the immune response at renal level, and by showing Ang II pro-inflammatory effects opposed to the anti-inflammatory actions of DA, the imbalance between both systems contributes to explain the development of inflammation and fibrosis detected at weeks 8 and 12 of FO, which progressively led to the appearance of renal structural damage at week 12, verified by the presence of microalbuminuria and the reduction of nephrin expression. Additionally, the metabolization of fructose at renal level was capable of exerting deleterious effects, with induction of inflammation and oxidative stress in this organ. Considering the complex interaction between RAS and RDS, it can be proposed that the imbalance between both systems led to the establishment of a positive feedback loop for the development of HT and renal inflammation. The alteration of L-dopa/DA index was early than the appearance of markers of inflammation and renal damage, so we postulate the utility of using this index as an early and potential biochemical biomarker of renal dysfunction in states of IR and HT. Ang II: angiotensin II; DA: dopamine; HOMA-IR: HOMA index of insulin resistance; HT: arterial hypertension; IR: insulin resistance; OCTs: transporters of organic electrogenic cations; OCTNs: electroneutral organic cation transporters; SBP: systolic blood pressure; RDS: renal dopaminergic system; RAS: renin angiotensin system.

diagnostic biomarker of renal alterations related to sodium retention, hypertension and kidney inflammatory damage, as well as a predictor of response to treatment and follow-up.

The results of this study may contribute to a better understanding of physiological relationship between RDS and RAS and their influence on renal sodium transport and regulation of natriuresis and diuresis. Moreover, new concepts about the imbalance between RDS and RAS in the experimental model of metabolic syndrome can provide new insights for the clinical treatment of the disease. The results also show new evidence supporting the role of DA as a nephroprotective agent and its potential use in future therapeutic strategies for the treatment of hypertension.

Whether the usefulness of urinary L-Dopa/DA ratio is confirmed, this study would set the first antecedent to propose the use of L-Dopa/DA index as an early marker to detect functional renal damage linked to sodium retention and/or hypertension.

Author Contributions

Planned and executed the experiments: Natalia L. Rukavina Mikusic and Nicolás M. Kouyoumdzian. Conceived the experimental design: Belisario E. Fernández and Marcelo R. Choi. Performed immunohistochemistry and immunofluorescence: Ana Uceda and Jorge E. Toblli. Performed the HPLC technique: Julieta S. Del Mauro. Performed analytical and metabolic determinations: Marcela Pandolfo. Contributed with reagents/materials/analysis tools: Ana M. Puyó and Mariela M. Gironacci. Analyzed the data: Natalia L. Rukavina Mikusic, Jorge E. Toblli, Belisario E. Fernández and Marcelo R. Choi. Wrote the paper: Natalia L. Rukavina Mikusic, Nicolás M. Kouyoumdzian, Belisario E. Fernández and Marcelo R. Choi.

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Disclosure Statement

None.

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Appendix A. Supplementary Experimental Procedures

Supplementary Experimental Procedures to this article can be found online at https://doi.org/10.1016/j.metabol.2018.04.010.

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