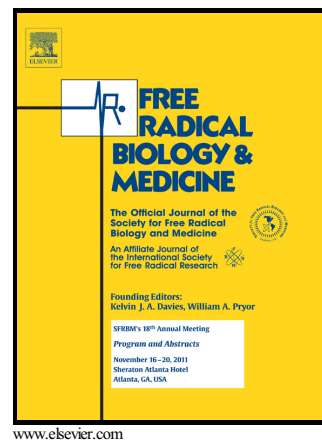


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**Dietary (-)-epicatechin mitigates oxidative stress, NO metabolism alterations,  
and inflammation in renal cortex from fructose-fed rats.**

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**Abstract**

High fructose consumption has been associated to deleterious metabolic conditions. In the kidney, high fructose causes renal alterations that contribute to the development of chronic kidney disease. Evidence suggests that dietary flavonoids have the ability to

prevent/attenuate risk factors of chronic diseases. This work investigated the capacity of (-)-epicatechin to prevent the renal damage induced by high fructose consumption in rats. Male Sprague Dawley rats received 10% (w/v) fructose in the drinking water for 8 w, with or without supplementation with (-)-epicatechin (20 mg/kg body weight/d) in the rat chow diet. Results showed that, in the presence of mild proteinuria, the renal cortex from fructose-fed rats exhibited fibrosis and decreases in nephrin, synaptopodin, and WT1, all indicators of podocyte function in association with: i) increased markers of oxidative stress; ii) modifications in the determinants of NO bioavailability, i.e., NO synthase (NOS) activity and expression; and iii) development of a pro-inflammatory condition, manifested as NF- $\kappa$ B activation, and associated with high expression of TNF $\alpha$ , iNOS, and IL-6. Dietary supplementation with (-)-epicatechin prevented or ameliorated the adverse effects of high fructose consumption. These results suggest that (-)-epicatechin ingestion would benefit when renal alterations occur associated with inflammation or metabolic diseases.

### **Keywords**

(-)-Epicatechin; high fructose consumption; superoxide anion; NADPH-oxidase; inflammation; nitric oxide; podocytes.

### **Abbreviations**

CAT, catalase; CuZnSOD, copper, zinc superoxide dismutase; eNOS, endothelial nitric oxide synthase; GPx, glutathione peroxidase; GSH, reduced glutathione; IL-6, interleukin-6; 4-HNE, 4-hydroxynonenal; hnRNP, heterogeneous nuclear ribonucleoprotein A1; iNOS, inducible nitric oxide synthase; L-NAME,  $N^{\omega}$ -nitro-L-arginine methyl ester; MAPKs, mitogen-activated protein kinases; MnSOD, manganese superoxide dismutase; NF- $\kappa$ B; nuclear factor kappa-light-chain-enhancer of activated B cells; nNOS, neuronal nitric oxide

synthase; NOS, nitric oxide synthase; NOX, NADPH oxidase; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNF $\alpha$ , tumor necrosis factor alpha; WT1, Wilms' tumor protein.

## Introduction

Epidemiological studies showed an increase in the worldwide prevalence of chronic kidney disease [1–3]. High fructose consumption has been linked with deleterious metabolic changes [4]. In terms of renal modifications, data from the National Health and Nutrition Examination Survey (NHANES) indicate a positive association between soda consumption, a major source of dietary fructose, and albuminuria in humans [5]. The administration of fructose to rats leads to renal hypertrophy, afferent arteriolar thickening, glomerular hypertension, and cortical vasoconstriction, all of them contributing to the development of chronic kidney disease [6].

Among several undesirable conditions developed in fructose fed-rats, elevated superoxide anion production seems to be a ubiquitous condition. In kidneys, an increase over physiological levels of superoxide anion has been related to oxidative stress, nitric oxide (NO) depletion, and inflammation. Furthermore, each of these conditions was associated to the loss of podocyte functionality, and the consequent alteration of the glomerular filtration barrier, which dysfunction is a major feature of kidney disease [7].

The beneficial effects of fruits and vegetables on human health can be in part attributed to their high content of flavonoids [8,9]. Increasing evidence demonstrates that dietary flavonoids and their metabolites contribute to the prevention and/or amelioration of the risk factors for cardiometabolic diseases [10–13]. The flavanol (–)-epicatechin is one of the most abundant flavonoids in human diets, being present in high concentrations in apples,

blackberries, broad beans, cherries, grapes, pears, raspberries, cocoa, and tea [14]. Protective effects of (–)-epicatechin in the kidney have been reported in pharmacological models of renal damage [15,16]. Dietary supplementation of (–)-epicatechin attenuated renal injury in rats chronically treated with the NO synthase (NOS) inhibitor *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) [15] and showed protective effects against the progression of mitochondrial injury in cisplatin-induced nephropathy in mice [16], but there are no reports on a diet-induced renal damage.

This work presents evidence that *in vivo* (–)-epicatechin supplementation was able to prevent kidney fibrosis and podocyte alterations in association with the prevention or amelioration of the increase superoxide anion production, the alterations in NO metabolism, and the inflammatory manifestations in the renal cortex from fructose-fed rats.

## Materials and Methods

### Materials

Primary antibodies for inducible nitric oxide synthase (iNOS) (#7271), p47<sup>phox</sup> (#7660), endothelial nitric oxide synthase (eNOS) (#654), neuronal nitric oxide synthase (nNOS) (#648), gp91<sup>phox</sup> (#5826), tumor necrosis factor alpha (TNFα) (#1350), manganese superoxide dismutase (MnSOD) (#133134), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 (#7151), heterogeneous nuclear ribonucleoprotein A1(hnRNP A1) (#32301), glutathione peroxidase (GPx) (#133160), Wilms' tumor protein (WT1) (#192), interleukin-6 (IL-6) (#1265), nephrin (#32530), synaptopodin (#21537), nitrotyrosine (#32731), and β-actin (#47778), and secondary antibodies rabbit anti-goat IgG-HRP (#2768), mouse anti-rabbit IgG-HRP (#2357), and goat anti-mouse IgG-HRP (#2005) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary

antibody for copper, zinc superoxide dismutase (CuZnSOD) (AB1237) was from Chemicon International (Temecula, CA, USA), the primary antibody for p-eNOS Ser(1177) (#9570) was from Cell Signaling Technology (Boston, USA), and the primary antibody for 4-hydroxynonenal (4-HNE) (ab46545) was from Abcam Inc. (Cambridge, MA, USA). (-)-Epicatechin, butylhydroxytoluene (BHT), 2-thiobarbituric acid, heparin, NADPH, glucose 6-phosphate, reduced glutathione (GSH), N,N'-dimethyl-9,9'-biacridinium dinitrate (lucigenin), bovine seroalbumin, superoxide dismutase (SOD) and glutathione reductase were from Sigma Chemical Co. (St. Louis, MO, USA). Fructose (purity  $\geq 99.5\%$ ) was obtained from Droguería Saporiti (Buenos Aires, Argentina). Commercial rat chow was from Gepsa-Feeds (Buenos Aires, Argentina). [ $^{14}\text{C}$ ] L-arginine was from PerkinElmer Life and Analytical Sciences (Boston, MA, USA).

#### *Animals, diets and experimental design*

All procedures were in agreement with standards for the care of laboratory animals as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and were approved by the Secretary of Science and Technology of the School of Medicine, National University of Cuyo. Male Sprague Dawley rats were housed under conditions of controlled temperature (21-25°C) and humidity, with a 12-h light/dark cycle. Rats weighing  $130 \pm 20$  g were randomly divided into the following groups (8 rats per group): control (C), receiving tap water *ad libitum* and standard rat chow diet; fructose (F), receiving 10% fructose (w/v) in the drinking water and standard rat chow diet; and fructose-(-)-epicatechin (FE), receiving 10% fructose (w/v) in the drinking water and the standard rat chow diet supplemented with (-)-epicatechin (20 mg/kg body weight/d) [17]. The (-)-epicatechin provided to the rats is equivalent to 200 mg/d for a 70-kg human, amount that is attainable through optimizing fruit and vegetable

intake and/or pharmacological strategies [18,19]. Food intake and beverage consumption were recorded throughout the experiment. Systolic blood pressure was measured at the beginning and at the end of the experimental period by tail plethysmography in conscious, prewarmed, slightly restrained rats, and recorded in a Grass Model 7 polygraph (Grass Instruments Co., Quincy, MA, USA). Four days before euthanasia, rats were placed in metabolic cages for 24 h for urine collection. After 8 w on the respective treatments, and after overnight fast, rats were weighed and anesthetized with ketamine (50 mg/kg of body weight) and acepromazine (1 mg/kg body weight). Blood was collected from the abdominal aorta into heparinized tubes and plasma was obtained after centrifugation (600 x g, 15 min, 4°C). Blood plasma samples were frozen at -80°C. Kidneys were excised immediately and processed for histological, immunohistochemical, and biochemical determinations.

#### *Sample processing*

For each animal, a portion of renal cortex was separated and fixed in phosphate-buffered 10% (v/v) formaldehyde (pH: 7.2) for further histological and immunohistochemical analysis. Another section of the renal cortex was excised and homogenized in PBS (7.6 mM  $\text{KH}_2\text{PO}_4$ , 42.4 mM  $\text{K}_2\text{HPO}_4$ , 150 mM NaCl, pH: 7.4), and centrifuged at 600 x g for 20 min at 4°C. Supernatants were used for the measurement of enzyme activities and for thiobarbituric acid reactive substances (TBARS) determination. For the determination of NADPH-dependent superoxide anion production, supernatants were centrifuged at 10000 x g for 20 min at 4°C to obtain mitochondria-free homogenates. For Western Blot determinations, renal cortex was homogenized in lysis buffer (150 mM NaCl, 50 mM Trizma-HCl, 1% (v/v) NP-40, pH: 8.0) in the presence of protease and phosphatase inhibitors, and centrifuged at 600 x g for 20 min. The supernatant was collected and defined as total fraction tissue. Cytosolic and nuclear fractions of kidney cortex were

isolated as described previously [20]. Protein content was measured by the Lowry method [21].

#### *Biochemical determinations*

All biochemical determinations were performed using commercially available kits: plasma glucose, triglycerides, total cholesterol and urinary protein levels (GTLab, Rosario, Argentina), and uric acid levels in plasma and creatinine levels in plasma and urine (Wiener Lab., Argentina). All the determinations were performed following the manufacturers' protocols.

#### *TBARS determination*

The assay was based on a previously described method [22]. Briefly, samples were mixed with 3% (w/v) sodium dodecyl sulfate (SDS), 0.1 N HCl, 10% (w/v) phosphotungstic acid, and 0.7% (w/v) 2-thiobarbituric acid, and heated for 45 min in boiling water. TBARS were extracted with 2 ml of n-butanol and detected fluorometrically ( $\lambda_{\text{ex}}$ : 515 nm;  $\lambda_{\text{em}}$ : 555 nm). 1, 1, 3, 3-Tetramethoxypropane was used to prepare the standard of malondialdehyde. The results were expressed as nmoles of TBARS (malondialdehyde equivalents)/mg protein.

#### *Determination of NADPH-dependent superoxide anion production*

Lucigenin-enhanced chemiluminescence was measured in renal cortex mitochondria-free homogenates according to Li *et al* [23]. Briefly, samples were added to vials containing 1 ml of 50 mM potassium phosphate at 37°C, pH: 7.4 containing lucigenin and NADPH (5  $\mu\text{M}$  and 40  $\mu\text{M}$  final concentrations, respectively) in the absence or the presence of SOD (200 U/ml). Light emission was measured every 10 s for 10 min using a LKB Wallac 1209



Rackbeta Liquid Scintillation Counter (Turku, Finland) in the chemiluminescence mode. Results were expressed as the difference between the areas under the curve in the absence and in the presence of SOD, which represents the amount of superoxide anion produced.

#### *Western Blot analysis*

Renal cortex homogenates were added with a 2X solution of sample buffer (62.5 mM Tris-HCl, pH: 6.8 containing 2% (w/v) SDS, 25% (w/v) glycerol, 5% (v/v)  $\beta$ -mercaptoethanol, and 0.01% (w/v) bromophenol blue) and heated at 95°C for 2 min. Sample aliquots containing 30  $\mu$ g of protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to polyvinylidenedifluoride membranes. Colored molecular weight standards (GE Healthcare, Piscataway, NJ, USA) were run simultaneously. Membranes were blotted for 2 h in 5% (w/v) nonfat milk and incubated overnight in the presence of the corresponding primary antibody (1:1000 dilution in PBS). After a subsequent incubation for 90 min at room temperature in the presence of the corresponding HRP-conjugated secondary antibody (1:5000 dilution in PBS), complexes were visualized by chemiluminescence. Films were scanned and a densitometric analysis was performed using Image J (National Institute of Health, Bethesda, Maryland, USA). Total and cytosolic extract band densities were normalized to the  $\beta$ -actin content. Bands from the nuclear fractions were normalized to the hnRNP A1 content.

#### *Determinations of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities*

Enzymes activities were measured in renal cortex homogenates. CAT activity was determined spectrophotometrically following the consumption of  $H_2O_2$  at 240 nm. Results were expressed as  $\mu$ mol  $H_2O_2$ /mg protein.min [24,25]. GPx activity was determined

spectrophotometrically following the enzymatic oxidation of NADPH at 340 nm in the presence of 1 mM GSH, 1 mM  $\text{NaN}_3$ , 0.15 mM NADPH, and 0.25 U/ml glutathione reductase. Results were expressed as  $\mu\text{mol}$  GSH oxidized/mg protein.min [26]. SOD activity was spectrophotometrically determined following the inhibition of the formation of adrenochrome. One unit of SOD is defined as the amount of enzyme that causes a 50% inhibition of the rate of this reaction. As CuZnSOD is inhibited by cyanide, the reactions were carried out in the absence or in the presence of 4  $\mu\text{M}$  KCN, to obtain total SOD or MnSOD activities, respectively. CuZnSOD activity was calculated as the difference between total and MnSOD activities. Results were expressed as units/mg protein [27].

#### *Determination of nitric oxide synthase (NOS) activity*

NOS activity was measured using [ $^{14}\text{C}$ ] L-arginine as substrate in renal cortex slices [28]. NO production, measured as pmol of [ $^{14}\text{C}$ ] L-citrulline, was normalized to the weight of the tissue slices incubated with the substrate during 30 min. Results were expressed as pmol [ $^{14}\text{C}$ ] L-citrulline/g.min.

#### *Histological and immunochemical analysis of the kidney*

Fixed kidney cortex were embedded in paraffin, and 3 micron sections were cut and stained with Sirius Red as described [29]. Histological evaluations were performed using a light microscope Nikon E400 (Nikon Instrument Group, Melville, NY, USA). Immunohistochemistry was performed with antibodies against IL-6 (1:100 in PBS), nephrin (1:400 in PBS), WT1 (1:400 in PBS) and synaptopodin (1: 300 in PBS). Staining was performed with a commercially modified avidin-biotin-peroxidase complex and hematoxylin counterstaining as described [30]. Immunostaining is quantified as percentage of positive staining per area from 20 random images viewed at x400 magnification. Measurements

were made using an image analyzer, Image-Pro Plus version 4.5 for Windows (Media Cybernetics, LP, Silver Spring, MD, USA).

### *Statistical analysis*

Results are shown as mean  $\pm$  SEM. Except for histology, data were analyzed by one-way analysis of variance (ANOVA) using StatView 5.0 (SAS Institute, Cary, NC, USA), and Fisher's significance difference test was used to examine differences between group means. Histological data were analyzed by nonparametric Kruskal-Wallis test using absolute values processed through GraphPad Prism 5.01 (GraphPad Software, Inc. San Diego, CA, USA), and Dunn's multiple comparison test. A value of  $p < 0.05$  was considered statistically significant.

### **Results**

Metabolic parameters are shown in **Table 1**. Body and kidney weights were similar among the three experimental groups. The two groups receiving fructose showed higher (43-47%,  $p < 0.05$ ) daily liquid intake and lower (29-18%,  $p < 0.05$ ) daily food intake compared with C. One reason for the increased liquid intake in the animals receiving fructose in the beverage could be the palatability of the fluid [31]. The decrease in the amount of solid diet consumed suggests a strategy to control the overall caloric intake [32]. Rats from F presented a systolic blood pressure 16 and 10 mmHg higher respect to C and FE ( $p < 0.05$ ), respectively. No differences were found in plasma glucose and total cholesterol levels among the three groups. Rats from F presented higher plasma triglyceride levels respect to C (1.7-fold increase,  $p < 0.05$ ); while FE rats presented values similar to C. No significant differences were found in uric acid and creatinine levels in serum among the three groups. Diuresis was higher (approximately 4.0-fold increase,  $p < 0.05$ ), in F and FE

compared to C. The amount of excreted protein was higher in F compared to C (71%,  $p < 0.05$ ) and FE (37%,  $p < 0.05$ ) (**Table 1**).

Collagen deposition, an index of fibrosis, was evaluated by Sirius Red staining in renal cortex tissue (Figure 1). Interstitial and perivascular collagen deposition was higher in F than in C (3.0-fold increase) indicating a high degree of fibrosis. FE showed a significantly lower collagen deposition, i.e. only 1.0-fold increase over C value. The expression of nephrin, one of the structural components of kidney glomerular slit diaphragms, was significantly lower in F respect to C (Figure 2A). Synaptopodin and WT1, both recognized markers of glomerular epithelial cells (podocytes), were also lower in F respect to C (Figure 2B and C). Taken together, these changes in protein expression demonstrated a podocyte injury in F. In FE, the expression of the three proteins was increased respect to F ( $p < 0.05$ ) tending to reach C values.

The content of TBARS, nitrotyrosine, and 4-HNE protein adducts in renal cortex were measured to evaluate oxidative stress. F rats showed higher values compared to C in the three parameters (29%, 48%, and 33%;  $p < 0.05$  for TBARS, nitrotyrosine, and 4-HNE protein adducts, respectively) (**Figure 3A, D, and F**). In FE the values of the three parameters were similar to those measured in C. Tissue capacity to produce superoxide anion was estimated measuring the SOD-inhibitable chemiluminescence of lucigenin in the presence of NADPH. Chemiluminescence was higher in F respect to C (107%;  $p < 0.05$ ), and in FE it was similar to C (**Figure 3B**). In terms of NADPH oxidase (NOX) protein subunits, the expression of the membrane catalytic subunit gp91<sup>phox</sup> was higher in F and FE respect to C (43% and 48% respectively;  $p < 0.05$ ), while the expression of the activator subunit p47<sup>phox</sup> was higher in F compared with C and FE (47% and 28% respectively;  $p < 0.05$ ) (**Figure 4**).

The activities of the antioxidant enzymes SOD, GPx, and CAT were measured in renal cortex as indexes of the capacity to cope with the increase in superoxide anion and hydrogen peroxide production. The activity and the expression of the cytosolic isoform (CuZnSOD) were higher in F compared to C (30% and 79%;  $p < 0.05$ ). In FE, CuZnSOD activity and expression showed values lower than in F, but did not reach statistical significance ( $p < 0.08$ ). No differences among the three groups were observed for the activity and expression of the mitochondrial isoform (MnSOD). GPx activity was higher in the renal cortex from F respect to C (32%;  $p < 0.05$ ), and was similar to C values in FE. CAT activity was similar among three groups (**Figure 5**).

In terms of NO metabolism in renal cortex, NOS activity was higher in F and FE compared to C (14% and 40%, respectively,  $p < 0.01$ ). nNOS protein levels in renal cortex homogenates, were significantly lower in F respect to C and FE (39% and 41%, respectively,  $p < 0.05$ ). eNOS protein expression was similar among the three experimental groups, but eNOS phosphorylation (Ser-1177) were significantly higher in F and FE respect to C (41% and 42%, respectively,  $p < 0.05$ ) (**Figure 6**).

In order to evaluate the occurrence of an inflammatory condition, the cytosolic and nuclear distribution of the transcription factor NF- $\kappa$ B subunit p65, and the expression of downstream pro-inflammatory targets TNF $\alpha$ , iNOS, and IL-6 were measured in renal cortex. The ratio nuclear/cytosolic p65 was higher in F respect to C and FE (31% and 27% respectively;  $p < 0.05$ ). TNF $\alpha$  protein level in F was higher in C and FE (47% and 53% respectively;  $p < 0.05$ ). iNOS protein content in renal cortex was higher in F respect to C and FE (9.0- and 2.6-fold increase, respectively;  $p < 0.05$ ) (**Figure 7**). IL-6 level in F was higher respect to C and FE (7.0- and 1.0-fold increase respectively;  $p < 0.05$ ) (**Figure 8**).

## Discussion

Fructose induced deleterious kidney changes that were associated with oxidative stress, alterations in NO metabolism, and a pro-inflammatory condition. All these adverse effects were absent or significantly reduced when the rat diets were supplemented with (–)-epicatechin. These results are in line with our previous work [17,19] showing that (–)-epicatechin prevents dysfunctional changes associated to high fructose treatment in the cardiovascular system, liver, and fat tissue.

Development of renal functional changes in fructose-fed rats is highly dependent on the amount of fructose administered and the time of exposure. In agreement with a previous report [6], under the experimental conditions used (10% (w/v) of fructose in the beverage for 8 w), renal functions were mildly affected. Even when the kidneys from F rats disposed normally nitrogenous waste products, an incipient loss of control of urinary protein excretion was observed. This renal condition, could be associated to both, interstitial and perivascular fibrosis, and the decrease of specific podocyte proteins, even no glomerular fibrosis was observed. This incipient damage to kidney was prevented by (–)-epicatechin. The decrease in fibrosis appears as a result of the attenuation in the pro-inflammatory condition. Meanwhile, the protective effects of (–)-epicatechin observed in podocytes could be related with alternative mechanisms that could occur in parallel or somehow related to inflammation, e.g. regulation of insulin signaling. In this regard, it has been reported that fructose consumption affects podocytes through an impairment of insulin signaling [33, 34], and that (–)-epicatechin improves insulin signaling in liver and adipose tissue [17].

In terms of mechanisms, both the high NADPH-dependent superoxide anion production, and the increased levels of oxidative damage observed in fructose-fed rats agree with previous observations in kidney [7, 35] and in other tissues [17, 19, 36]. The action of (–)-

epicatechin preventing the increases in oxidative stress, suggests an antioxidant action. (-)-Epicatechin shares with other flavonoids the ability to scavenge free radicals being effective *ex vivo* at concentrations of 25-100  $\mu\text{M}$  [37–39]. However, due to the sub micromolar concentration of this compound found in plasma and in other tissues [40, 41], the possibility that (-)-epicatechin could scavenge radicals *in vivo* with a physiological relevance is unlikely [9, 42]. Then, indirect antioxidant mechanisms, operative at the amount of (-)-epicatechin expected to be present in kidneys, must be proposed to explain the observed effects [43, 44]. One mechanism could be the regulation of the source of superoxide anion production in renal cortex. In the kidney, NOXs are a major source of both superoxide anion and hydrogen peroxide [45]. In this work, we observed that fructose treatment increased the protein expressions of the NOX2 subunits gp91<sup>phox</sup> and p47<sup>phox</sup>. (-)-Epicatechin partially prevented the overexpression of p47<sup>phox</sup> and this prevention would result in a normal superoxide anion production.

The production of superoxide anion is tightly regulated given its role in signaling pathways and the need to prevent uncontrolled oxidation of cell components [46]. The observed increased activity and expression of the CuZnSOD in F would reflect an adaptive mechanism against increased superoxide anion cellular levels. Superoxide metabolism by SOD leads to an increase in hydrogen peroxide production, which explain the increased GPx activity that can occur via posttranslational modifications, e.g. phosphorylation at activators sites [47]. The inhibition by (-)-epicatechin of the excessive superoxide anion production triggered by fructose may explain the partial prevention of the increases in CuZnSOD and GPx activities.

In terms of NO metabolism, in F rats NOS activation would justify a moderate increase of NO production; at the same time superoxide anion production could be doubled. These altered rates of production, and the high rate of the reaction between NO and superoxide

anion [48, 49] would result in a lower overall NO bioavailability that will compromise normal kidney function. In addition, being peroxynitrite, the product of the reaction between NO and superoxide anion, a strong oxidant, it would further contribute to the observed oxidative damage. In the rats receiving (–)-epicatechin, a higher NO bioavailability would result of the moderate increase in NOS, and mostly by a return to normal values of superoxide anion production determinants. The restoration of a physiologically appropriate NO bioavailability by (–)-epicatechin associated to normal tissue function has been previously described in other experimental models and tissues [19, 50, 51].

Impaired oxidant and NO metabolisms can lead to an altered cell redox status, triggering or amplifying inflammatory events [17, 52]. The occurrence of renal oxidative stress in fructose-fed rats could be the cause for the activation of the redox-sensitive and pro-inflammatory NF- $\kappa$ B transcription factor. Furthermore, the observed high levels of the pro-inflammatory agents, i.e. TNF $\alpha$ , iNOS and IL-6, are indicative that fructose consumption led to an inflammatory condition in the kidney. (–)-Epicatechin supplementation mitigated fructose-mediated inflammation by decreasing the expression of the measured cytokines and proteins. These results in kidney are in agreement with those found in rats subjected to the same fructose overload showing that (–)-epicatechin mitigated NF- $\kappa$ B activation, and TNF $\alpha$ , and monocyte chemoattractant protein-1 (MCP-1) expressions in both adipose tissue and liver [17]. In addition, in 3T3-L1 adipocytes in culture (–)-epicatechin suppressed the TNF $\alpha$ -mediated activation of NF- $\kappa$ B, activator protein-1 (AP-1), and the mitogen-activated protein kinases (MAPKs) [44]. Moreover, direct evidence showing that (–)-epicatechin acts decreasing NF- $\kappa$ B activation in vivo has been recently published [53]. Molecular mechanisms involved in the effects of (–)-epicatechin on NF- $\kappa$ B activation could be either, secondarily to an inhibition of oxidant production, and/or through specific



interactions of (–)-epicatechin with NF- $\kappa$ B proteins p50 and p65, preventing their binding to NF- $\kappa$ B sites in the promoter of target genes [54, 55].

It is well known that (–)-epicatechin is extensively metabolized after ingestion, allowing very low concentrations of the parent compound (< 500 nM) in human blood after the consumption of flavanol-rich foods. It is very likely that metabolites of (–)-epicatechin are the molecular effectors of the observed effects. For example, 3'- and 4'-O-methyl(–)-epicatechin were postulated to interact and inhibit NOX activity in endothelial cells [56], and 3'- and 4'-O-methyl(–)-epicatechin-5/7-sulfates are major metabolites present in plasma after (–)-epicatechin administration to humans [40, 41].

Integrating the present results, we propose that the oxidative stress triggered by NOX overexpression and superoxide anion production increase is one of the main underlying causes of the incipient kidney changes associated with high fructose intake. In the long term, this would lead to overt kidney dysfunction. This hypothesis is supported by results showing that: i) a fructose-induced oxidant production, especially superoxide anion, participates in podocyte injury in rodents [7]; and ii) fructose activates the superoxide anion/p38/MAPK/TXNIP/NLRP3 inflammasome pathway resulting in glomerular podocyte injury [57]. Consequently, a down-regulation of NOX, as is that observed following (–)-epicatechin supplementation, could be a central event in the control of fructose-associated and inflammation-mediated kidney damage.

Taken as a whole, dietary supplementation with (–)-epicatechin mitigates renal modifications observed in fructose-fed rats through the modulation of oxidant production, improvement of NO bioavailability, and down-regulation of redox-sensitive pro-inflammatory pathways. Overall (–)-epicatechin emerges as a bioactive which consumption, as part of a flavanol-rich diet, could help to prevent the renal alterations found in subjects suffering metabolic and other inflammatory diseases.

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**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the authors.

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**Table 1. Effects of (–)-epicatechin on metabolic and renal function parameters and systolic blood pressure of fructose-fed rats**

Parameter	C	F	FE
Body weight (g)	393 ± 17	365 ± 12	389 ± 15
Kidney weight (g)	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
Liquid intake (ml/24 h)	51 ± 4	73 ± 3*	75 ± 3*
Food intake (g/24 h)	28 ± 1	20 ± 1*	23 ± 2*
Systolic blood pressure (mmHg)	129 ± 3	145 ± 3*	135 ± 2 <sup>†</sup>
Plasma glucose (mg/dl)	90 ± 2	85 ± 2	90 ± 2
Plasma cholesterol (mg/dl)	43 ± 2	45 ± 3	47 ± 4
Plasma triglycerides (mg/dl)	55 ± 4	148 ± 25*	85 ± 7 <sup>†</sup>
Serum uric acid (mg/dl)	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Serum creatinine (mg/dl)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Diuresis (ml/24 h)	6 ± 1	28 ± 3*	30 ± 3*
Proteinuria (mg/mg creatinine)	2.4 ± 0.4	4.1 ± 0.6*	3.0 ± 0.2 <sup>†</sup>

C: control, F: fructose, and FE: fructose-(–)-epicatechin groups. Values are expressed as means ± SEM; n = 8 animals/group. \*p < 0.05 respect to C, <sup>†</sup>p < 0.05 respect to F.

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**Figure captions**

**Figure 1: Effects of dietary (-)-epicatechin on collagen deposition in the renal cortex from fructose-fed rats. (A-C)** Representative images for renal cortex collagen III staining using Sirius Red in the different experimental groups. **(D)** After quantification of collagen III staining degree in samples from C: control, F: fructose, and FE: fructose-(-)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.01 respect to C, †p < 0.01 respect to C and F.

**Figure 2: Effects of dietary (-)-epicatechin on nephrin, synaptopodin and WT1 immunostaining in the renal cortex from fructose-fed rats. (A-C)** Representative images of proteins nephrin, synaptopodin and WT1 in renal cortex from the different experimental groups. **(D)** After quantification of the three proteins in samples from C: control, F: fructose, and FE: fructose-(-)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.01 respect to C, †p < 0.05 respect to F, ‡p < 0.01 respect to C and F.

**Figure 3: Effects of dietary (-)-epicatechin on the levels of TBARS and nitrotyrosine and 4-HNE protein adducts and on NADPH-dependent superoxide anion production in the renal cortex from fructose-fed rats. (A)** TBARS were measured in renal cortex homogenates, **(B)** NADPH-dependent superoxide anion production was measured as SOD-inhibitable lucigenin chemiluminescence in mitochondria-free renal cortex homogenates, **(C-D)** nitrotyrosine protein adducts were measured by Western Blot in renal cortex homogenates (Left panel shows a representative image), and **(E-F)** 4-HNE protein adducts were measured by Western Blot in the cytosolic fraction of renal cortex homogenates (Left panel shows a representative image). C: control, F: fructose, FE:

fructose-(–)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.05 respect to C, †p < 0.05 respect to F.

**Figure 4: Effects of dietary (–)-epicatechin on gp91<sup>phox</sup> and p47<sup>phox</sup> expression in the renal cortex from fructose-fed rats. (A) gp91<sup>phox</sup> and (B) p47<sup>phox</sup> protein levels were measured by Western Blot in renal cortex homogenates from C: control, F: fructose, FE: fructose-(–)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.05 respect to C, †p < 0.05 respect to F.**

**Figure 5: Effects of dietary (–)-epicatechin on the activity and expression of SOD and GPx, and the activity of CAT in the renal cortex from fructose-fed rats. (A) SOD activity and (B) expression, (C) GPx activity and (D) expression and (E) CAT activity were measured in renal cortex homogenates from C: control, F: fructose, FE: fructose-(–)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.05 respect to C.**

**Figure 6: Effects of dietary (–)-epicatechin on NOS activity, nNOS and eNOS expression and eNOS phosphorylation in the renal cortex from fructose-fed rats. (A) NOS activity measured in renal cortex slices. (B) nNOS, (C) eNOS protein levels, and (D) eNOS phosphorylation in Ser(1177) were measured in renal cortex homogenates from C: control, F: fructose, FE: fructose-(–)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.05 respect to C, †p < 0.05 respect to F, ‡p < 0.05 respect to C and F.**

**Figure 7: Effects of dietary (–)-epicatechin on the ratio nuclear/cytosolic p65 and on TNF $\alpha$  and iNOS expression in the renal cortex from fructose-fed rats. (A) The ratio**

nuclear/cytosolic p65, **(B)** TNF $\alpha$  and **(C)** iNOS expressions were measured in renal cortex homogenates from C: control, F: fructose, FE: fructose(-)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.05 respect to C, †p < 0.05 respect to F.

**Figure 8: Effects of dietary (-)-epicatechin on IL-6 immunostaining in the renal cortex from fructose-fed rats. (A-C)** Representative images for IL-6 in the renal cortex from the different experimental groups. **(D)** After quantification of IL-6 expression immunostaining in C: control, F: fructose, and FE: fructose(-)-epicatechin. Values are expressed as means  $\pm$  SEM; n = 8 animals/group. \*p < 0.01 respect to C, †p < 0.01 respect to C and F.

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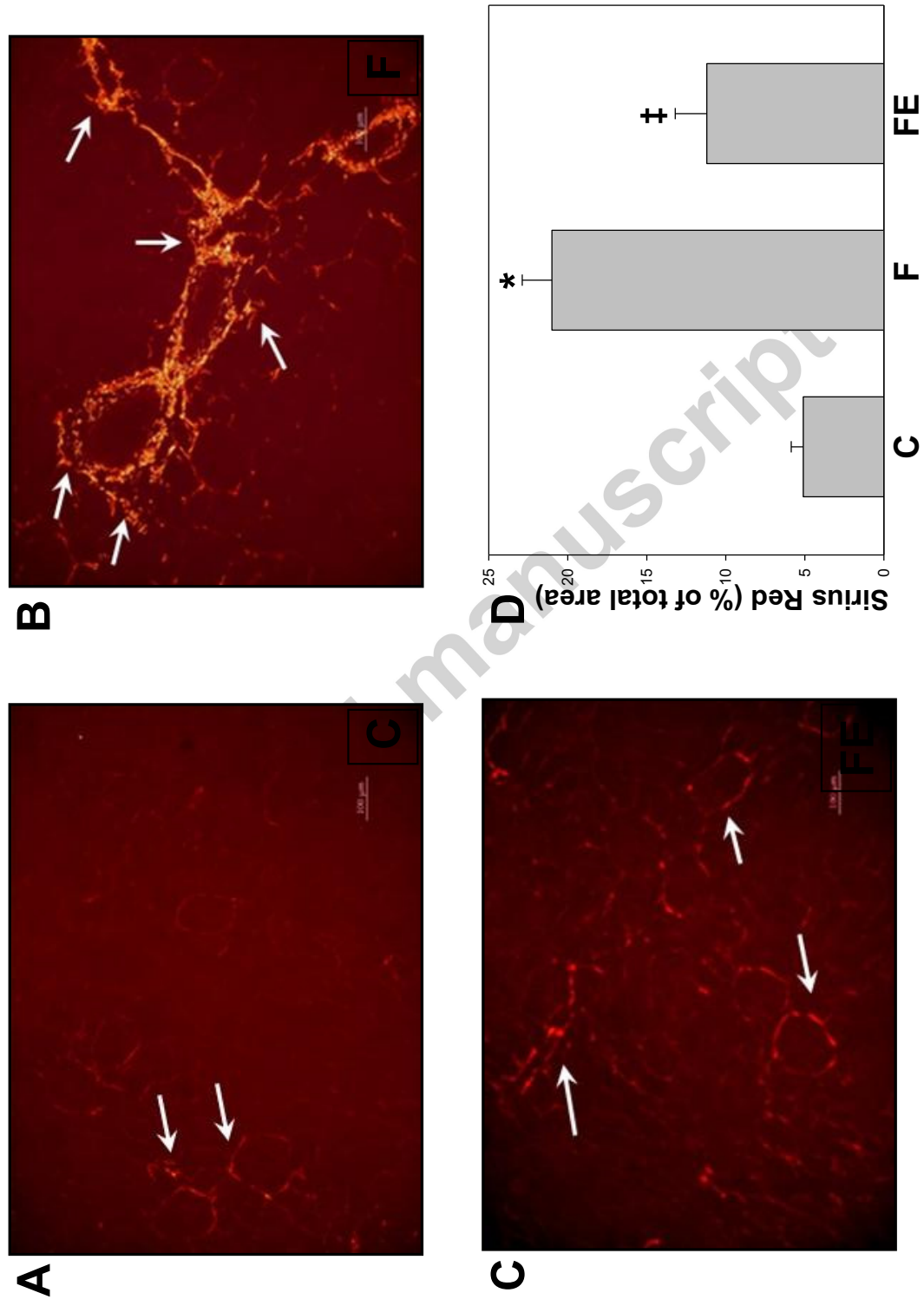
**Highlights**

- (-)-Epicatechin reduced alterations in podocytes induced by fructose in rats.
- (-)-Epicatechin reduced oxidative stress in renal cortex of fructose-fed rats.
- (-)-Epicatechin restored NO bioavailability in renal cortex of fructose-fed rats.
- (-)-Epicatechin ameliorated inflammation in renal cortex of fructose-fed rats.

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Figure 1



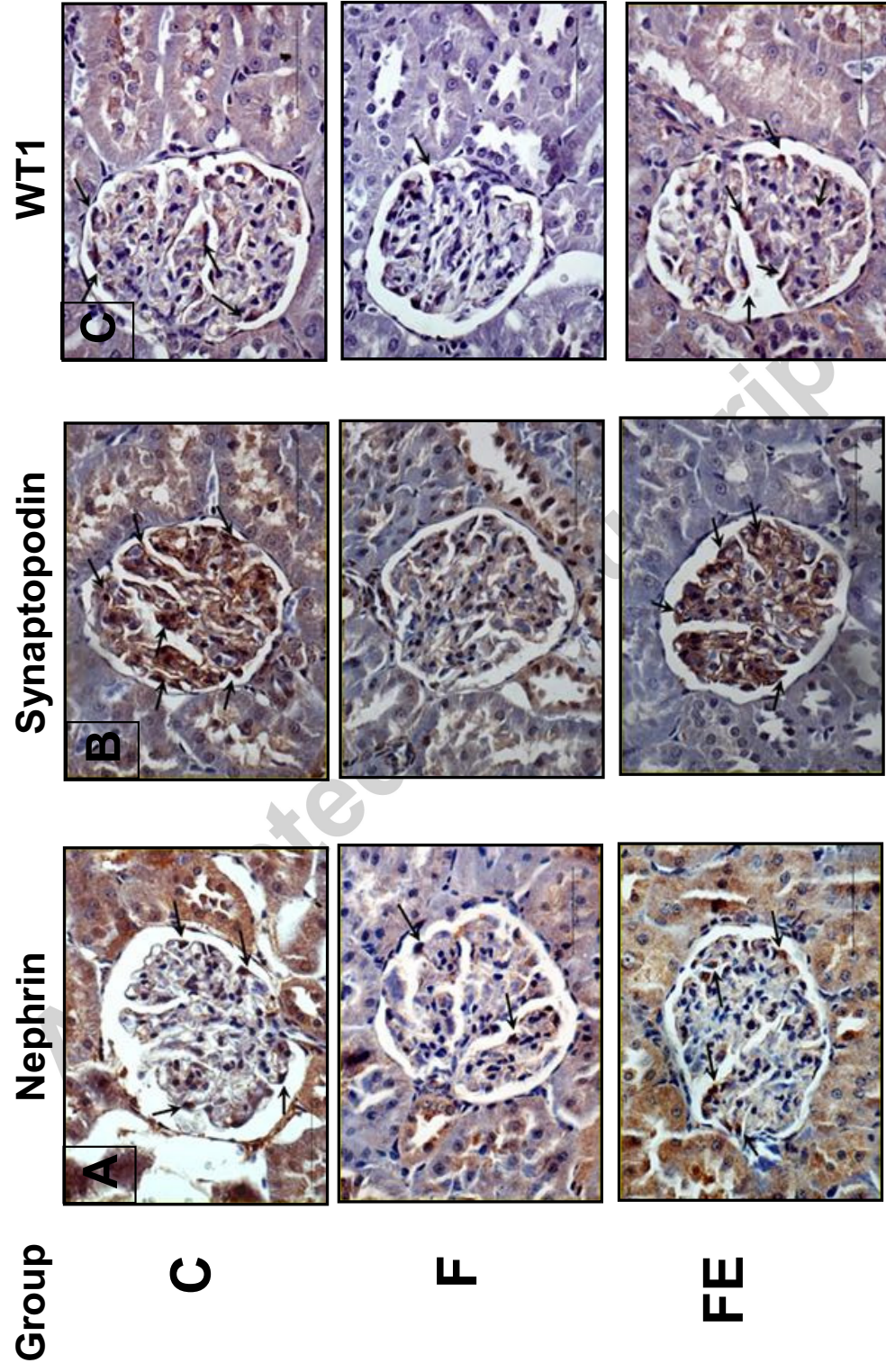


Figure 2

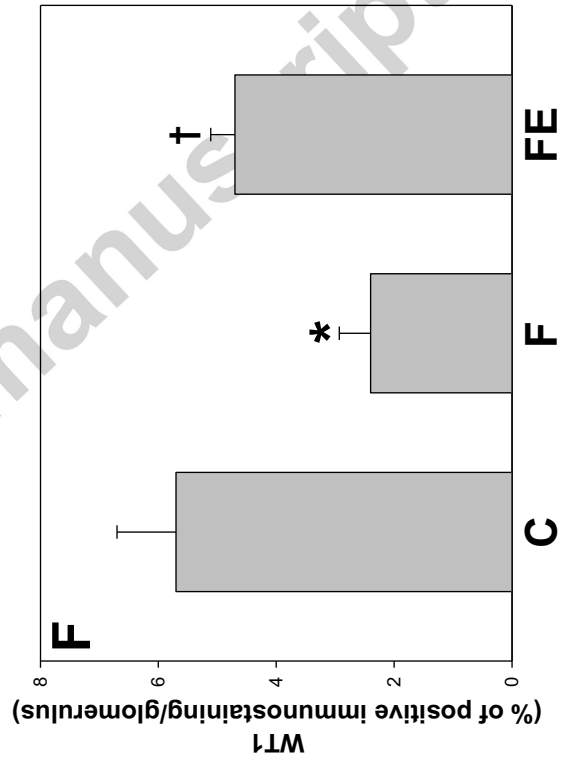
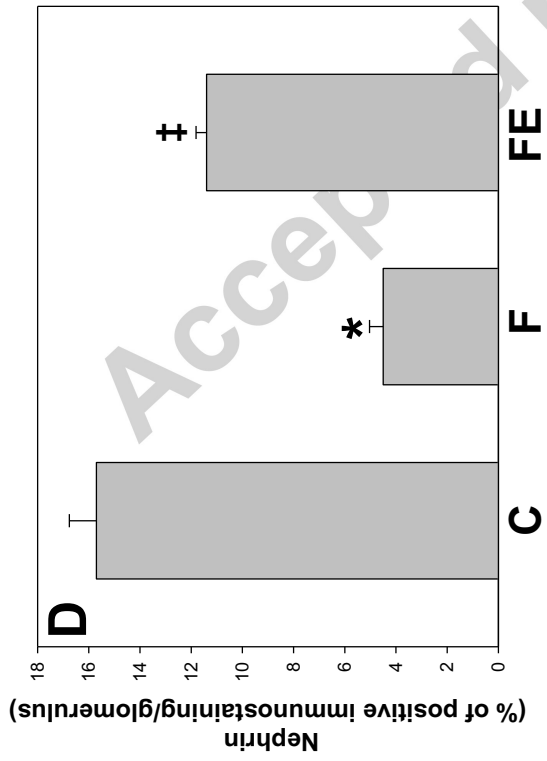
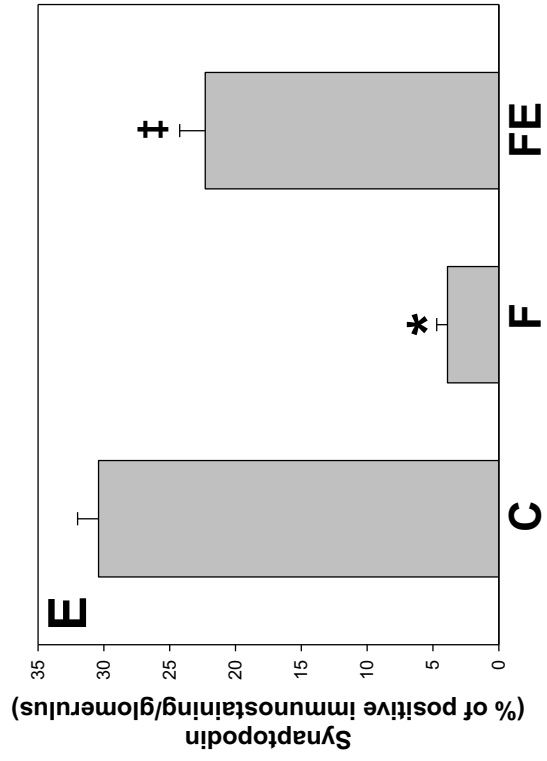
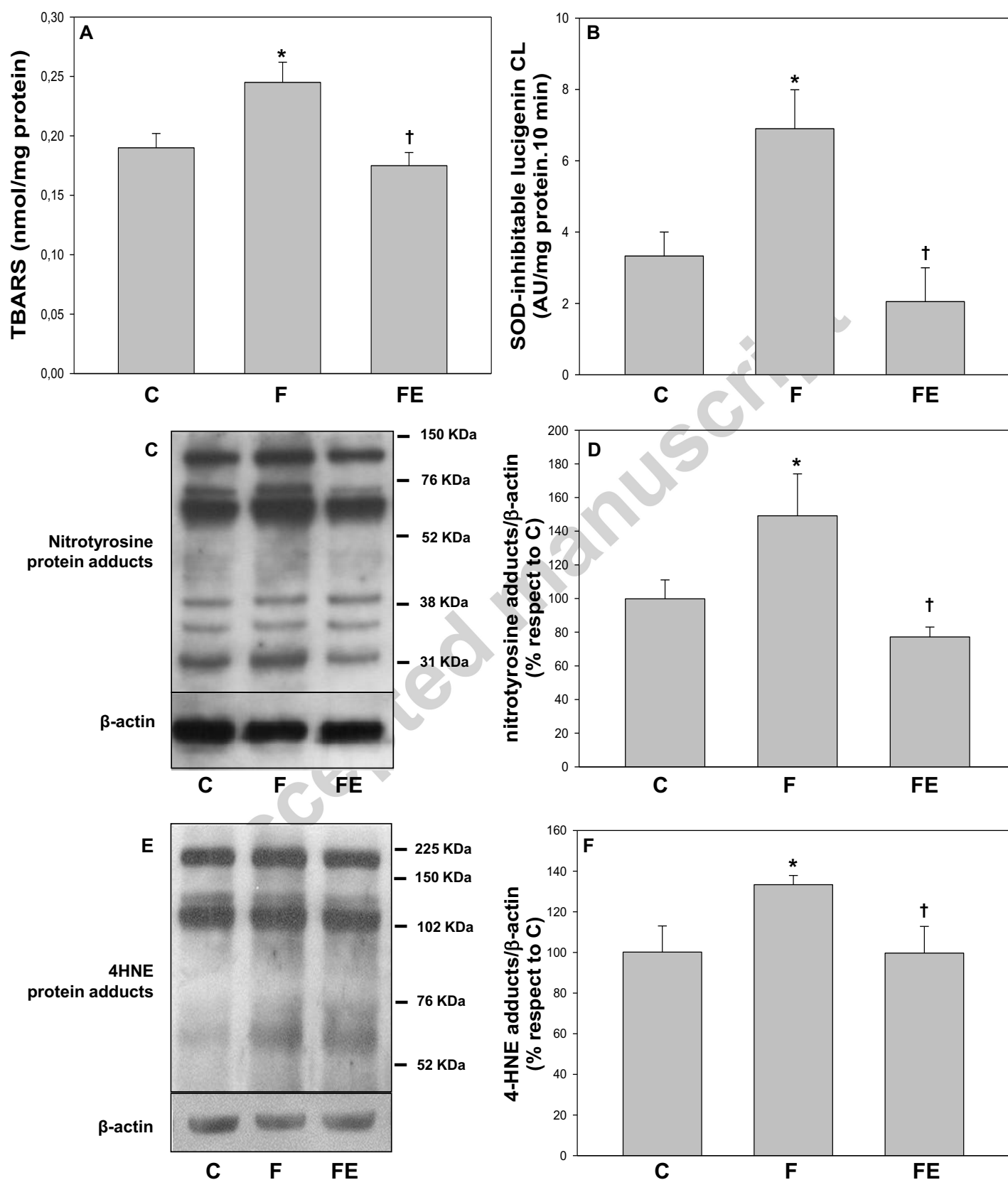


Figure 3



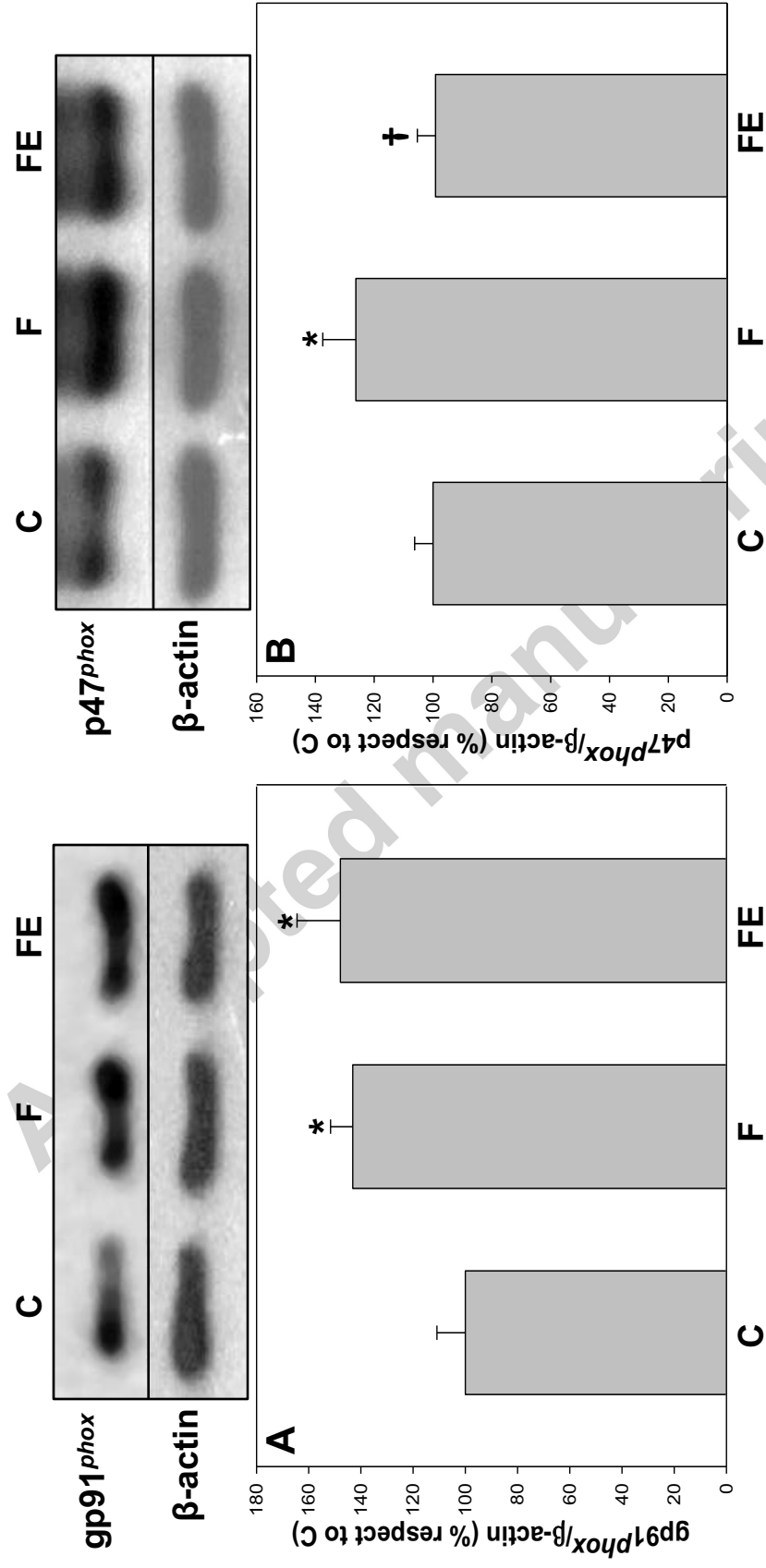


Figure 4

Figure 5

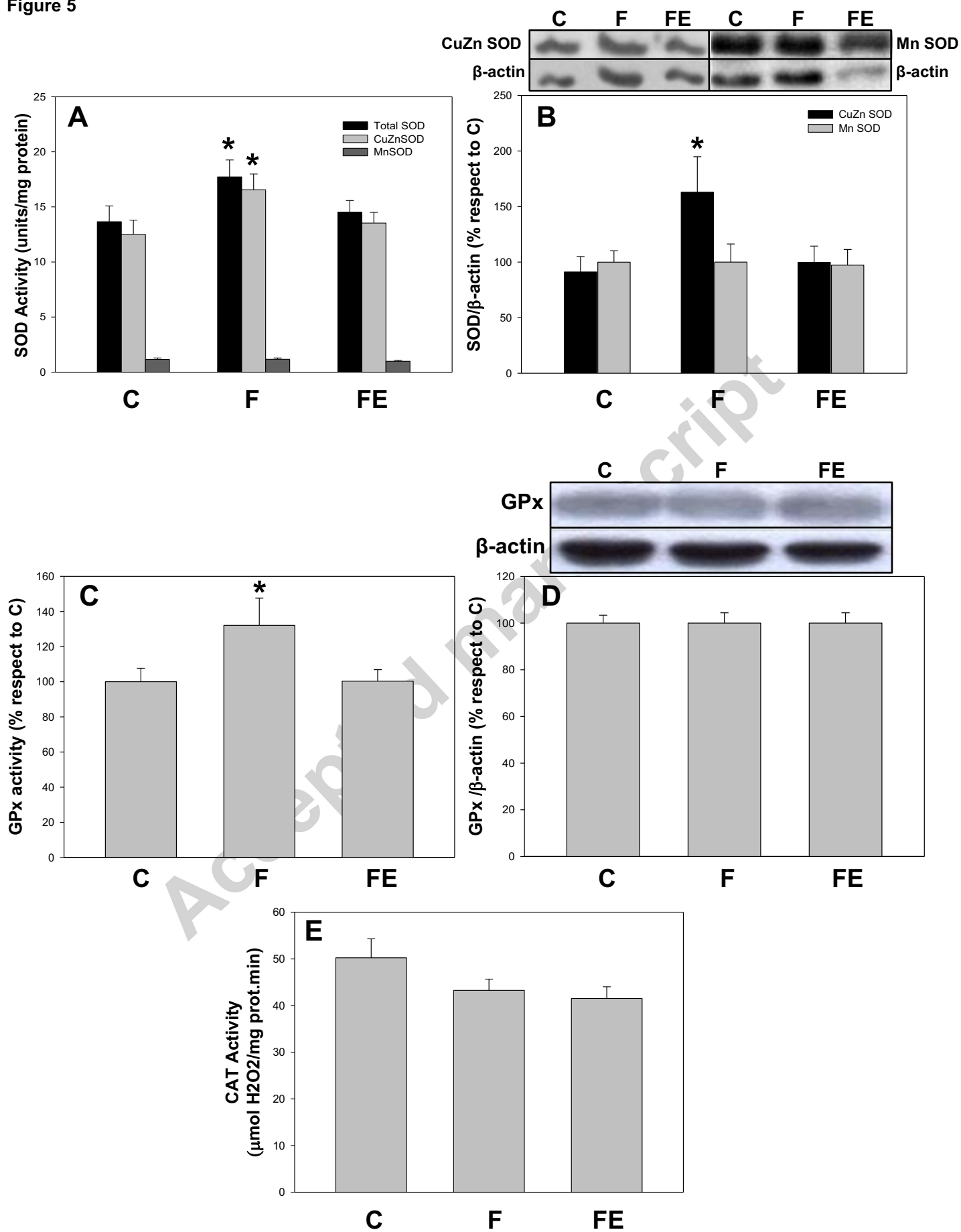
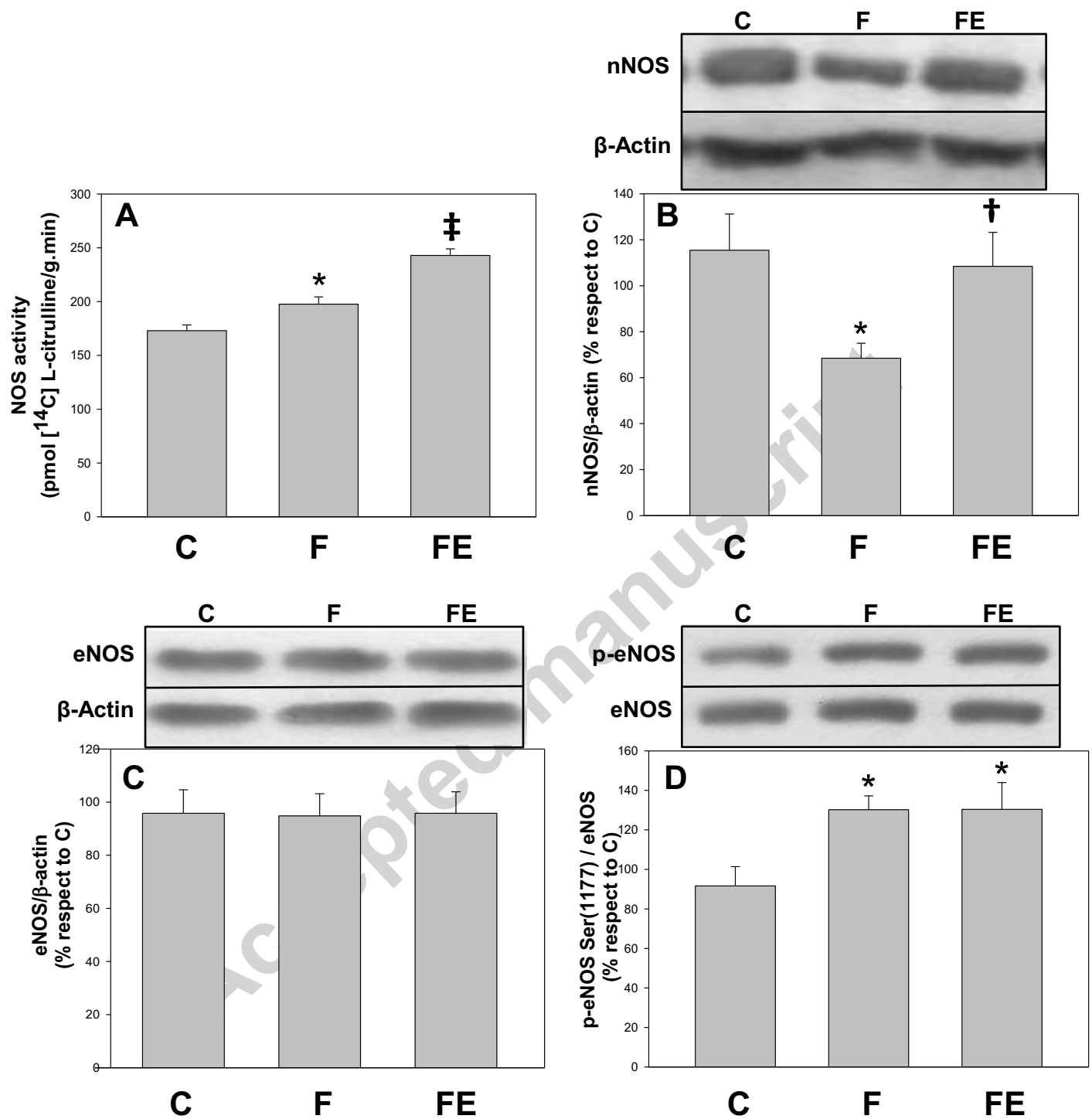
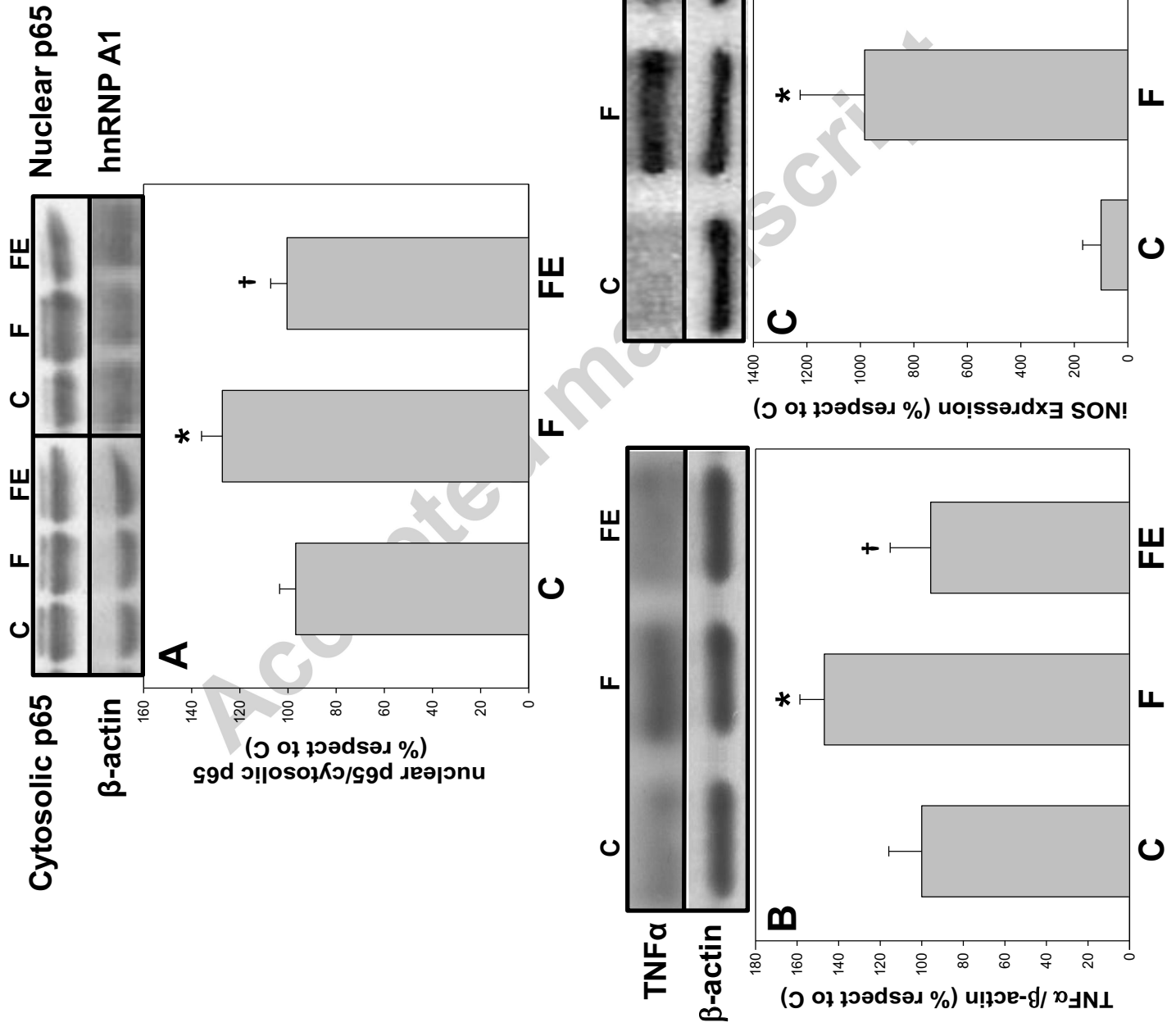


Figure 6







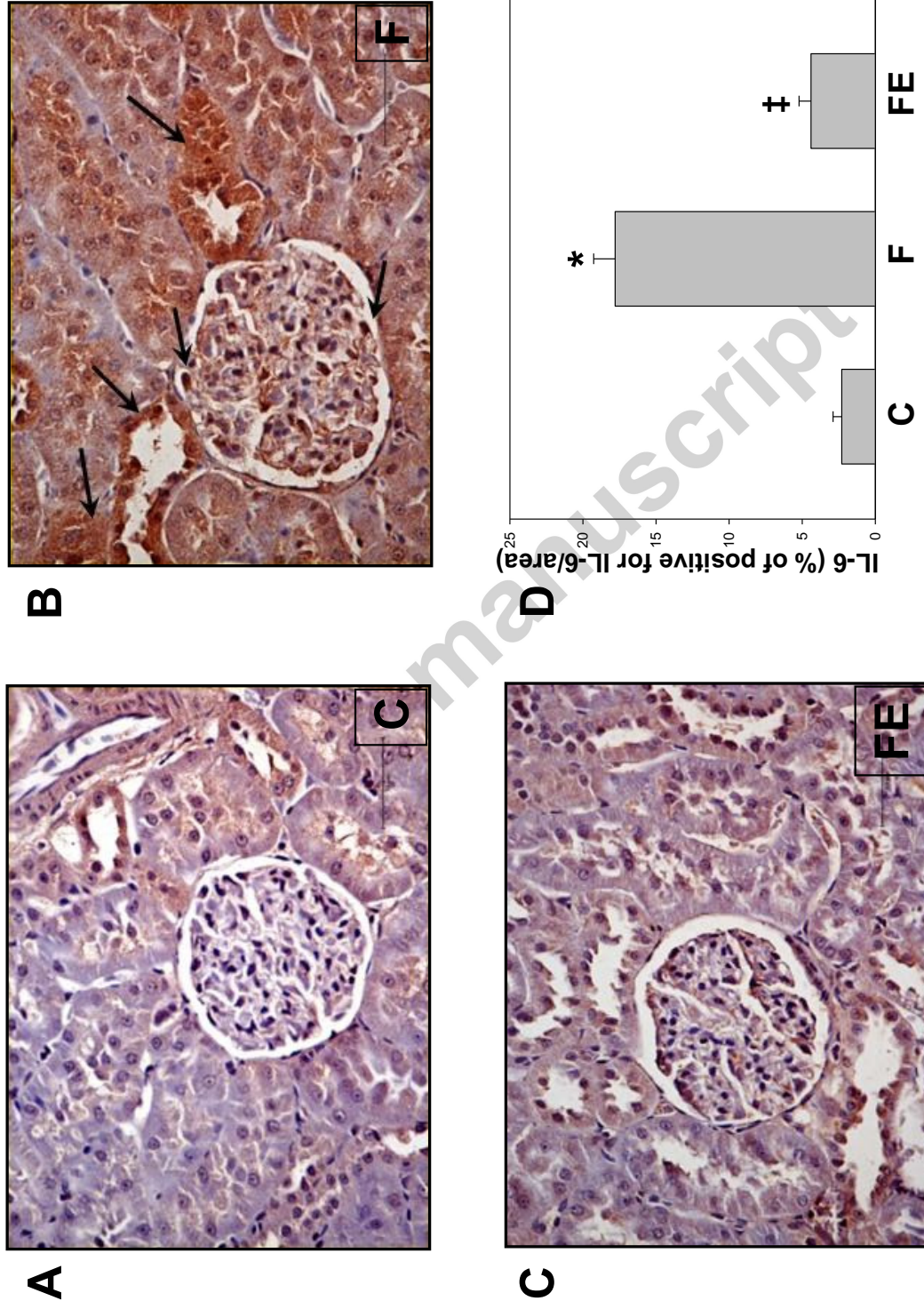


Figure 8