Article

Comparison of the renal effects of bisphenol A in mice with and without experimental diabetes. Role of sexual dimorphism

Rafael Moreno-Gómez-Toledano[1*], María I. Arenas[2*], Carmen Muñoz-Moreno[1], Nuria Olea-Herrero[1], Paula Reventun[3], Adriana Izquierdo-Lahuerta[5], Alba Antón-Cornejo[6], Marta González-Santander[1], Carlos Zaragoza[7], Marta Saura[4] and Ricardo J. Bosch[1‡§]

[1] Universidad de Alcalá, Laboratory of Renal Physiology and Experimental Nephrology, Group of Pathophysiology of the cardiovascular, renal and nervous systems, Department of Biological Systems/Physiology Unit, Alcalá de Henares, Spain

[2] Universidad de Alcalá, Department of Biomedicine and Biotechnology, Alcalá de Henares, Spain

[3] Department of Medicine, Division of Cardiology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[4] Universidad de Alcalá, Laboratory of Pathophysiology of the Vascular Wall, Group of Pathophysiology of the cardiovascular, renal and nervous systems, IRICYS, Department of System Biology/Physiology Unit, Alcalá de Henares, Spain

[5] University Rey Juan Carlos, Biochemistry and Molecular Biology area, Department of Basic Sciences of Health, Alcorcon, Spain

[6] Clinical Analysis Service, Principe de Asturias Hospital, Alcalá de Henares, Spain

[7] Laboratory of Cardiovascular Pathophysiology, Joint Translational Research Unit, University Francisco de Vitoria School of Medicine and Division of Cardiology, University Hospital Ramón y Cajal, Madrid, Spain

[*] These authors contributed equally to this work

[‡] Corresponding author
[§]E-mail: ricardoj.bosch@uah.es

Abstract

Bisphenol-A (BPA), a chemical -xenoestrogen- used in the production of the plastic lining of food and beverage containers, is present in the urine of almost the entire population. Recent studies have shown that BPA exposure is associated with podocytopathy, increased urinary albumin excretion (UAE), and hypertension. Since these changes are characteristic of early diabetic nephropathy (DN), we explored the renal effects of BPA and diabetes including the potential role of sexual dimorphism. Male and female mice were included in the following animals' groups: control mice (C), mice treated with 21.2 mg/Kg of BPA in the drinking water (BPA), diabetic mice induced by streptozotocin (D), and D mice treated with BPA (D+BPA). Male mice form the D+BPA group died by the tenth week of the study due probably to hydro-electrolytic disturbances.

Although BPA treated mice did not show an increase in serum creatinine, as observed in D and D+BPA groups, they displayed similar alteration to those of the D group, including increased in kidney damage biomarkers NGAL and KIM-1, UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli, as well as TGF-β, CHOP and PCNA upregulation. UAE, collapsed glomeruli, PCNA staining, TGF-β, NGAL and animal survival, significantly impaired in D+BPA animals. Moreover, UAE, collapsed glomeruli and animal survival also displayed a sexual dimorphism pattern.

In conclusion, oral administration of BPA is capable of promoting in the kidney alterations that resemble early DN. Further translational studies are needed to clarify the potential role of BPA in renal diseases, particularly in diabetic patients.

Keywords: Bisphenol A, mouse, diabetes, diabetic nephropathy.

Highlights

- Oral administration of BPA at a concentration below the "no observed adverse effect level" (NOAEL) promotes in the mice alterations that resemble early diabetic nephropathy.

-Mice receiving BPA develop proteinuria, hypertension, podocytopenia, apoptosis, collapsed glomeruli, as well as the upregulation of kidney damage biomarkers NGAL and KIM-1, and the TGF-β system.

- Proteinuria, collapsed glomeruli, and animal survival displayed a sexual dimorphism pattern in diabetic mice receiving BPA.

1. Introduction

Diabetes mellitus (DM) is a growing disease that affects an increasing number of people. In 1980 it affected 108 million people globally [1], increasing to 171 million in 2000 [2]. Interestingly, in 2014, the number of patients augmented to 422 million [1]. According to studies conducted by the International Diabetes Federation, it is estimated that almost 500 million people in the world are currently living with diabetes. That number is projected to reach 578 million in 2030 and 700 million in 2045 [3]. Of all of them, it is estimated that about half of patients with type 1 diabetes develop diabetic nephropathy (DN) throughout their lives, being 30-50% in the case of patients with type 2 diabetes [4].

In the same way, an increase in the prevalence of diabetic kidney disease has also been observed. Gheith et al. [4] determined an increase from 7.1% (1988-1994) to 10.7% (2005-2008) in people over 65 years of age. DN is also the most common cause of end-stage renal disease, having been observed in the United States to cause up to 80% of cases [5]. DN has been recognized as a worldwide medical catastrophe and is now the most important etiology of end-

stage renal failure in developed countries [6]. However, since not all diabetic patients suffer renal alterations, it is considered that there must be numerous environmental and biological factors yet to be determined.

Parallel to the increase in chronic diseases, an increase in exposure to xenobiotic compounds, such as bisphenol A (BPA), has been observed [7,8]. BPA is an essential element in manufacturing PVC and epoxy resins, used in numerous sectors such as the food industry, medical-surgical material, thermal paper cash register receipts, or even clothes [9–12]. In this way, BPA levels have been detected in the vast majority of foods, which manifests as significant levels of BPA in almost the entire human population studied, reaching percentages of 95% in the United States [13], 97% in Spain [14], 90-100% in China [15,16] or even 100% in Canada [17].

BPA is known to act as an endocrine disruptor, and due to its physical properties, it is capable of crossing any physiological barrier, such as the blood-brain or placental barrier [18]. In the last 20 years, various studies have determined that BPA can affect numerous organs and systems, such as the female and male reproductive system, the cardiovascular system and the endocrine system, or even the correct fetal development, to name a few examples [19–22]. In this sense, a pattern of sexual dimorphism has been observed during the gestational or perinatal period, compared to the same exposure to BPA, reaching differences on hepatic [23,24], adipogenic [25], neurological [26–28], endocrine [29], immunological [30], and renal development [31]. Pollock et al. [32] determined that the kidney is one of the organs where more BPA can be quantified, and this concentration rises before prolonged exposure over time, which also suggests the existence of a possible accumulation.

Epidemiological studies in humans suggest the possible association between high urinary BPA levels and predisposition to albuminuria [33,34] or hypertension [35,36]. Moreover, Olea et al. [37] in an experimental animal model have shown the development of podocytopathy and increased urinary albumin excretion (UAE) in mice after five weeks of i.p. exposure of BPA. Furthermore, BPA exposure has been associated with hypertension in mice [22].

Since these changes are characteristic of early DN, herein we explored the hypothesis that oral BPA exposure, being the most important one in mammals, might resemble DN and promote the progression of kidney damage. Moreover, we also studied well-characterized molecules involved in the pathophysiology of DN, such as the proapoptotic and profibrogenic TGF-β, and the well-known inflammatory mediators MCP-1 and TNF-α.

2. Material and methods

2.1. Animal model. CD1 mice (25–30 g) were used in all the experiments. All the studies were performed following guidelines established by Institutional Animal Care and Use Committees at the University of Alcalá. Mice were housed in a temperature-controlled room (21 ± 2°C) on a 14/10 h light/dark cycle under pathogen-free conditions and food ad libitum. Mice were given free access to water or BPA-containing water at a dose of 150 μg/ml (Sigma, Saint Louis, MO).

Four different animal groups were studied: Control (C), non-diabetic animals with BPA in the drinking water (BPA) 150 μg/ml, (21.2 mg/kg/day), diabetic animals without BPA (D), and diabetic animals with BPA in the drinking water (D+BPA).

In total forty non-diabetic mice were used, 20 mice as control and 20 mice treated with BPA. Diabetes was induced by three daily consecutive intraperitoneal injections of streptozotocin (STZ) (Sigma), 65 mg/kg body weight in citrate buffer, pH 4.5 (vehicle) [38]. Mice with blood glucose >300 mg/dL were included in the study (n=45). Male and female mice were used in approximately equal number with the exception of the male D+BPA group (17 male and 8 female) due to the mortality of the former group at 10 weeks. For this reason, the period of the

study was limited to eight weeks using 5 to 7 animals per group except for the Kaplan-Mayer survival table.

Mice were placed in metabolic cages, and 24-h urine was collected for biochemical measurement as previously reported [39]. After eight weeks of treatment and development of the diabetic model, the animals were sacrificed under isoflurane. Blood was taken by cardiac puncture under ether anesthesia for biochemical measurements. The right kidney of each animal was removed, weighed, frozen in liquid nitrogen, and stored at −80 °C for subsequent total RNA extraction. The left kidney was weighed and fixed in 10% buffered formaldehyde for morphological and immunohistochemistry studies. The degree of renal hypertrophy was expressed as an index, the ratio of kidney weight to total body weight.

2.2. Determination of biochemical test. Quantifications of plasma sodium, potassium, albumin, creatinine, and urine albumin and creatinine were performed with an ADVIA Chemistry system (Siemens Healthineers).

2.3. Immunohistochemistry. Serial sections (4 μm thicknesses) from mice kidneys formalin-fixed and embedded in paraffin were deparaffinized with xylene, rehydrated, and placed in 10 mM sodium citrate buffer, pH 6.0, and heated in a conventional pressure cooker for 2 min. The sections were allowed to cool for 20 min. After rinsing with distilled water, the sections were washed twice in Tris buffer saline (TBS), pH 7.6, for 5 min. Endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2 for 20 min. Sections were subsequently washed with distilled H_2O and TBS and incubated with 3% normal donkey serum plus 0.05% Triton X-100 in TBS (blocking solution) at room temperature for 30 min to prevent nonspecific binding of the first antibody. Afterward, sections were incubated overnight at 4°C with primary antibodies diluted in blocking solution, which was diluted 1:9 in TBS. (WT-1, 1:1000; CHOP, 1:300; PCNA, 1:1500; MCP-1, 1:400; 4-Hydroxynonenal (4-HNE) 1:250). Then, the sections were washed and incubated in primary antibodies amplifier Quanto (Ultravision Quanto detection system–peroxidase, Master Diagnóstica, Granada, Spain) for 10 min. After an extensive wash in TBS, the sections were incubated in polymer Quanto for 10 min. The peroxidase activity was detected using the DAB kit (Master Diagnóstica). Tissue sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in Entellan®.

The immunoreactivity of each focus of interest was measured using the stereological software Motic Images Advanced 3.2 (Motic China Group Co, Ltd). This program allows the selection of fields to be studied by systematic random sampling after the input of an appropriate sampling fraction. An average of 10 light microscopic fields per section was scanned using the x20 objective. The staining intensity was measured using a grey level scale (0: white; 255: black) with a negative image. All results are expressed as mean ± SEM.

2.4. TUNEL assay. For the detection of apoptotic cells, the DeadEndTM Fluorometric TUNEL System was applied (Promega, Madison, WI, USA) to paraffin-embedded sections according to the manufacturer's instructions. Then, the sections were incubated with an anti-FITC-HRP for 30 min. and the reaction product in nuclei was developed with DAB.

2.5. Total RNA extraction and quantitative RT-PCR. Total RNA was isolated from each mouse kidney homogenate obtained using TriReagent (Sigma-Aldrich). Total RNA was quantified by Nanodrop 1000 Spectrophotometer (Thermo Scientific). A total of 500 μg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Promega Kit according to the manufacturer's instructions (5 minutes 65°C and 1 hour 37°C). PCR was performed duplicated for each sample using an adequate dilution of cDNA as a template for different genes (see supplementary material Table S1 for SYBR Green primers and TaqMan probes). β-actin and 36B4 were used as housekeeping genes. The amplification was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) by using the following conditions: 2 minutes 50°C, 10 minutes 95°C, 40 cycles (15 seconds 95°C, 1 minute 60°C). Reagents were from Applied

Biosystems (for TaqMan probes) and SYBR Green (for SYBR Green primers). We used a standard curve method, using the untreated samples as a calibrator, to calculate the relative quantity of gene expression. We used the BestKeeper software tool (http://www.gene-quantification.info/) to validate housekeeping genes [40].

2.6. Measurement of blood pressure (tail cuff method). We used a non-invasive pressure gauge (LE 5001 Pressure Meter; Letica Scientific Instruments, Hospitalet, Spain) to measure the blood pressure. The measurements were always made at the same time of day, with the least possible stress for the animal (acoustic and luminous). Being nocturnal animals and having a high inactivity peak at 8 a.m., they were not manipulated until at least two hours later. The animals were prewarmed to 30°C with a heater (LE5660/6, Letica Scientific Instruments). The sphygmomanometer and the pulse transducer were never placed in the area near the animal's body because they can cause damage.

2.7. Statistical analysis. All results were expressed as mean ± SEM. $p < 0.05$ was considered statistically significant. To determine the effects of treatments, one-way ANOVA or Kruskal-Wallis followed by a Bonferroni or Dunns test, respectively, were carried out. To determine sex differences in the BPA and diabetes treatment, two-way ANOVA followed by a Tukey's multiple comparisons test or Sidak's multiple comparisons test were carried out. The *p*-values presented in figures and tables corresponded to post hoc test. All statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Animals display a sexual survival dimorphism pattern. As shown in figure 1A, the Kaplan-Meier animal survival analysis showed that all-male D+BPA mice died by the tenth week of treatment while no animal loses were found among the other experimental animal groups. In order to obtain all the biological samples needed to complete the animal's studies, some male animals were included in the studied groups as described in the methods section.

Besides dehydration, no other potential cause of death was observed in the bodies of dead mice. As shown in figure 1B, both D and D+BPA groups showed a significant increase in urinary output than the control littermates. However, only D group showed significant differences between males and females. As expected, these animal groups display an increased value of plasma sodium together with a decreased potassium (figures 1C and 1D), albeit these values did not display a sexual dimorphism pattern. Unfortunately, one limitation of the study is the lack of **biochemical** data beyond the study's eight weeks. Therefore, although the cause of the male D+BPA group's death cannot be determined with certainty, we can only assume that it was related to hydroelectrolytic disturbances.

3.2. Animal body weight and renal functional parameters. As previously described [41–44], control male mice showed a significant increase in body weight and renal hypertrophy compared to females ´littermates (figures 1E and 1F). As shown in figure 1E, compared to control mice, the average animal body weight was not affected in the BPA group, while both D and D+BPA groups showed a significant decrease in this parameter. Moreover, both control male and D male mice showed a significant increase in body weight compared to females' littermates. The D and D+BPA mice showed a significant increase in the renal hypertrophy index compared to the control group. Of note is that the females of the D+BPA group develop an increase in this parameter similar to males. In the rest of the groups, the females showed a lower hypertrophy index than their male littermates (figure 1F).

At 8 weeks of treatment, the glycemia of diabetic animals remained above 400 mg/dl in all animals, while BPA group were not significantly different from those of the C group (figure 2A).

As expected, all animal groups studied display a significant increase in UAE compared to the C group (figure 2B). UAE was significantly higher in the D group compared to the BPA group. Interestingly, male D+BPA mice display a significant increase in UAE compared to the BPA group, demonstrating a sexual dimorphism pattern compared to their female littermates.

Moreover, a significant decrease in plasma albumin was observed in D and D+BPA (figure 2C). Although endogenous creatinine clearance not showed significant differences among animal groups studied, plasma creatinine displayed a statistically significant increase in both D and D+BPA groups compared to their correspondent's controls (figure 2D and 2E). To further investigate BPA effects **at the molecular level on the kidney**, the gene expression of the kidney damage biomarkers Havcr-1 and Lidocalpin-2, which codify NGAL and KIM-1, respectively, were evaluated [45,46] (figure 2F and 2G). Both mRNAs were significantly increased in all treated mice in comparison to their control littermates. Interestingly, NGAL also showed a significant further increase in diabetic animals receiving BPA. Thus, kidney damage biomarkers supported the notion that BPA exposures to mice induced kidney damage in non-diabetic mice and exacerbated injury in diabetic mice. No sexual dimorphism was observed in these parameters.

As shown in figure 2H and 2I, systolic and diastolic arterial blood pressures were substantially higher in the three experimental models. No sexual dimorphism in this parameter was observed.

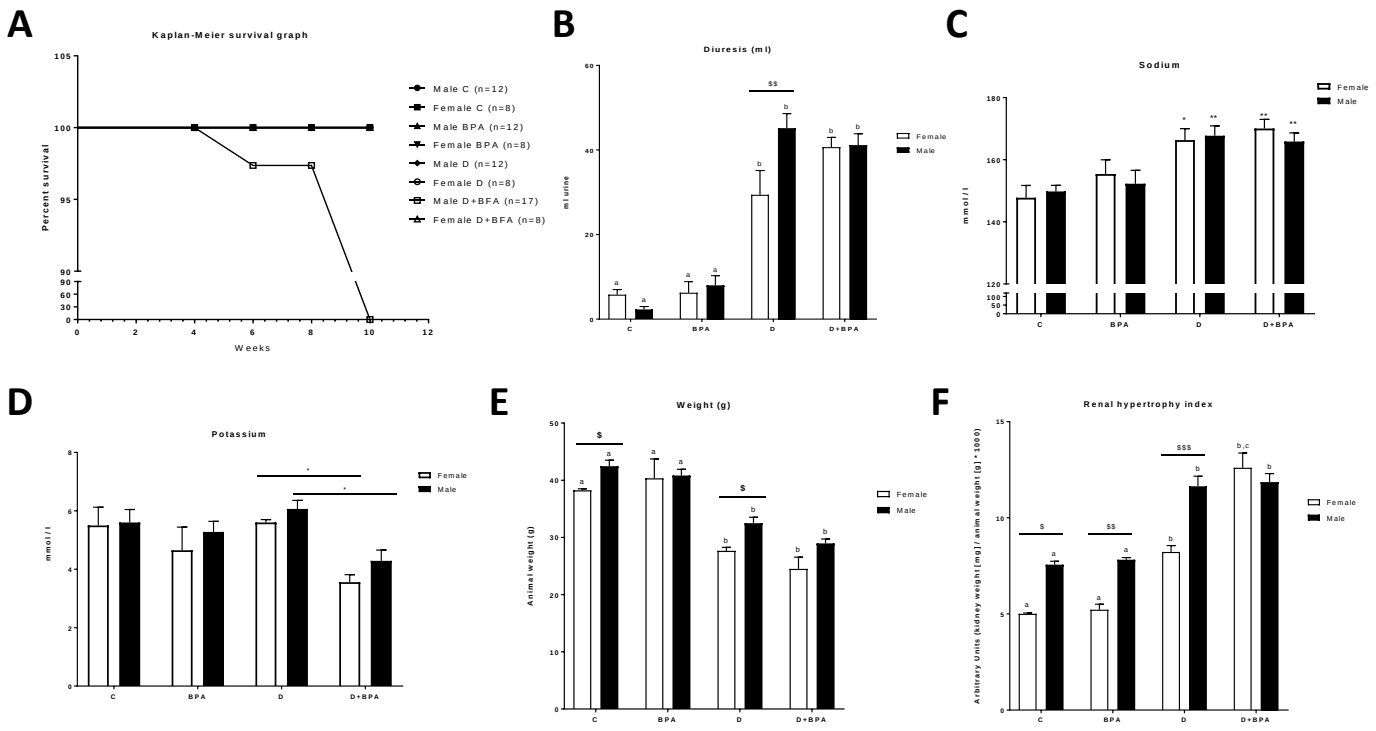


Figure 1. Survival, plasma ions, and physiological study. A. Kaplan-Meier survival graph. Note that the survival of all groups is 100%, except for the D+BPA group. For this reason, all symbols and lines overlap at the same height, preventing correct viewing. **B.** Body weight, in grams. Diabetic animals showed a significant decrease in body weight than C and BPA groups. Note that only C and D groups showed sexual dimorphism. **C.** Renal hypertrophy index (kidney weight [mg] / animal weight [g] * 1000). All diabetic animals showed a significant increase in renal hypertrophy index. Note that C, BPA, and D groups showed sexual dimorphism. **D.** 24-hour diuresis, in ml. All diabetic animals excreted volumes of urine between 5 and 10 times higher than control and BPA groups. **E.** Plasma sodium, in mmol/l. All diabetic animals showed elevated plasma sodium concentrations. **F.** Plasma potassium, in mmol/l. Only the D+BPA group showed low plasma potassium concentrations. All groups with different letters have significant differences ($p<0.01$). Letter c represents significant differences with diabetic female ($p<0.0001$). *$p<0.05$;

**p<0.01 comparing to their respective control group. $$p$<0.05; $$$p$<0.01; $$$$p$<0.001 comparing between males and females of the same group. The graph represents mean values ± SEM. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

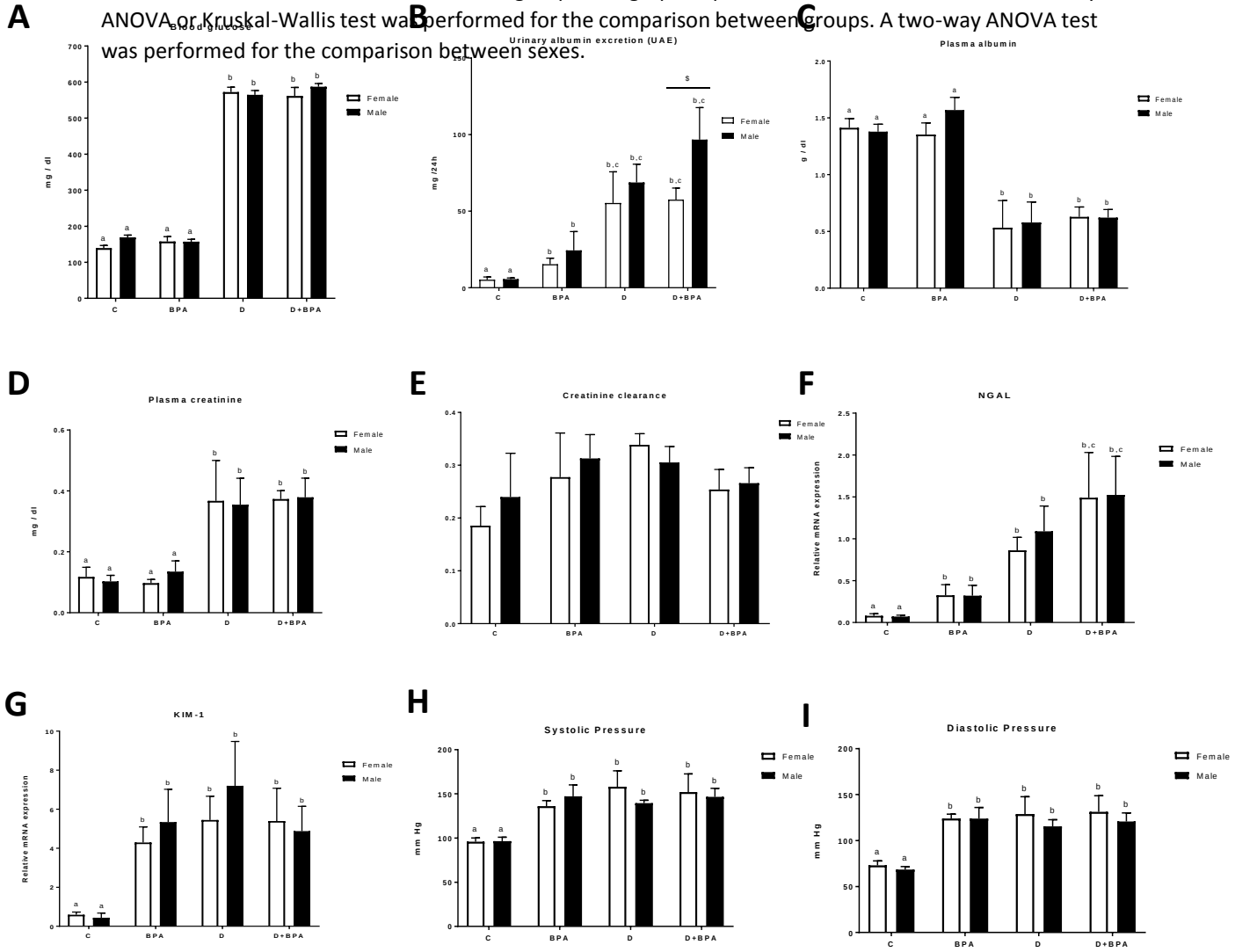


Figure 2. Biochemical study of the kidney and arterial pressure. A. Blood glucose. All diabetic animals showed higher blood glucose at the end of the experiment (8 weeks). **B.** Albuminuria, expressed in mg of albumin excreted in 24 hours. Note that all groups showed elevated levels of albuminuria compared with the control group. **C.** Plasma albumin, in g/dl. All diabetic animals showed low values of plasma albumin, in the nephrotic range. **D.** Plasma creatinine, in mg/dl. All diabetic animals showed high plasma creatinine values. **E.** Creatinine clearance. All groups showed similar creatinine clearance values. **F.** NGAL qPCR. **G.** KIM-1 qPCR. **H.** After eight weeks of treatment, all groups showed a significant increase in systolic pressure than the control group. **I.** Diastolic pressure showed the same pattern than systolic pressure. $$p$<0.05 comparing between males and females of the same group. All groups with different letters have significant differences (p<0.05). Letter c represents significant differences with BPA group (p<0.05). The graph represents mean values ± SEM. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

3.3. Histological, immunohistochemical, and Q-PCR studies. BPA and D groups display similar renal histological changes at both glomerular and tubular compartments, including collapsed glomeruli, tubular dilatation, sloughing off the tubuloepithelial cells, and hyaline casts in the tubules' lumen. Diabetic mice treated with BPA showed similar histological alterations observed

in these groups. Of note, glomerular damage analyzed as collapsed corpuscles percentage showed a sexual dimorphism pattern in BPA and D+BPA groups being male mice significantly impaired (figure 3).

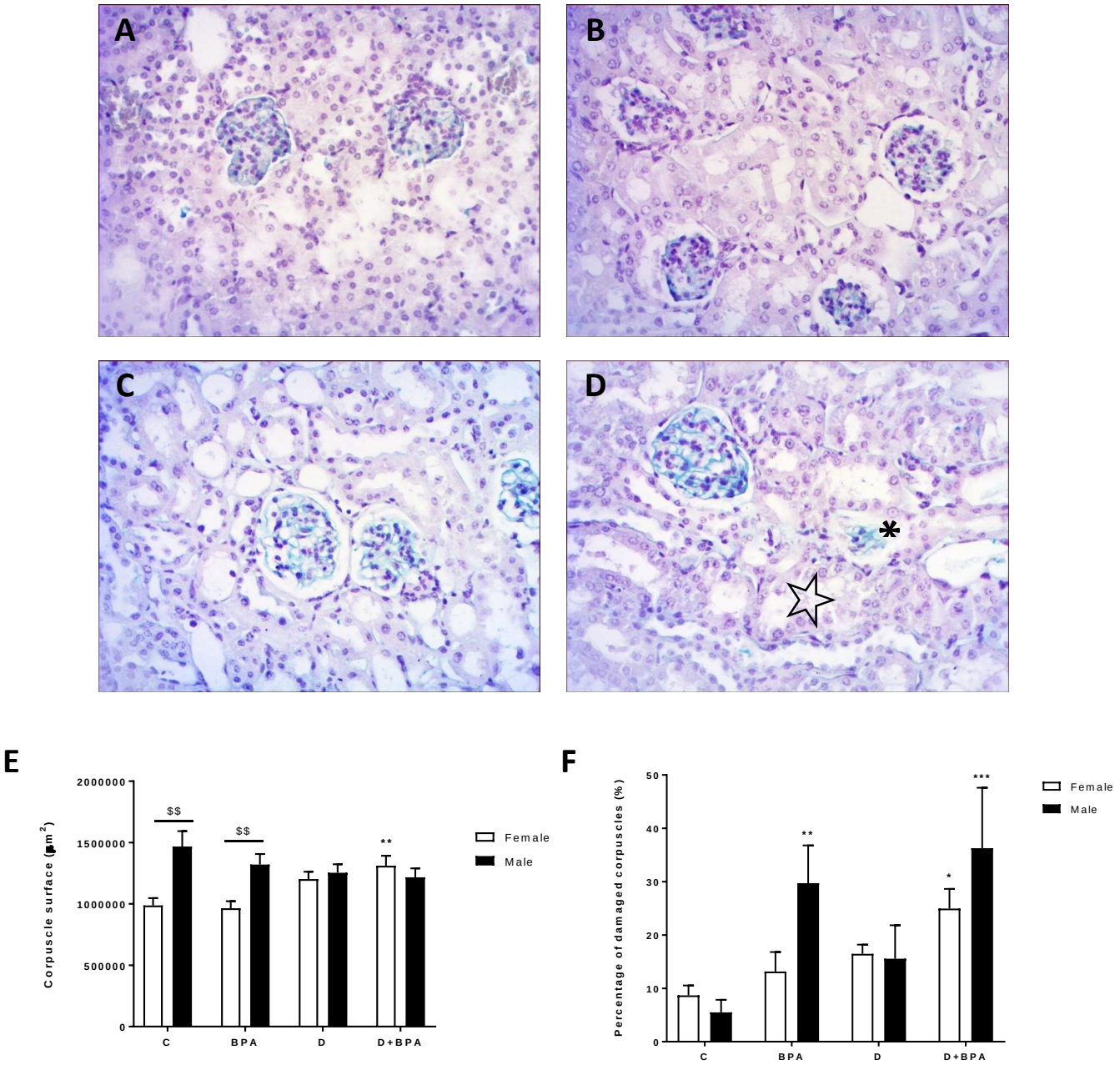


Figure 3. Histological examination (PAS-Alcian blue staining). A. Control kidney. **B.** Mice BPA-treated showed altered renal corpuscles and dilated convoluted tubules. **C.** The kidney of the diabetic mice showed both Bowman's capsules and convoluted tubules extremely dilated. **D.** D+BPA mice showed mesangial expansion, some corpuscles were destroyed (*), and the epithelium in some tubules was damaged (star) (X300). **E.** Histogram compares the corpuscular surface in each group observing statistically significant differences between females and males in both control and BPA-treated groups. **F.** Histogram represents the collapsed renal corpuscles percentage, showing a sexual dimorphism pattern in BPA and D+BPA groups. *p<0.05; **p<0.01 using one-way ANOVA or Kruskal-Wallis test for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes. $$$p$<0.01 comparing between males and females of the same group.

Immunolabeling with WT-1, a specific marker of podocytes, demonstrated that podocytes cell number was significantly decreased in all groups compared to their corresponding control

littermates (figure 4). Interestingly, D+BPA animals display a further decrease in this parameter compared to both BPA and D groups. No sexual dimorphisms were observed on this parameter.

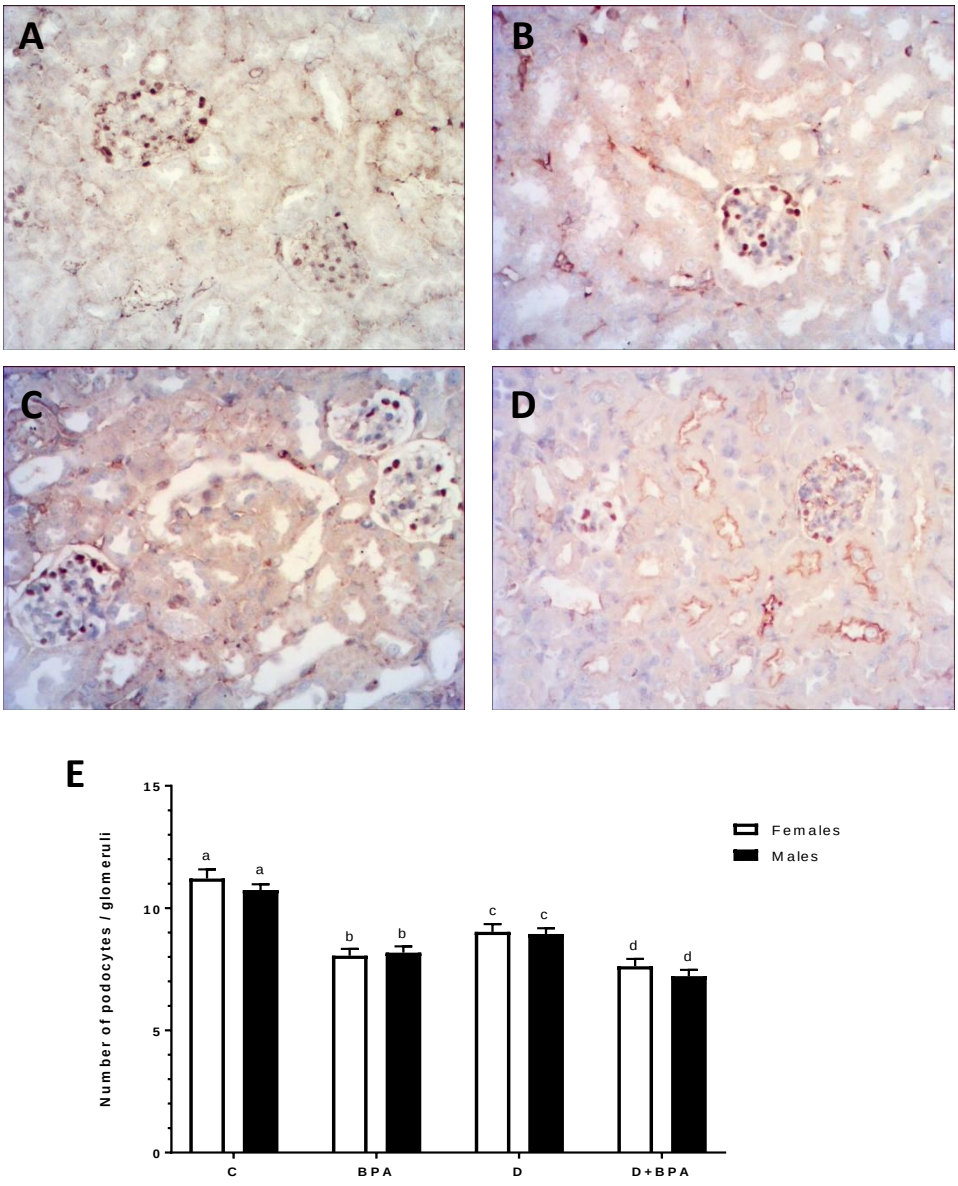


Figure 4. WT-1 immunostaining. A. Control kidney. **B.** Mice BPA-treated is showing a reduction in the podocytes number vs. control animal. **C.** Diabetic mice presented a higher podocyte number than BPA-treated animals but lower than control. **D.** D+BPA mice had the lowest number of podocytes (X300). **E.** The histogram shows statistically significant differences between all experimental groups. All groups with different letters have significant differences ($p<0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

Cell death, including apoptosis, as analyzed by CHOP immunohistochemistry and TUNEL assay, respectively, were significantly increased in all group studies in comparison to their control littermates. D group presented the highest optical density in CHOP immunostaining (figure 5). D+BPA group presented the highest number of apoptotic cells (figure 6).

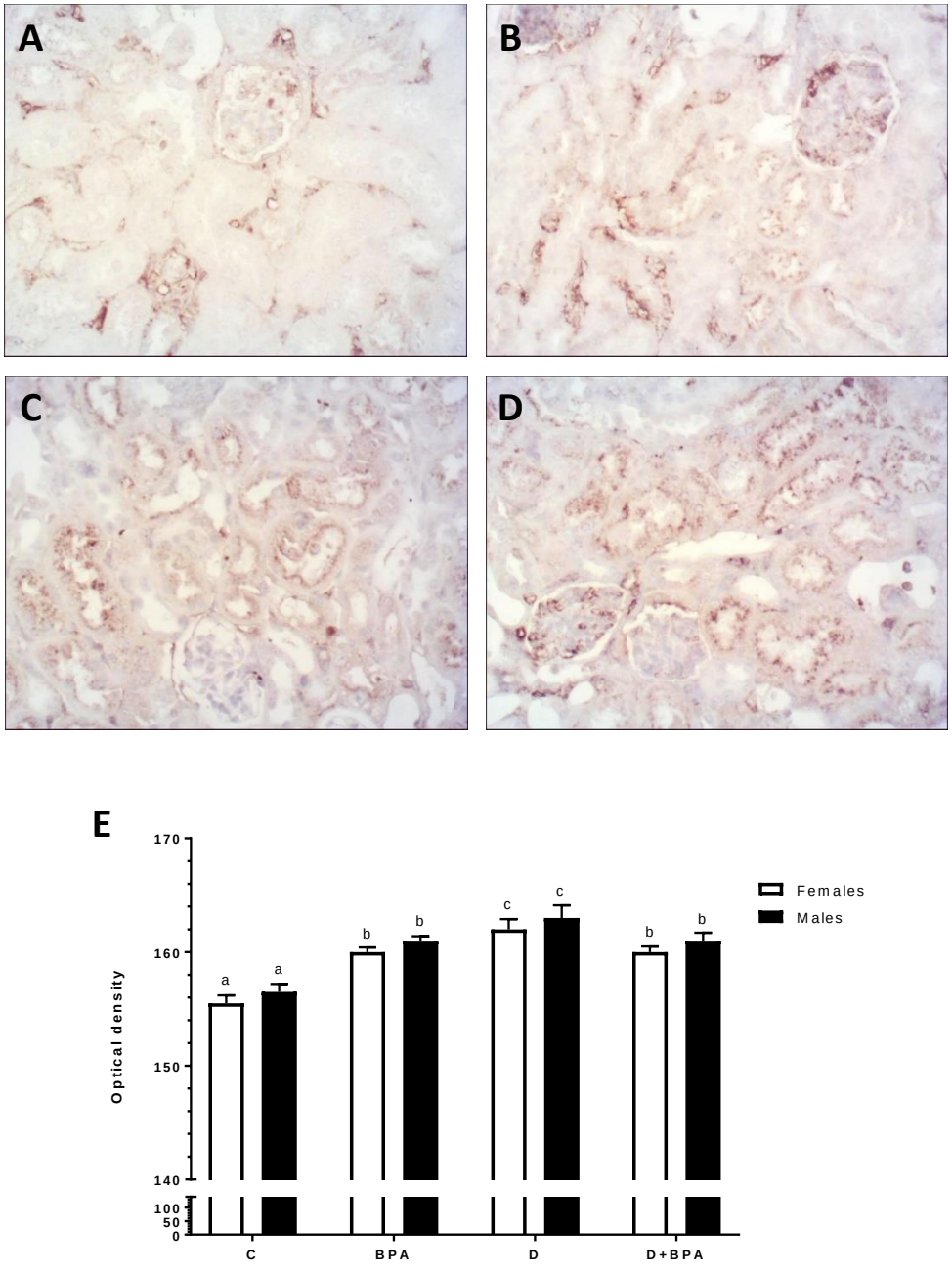


Figure 5. CHOP immunostaining. A. In the control group, the CHOP labeling was observed in the blood vessel endothelial cells. **B.** In BPA-treated kidneys, the immunolabeling was increased and appeared in the apical cytoplasm of the tubuloepithelial cells. **C.** Diabetic mice presented the highest immunoreaction to CHOP antibody. **D.** D+BPA mice presented elevated immunostaining but lower than in diabetic mice (X300). **E.** Histogram showing the optical density of CHOP immunostaining. All groups with different letters have significant differences ($p<0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

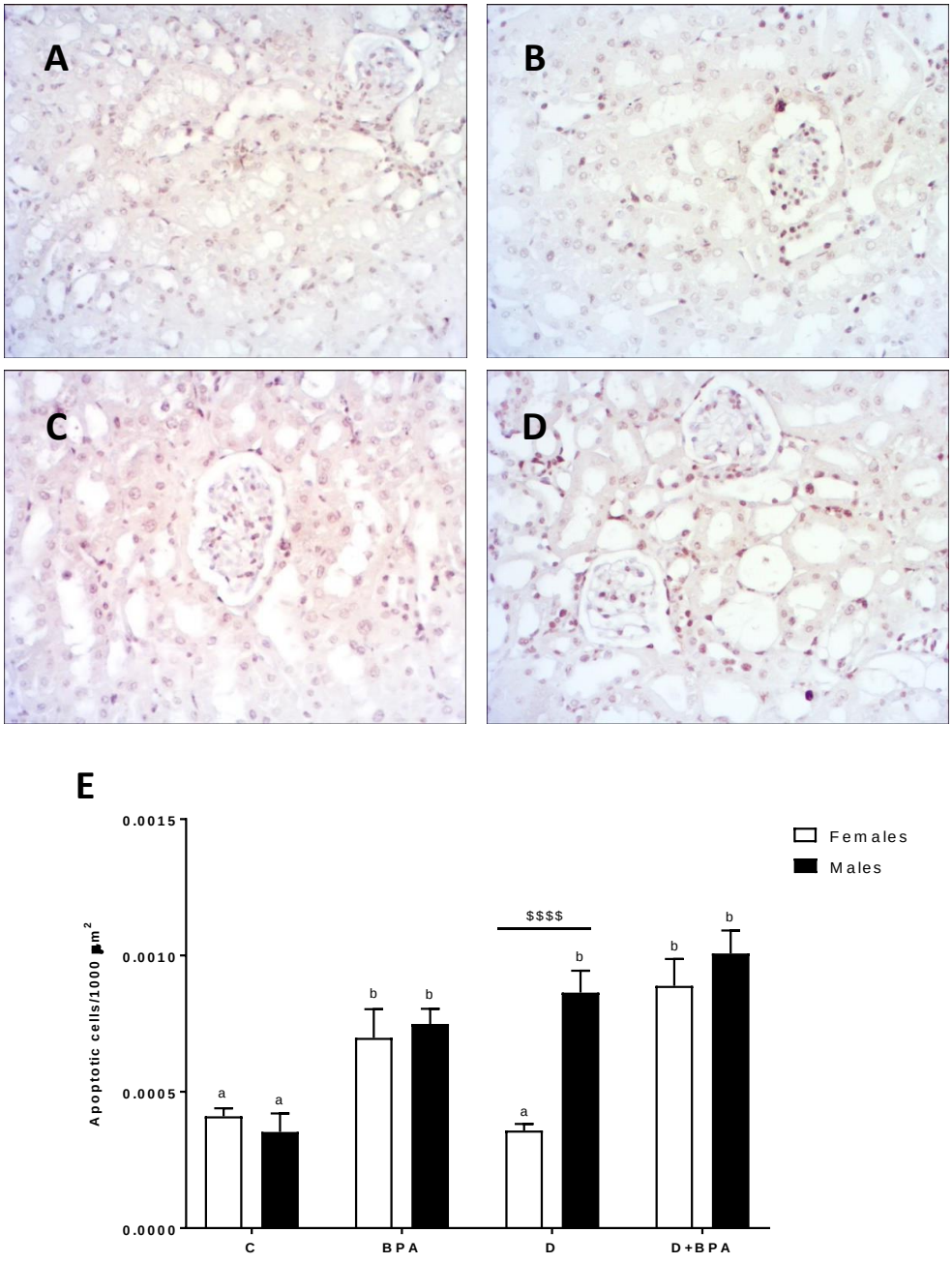


Figure 6. TUNEL assay. A. Kidneys from control the group presented a small number of apoptotic cells. **B.** BPA-treated mice presented a higher number of apoptotic cells than controls. **C.** In diabetic mice, kidneys from males had a high number of dead cells; however, the female mice were similar to the control group. **D.** D+BPA mice showed the highest number of apoptotic cells (X300). **E.** Histogram shows the gender differences in each group. $$$$ p<0.0001 females vs. males. All groups with different letters have significant differences (p<0.05). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

To explore if there was a compensatory mitotic response to the observed increase in cell death, cell proliferation was analyzed by PCNA immunostaining. Both BPA and D groups develop a significant proliferative response, even higher in female mice than their male littermates (figure 7). Interestingly, D+BPA group also showed PCNA-upregulation compared to their control littermates, albeit without sexual dimorphism.

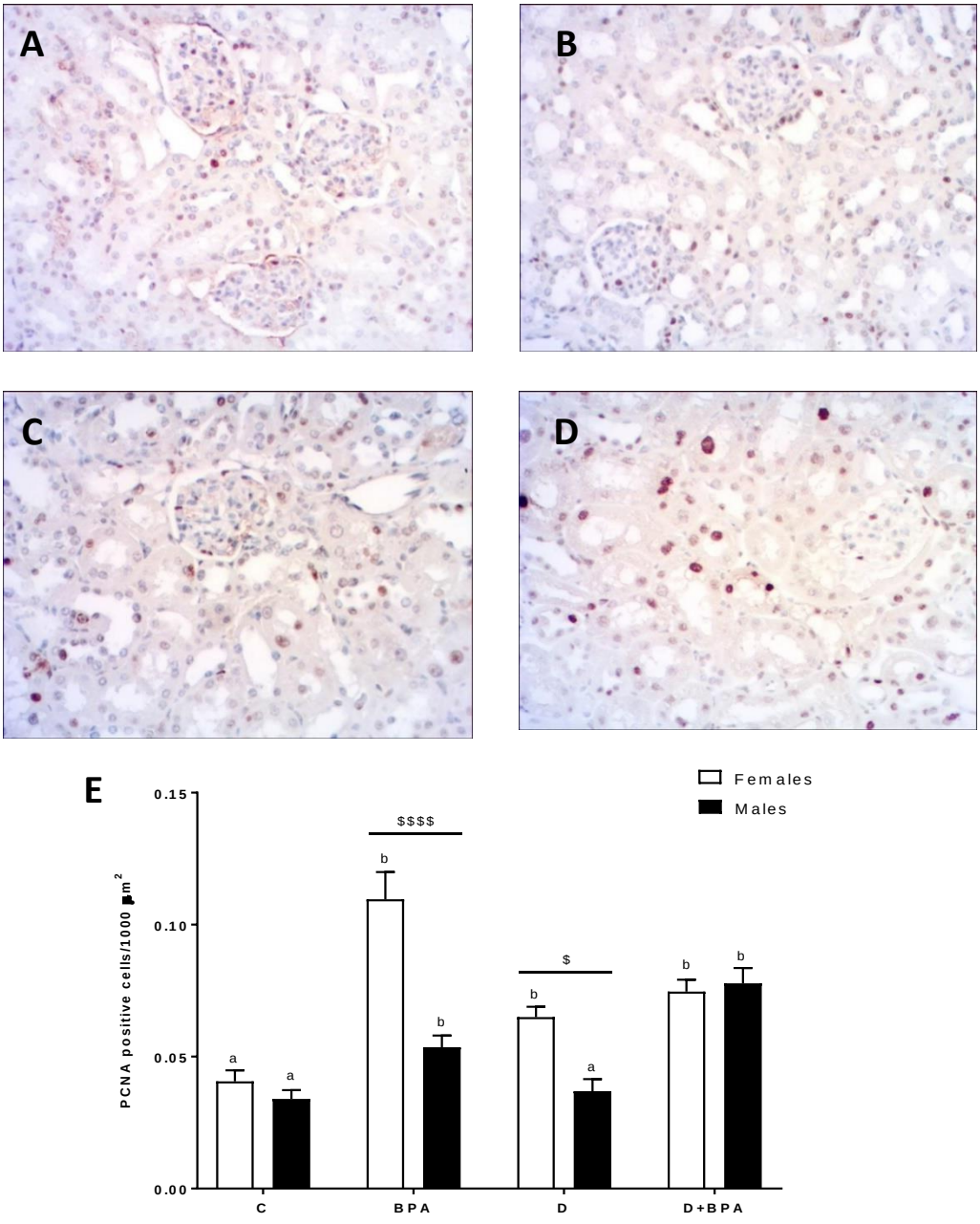


Figure 7. PCNA immunostaining. A. Control groups showed a low number of proliferating cells. **B.** Kidneys from BPA-treated mice presented a higher number of PCNA positive cells than control groups, especially females. **C.** Diabetic mice showed a number similar to control in males and higher in females. **D.** D+BPA mice presented a high number of proliferating cells without any difference between gender (X300). **E.** Histogram representing the gender differences in each group. All groups with different letters have significant differences ($p<0.05$). $\$p<0.05$; $\$\$\$\$p<0.0001$ females vs. males. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

We then explored the potential role of oxidative stress as an inflammatory mediator by analyzing lipid peroxidation as 4-HNE staining (figure 8). Control and BPA animals showed a sexual dimorphism pattern being significantly higher in the male. All treated mice, except D+BPA male displayed a significant increase in comparison to their control littermates.

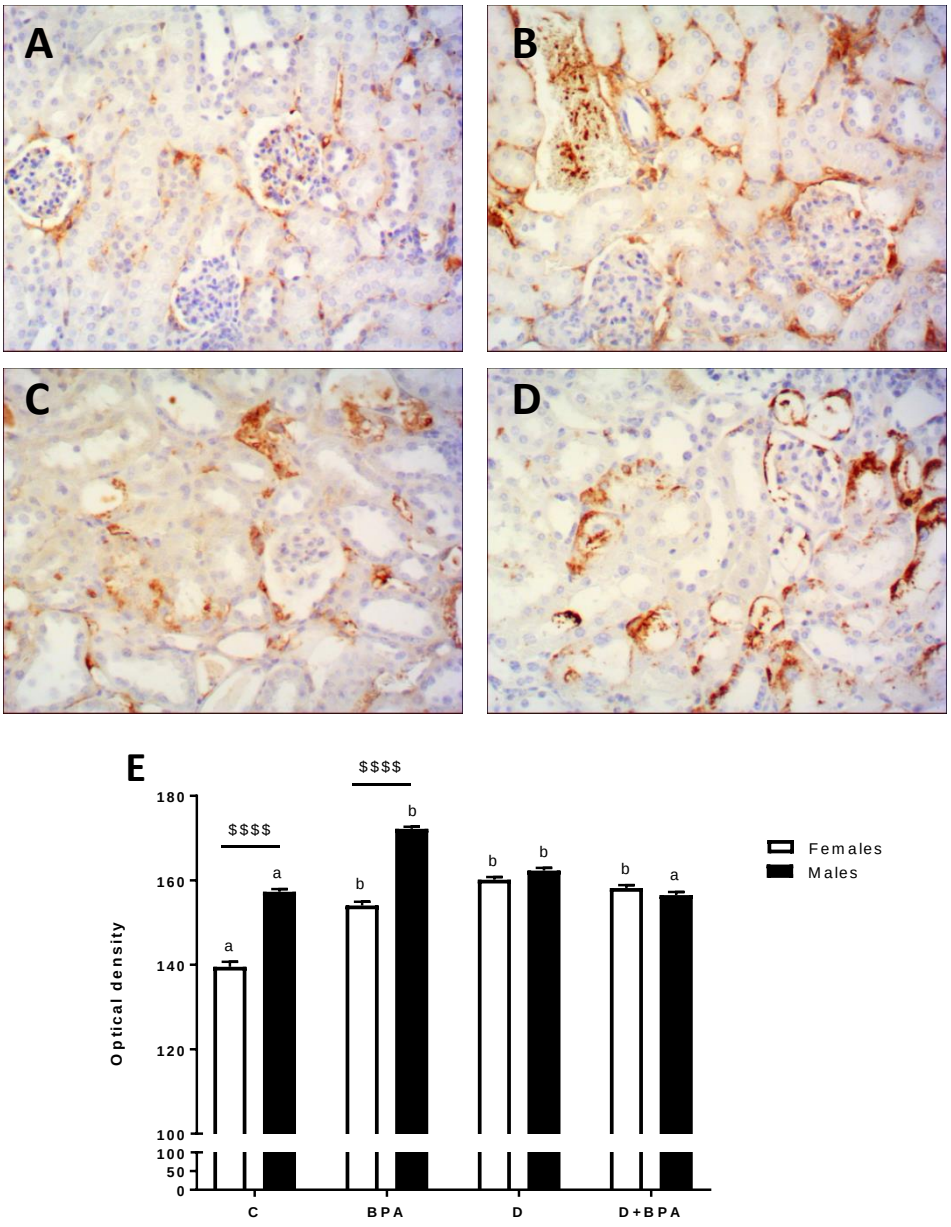


Figure 8. 4-HNE immunolabeling. **A.** Control group. **B.** BPA-mice showed an important 4-HNE elevation, compared to their control group. Furthermore, BPA was the only treated group with sexual dimorphism pattern, similar than control. **C.** D group showed an important elevation without sexual dimorphism pattern. **D.** Only females D+BPA showed higher 4-HNE staining. **E.** Histogram represents the optical density of 4-HNE immunostaining. All groups with different letters have significant differences ($p<0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

We finally explored the potential role of several inflammatory mediators in the present experimental model (figures 9 and 10). No significant changes in the expression of TNF-α, MCP-1, and IL-1β were observed in BPA-treated groups (figures 9A, B, C). The same expression pattern was observed in MCP-1 immunolabeling (figure 10). By contrast, all of them were significantly upregulated in D animal group. Moreover, D+BPA group showed a significant upregulation of MCP-1. Figure 9D showed a significant upregulation of IL-10 restricted to BPA animal group. TGF-β and its receptor, a well-characterized system involved in both BPA and renal diabetic damage, were analyzed by Q-PCR. TGF-β was significantly upregulated in all animal groups, being significantly higher in the D+BPA group. The latter observation suggests that BPA might potentiate TGF-β upregulation in diabetic mice (figure 9E). Interestingly, the D+BPA group also displays the upregulation of the TGF-β receptor (figure 9F). No sexual dimorphisms were observed on these parameters.

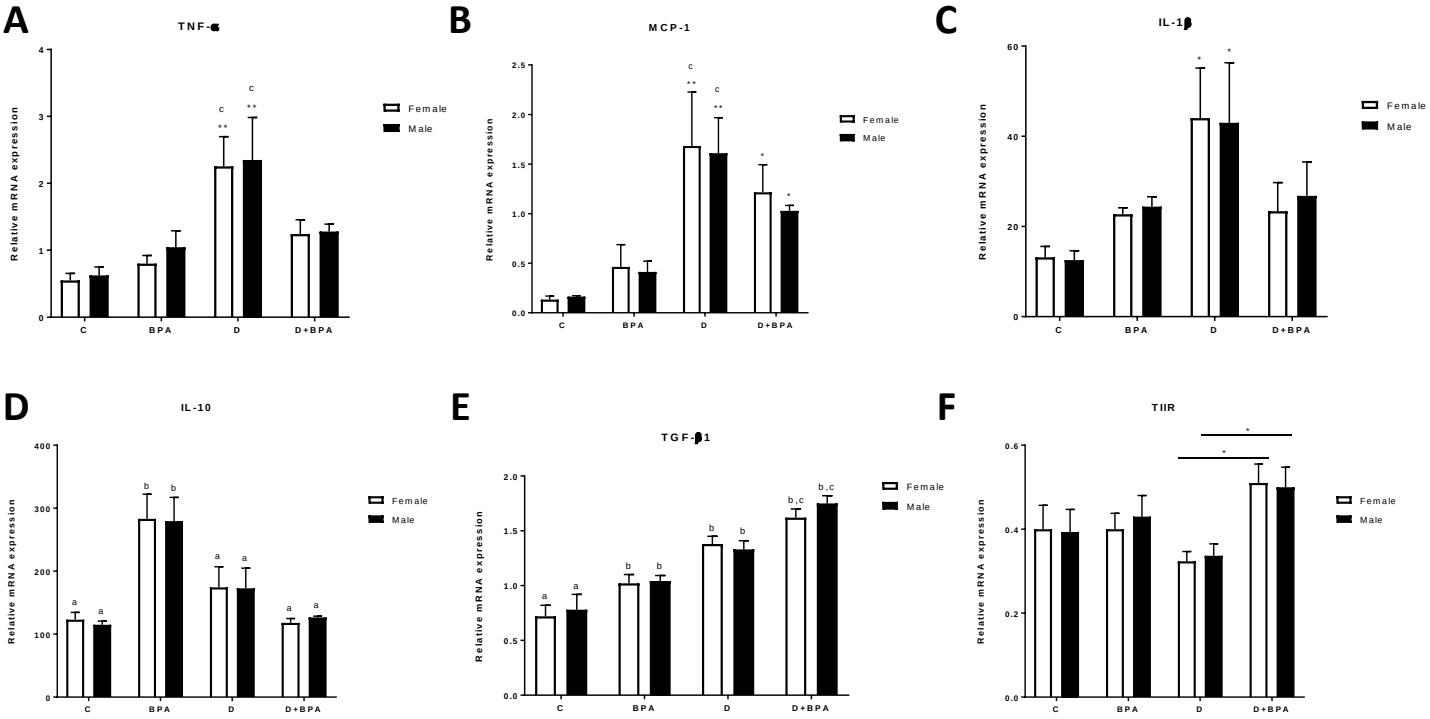


Figure 9. Quantitative RT-PCR. A. TNF-α. The highest relative expression of the inflammatory messenger corresponds to the D group. **B.** MCP-1. As in the previous case, the highest relative expression of the inflammatory marker MCP-1 corresponds to the D group. **C.** IL-1β. The same expression pattern was observed as in the other two pro-inflammatory cytokines. **D.** IL-10. The expression of this anti-inflammatory cytokine only increased in the BPA group **E.** TGF-β1. D+BPA group showed the highest relative expression of TGF-β1 mRNA. **F.** TIIR. The only differences are observed between groups D and D+BPA. *p<0.05; **p<0.01 using the Kruskal-Wallis test for the comparison between groups. All groups with different letters have significant differences (p<0.05). Letter c represents significant differences with BPA group (p<0.05). A two-way ANOVA test was performed for the comparison between sexes.

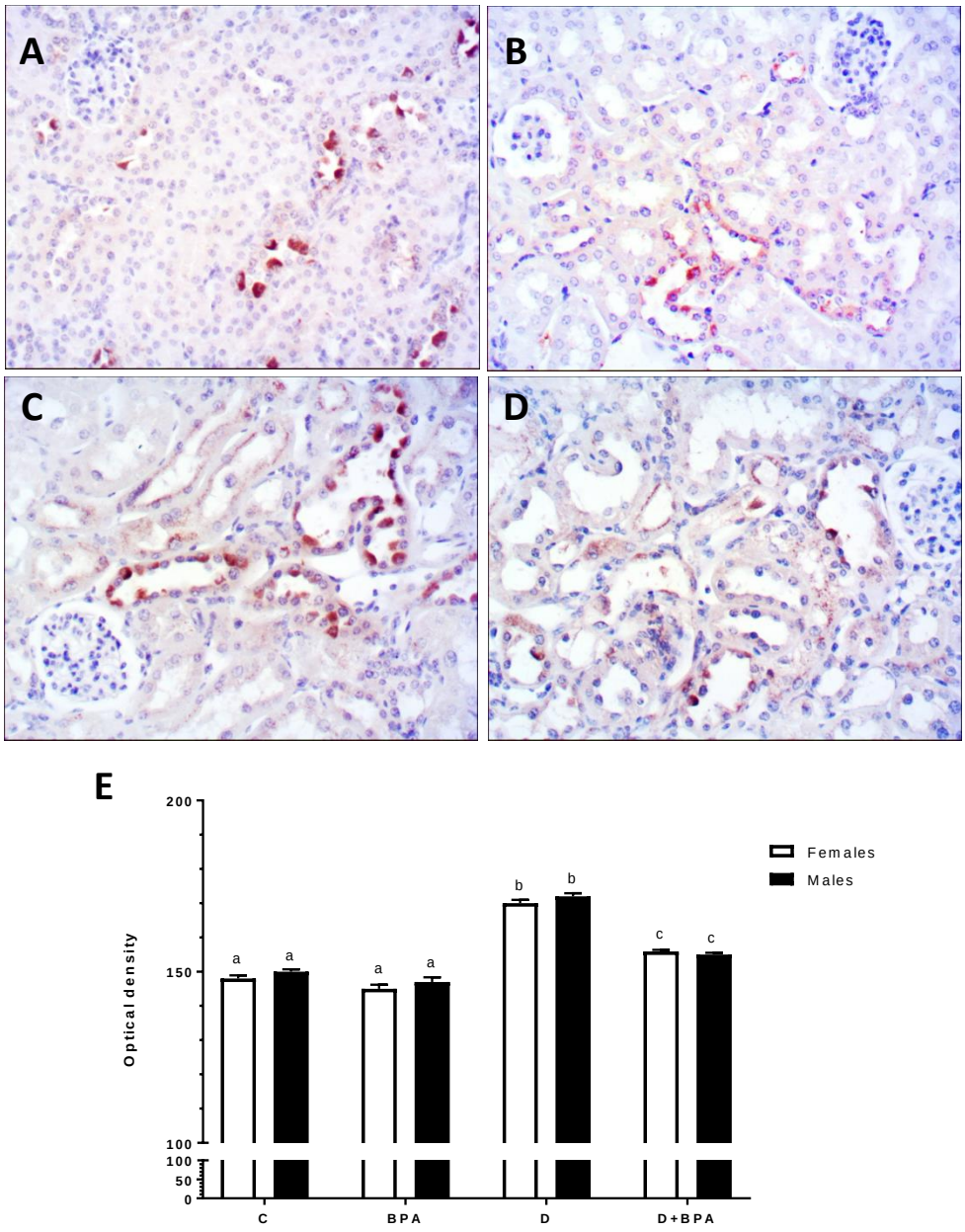


Figure 10. MCP-1 immunolabeling. A. In control kidneys, MCP-1 was observed in the cytoplasm of some epithelial cells of the collecting tubules. **B**. In the BPA group, the labeling was similar to the control group. **C.** Diabetic mice showed a high number of labeled cells and higher intensity in the MCP-1 expression. **D.** Kidneys from D+BPA mice presented an elevated immunolabeling to MCP-1 antibody but with less intensity than diabetic animals (X300). **E.** Histogram represents the optical density of MCP-1 immunostaining. All groups with different letters have significant differences ($p<0.0001$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

4. Discussion

Experimental and human studies have demonstrated a significant association between urinary excretion of BPA and albuminuria, a well-known factor involved in the mechanism of renal disease progression [33,34,47,48]. In this regard, Hu et al. [47], in a prospective study of 302 patients followed for six years, have demonstrated serum BPA as a predictor of chronic kidney disease in primary hypertension. Serum BPA was also described as a risk factor in the progression of diabetic nephropathy in patients with type 2 diabetes [48]. Our present study provides an experimental animal model to support these findings since we describe that the oral administration of BPA is capable of promoting in the kidney pathophysiological and molecular alterations that resemble early DN, such as increased in UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli as well as CHOP and TGF-β system upregulation.

We presently used the STZ model, which is considered the 'work horse' for experimental studies in diabetic nephropathy [49,50]. Consistent with previous findings in this model, diabetic mice developed renal hypertrophy and an increase in UAE, hypertension, podocytopenia, apoptosis [39,51–55], and the upregulation of CHOP, PCNA, MCP-1, and TGF-β throughout this study [51,52].

Regarding BPA doses used in our study's, animals received 150 µg/ml (21.2 mg/kg). Currently, it is accepted that the "no observed adverse effect level", known as NOAEL, in the renal system is 50 mg/kg [56,57], which is at least twice times higher than the dose used. European Food Safety Authority (EFSA) currently uses this NOAEL dose to estimate the tolerable daily intake (TDI) of 4 µg/kg in humans [57–59]. In the EFSA 2015 report, it is estimated that the total exposure to BPA in the population could reach almost half of the TDI (around 1.5 µg/kg) [57], which is consistent with using an equivalent concentration half of the NOAEL in animal models.

BPA-treated mice did not display significant polyuria as expected from previous publish [37] and unpublished observations using BPA concentration higher than renal NOAEL. Therefore, polyuria and the risk of higher BPA exposure were restricted to D+BPA mice. In any case, the BPA group developed similar renal changes to those of the diabetic group. Although this represents a limitation in our studies, it emphasizes the role of BPA exposure and the importance of maintaining strict metabolic control of diabetic patients to avoid osmotic diuresis and BPA overexposure.

One unexpected finding of this study was that all male mice from the D+BPA group died by the tenth week of the experimental procedure. Unfortunately, a limitation of the study is the lack of data beyond eight weeks. Therefore, albeit the cause of the death cannot be determined with certainty, we can only assume that it was related to hydroelectrolytic disturbances in animals with impaired renal function.

In accordance with previous works [41–44], control male mice showed increased in body weight and renal hypertrophy compared to female littermates. Besides the observed sexual survival dimorphism pattern, other significant pathophysiological differences between male and female animals were found. Treated male animals display significant increases UAE, and renal damage analyzed as collapsed glomeruli. Renal cell proliferation, as analyzed by PCNA staining, was the only parameter found to be significantly higher in female animals than in the males' littermates.

Although endogenous creatinine clearance showed no significant differences among animal groups studied, serum creatinine displayed a statistically significant increase in both D and D+BPA groups compared to their correspondent's controls. It is known that to a variable degree, tubules secrete creatinine, which, by itself, would lead to an overestimation of renal function [60]. To further investigate BPA effects at the molecular level on the kidney, the gene expression of the kidney damage biomarkers Havcr-1 and Lidocalpin-2, which codify KIM-1 and NGAL, respectively, were evaluated [45,46]. Both genes were significantly increased in all treated mice in comparison to their control littermates. Interestingly, NGAL also showed a significant further

increase in diabetic animals receiving BPA. All renal functional data studied supported the notion that BPA exposures to mice induced kidney damage in control mice and exacerbated injury in diabetic mice.

All treated male and female mice showed similar impairment of renal function. In contrast, male mice displayed a significant increase in urinary output restricted to D animal group, and female mice showed a significant increase in cell proliferation in this animal group. It is thus tempering to speculate, albeit not probed in the present setting, that increased urinary output could predispose to hydro electrolyte disturbances while increased in renal cell proliferation could, at least in theory, partially explain differences in sexual survival dimorphism pattern. On the other hand, D+BPA animal group did not display an additional effect on cell proliferation.

Several studies have demonstrated that most renal cells grown in high glucose conditions initially present a self-limited proliferation, followed by cell cycle arrest in the G1-phase, undergoing cellular hypertrophy [51,61]. These cellular events require the combined effect of mitogen-induced entry into the cell cycle and subsequent arrest at the G1 modulated by cell cycle regulatory proteins, including TGF-β1. Therefore, it is possible to speculate that the intense hypertrophy response observed in the D+BPA group may abrogate the mitogenic effect induced by BPA.

Sexual dimorphism could be due to different susceptibility to BPA, physiological differences, including sexual hormones, particularly androgen [62], or a combination of both. Indeed, different responses in males and females to the same exposure to BPA have been found. Most of the studies have been carried out mainly on the gestational or perinatal effect, where it has been determined that BPA can exert a different effect in males and females at the hepatic [23,24], adipogenic [25], neurological [26–28], endocrine [29], immunological [30], or renal level [31]. Furthermore, it has been observed that BPA is capable of inducing an agonist action on the estrogen receptor and an antagonist on the androgen receptor [63].

On the other hand, there is evidence that suggests that females have higher nephroprotection than males. In experimental animal models, female rats have increased protection against post-ischemic renal failure [64], doxorubicin treatment [65], and cisplatin treatment [66]. Epidemiological studies in humans also suggest the existence of nephroprotection in women. In this sense, one of the most extensive studies carried out is that of Neugarten et al. [67], where after performing a meta-analysis with 68 different studies and a total of 11,345 patients, they determined that men with chronic kidney disease caused by various etiologies show a much faster decline in kidney function than women. In another epidemiological study with 27,805 patients with type I diabetes, a statistically significant relationship was observed between the male sex and microalbuminuria development, an early marker of kidney disease [68].

Furthermore, probably because a BPA dose equivalent to half the NOAEL was used, the BPA group results do not show remarkable differences to the control group at the biometric and biochemical levels. However, an increase in albuminuria (less than in the diabetic groups, but equally significant) and a significant increase in blood pressure were observed. On the other hand, the histological studies showed interesting differences to the control group by showing the collapsed glomeruli, and a reduction in the number of cells, with the subsequent confirmation by TUNEL of the increase of apoptotic cells and a reduction in the number of podocytes labeled with WT-1.

Regarding sexual dimorphism, were observed differences in the renal hypertrophy index and albuminuria, with males presenting a higher number in both parameters. Although podocyte (WT-1) immunostaining showed no sexual dimorphism, particularly in the D+BPA group, the number collapsing glomerulus did show sexual dimorphism. Thus, damaged glomeruli could account for the observed increase in the UAE in the male D+BPA group.

In general, the results show that the route of administration of BPA and the dose used after eight weeks of treatment can exert an effect on the renal system similar to that described in the early stages of the development of diabetic nephropathy [69,70] at a dose lower than that considered "NOAEL" of the renal system [56].

Although there are limitations when using mouse models for assessing renal failure or long-term histomorphological changes [71], our findings may have pathophysiological implications since the amount of proteinuria, podocyte number and the upregulation of kidney damage biomarkers are reliable predictors of the progression of renal disease [37,72–75]. Together with our present findings, all the available data strongly suggest that even low-grade proteinuria associated with BPA exposure might involve podocyte damage of an uncertain (or as yet unexplored) outcome, jointly with the biomarkers mentioned above are indicators of the need for future studies and raising a red flag of caution against increasing BPA exposure. Our data agree with Ruiz-Priego et al. [76], supporting the use of NGAL and KIM-1 as biomarkers of BPA-nephropathy.

Animals treated with BPA showed a significant increase in the expression of the CHOP protein. The kidney is one of the organs with the highest ER stress susceptibility because the fractional protein synthesis rates are almost half the total body load daily [77]. The possibility that ER stress is involved in the development of diabetic nephropathy has been described. Among the three signaling pathways that make up the ER stress response system, two of them are protective, and the remaining one, ERK-ATF4-CHOP, induces apoptosis in some kidney diseases [78]. It is interesting to note that both diabetic animals (as in other publications [79]) and those treated with BPA showed high CHOP levels. In this way, the hypothesis that BPA can induce similar damage to diabetic nephropathy is reaffirmed.

In order to get inside into the molecular mechanisms involves in the renal changes observed, we first analyzed the proapoptotic and profibrogenic TGF-β system. As expected, diabetic and BPA groups display the upregulation of TGF-β, a cytokine involved in hypertrophy, apoptosis, and renal fibrosis. Interestingly, this upregulation was significantly higher in the D+BPA group, thus suggesting a molecular mechanism for the observed renal parameters impairment in the latter group.

We then analyzed the renal expression of MCP-1, TNF-α and IL-1β well-known pro-inflammatory mediators in DN [80]. As expected, these molecules were found to be upregulated in D mice. On the contrary, BPA mice did not show changes in the expression of these pro-inflammatory mediators. It is well stablished that BPA can promote an inflammatory response by upregulating inflammatory mediators such as IL-1β, TNF-α, or MCP-1 [76,81,82], it is also known that it can also trigger the expression of anti-inflammatory molecules like IL-10 or TGF-β [83,84]. Herein we observed a significant upregulation of IL-10 restricted to BPA-treated mice. This finding could account, at least in part, for the lack of activation of proinflammatory mediators in this animal group.

In any case, recent observations using a BPA concentration higher than renal NOAEL, demonstrate the upregulation of several proinflammatory mediators (including MCP-1, RANTES or IL-6) [76]. All data available strongly suggest that the level of BPA exposure might be critical to promote renal inflammation.

In conclusion, we observed that oral administration of BPA is capable of promoting in the kidney alterations that resemble early DN, such as increased in kidney damage biomarkers NGAL and KIM-1, UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli as well as TGF-β, CHOP and PCNA upregulation, albeit they did not develop changes in serum creatinine as observed in D mice. Moreover, UAE, collapsed glomeruli, PCNA staining, TGF-β (TIIR), and animal

survival, significantly impaired in diabetic animals receiving BPA. Furthermore, UAE, collapsed glomeruli and animal survival also displayed a sexual dimorphism pattern.

Collectively, these data show that further translational studies are needed to clarify the potential role of BPA in renal diseases, particularly in diabetic patients.

5. Funding

This work was supported in part by grants from Instituto de Salud Carlos III (PI15/02139) -Fondo Europeo de Desarrollo Regional (FEDER)-.

R. Moreno-Gómez-Toledano is recipient of a research contract from CAM (B2017-BMD-3686).

6. Acknowledgments

We want to thank M. Bosch for proofreading of the manuscript. We also want to thank Dr. Jordi Bover (Nephrology Service, Germans Trias i Pujol Hospital, Universitat Autònoma de Barcelona) for his suggestions and comments.

7. References

[1] B. Zhou, Y. Lu, K. Hajifathalian, J. Bentham, M. Di Cesare, G. Danaei, H. Bixby, M.J. Cowan, M.K. Ali, C. Taddei, W.C. Lo, B. Reis-Santos, G.A. Stevens, L.M. Riley, J.J. Miranda, P. Bjerregaard, J.A. Rivera, H.M. Fouad, G. Ma, J.Z. Cisneros, Worldwide trends in diabetes since 1980: A pooled analysis of 751 population-based studies with 4.4 million participants, Lancet. 387 (2016) 1513–1530. https://doi.org/10.1016/S0140-6736(16)00618-8.

[2] S. Wild, G. Roglic, A. Green, R. Sicree, H. King, Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030, Diabetes Care. 27 (2004) 1047–1053. https://doi.org/10.2337/diacare.27.5.1047.

[3] P. Saeedi, I. Petersohn, P. Salpea, B. Malanda, S. Karuranga, N. Unwin, S. Colagiuri, L. Guariguata, A.A. Motala, K. Ogurtsova, J.E. Shaw, D. Bright, R. Williams, Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition, Diabetes Res. Clin. Pract. 157 (2019). https://doi.org/10.1016/j.diabres.2019.107843.

[4] O. Gheith, N. Farouk, N. Nampoory, M.A. Halim, T. Al-Otaibi, Diabetic kidney disease: world wide difference of prevalence and risk factors., J. Nephropharmacology. 5 (2016) 49–56. http://www.ncbi.nlm.nih.gov/pubmed/28197499.

[5] M. Narres, H. Claessen, S. Droste, T. Kvitkina, M. Koch, O. Kuss, A. Icks, The incidence of end-stage renal disease in the diabetic (compared to the non-diabetic) population: A systematic review, PLoS One. 11 (2016). https://doi.org/10.1371/journal.pone.0147329.

[6] E. Ritz, I. Rychlik, F. Locatelli, S. Halimi, End-stage renal failure in type 2 diabetes: A medical catastrophe of worldwide dimensions, Am. J. Kidney Dis. 34 (1999) 795–808. https://doi.org/10.1016/S0272-6386(99)70035-1.

[7] E. Ribeiro, C. Ladeira, S. Viegas, Occupational Exposure to Bisphenol A (BPA): A Reality That Still Needs to Be Unveiled, Toxics. 5 (2017) 22. https://doi.org/10.3390/toxics5030022.

[8] P. Allard, M.P. Colaiácovo, Bisphenol A, in: R.C. Gupta (Ed.), Reproductive and Developmental Toxicology , Elsevier, Amsterdam, 2011: p. 1202. https://doi.org/10.1016/B978-0-12-382032-7.10050-5.

[9] J. Corrales, L.A. Kristofco, W. Baylor Steele, B.S. Yates, C.S. Breed, E. Spencer Williams,

B.W. Brooks, Global assessment of bisphenol a in the environment: Review and analysis of its occurrence and bioaccumulation, Dose-Response. 13 (2015) 1–29. https://doi.org/10.1177/1559325815598308.

[10] A. Bacle, S. Thevenot, C. Grignon, M. Belmouaz, M. Bauwens, B. Teychene, N. Venisse, V. Migeot, A. Dupuis, Determination of bisphenol A in water and the medical devices used in hemodialysis treatment, Int. J. Pharm. 505 (2016) 115–121. https://doi.org/10.1016/j.ijpharm.2016.03.003.

[11] M.K. Björnsdotter, J. de Boer, A. Ballesteros-Gómez, Bisphenol A and replacements in thermal paper: A review, Chemosphere. 182 (2017) 691–706. https://doi.org/10.1016/j.chemosphere.2017.05.070.

[12] C. Freire, J.-M. Molina-Molina, L.M. Iribarne-Duran, I. Jimenez-Diaz, F. Vela-Soria, V. Mustieles, J. Pedro Arrebola, M.F. Fernandez, F. Artacho-Cordon, N. Olea, Concentrations of bisphenol A and parabens in socks for infants and young children in Spain and their hormone-like activities, Environ. Int. 127 (2019) 592–600. https://doi.org/10.1016/j.envint.2019.04.013.

[13] A.M. Calafat, Z. Kuklenyik, J.A. Reidy, S.P. Caudill, J. Ekong, L.L. Needham, Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population, Environ. Health Perspect. 113 (2005) 391–395. https://doi.org/10.1289/ehp.7534.

[14] A. Covaci, E. Den Hond, T. Geens, E. Govarts, G. Koppen, H. Frederiksen, L.E. Knudsen, T.A. Morck, A.C. Gutleb, C. Guignard, E. Cocco, M. Horvat, E. Heath, T. Kosjek, D. Mazej, J.S. Tratnik, A. Castano, M. Esteban, F. Cutanda, J.J. Ramos, M. Berglund, K. Larsson, B.A.G. Jonsson, P. Biot, L. Casteleyn, R. Joas, A. Joas, L. Bloemen, O. Sepai, K. Exley, G. Schoeters, J. Angerer, M. Kolossa-Gehring, U. Fiddicke, D. Aerts, H.M. Koch, J. Jose Ramos, M. Berglund, K. Larsson, B.A.G. Jonsson, P. Biot, L. Casteleyn, R. Joas, A. Joas, L. Bloemen, O. Sepai, K. Exley, G. Schoeters, J. Angerer, M. Kolossa-Gehring, U. Fiddicke, D. Aerts, H.M. Koch, Urinary BPA measurements in children and mothers from six European member states: Overall results and determinants of exposure, Environ. Res. 141 (2015) 77–85. https://doi.org/10.1016/j.envres.2014.08.008.

[15] Z. Zhang, H. Alomirah, H.S. Cho, Y.F. Li, C. Liao, T.B. Minh, M.A. Mohd, H. Nakata, N. Ren, K. Kannan, Urinary bisphenol a concentrations and their implications for human exposure in several Asian countries, Environ. Sci. Technol. 45 (2011) 7044–7050. https://doi.org/10.1021/es200976k.

[16] X. Li, G.-G.G. Ying, J.-L.L. Zhao, Z.-F.F. Chen, H.-J.J. Lai, H.-C.C. Su, 4-Nonylphenol, bisphenol-A and triclosan levels in human urine of children and students in China, and the effects of drinking these bottled materials on the levels, Environ. Int. 52 (2013) 81–86. https://doi.org/10.1016/j.envint.2011.03.026.

[17] T. Bushnik, D. Haines, P. Levallois, J. Levesque, J. Van Oostdam, C. Viau, Lead and bisphenol A concentrations in the Canadian population, Heal. REPORTS. 21 (2010) 7–18.

[18] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J.R.R. Paumgartten, G. Schoenfelder, Urinary, Circulating, and Tissue Biomonitoring Studies Indicate Widespread Exposure to Bisphenol A, Environ. Health Perspect. 118 (2010) 1055–1070. https://doi.org/10.1289/ehp.0901716.

[19] A.C. Gore, V.A. Chappell, S.E. Fenton, J.A. Flaws, A. Nadal, G.S. Prins, J. Toppari, R.T. Zoeller, Executive Summary to EDC-2: The Endocrine Society's second Scientific Statement on endocrine-disrupting chemicals, Endocr. Rev. 36 (2015) 593–602. https://doi.org/10.1210/er.2015-1093.

[20] E. Testai, P. Hartemann, E. Rodriguez-Farre, S.C. Rastogi, J. Bustos, U. Gundert-Remy, A. Hensten, H.M. Kopperud, N.N. Olea, A. Piersma, W. De Jong, The safety of the use of bisphenol A in medical devices., Regul. Toxicol. Pharmacol. 79 (2016) 106–107. https://doi.org/10.1016/j.yrtph.2016.01.014.

[21] J.R. Rochester, Bisphenol A and human health: A review of the literature, Reprod. Toxicol. 42 (2013) 132–155. https://doi.org/10.1016/j.reprotox.2013.08.008.

[22] M. Saura, S. Marquez, P. Reventun, N. Olea-Herrero, M.I. Arenas, R. Moreno-Gómez-Toledano, M. Gómez-Parrizas, C. Muñóz-Moreno, M. González-Santander, C. Zaragoza, R.J. Bosch, Oral administration of bisphenol A induces high blood pressure through angiotensin II/CaMKII-dependent uncoupling of eNOS, FASEB J. 28 (2014) 4719–4728. https://doi.org/10.1096/fj.14-252460.

[23] Y. Ilagan, R. Mamillapalli, L.G. Goetz, J. Kayani, H.S. Taylor, Bisphenol-A exposure in utero programs a sexually dimorphic estrogenic state of hepatic metabolic gene expression, Reprod. Toxicol. 71 (2017) 84–94.

[24] B. Debenedictis, H. Guan, K. Yang, Prenatal Exposure to Bisphenol A Disrupts Mouse Fetal Liver Maturation in a Sex-Specific Manner, J. Cell. Biochem. 117 (2016) 344–350. https://doi.org/10.1002/jcb.25276.

[25] Y. Pu, J.D. Gingrich, J.P. Steibel, A. Veiga-Lopez, Sex-Specific Modulation of Fetal Adipogenesis by Gestational Bisphenol A and Bisphenol S Exposure, Endocrinology. 158 (2017) 3844–3858. https://doi.org/10.1210/en.2017-00615.

[26] V. Mustieles, R. Pérez-Lobato, N. Olea, M.F. Fernández, Bisphenol A: Human exposure and neurobehavior, Neurotoxicology. 49 (2015) 174–184. https://doi.org/10.1016/j.neuro.2015.06.002.

[27] K.A. McCaffrey, B. Jones, N. Mabrey, B. Weiss, S.H. Swan, H.B. Patisaul, Sex specific impact of perinatal bisphenol A (BPA) exposure over a range of orally administered doses on rat hypothalamic sexual differentiation, Neurotoxicology. 36 (2013) 55–62. https://doi.org/10.1016/j.neuro.2013.03.001.

[28] S. Mhaouty-Kodja, L.P. Belzunces, M.C. Canivenc, H. Schroeder, C. Chevrier, E. Pasquier, Impairment of learning and memory performances induced by BPA: Evidences from the literature of a MoA mediated through an ED, Mol. Cell. Endocrinol. 475 (2018) 54–73. https://doi.org/10.1016/j.mce.2018.03.017.

[29] S.E. Arambula, D. Jima, H.B. Patisaul, Prenatal bisphenol A (BPA) exposure alters the transcriptome of the neonate rat amygdala in a sex-specific manner: a CLARITY-BPA consortium study, Neurotoxicology. 65 (2018) 207–220. https://doi.org/10.1016/j.neuro.2017.10.005.

[30] R.B. Gear, S.M. Belcher, Impacts of Bisphenol A and Ethinyl Estradiol on Male and Female CD-1 Mouse Spleen, Sci. Rep. 7 (2017) 856. https://doi.org/10.1038/s41598-017-00961-8.

[31] P. Nuñez, T. Fernandez, M. García-Arévalo, P. Alonso-Magdalena, A. Nadal, C. Perillan, J. Arguelles, Effects of bisphenol A treatment during pregnancy on kidney development in mice: a stereological and histopathological study., J. Dev. Orig. Health Dis. 9 (2018) 208–214. https://doi.org/10.1017/S2040174417000939.

[32] T. Pollock, D. deCatanzaro, Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses, Reprod.

Toxicol. 49 (2014) 145–154. https://doi.org/10.1016/j.reprotox.2014.08.005.

[33] M. Li, Y. Bi, L. Qi, T. Wang, M. Xu, Y. Huang, Y. Xu, Y. Chen, J. Lu, W. Wang, G. Ning, Exposure to bisphenol A is associated with low-grade albuminuria in Chinese adults, Kidney Int. 81 (2012) 1131–1139. https://doi.org/10.1038/ki.2012.6.

[34] L. Trasande, T.M. Attina, H. Trachtman, Bisphenol A exposure is associated with low-grade urinary albumin excretion in children of the United States, Kidney Int. 83 (2013) 741–748. https://doi.org/10.1038/ki.2012.422.

[35] A. Shankar, S. Teppala, Urinary bisphenol A and hypertension in a multiethnic sample of US adults., J. Environ. Public Health. 2012 (2012) 481641. https://doi.org/10.1155/2012/481641.

[36] S. Bae, Y.-C.C. Hong, Exposure to bisphenol a from drinking canned beverages increases blood pressure: Randomized crossover trial, Hypertension. 65 (2015) 313–319. https://doi.org/10.1161/HYPERTENSIONAHA.114.04261.

[37] N. Olea-Herrero, M.I. Arenas, C. Munoz-Moreno, R. Moreno-Gómez-Toledano, M. Gonzalez-Santander, I. Arribas, R.J. Bosch, Bisphenol-A Induces Podocytopathy With Proteinuria in Mice, J. Cell. Physiol. 229 (2014) 2057–2066. https://doi.org/10.1002/jcp.24665.

[38] M. Kitada, Y. Ogura, D. Koya, Rodent models of diabetic nephropathy: Their utility and limitations, Int. J. Nephrol. Renovasc. Dis. 9 (2016) 279–290. https://doi.org/10.2147/IJNRD.S103784.

[39] A. Izquierdo, P. López-Luna, A. Ortega, M. Romero, M.A. Gutiérrez-Tarrés, I. Arribas, M.J.R. Álvarez, P. Esbrit, R.J. Bosch, The parathyroid hormone-related protein system and diabetic nephropathy outcome in streptozotocin-induced diabetes, Kidney Int. 69 (2006) 2171–2178. https://doi.org/10.1038/sj.ki.5000195.

[40] M.W. Pfaffl, A. Tichopad, C. Prgomet, T.P. Neuvians, Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations, Biotechnol. Lett. 26 (2004) 509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47.

[41] C. Jean-Faucher, M. Berger, C. Gallon, M. De Turckheim, G. Veyssiere, C. Jean, Sex-related differences in renal size in mice: Ontogeny and influence of neonatal androgens, J. Endocrinol. 115 (1987) 241–246. https://doi.org/10.1677/joe.0.1150241.

[42] A. Yabuki, S. Tanaka, M. Matsumoto, S. Suzuki, Morphometric study of gender differences with regard to age-related changes in the C57BL/6 mouse kidney, Exp. Anim. 55 (2006) 399–404. https://doi.org/10.1538/expanim.55.399.

[43] L. Huang, A. Scarpellini, M. Funck, E.A.M. Verderio, T.S. Johnson, Development of a Chronic Kidney Disease Model in C57BL/6 Mice with Relevance to Human Pathology, Nephron Extra. 3 (2013) 12–29. https://doi.org/10.1159/000346180.

[44] A. Yoldas, M.O. Dayan, Morphological characteristics of renal artery and kidney in rats, Sci. World J. 2014 (2014). https://doi.org/10.1155/2014/468982.

[45] R.P. Amin, A.E. Vickers, F. Sistare, K.L. Thompson, R.J. Roman, M. Lawton, J. Kramer, H.K. Hamadeh, J. Collins, S. Grissom, L. Bennett, C.J. Tucker, S. Wild, C. Kind, V. Oreffo, J.W. Davis, S. Curtiss, J.M. Naciff, M. Cunningham, R. Tennant, J. Stevens, B. Car, T.A. Bertram, C.A. Afshari, Identification of putative gene-based markers of renal toxicity, Environ. Health Perspect. 112 (2004) 465–479. https://doi.org/10.1289/ehp.6683.

[46] W.C. Prozialeck, V.S. Vaidya, J. Liu, M.P. Waalkes, J.R. Edwards, P.C. Lamar, A.M. Bernard, X. Dumont, J. V. Bonventre, Kidney injury molecule-1 is an early biomarker of cadmium nephrotoxicity, Kidney Int. 72 (2007) 985–993. https://doi.org/10.1038/sj.ki.5002467.

[47] J. Hu, Y. Wang, X. Xiang, C. Peng, R. Gao, R. Goswami, H. Zhou, Y. Zhang, Q. Zhen, Q. Cheng, S. Yang, Q. Li, Serum bisphenol A as a predictor of chronic kidney disease progresion in primary hypertension: A 6-year prospective study, J. Hypertens. 34 (2016) 332–337. https://doi.org/10.1097/HJH.0000000000000780.

[48] J. Hu, S. Yang, Y. Wang, R. Goswami, C. Peng, R. Gao, H. Zhou, Y. Zhang, Q. Cheng, Q. Zhen, Q. Li, Serum bisphenol A and progression of type 2 diabetic nephropathy: a 6-year prospective study, ACTA Diabetol. 52 (2015) 1135–1141. https://doi.org/10.1007/s00592-015-0801-5.

[49] M.L. Gross, E. Ritz, A. Schoof, M. Adamczak, A. Koch, O. Tulp, A. Parkman, A. El-Shakmak, A. Szabo, K. Amann, Comparison of renal morphology in the Streptozotocin and the SHR/N-cp models of diabetes, Lab. Investig. 84 (2004) 452–464. https://doi.org/10.1038/labinvest.3700052.

[50] M.P. O'Donnell, B.L. Kasiske, W.F. Keane, Glomerular hemodynamic and structural alterations in experimental diabetes mellitus, FASEB J. 2 (1988) 2339–2347. https://doi.org/10.1096/fasebj.2.8.3282959.

[51] M. Romero, A. Ortega, A. Izquierdo, P. López-Luna, R.J. Bosch, Parathyroid hormone-related protein induces hypertrophy in podocytes via TGF-β1 and p27Kip1: Implications for diabetic nephropathy, Nephrol. Dial. Transplant. 25 (2010) 2447–2457. https://doi.org/10.1093/ndt/gfq104.

[52] Tisher C.C., T.H. Hostetter, Diabetic Nephropathy, in: T. CC, B. BM (Eds.), Renal Pathology (vol.2), JB Lippincott Co. Ed, Philadelphia, 1994: p. 1387.

[53] M.E. Cooper, D. Vranes, S. Youssef, S.A. Stacker, A.J. Cox, B. Rizkalla, D.J. Casley, L.A. Bach, D.J. Kelly, R.E. Gilbert, Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes, Diabetes. 48 (1999) 2229–2239. https://doi.org/10.2337/diabetes.48.11.2229.

[54] M. Katoh, Y. Ohmachi, Y. Kurosawa, H. Yoneda, N. Tanaka, H. Narita, Effects of imidapril and captopril on streptozotocin-induced diabetic nephropathy in mice, Eur. J. Pharmacol. 398 (2000) 381–387. https://doi.org/10.1016/S0014-2999(00)00320-4.

[55] T. Yotsumoto, T. Naitoh, K.I. Shikada, S. Tanaka, Effects of specific antagonists of angiotensin II receptors and captopril on diabetic nephropathy in mice, Jpn. J. Pharmacol. 75 (1997) 59–64. https://doi.org/10.1254/jjp.75.59.

[56] R.W. Tyl, C.B. Myers, M.C. Marr, C.S. Sloan, N.P. Castillo, M.M. Veselica, J.C. Seely, S.S. Dimond, J.P. Van Miller, R.N. Shiotsuka, D. Beyer, S.G. Hentges, J.M. Waechter Jr., J.M.J. Waechter, Two-generation reproductive toxicity study of dietary bisphenol a in CD-1 (Swiss) mice, Toxicol. Sci. 104 (2008) 362–384. https://doi.org/10.1093/toxsci/kfn084.

[57] EFSA, Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA J. 13 (2016) 3978. https://doi.org/10.2903/j.efsa.2015.3978.

[58] EFSA, What potential health effects of BPA has EFSA identified? EFSA's risk assessment in more detail How did EFSA's experts calculate the new TDI?, (n.d.). https://doi.org/10.2805/075460.

[59] K. Ćwiek-Ludwicka, Bisphenol A (BPA) in food contact materials - new scientific opinion

from EFSA regarding public health risk, Rocz. Panstw. Zakl. Hig. 66 (2015) 299–307. https://doi.org/10.1016/j.reprotox.2016.06.020.

[60] G. Giebisch, E.E. Windhager, P.S. Aronson, Glomerular filtration and renal blood flow, in: W.F. Boron, E.L. Boulpaep (Eds.), Medical Physiology, 3rd ed., Elsevier, Amsterdam, 2016: p. 1312.

[61] A. Ortega, M. Romero, A. Izquierdo, N. Troyano, Y. Arce, J.A. Ardura, M.I. Arenas, J. Bover, P. Esbrit, R.J. Bosch, Parathyroid hormone-related protein is a hypertrophy factor for human mesangial cells: Implications for diabetic nephropathy, J. Cell. Physiol. 227 (2012) 1980–1987. https://doi.org/10.1002/jcp.22926.

[62] L. Caporossi, B. Papaleo, Exposure to Bisphenol a and Gender Differences: From Rodents to Humans Evidences and Hypothesis about the Health Effects, J. Xenobiotics. 5 (2015) 5264. https://doi.org/10.4081/xeno.2015.5264.

[63] C. Teng, B. Goodwin, K. Shockley, M. Xia, R. Huang, J. Norris, B.A. Merrick, A.M. Jetten, C.P. Austin, R.R. Tice, Bisphenol A affects androgen receptor function via multiple mechanisms, Chem. Biol. Interact. 203 (2013) 556–564. https://doi.org/10.1016/j.cbi.2013.03.013.

[64] V. Müller, G. Losonczy, U. Heemann, Á. Vannay, A. Fekete, G. Reusz, T. Tulassay, A.J. Szabó, Sexual dimorphism in renal ischemia-reperfusion injury in rats: Possible role of endothelin, Kidney Int. 62 (2002) 1364–1371. https://doi.org/10.1046/j.1523-1755.2002.00590.x.

[65] M.K.O. Grant, D.M. Seelig, L.C. Sharkey, W.S.V. Choi, I.Y. Abdelgawad, B.N. Zordoky, Sexual dimorphism of acute doxorubicin-induced nephrotoxicity in C57Bl/6 mice, PLoS One. 14 (2019) 1–19. https://doi.org/10.1371/journal.pone.0212486.

[66] M. Shi, K.L. McMillan, J. Wu, N. Gillings, B. Flores, O.W. Moe, M.C. Hu, Cisplatin nephrotoxicity as a model of chronic kidney disease, Lab. Investig. 98 (2018) 1105–1121. https://doi.org/10.1038/s41374-018-0063-2.

[67] J. Neugarten, A. Acharya, S.R. Silbiger, Effect of Gender on the Progression of Nondiabetic Renal Disease.pdf, J Am Soc Nephrol. 11 (2000) 319–329.

[68] J. Neugarten, L. Golestaneh, Gender and the prevalence and progression of renal disease, Adv. Chronic Kidney Dis. 20 (2013) 390–395. https://doi.org/10.1053/j.ackd.2013.05.004.

[69] F. de Álvaro Moreno, A. Aguilera Peralta, E. Valdés Chiong, Nefropatía diabética, Med. - Programa Form. Médica Contin. Acreditado. 8 (2000) 1001–1008. https://doi.org/10.1016/S0304-5412(00)70188-0.

[70] A. Martínez-Castelao, Nefropatía diabética, in: M. Ed (Ed.), Manual de Nefrología clínica, díalisis y transporte renal, 1998: pp. 1043–1059.

[71] M.D. Breyer, E. Böttinger, F.C. Brosius, T.M. Coffman, R.C. Harris, C.W. Heilig, K. Sharma, AMDCC, Mouse Models of Diabetic Nephropathy, J. Am. Soc. Nephrol. 16 (2005) 27–45. https://doi.org/10.1681/ASN.2004080648.

[72] G. D'Amico, C. Bazzi, Pathophysiology of proteinuria, Kidney Int. 63 (2003) 809–825. https://doi.org/10.1046/j.1523-1755.2003.00840.x.

[73] T.W. Meyer, P.H. Bennett, R.G. Nelson, Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria,

Diabetologia. 42 (1999) 1341–1344. https://doi.org/10.1007/s001250051447.

[74] H. V. Alderson, J.P. Ritchie, S. Pagano, R.J. Middleton, M. Pruijm, N. Vuilleumier, P.A. Kalra, The associations of blood kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin with progression from CKD to ESRD, Clin. J. Am. Soc. Nephrol. 11 (2016) 2141–2149. https://doi.org/10.2215/CJN.02670316.

[75] K.G. Garlo, W.B. White, G.L. Bakris, F. Zannad, C.A. Wilson, S. Kupfer, M. Vaduganathan, D.A. Morrow, C.P. Cannon, D.M. Charytan, Kidney biomarkers and decline in eGFR in patients with type 2 diabetes, Clin. J. Am. Soc. Nephrol. 13 (2018) 398–405. https://doi.org/10.2215/CJN.05280517.

[76] A.R. Priego, E.G. Parra, S. Mas, J.L. Morgado-Pascual, M. Ruiz-Ortega, S. Rayego-Mateos, Bisphenol a modulates autophagy and exacerbates chronic kidney damage in mice, Int. J. Mol. Sci. 22 (2021). https://doi.org/10.3390/ijms22137189.

[77] A. Zhuang, J.M. Forbes, Stress in the kidney is the road to pERdition: Is endoplasmic reticulum stress a pathogenic mediator of diabetic nephropathy?, J. Endocrinol. 222 (2014) R97–R111. https://doi.org/10.1530/JOE-13-0517.

[78] M. Taniguchi, H. Yoshida, Endoplasmic reticulum stress in kidney function and disease, Curr. Opin. Nephrol. Hypertens. 24 (2015) 345–350. https://doi.org/10.1097/MNH.0000000000000141.

[79] H. Xie, L. Huang, Y. Li, H. Zhang, H. Liu, Endoplasmic reticulum stress and renal lesion in mice with combination of high-fat diet and streptozotocin-induced diabetes, Acta Cir. Bras. 31 (2016) 150–155. https://doi.org/10.1590/s0102-865020160030000001.

[80] J.F. Navarro, C. Mora, M. Macía, J. García, Inflammatory parameters are independently associated with urinary albumin in type 2 diabetes mellitus, Am. J. Kidney Dis. 42 (2003) 53–61. https://doi.org/10.1016/S0272-6386(03)00408-6.

[81] M. Murata, J.H. Kang, Bisphenol A (BPA) and cell signaling pathways, Biotechnol. Adv. 36 (2018) 311–327. https://doi.org/10.1016/j.biotechadv.2017.12.002.

[82] G.J. Alekhya Sita, M. Gowthami, G. Srikanth, M.M. Krishna, K. Rama Sireesha, M. Sajjarao, K. Nagarjuna, M. Nagarjuna, G.K. Chinnaboina, A. Mishra, N. SreeHarsha, Protective role of luteolin against bisphenol A-induced renal toxicity through suppressing oxidative stress, inflammation, and upregulating Nrf2/ARE/ HO-1 pathway, IUBMB Life. 71 (2019) 1041–1047. https://doi.org/10.1002/iub.2066.

[83] J. Xu, G. Huang, T.L. Guo, Developmental bisphenol a exposure modulates immune-related diseases, Toxics. 4 (2016). https://doi.org/10.3390/toxics4040023.

[84] X. Tian, M. Takamoto, K. Sugane, Bisphenol A promotes IL-4 production by Th2 cells, Int. Arch. Allergy Immunol. 132 (2003) 240–247. https://doi.org/10.1159/000074305.

Supplementary Table 1. SYBR Green primers and TaqMan probes.

RT-PCR primers		
TNF-α	Forward	5´- AGGCACTCCCCCAAAAGATG-3´
	Reverse	5´-TGAGGGTCTGGGCCATAGAA
TGF-β1	Forward	5´-GCAACATGTGGAACTCTACCAG-3’
	Reverse	5´-CAGCCACTCAGGCGTATCA-3’
TIIR	Forward	5’- CCTACTCTGTCTGTGGATGACCT - 3’
	Reverse	5´- ACTTCCGGGGCCATGTAT - 3’
MCP-1	Forward	5’- CACTCACTCCACAACCCAAGA - 3’
	Reverse	5’- CAAAGACCCTCAAAACATCCC - 3’
IL-10	Forward	5’- AGGGTGTCTCCTTCCTCACA - 3’
	Reverse	5’- TGTTACACTCGCCCCCTTTG - 3’
IL-1β	Forward	5’- TGGGCCTCAAAGGAAAGAAT - 3’
	Reverse	5’- CAGGCTTGTGCTCTGCTTGT - 3’
KIM-1	Forward	5’- ATGAATCAGATTCAAGTCTTC - 3’
	Reverse	5’- TCTGGTTTGTGAGTCCATGTG - 3’
NGAL	Forward	5’- CACCACGGACTACAACCAGTTCGC - 3’
	Reverse	5’- TCAGTTGTCAATGCATTGGTCGGTG - 3’
TaqMan probes		
β-Actin	Forward	5´- GCTCTGGCTCCTAGCACCAT- 3´
	Reverse	5´- GCCACCGATCCACACAGAGT- 3´
	Probe	5´- ATCAAGATCATTGCTCCTCCTGAGCGC- 3´

Article

Comparison of the renal effects of bisphenol A in mice with and without experimental diabetes. Role of sexual dimorphism

Rafael Moreno-Gómez-Toledano[1*], María I. Arenas[2*], Carmen Muñoz-Moreno[1], Nuria Olea-Herrero[1], Paula Reventun[3], Adriana Izquierdo-Lahuerta[5], Alba Antón-Cornejo[6], Marta González-Santander[1], Carlos Zaragoza[7], Marta Saura[4] and Ricardo J. Bosch[1‡§]

[1] Universidad de Alcalá, Laboratory of Renal Physiology and Experimental Nephrology, Group of Pathophysiology of the cardiovascular, renal and nervous systems, Department of Biological Systems/Physiology Unit, Alcalá de Henares, Spain

[2] Universidad de Alcalá, Department of Biomedicine and Biotechnology, Alcalá de Henares, Spain

[3] Department of Medicine, Division of Cardiology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[4] Universidad de Alcalá, Laboratory of Pathophysiology of the Vascular Wall, Group of Pathophysiology of the cardiovascular, renal and nervous systems, IRICYS, Department of System Biology/Physiology Unit, Alcalá de Henares, Spain

[5] University Rey Juan Carlos, Biochemistry and Molecular Biology area, Department of Basic Sciences of Health, Alcorcon, Spain

[6] Clinical Analysis Service, Principe de Asturias Hospital, Alcalá de Henares, Spain

[7] Laboratory of Cardiovascular Pathophysiology, Joint Translational Research Unit, University Francisco de Vitoria School of Medicine and Division of Cardiology, University Hospital Ramón y Cajal, Madrid, Spain

[*] These authors contributed equally to this work

[‡] Corresponding author
[§]E-mail: ricardoj.bosch@uah.es

Abstract

Bisphenol-A (BPA), a chemical -xenoestrogen- used in the production of the plastic lining of food and beverage containers, is present in the urine of almost the entire population. Recent studies have shown that BPA exposure is associated with podocytopathy, increased urinary albumin excretion (UAE), and hypertension. Since these changes are characteristic of early diabetic nephropathy (DN), we explored the renal effects of BPA and diabetes including the potential role of sexual dimorphism. Male and female mice were included in the following animals' groups: control mice (C), mice treated with 21.2 mg/Kg of BPA in the drinking water (BPA), diabetic mice induced by streptozotocin (D), and D mice treated with BPA (D+BPA). Male mice form the D+BPA group died by the tenth week of the study due probably to hydro-electrolytic disturbances.

Although BPA treated mice did not show an increase in serum creatinine, as observed in D and D+BPA groups, they displayed similar alteration to those of the D group, including increased in kidney damage biomarkers NGAL and KIM-1, UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli, as well as TGF-β, CHOP and PCNA upregulation. UAE, collapsed glomeruli, PCNA staining, TGF-β, NGAL and animal survival, significantly impaired in D+BPA animals. Moreover, UAE, collapsed glomeruli and animal survival also displayed a sexual dimorphism pattern.

In conclusion, oral administration of BPA is capable of promoting in the kidney alterations that resemble early DN. Further translational studies are needed to clarify the potential role of BPA in renal diseases, particularly in diabetic patients.

Keywords: Bisphenol A, mouse, diabetes, diabetic nephropathy.

Highlights

- Oral administration of BPA at a concentration below the "no observed adverse effect level" (NOAEL) promotes in the mice alterations that resemble early diabetic nephropathy.

-Mice receiving BPA develop proteinuria, hypertension, podocytopenia, apoptosis, collapsed glomeruli, as well as the upregulation of kidney damage biomarkers NGAL and KIM-1, and the TGF-β system.

- Proteinuria, collapsed glomeruli, and animal survival displayed a sexual dimorphism pattern in diabetic mice receiving BPA.

1. Introduction

Diabetes mellitus (DM) is a growing disease that affects an increasing number of people. In 1980 it affected 108 million people globally [1], increasing to 171 million in 2000 [2]. Interestingly, in 2014, the number of patients augmented to 422 million [1]. According to studies conducted by the International Diabetes Federation, it is estimated that almost 500 million people in the world are currently living with diabetes. That number is projected to reach 578 million in 2030 and 700 million in 2045 [3]. Of all of them, it is estimated that about half of patients with type 1 diabetes develop diabetic nephropathy (DN) throughout their lives, being 30-50% in the case of patients with type 2 diabetes [4].

In the same way, an increase in the prevalence of diabetic kidney disease has also been observed. Gheith et al. [4] determined an increase from 7.1% (1988-1994) to 10.7% (2005-2008) in people over 65 years of age. DN is also the most common cause of end-stage renal disease, having been observed in the United States to cause up to 80% of cases [5]. DN has been recognized as a worldwide medical catastrophe and is now the most important etiology of end-

stage renal failure in developed countries [6]. However, since not all diabetic patients suffer renal alterations, it is considered that there must be numerous environmental and biological factors yet to be determined.

Parallel to the increase in chronic diseases, an increase in exposure to xenobiotic compounds, such as bisphenol A (BPA), has been observed [7,8]. BPA is an essential element in manufacturing PVC and epoxy resins, used in numerous sectors such as the food industry, medical-surgical material, thermal paper cash register receipts, or even clothes [9–12]. In this way, BPA levels have been detected in the vast majority of foods, which manifests as significant levels of BPA in almost the entire human population studied, reaching percentages of 95% in the United States [13], 97% in Spain [14], 90-100% in China [15,16] or even 100% in Canada [17].

BPA is known to act as an endocrine disruptor, and due to its physical properties, it is capable of crossing any physiological barrier, such as the blood-brain or placental barrier [18]. In the last 20 years, various studies have determined that BPA can affect numerous organs and systems, such as the female and male reproductive system, the cardiovascular system and the endocrine system, or even the correct fetal development, to name a few examples [19–22]. In this sense, a pattern of sexual dimorphism has been observed during the gestational or perinatal period, compared to the same exposure to BPA, reaching differences on hepatic [23,24], adipogenic [25], neurological [26–28], endocrine [29], immunological [30], and renal development [31]. Pollock et al. [32] determined that the kidney is one of the organs where more BPA can be quantified, and this concentration rises before prolonged exposure over time, which also suggests the existence of a possible accumulation.

Epidemiological studies in humans suggest the possible association between high urinary BPA levels and predisposition to albuminuria [33,34] or hypertension [35,36]. Moreover, Olea et al. [37] in an experimental animal model have shown the development of podocytopathy and increased urinary albumin excretion (UAE) in mice after five weeks of i.p. exposure of BPA. Furthermore, BPA exposure has been associated with hypertension in mice [22].

Since these changes are characteristic of early DN, herein we explored the hypothesis that oral BPA exposure, being the most important one in mammals, might resemble DN and promote the progression of kidney damage. Moreover, we also studied well-characterized molecules involved in the pathophysiology of DN, such as the proapoptotic and profibrogenic TGF-β, and the well-known inflammatory mediators MCP-1 and TNF-α.

2. Material and methods

2.1. Animal model. CD1 mice (25–30 g) were used in all the experiments. All the studies were performed following guidelines established by Institutional Animal Care and Use Committees at the University of Alcalá. Mice were housed in a temperature-controlled room (21 ± 2°C) on a 14/10 h light/dark cycle under pathogen-free conditions and food ad libitum. Mice were given free access to water or BPA-containing water at a dose of 150 µg/ml (Sigma, Saint Louis, MO).

Four different animal groups were studied: Control (C), non-diabetic animals with BPA in the drinking water (BPA) 150 µg/ml, (21.2 mg/kg/day), diabetic animals without BPA (D), and diabetic animals with BPA in the drinking water (D+BPA).

In total forty non-diabetic mice were used, 20 mice as control and 20 mice treated with BPA. Diabetes was induced by three daily consecutive intraperitoneal injections of streptozotocin (STZ) (Sigma), 65 mg/kg body weight in citrate buffer, pH 4.5 (vehicle) [38]. Mice with blood glucose >300 mg/dL were included in the study (n=45). Male and female mice were used in approximately equal number with the exception of the male D+BPA group (17 male and 8 female) due to the mortality of the former group at 10 weeks. For this reason, the period of the

study was limited to eight weeks using 5 to 7 animals per group except for the Kaplan-Mayer survival table.

Mice were placed in metabolic cages, and 24-h urine was collected for biochemical measurement as previously reported [39]. After eight weeks of treatment and development of the diabetic model, the animals were sacrificed under isoflurane. Blood was taken by cardiac puncture under ether anesthesia for biochemical measurements. The right kidney of each animal was removed, weighed, frozen in liquid nitrogen, and stored at −80 °C for subsequent total RNA extraction. The left kidney was weighed and fixed in 10% buffered formaldehyde for morphological and immunohistochemistry studies. The degree of renal hypertrophy was expressed as an index, the ratio of kidney weight to total body weight.

2.2. Determination of biochemical test. Quantifications of plasma sodium, potassium, albumin, creatinine, and urine albumin and creatinine were performed with an ADVIA Chemistry system (Siemens Healthineers).

2.3. Immunohistochemistry. Serial sections (4 µm thicknesses) from mice kidneys formalin-fixed and embedded in paraffin were deparaffinized with xylene, rehydrated, and placed in 10 mM sodium citrate buffer, pH 6.0, and heated in a conventional pressure cooker for 2 min. The sections were allowed to cool for 20 min. After rinsing with distilled water, the sections were washed twice in Tris buffer saline (TBS), pH 7.6, for 5 min. Endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2 for 20 min. Sections were subsequently washed with distilled H_2O and TBS and incubated with 3% normal donkey serum plus 0.05% Triton X-100 in TBS (blocking solution) at room temperature for 30 min to prevent nonspecific binding of the first antibody. Afterward, sections were incubated overnight at 4°C with primary antibodies diluted in blocking solution, which was diluted 1:9 in TBS. (WT-1, 1:1000; CHOP, 1:300; PCNA, 1:1500; MCP-1, 1:400; 4-Hydroxynonenal (4-HNE) 1:250). Then, the sections were washed and incubated in primary antibodies amplifier Quanto (Ultravision Quanto detection system–peroxidase, Master Diagnóstica, Granada, Spain) for 10 min. After an extensive wash in TBS, the sections were incubated in polymer Quanto for 10 min. The peroxidase activity was detected using the DAB kit (Master Diagnóstica). Tissue sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in Entellan®.

The immunoreactivity of each focus of interest was measured using the stereological software Motic Images Advanced 3.2 (Motic China Group Co, Ltd). This program allows the selection of fields to be studied by systematic random sampling after the input of an appropriate sampling fraction. An average of 10 light microscopic fields per section was scanned using the x20 objective. The staining intensity was measured using a grey level scale (0: white; 255: black) with a negative image. All results are expressed as mean ± SEM.

2.4. TUNEL assay. For the detection of apoptotic cells, the DeadEndTM Fluorometric TUNEL System was applied (Promega, Madison, WI, USA) to paraffin-embedded sections according to the manufacturer's instructions. Then, the sections were incubated with an anti-FITC-HRP for 30 min. and the reaction product in nuclei was developed with DAB.

2.5. Total RNA extraction and quantitative RT-PCR. Total RNA was isolated from each mouse kidney homogenate obtained using TriReagent (Sigma-Aldrich). Total RNA was quantified by Nanodrop 1000 Spectrophotometer (Thermo Scientific). A total of 500 µg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Promega Kit according to the manufacturer's instructions (5 minutes 65°C and 1 hour 37°C). PCR was performed duplicated for each sample using an adequate dilution of cDNA as a template for different genes (see supplementary material Table S1 for SYBR Green primers and TaqMan probes). β-actin and 36B4 were used as housekeeping genes. The amplification was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) by using the following conditions: 2 minutes 50°C, 10 minutes 95°C, 40 cycles (15 seconds 95°C, 1 minute 60°C). Reagents were from Applied

Biosystems (for TaqMan probes) and SYBR Green (for SYBR Green primers). We used a standard curve method, using the untreated samples as a calibrator, to calculate the relative quantity of gene expression. We used the BestKeeper software tool (http://www.gene-quantification.info/) to validate housekeeping genes [40].

2.6. Measurement of blood pressure (tail cuff method). We used a non-invasive pressure gauge (LE 5001 Pressure Meter; Letica Scientific Instruments, Hospitalet, Spain) to measure the blood pressure. The measurements were always made at the same time of day, with the least possible stress for the animal (acoustic and luminous). Being nocturnal animals and having a high inactivity peak at 8 a.m., they were not manipulated until at least two hours later. The animals were prewarmed to 30°C with a heater (LE5660/6, Letica Scientific Instruments). The sphygmomanometer and the pulse transducer were never placed in the area near the animal's body because they can cause damage.

2.7. Statistical analysis. All results were expressed as mean ± SEM. $p < 0.05$ was considered statistically significant. To determine the effects of treatments, one-way ANOVA or Kruskal-Wallis followed by a Bonferroni or Dunns test, respectively, were carried out. To determine sex differences in the BPA and diabetes treatment, two-way ANOVA followed by a Tukey's multiple comparisons test or Sidak's multiple comparisons test were carried out. The *p*-values presented in figures and tables corresponded to post hoc test. All statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Animals display a sexual survival dimorphism pattern. As shown in figure 1A, the Kaplan-Meier animal survival analysis showed that all-male D+BPA mice died by the tenth week of treatment while no animal loses were found among the other experimental animal groups. In order to obtain all the biological samples needed to complete the animal's studies, some male animals were included in the studied groups as described in the methods section.

Besides dehydration, no other potential cause of death was observed in the bodies of dead mice. As shown in figure 1B, both D and D+BPA groups showed a significant increase in urinary output than the control littermates. However, only D group showed significant differences between males and females. As expected, these animal groups display an increased value of plasma sodium together with a decreased potassium (figures 1C and 1D), albeit these values did not display a sexual dimorphism pattern. Unfortunately, one limitation of the study is the lack of **biochemical** data beyond the study's eight weeks. Therefore, although the cause of the male D+BPA group's death cannot be determined with certainty, we can only assume that it was related to hydroelectrolytic disturbances.

3.2. Animal body weight and renal functional parameters. As previously described [41–44], control male mice showed a significant increase in body weight and renal hypertrophy compared to females ´littermates (figures 1E and 1F). As shown in figure 1E, compared to control mice, the average animal body weight was not affected in the BPA group, while both D and D+BPA groups showed a significant decrease in this parameter. Moreover, both control male and D male mice showed a significant increase in body weight compared to females' littermates. The D and D+BPA mice showed a significant increase in the renal hypertrophy index compared to the control group. Of note is that the females of the D+BPA group develop an increase in this parameter similar to males. In the rest of the groups, the females showed a lower hypertrophy index than their male littermates (figure 1F).

At 8 weeks of treatment, the glycemia of diabetic animals remained above 400 mg/dl in all animals, while BPA group were not significantly different from those of the C group (figure 2A).

As expected, all animal groups studied display a significant increase in UAE compared to the C group (figure 2B). UAE was significantly higher in the D group compared to the BPA group. Interestingly, male D+BPA mice display a significant increase in UAE compared to the BPA group, demonstrating a sexual dimorphism pattern compared to their female littermates.

Moreover, a significant decrease in plasma albumin was observed in D and D+BPA (figure 2C). Although endogenous creatinine clearance not showed significant differences among animal groups studied, plasma creatinine displayed a statistically significant increase in both D and D+BPA groups compared to their correspondent's controls (figure 2D and 2E). To further investigate BPA effects **at the molecular level on the kidney**, the gene expression of the kidney damage biomarkers Havcr-1 and Lidocalpin-2, which codify NGAL and KIM-1, respectively, were evaluated [45,46] (figure 2F and 2G). Both mRNAs were significantly increased in all treated mice in comparison to their control littermates. Interestingly, NGAL also showed a significant further increase in diabetic animals receiving BPA. Thus, kidney damage biomarkers supported the notion that BPA exposures to mice induced kidney damage in non-diabetic mice and exacerbated injury in diabetic mice. No sexual dimorphism was observed in these parameters.

As shown in figure 2H and 2I, systolic and diastolic arterial blood pressures were substantially higher in the three experimental models. No sexual dimorphism in this parameter was observed.

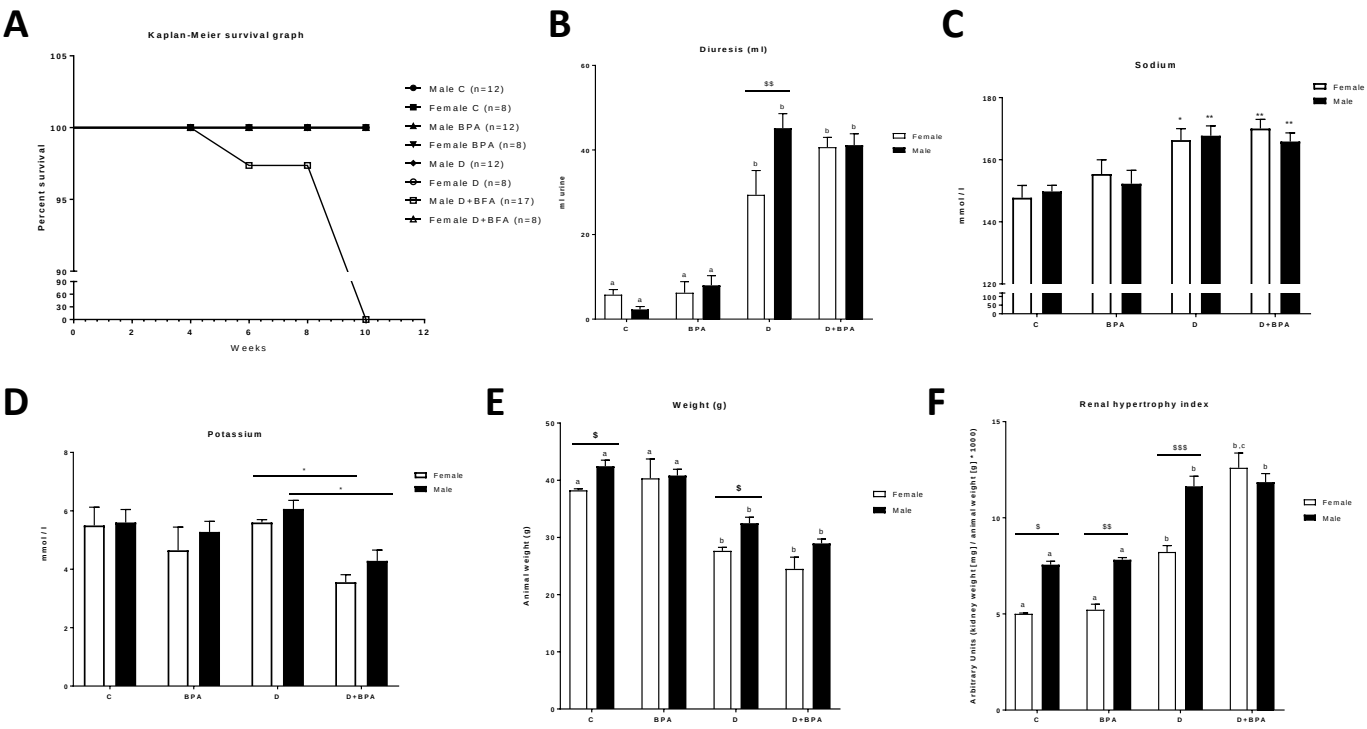


Figure 1. Survival, plasma ions, and physiological study. A. Kaplan-Meier survival graph. Note that the survival of all groups is 100%, except for the D+BPA group. For this reason, all symbols and lines overlap at the same height, preventing correct viewing. **B.** Body weight, in grams. Diabetic animals showed a significant decrease in body weight than C and BPA groups. Note that only C and D groups showed sexual dimorphism. **C.** Renal hypertrophy index (kidney weight [mg] / animal weight [g] * 1000). All diabetic animals showed a significant increase in renal hypertrophy index. Note that C, BPA, and D groups showed sexual dimorphism. **D.** 24-hour diuresis, in ml. All diabetic animals excreted volumes of urine between 5 and 10 times higher than control and BPA groups. **E.** Plasma sodium, in mmol/l. All diabetic animals showed elevated plasma sodium concentrations. **F.** Plasma potassium, in mmol/l. Only the D+BPA group showed low plasma potassium concentrations. All groups with different letters have significant differences ($p<0.01$). Letter c represents significant differences with diabetic female ($p<0.0001$). *$p<0.05$;

**p<0.01 comparing to their respective control group. $$p$<0.05; $$$p$<0.01; $$$$p$<0.001 comparing between males and females of the same group. The graph represents mean values ± SEM. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

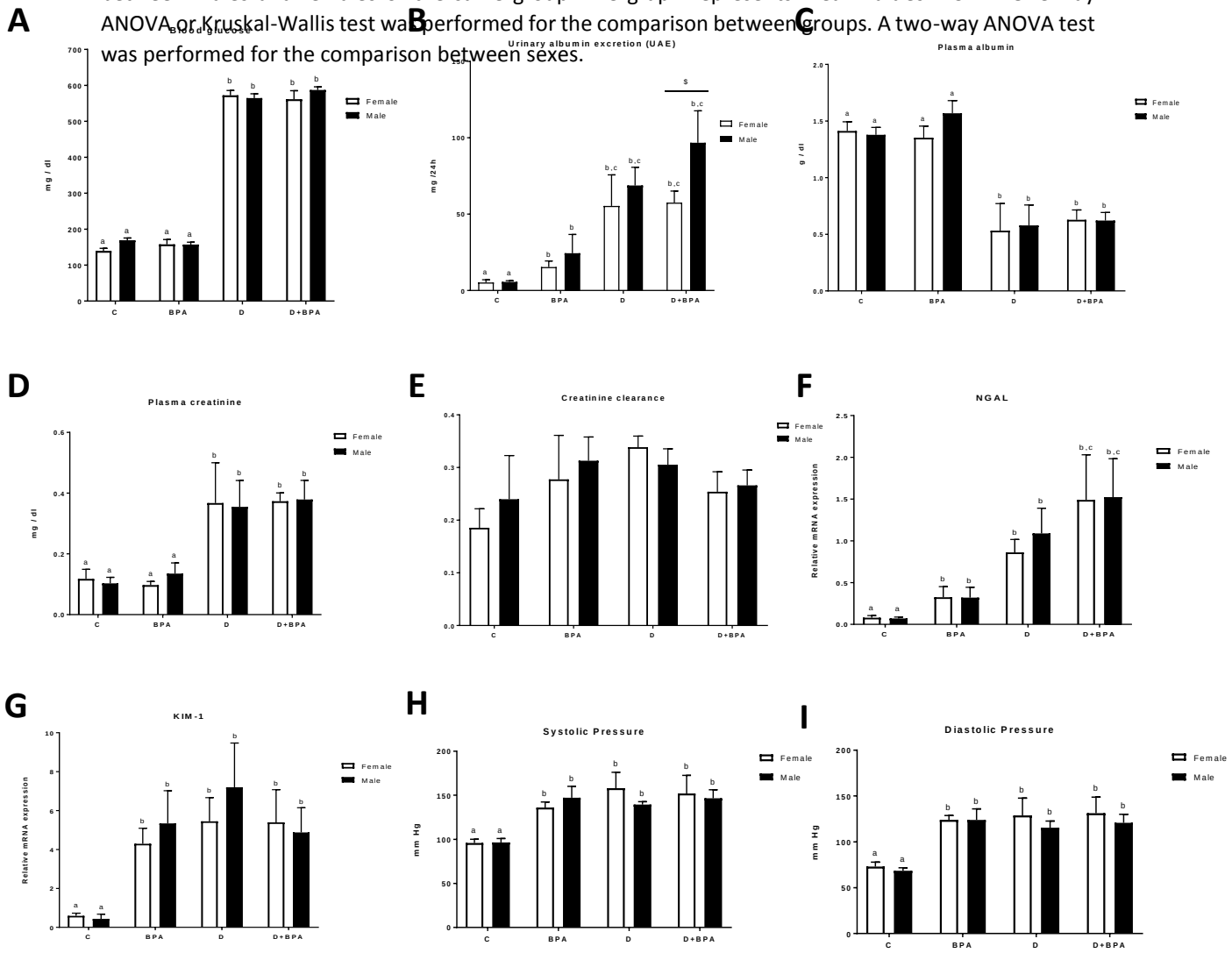


Figure 2. Biochemical study of the kidney and arterial pressure. A. Blood glucose. All diabetic animals showed higher blood glucose at the end of the experiment (8 weeks). **B.** Albuminuria, expressed in mg of albumin excreted in 24 hours. Note that all groups showed elevated levels of albuminuria compared with the control group. **C.** Plasma albumin, in g/dl. All diabetic animals showed low values of plasma albumin, in the nephrotic range. **D.** Plasma creatinine, in mg/dl. All diabetic animals showed high plasma creatinine values. **E.** Creatinine clearance. All groups showed similar creatinine clearance values. **F.** NGAL qPCR. **G.** KIM-1 qPCR. **H.** After eight weeks of treatment, all groups showed a significant increase in systolic pressure than the control group. **I.** Diastolic pressure showed the same pattern than systolic pressure. $$p$<0.05 comparing between males and females of the same group. All groups with different letters have significant differences (p<0.05). Letter c represents significant differences with BPA group (p<0.05). The graph represents mean values ± SEM. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

3.3. Histological, immunohistochemical, and Q-PCR studies. BPA and D groups display similar renal histological changes at both glomerular and tubular compartments, including collapsed glomeruli, tubular dilatation, sloughing off the tubuloepithelial cells, and hyaline casts in the tubules' lumen. Diabetic mice treated with BPA showed similar histological alterations observed

in these groups. Of note, glomerular damage analyzed as collapsed corpuscles percentage showed a sexual dimorphism pattern in BPA and D+BPA groups being male mice significantly impaired (figure 3).

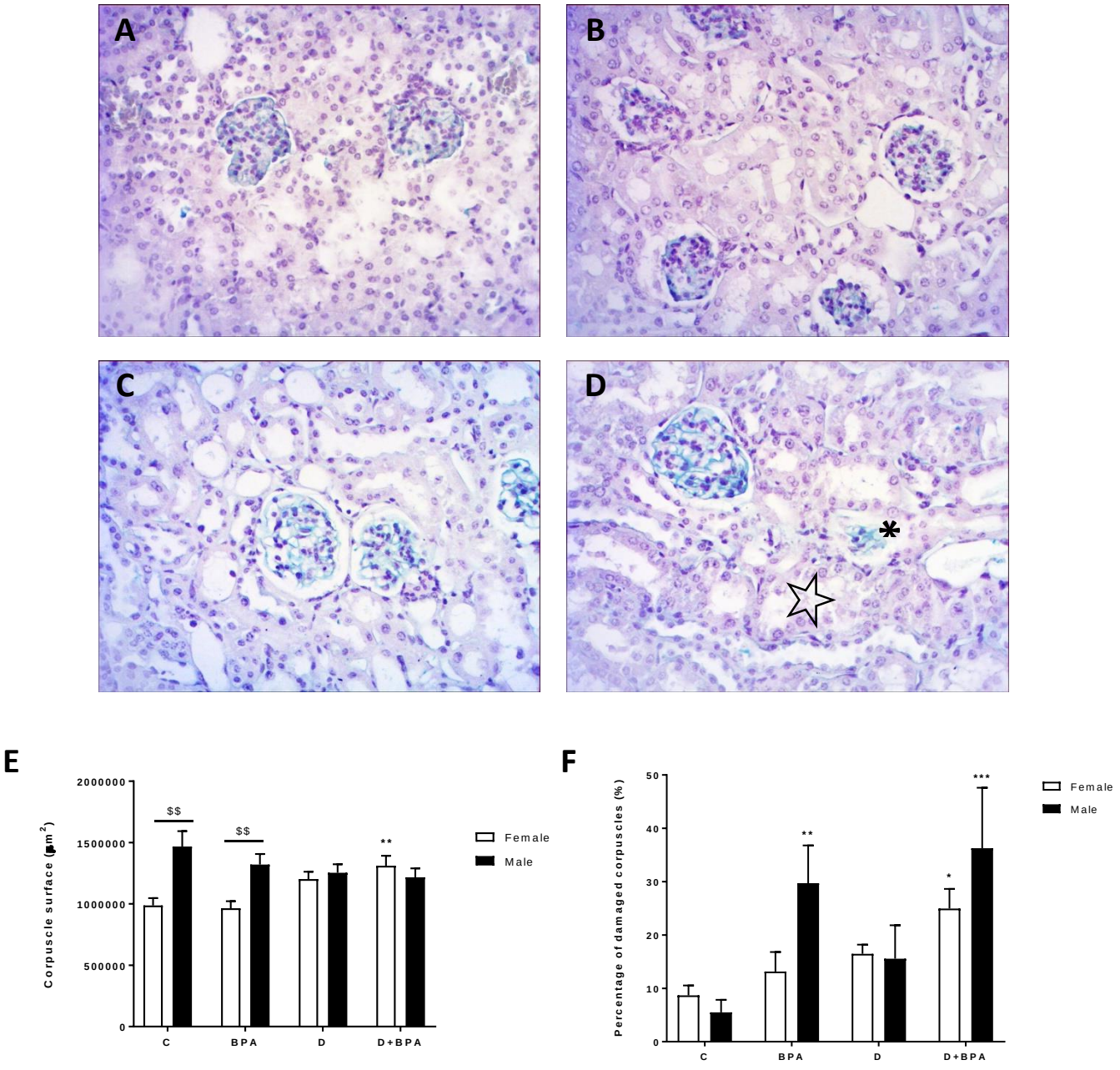


Figure 3. Histological examination (PAS-Alcian blue staining). A. Control kidney. **B.** Mice BPA-treated showed altered renal corpuscles and dilated convoluted tubules. **C.** The kidney of the diabetic mice showed both Bowman's capsules and convoluted tubules extremely dilated. **D.** D+BPA mice showed mesangial expansion, some corpuscles were destroyed (*), and the epithelium in some tubules was damaged (star) (X300). **E.** Histogram compares the corpuscular surface in each group observing statistically significant differences between females and males in both control and BPA-treated groups. **F.** Histogram represents the collapsed renal corpuscles percentage, showing a sexual dimorphism pattern in BPA and D+BPA groups. *p<0.05; **p<0.01 using one-way ANOVA or Kruskal-Wallis test for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes. $$$p$<0.01 comparing between males and females of the same group.

Immunolabeling with WT-1, a specific marker of podocytes, demonstrated that podocytes cell number was significantly decreased in all groups compared to their corresponding control

littermates (figure 4). Interestingly, D+BPA animals display a further decrease in this parameter compared to both BPA and D groups. No sexual dimorphisms were observed on this parameter.

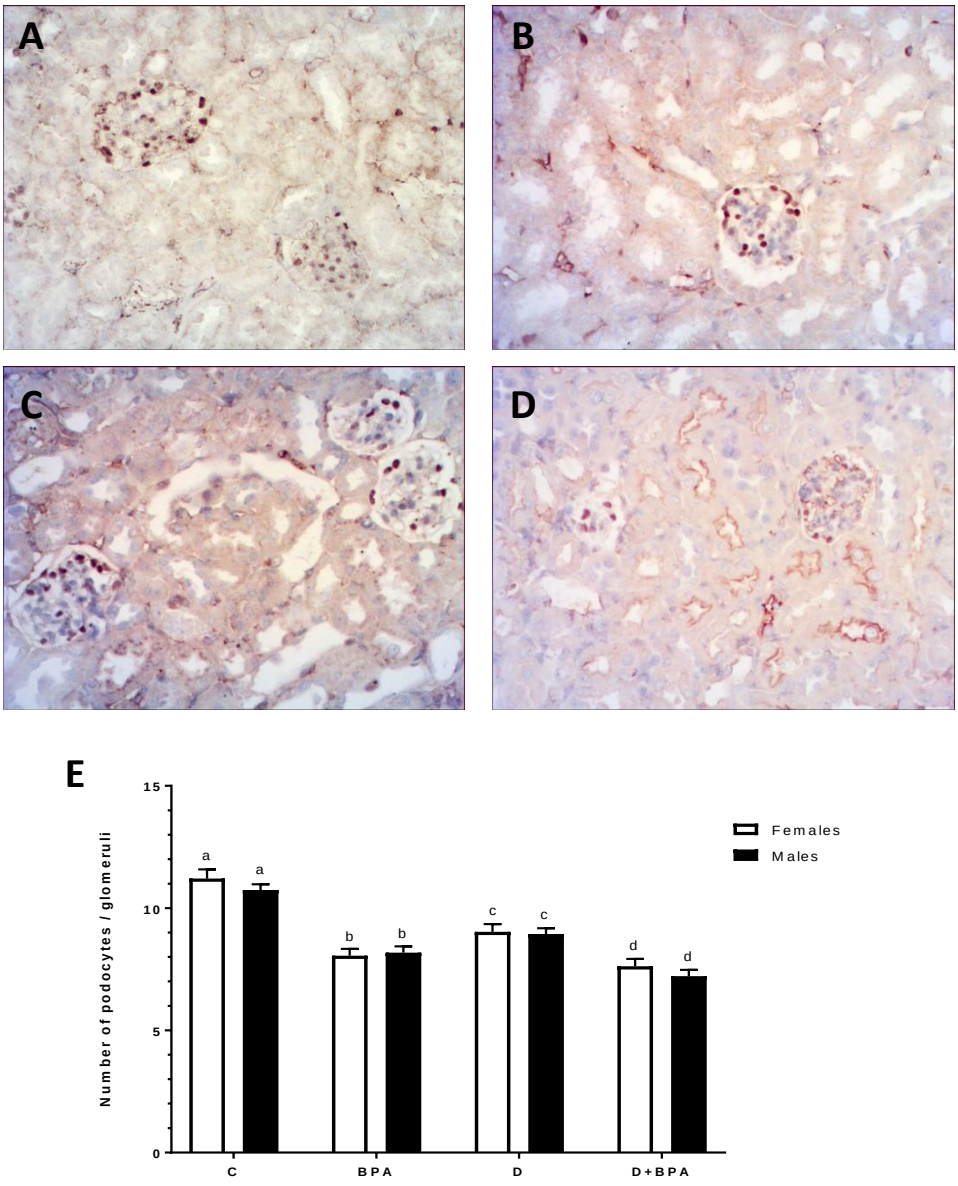


Figure 4. WT-1 immunostaining. A. Control kidney. **B.** Mice BPA-treated is showing a reduction in the podocytes number vs. control animal. **C.** Diabetic mice presented a higher podocyte number than BPA-treated animals but lower than control. **D.** D+BPA mice had the lowest number of podocytes (X300). **E.** The histogram shows statistically significant differences between all experimental groups. All groups with different letters have significant differences ($p<0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

Cell death, including apoptosis, as analyzed by CHOP immunohistochemistry and TUNEL assay, respectively, were significantly increased in all group studies in comparison to their control littermates. D group presented the highest optical density in CHOP immunostaining (figure 5). D+BPA group presented the highest number of apoptotic cells (figure 6).

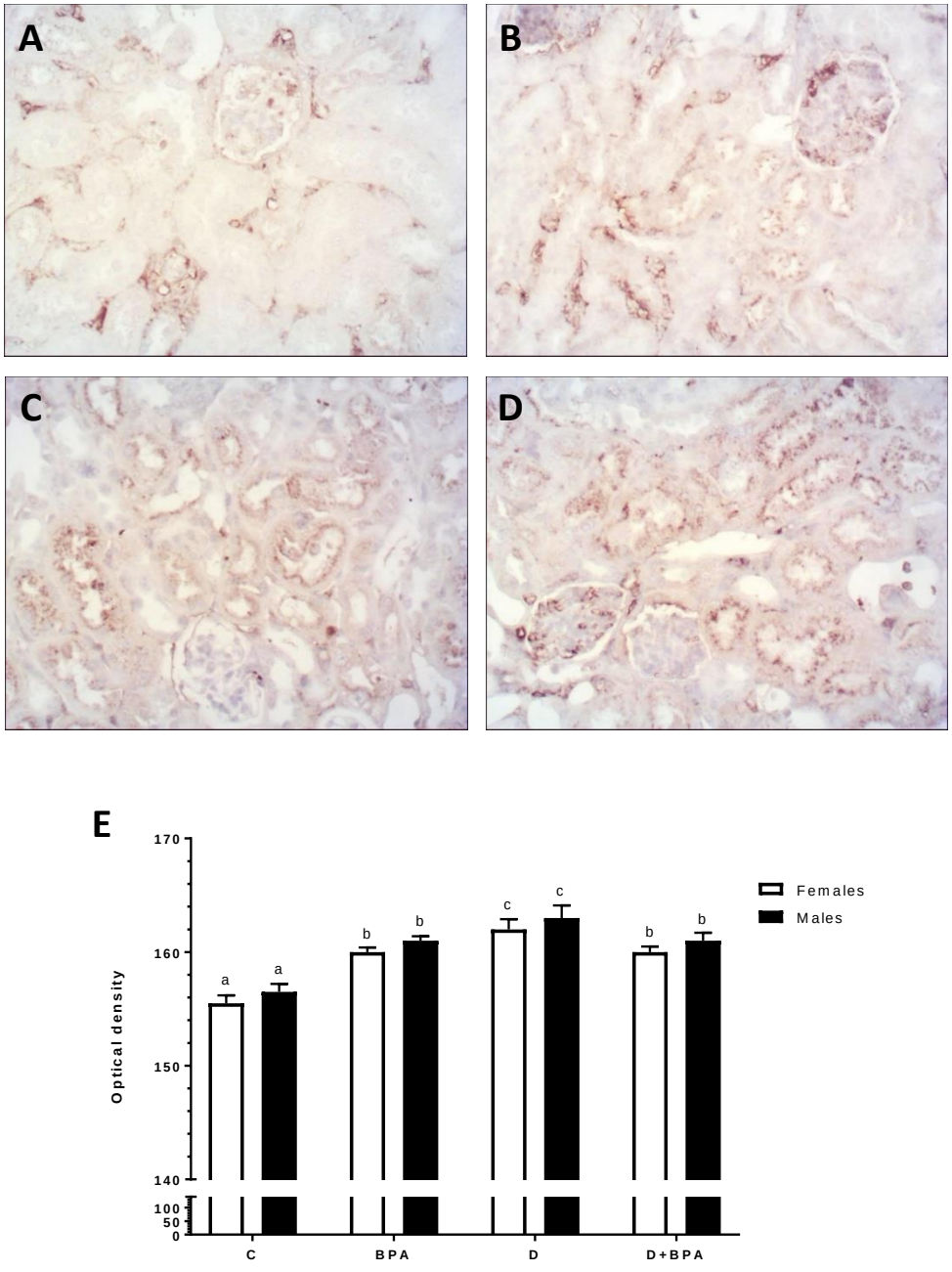


Figure 5. CHOP immunostaining. A. In the control group, the CHOP labeling was observed in the blood vessel endothelial cells. **B.** In BPA-treated kidneys, the immunolabeling was increased and appeared in the apical cytoplasm of the tubuloepithelial cells. **C.** Diabetic mice presented the highest immunoreaction to CHOP antibody. **D.** D+BPA mice presented elevated immunostaining but lower than in diabetic mice (X300). **E.** Histogram showing the optical density of CHOP immunostaining. All groups with different letters have significant differences ($p<0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

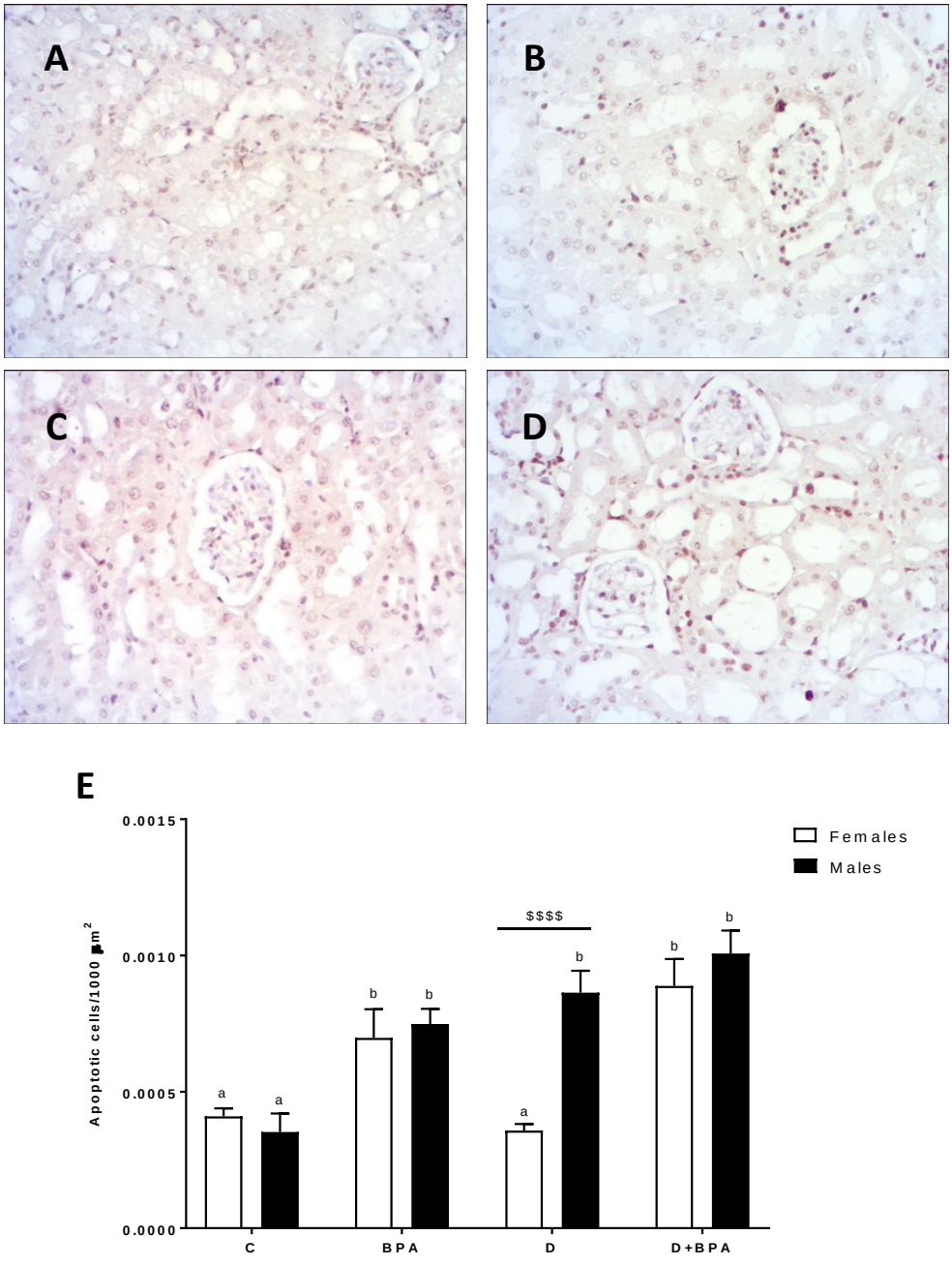


Figure 6. TUNEL assay. A. Kidneys from control the group presented a small number of apoptotic cells. **B.** BPA-treated mice presented a higher number of apoptotic cells than controls. **C.** In diabetic mice, kidneys from males had a high number of dead cells; however, the female mice were similar to the control group. **D.** D+BPA mice showed the highest number of apoptotic cells (X300). **E.** Histogram shows the gender differences in each group. $$$$ p<0.0001 females vs. males. All groups with different letters have significant differences (p<0.05). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

To explore if there was a compensatory mitotic response to the observed increase in cell death, cell proliferation was analyzed by PCNA immunostaining. Both BPA and D groups develop a significant proliferative response, even higher in female mice than their male littermates (figure 7). Interestingly, D+BPA group also showed PCNA-upregulation compared to their control littermates, albeit without sexual dimorphism.

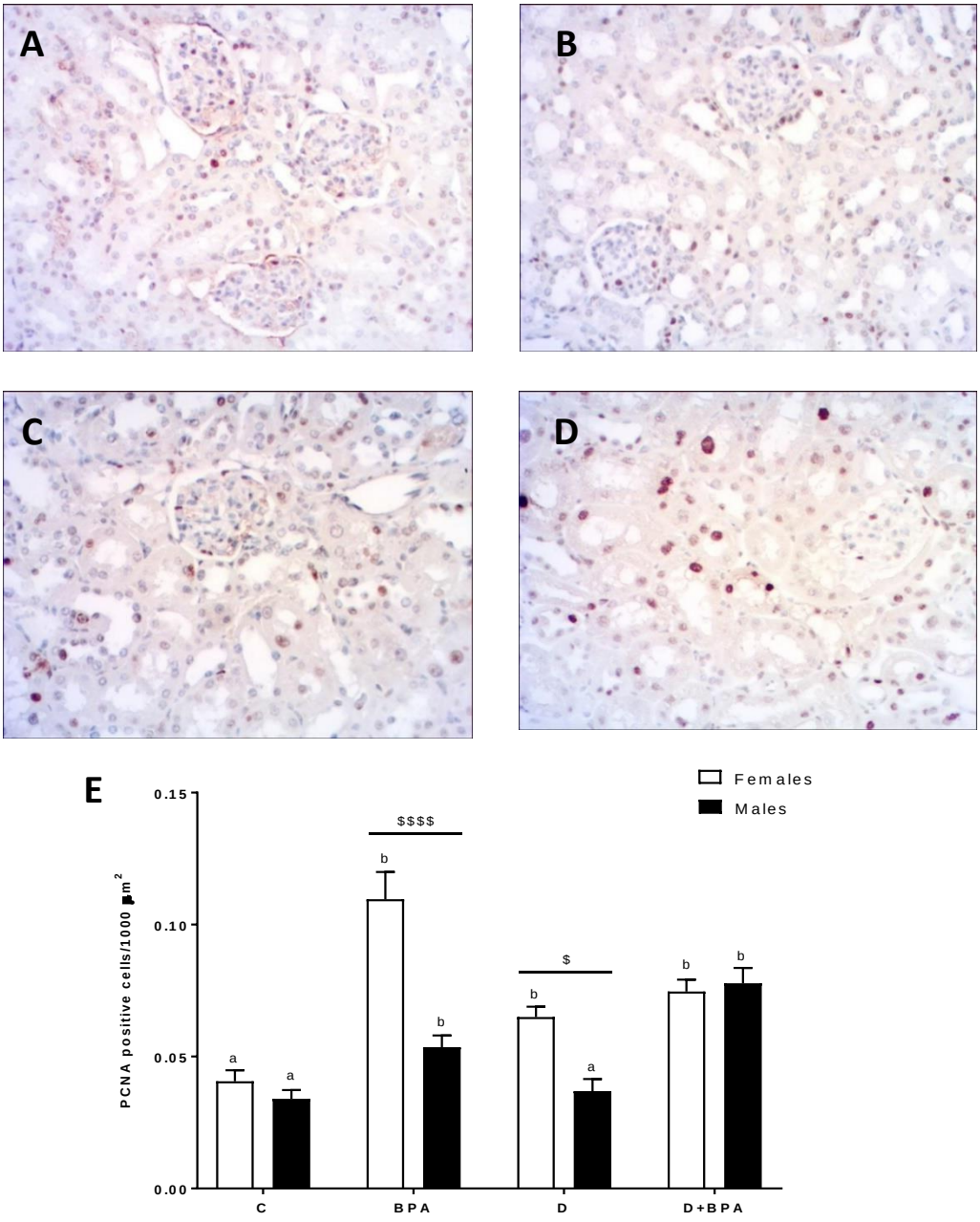
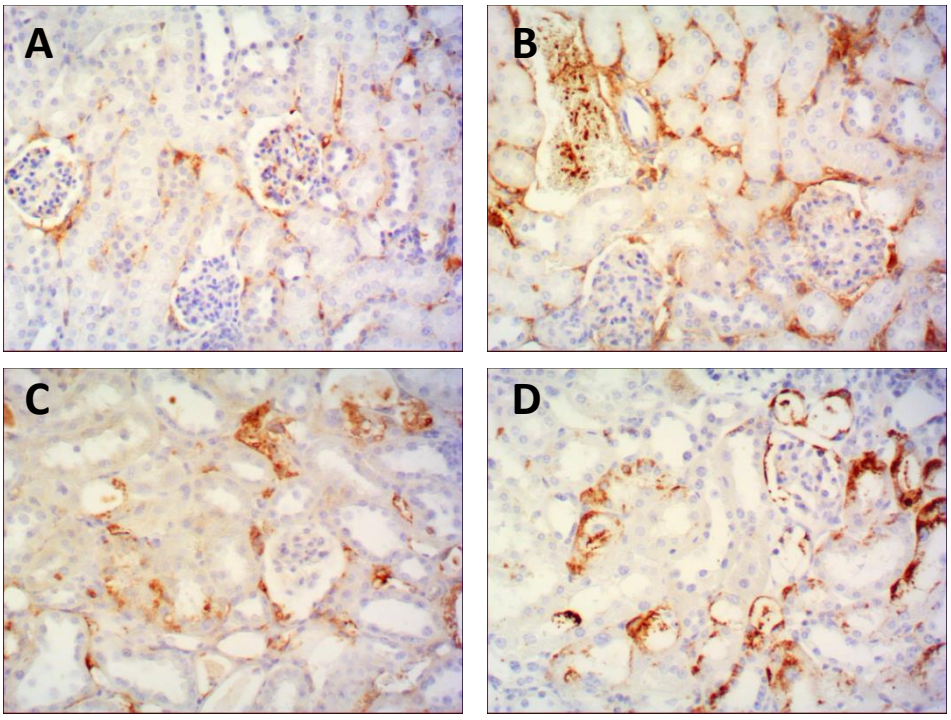


Figure 7. PCNA immunostaining. A. Control groups showed a low number of proliferating cells. **B.** Kidneys from BPA-treated mice presented a higher number of PCNA positive cells than control groups, especially females. **C.** Diabetic mice showed a number similar to control in males and higher in females. **D.** D+BPA mice presented a high number of proliferating cells without any difference between gender (X300). **E.** Histogram representing the gender differences in each group. All groups with different letters have significant differences ($p<0.05$). $\$p<0.05$; $\$\$\$\$p<0.0001$ females vs. males. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

We then explored the potential role of oxidative stress as an inflammatory mediator by analyzing lipid peroxidation as 4-HNE staining (figure 8). Control and BPA animals showed a sexual dimorphism pattern being significantly higher in the male. All treated mice, except D+BPA male displayed a significant increase in comparison to their control littermates.



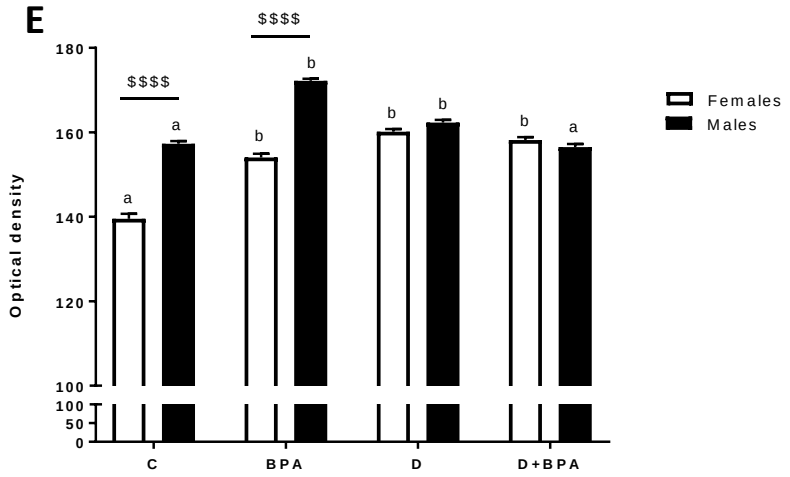


Figure 8. 4-HNE immunolabeling. **A.** Control group. **B.** BPA-mice showed an important 4-HNE elevation, compared to their control group. Furthermore, BPA was the only treated group with sexual dimorphism pattern, similar than control. **C.** D group showed an important elevation without sexual dimorphism pattern. **D.** Only females D+BPA showed higher 4-HNE staining. **E.** Histogram represents the optical density of 4-HNE immunostaining. All groups with different letters have significant differences ($p<0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

We finally explored the potential role of several inflammatory mediators in the present experimental model (figures 9 and 10). No significant changes in the expression of TNF-α, MCP-1, and IL-1β were observed in BPA-treated groups (figures 9A, B, C). The same expression pattern was observed in MCP-1 immunolabeling (figure 10). By contrast, all of them were significantly upregulated in D animal group. Moreover, D+BPA group showed a significant upregulation of MCP-1. Figure 9D showed a significant upregulation of IL-10 restricted to BPA animal group. TGF-β and its receptor, a well-characterized system involved in both BPA and renal diabetic damage, were analyzed by Q-PCR. TGF-β was significantly upregulated in all animal groups, being significantly higher in the D+BPA group. The latter observation suggests that BPA might potentiate TGF-β upregulation in diabetic mice (figure 9E). Interestingly, the D+BPA group also displays the upregulation of the TGF-β receptor (figure 9F). No sexual dimorphisms were observed on these parameters.

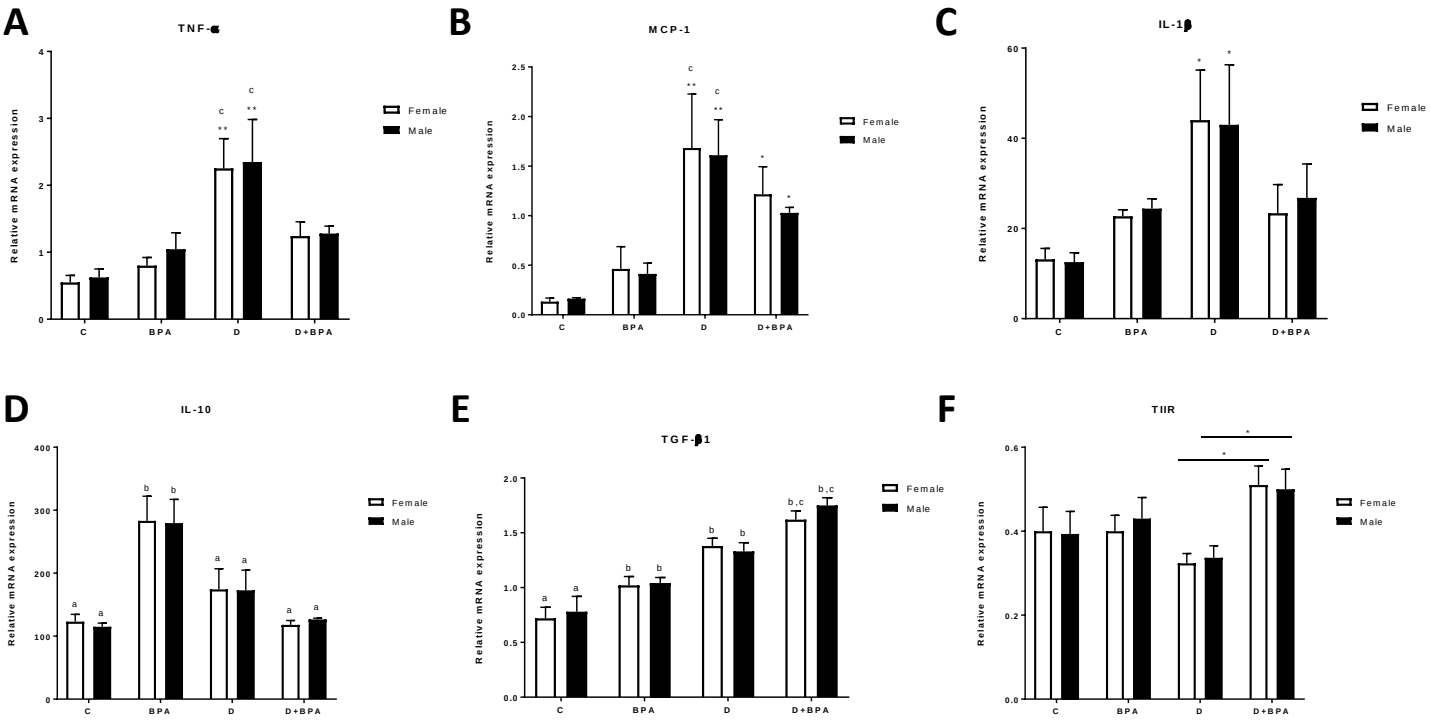


Figure 9. Quantitative RT-PCR. A. TNF-α. The highest relative expression of the inflammatory messenger corresponds to the D group. **B.** MCP-1. As in the previous case, the highest relative expression of the inflammatory marker MCP-1 corresponds to the D group. **C.** IL-1β. The same expression pattern was observed as in the other two pro-inflammatory cytokines. **D.** IL-10. The expression of this anti-inflammatory cytokine only increased in the BPA group **E.** TGF-β1. D+BPA group showed the highest relative expression of TGF-β1 mRNA. **F.** TIIR. The only differences are observed between groups D and D+BPA. *p<0.05; **p<0.01 using the Kruskal-Wallis test for the comparison between groups. All groups with different letters have significant differences (p<0.05). Letter c represents significant differences with BPA group (p<0.05). A two-way ANOVA test was performed for the comparison between sexes.

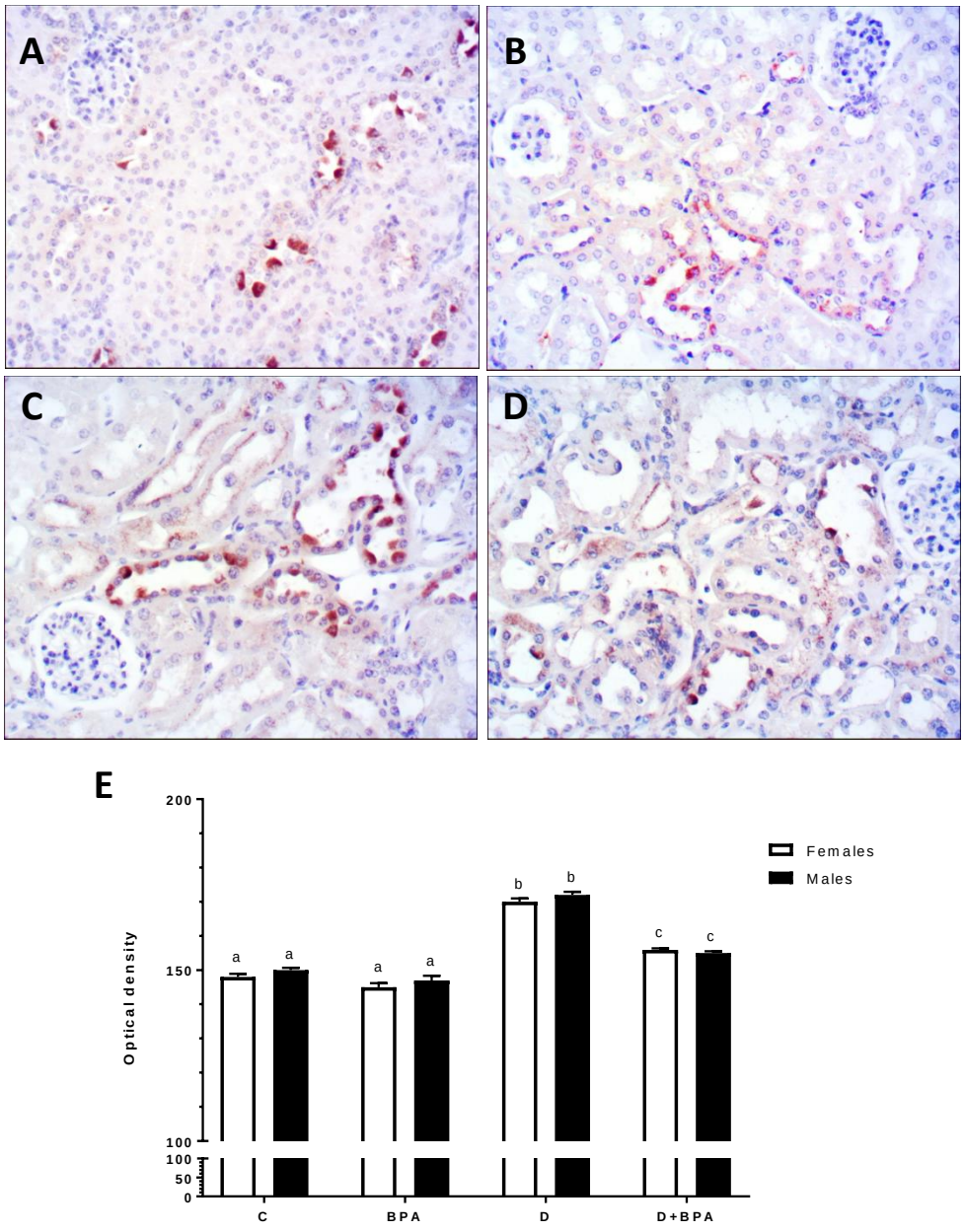


Figure 10. MCP-1 immunolabeling. A. In control kidneys, MCP-1 was observed in the cytoplasm of some epithelial cells of the collecting tubules. **B**. In the BPA group, the labeling was similar to the control group. **C.** Diabetic mice showed a high number of labeled cells and higher intensity in the MCP-1 expression. **D.** Kidneys from D+BPA mice presented an elevated immunolabeling to MCP-1 antibody but with less intensity than diabetic animals (X300). **E.** Histogram represents the optical density of MCP-1 immunostaining. All groups with different letters have significant differences ($p<0.0001$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

4. Discussion

Experimental and human studies have demonstrated a significant association between urinary excretion of BPA and albuminuria, a well-known factor involved in the mechanism of renal disease progression [33,34,47,48]. In this regard, Hu et al. [47], in a prospective study of 302 patients followed for six years, have demonstrated serum BPA as a predictor of chronic kidney disease in primary hypertension. Serum BPA was also described as a risk factor in the progression of diabetic nephropathy in patients with type 2 diabetes [48]. Our present study provides an experimental animal model to support these findings since we describe that the oral administration of BPA is capable of promoting in the kidney pathophysiological and molecular alterations that resemble early DN, such as increased in UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli as well as CHOP and TGF-β system upregulation.

We presently used the STZ model, which is considered the 'work horse' for experimental studies in diabetic nephropathy [49,50]. Consistent with previous findings in this model, diabetic mice developed renal hypertrophy and an increase in UAE, hypertension, podocytopenia, apoptosis [39,51–55], and the upregulation of CHOP, PCNA, MCP-1, and TGF-β throughout this study [51,52].

Regarding BPA doses used in our study's, animals received 150 µg/ml (21.2 mg/kg). Currently, it is accepted that the "no observed adverse effect level", known as NOAEL, in the renal system is 50 mg/kg [56,57], which is at least twice times higher than the dose used. European Food Safety Authority (EFSA) currently uses this NOAEL dose to estimate the tolerable daily intake (TDI) of 4 µg/kg in humans [57–59]. In the EFSA 2015 report, it is estimated that the total exposure to BPA in the population could reach almost half of the TDI (around 1.5 µg/kg) [57], which is consistent with using an equivalent concentration half of the NOAEL in animal models.

BPA-treated mice did not display significant polyuria as expected from previous publish [37] and unpublished observations using BPA concentration higher than renal NOAEL. Therefore, polyuria and the risk of higher BPA exposure were restricted to D+BPA mice. In any case, the BPA group developed similar renal changes to those of the diabetic group. Although this represents a limitation in our studies, it emphasizes the role of BPA exposure and the importance of maintaining strict metabolic control of diabetic patients to avoid osmotic diuresis and BPA overexposure.

One unexpected finding of this study was that all male mice from the D+BPA group died by the tenth week of the experimental procedure. Unfortunately, a limitation of the study is the lack of data beyond eight weeks. Therefore, albeit the cause of the death cannot be determined with certainty, we can only assume that it was related to hydroelectrolytic disturbances in animals with impaired renal function.

In accordance with previous works [41–44], control male mice showed increased in body weight and renal hypertrophy compared to female littermates. Besides the observed sexual survival dimorphism pattern, other significant pathophysiological differences between male and female animals were found. Treated male animals display significant increases UAE, and renal damage analyzed as collapsed glomeruli. Renal cell proliferation, as analyzed by PCNA staining, was the only parameter found to be significantly higher in female animals than in the males' littermates.

Although endogenous creatinine clearance showed no significant differences among animal groups studied, serum creatinine displayed a statistically significant increase in both D and D+BPA groups compared to their correspondent's controls. It is known that to a variable degree, tubules secrete creatinine, which, by itself, would lead to an overestimation of renal function [60]. To further investigate BPA effects at the molecular level on the kidney, the gene expression of the kidney damage biomarkers Havcr-1 and Lidocalpin-2, which codify KIM-1 and NGAL, respectively, were evaluated [45,46]. Both genes were significantly increased in all treated mice in comparison to their control littermates. Interestingly, NGAL also showed a significant further

increase in diabetic animals receiving BPA. All renal functional data studied supported the notion that BPA exposures to mice induced kidney damage in control mice and exacerbated injury in diabetic mice.

All treated male and female mice showed similar impairment of renal function. In contrast, male mice displayed a significant increase in urinary output restricted to D animal group, and female mice showed a significant increase in cell proliferation in this animal group. It is thus tempering to speculate, albeit not probed in the present setting, that increased urinary output could predispose to hydro electrolyte disturbances while increased in renal cell proliferation could, at least in theory, partially explain differences in sexual survival dimorphism pattern. On the other hand, D+BPA animal group did not display an additional effect on cell proliferation.

Several studies have demonstrated that most renal cells grown in high glucose conditions initially present a self-limited proliferation, followed by cell cycle arrest in the G1-phase, undergoing cellular hypertrophy [51,61]. These cellular events require the combined effect of mitogen-induced entry into the cell cycle and subsequent arrest at the G1 modulated by cell cycle regulatory proteins, including TGF-β1. Therefore, it is possible to speculate that the intense hypertrophy response observed in the D+BPA group may abrogate the mitogenic effect induced by BPA.

Sexual dimorphism could be due to different susceptibility to BPA, physiological differences, including sexual hormones, particularly androgen [62], or a combination of both. Indeed, different responses in males and females to the same exposure to BPA have been found. Most of the studies have been carried out mainly on the gestational or perinatal effect, where it has been determined that BPA can exert a different effect in males and females at the hepatic [23,24], adipogenic [25], neurological [26–28], endocrine [29], immunological [30], or renal level [31]. Furthermore, it has been observed that BPA is capable of inducing an agonist action on the estrogen receptor and an antagonist on the androgen receptor [63].

On the other hand, there is evidence that suggests that females have higher nephroprotection than males. In experimental animal models, female rats have increased protection against post-ischemic renal failure [64], doxorubicin treatment [65], and cisplatin treatment [66]. Epidemiological studies in humans also suggest the existence of nephroprotection in women. In this sense, one of the most extensive studies carried out is that of Neugarten et al. [67], where after performing a meta-analysis with 68 different studies and a total of 11,345 patients, they determined that men with chronic kidney disease caused by various etiologies show a much faster decline in kidney function than women. In another epidemiological study with 27,805 patients with type I diabetes, a statistically significant relationship was observed between the male sex and microalbuminuria development, an early marker of kidney disease [68].

Furthermore, probably because a BPA dose equivalent to half the NOAEL was used, the BPA group results do not show remarkable differences to the control group at the biometric and biochemical levels. However, an increase in albuminuria (less than in the diabetic groups, but equally significant) and a significant increase in blood pressure were observed. On the other hand, the histological studies showed interesting differences to the control group by showing the collapsed glomeruli, and a reduction in the number of cells, with the subsequent confirmation by TUNEL of the increase of apoptotic cells and a reduction in the number of podocytes labeled with WT-1.

Regarding sexual dimorphism, were observed differences in the renal hypertrophy index and albuminuria, with males presenting a higher number in both parameters. Although podocyte (WT-1) immunostaining showed no sexual dimorphism, particularly in the D+BPA group, the number collapsing glomerulus did show sexual dimorphism. Thus, damaged glomeruli could account for the observed increase in the UAE in the male D+BPA group.

In general, the results show that the route of administration of BPA and the dose used after eight weeks of treatment can exert an effect on the renal system similar to that described in the early stages of the development of diabetic nephropathy [69,70] at a dose lower than that considered "NOAEL" of the renal system [56].

Although there are limitations when using mouse models for assessing renal failure or long-term histomorphological changes [71], our findings may have pathophysiological implications since the amount of proteinuria, podocyte number and the upregulation of kidney damage biomarkers are reliable predictors of the progression of renal disease [37,72–75]. Together with our present findings, all the available data strongly suggest that even low-grade proteinuria associated with BPA exposure might involve podocyte damage of an uncertain (or as yet unexplored) outcome, jointly with the biomarkers mentioned above are indicators of the need for future studies and raising a red flag of caution against increasing BPA exposure. Our data agree with Ruiz-Priego et al. [76], supporting the use of NGAL and KIM-1 as biomarkers of BPA-nephropathy.

Animals treated with BPA showed a significant increase in the expression of the CHOP protein. The kidney is one of the organs with the highest ER stress susceptibility because the fractional protein synthesis rates are almost half the total body load daily [77]. The possibility that ER stress is involved in the development of diabetic nephropathy has been described. Among the three signaling pathways that make up the ER stress response system, two of them are protective, and the remaining one, ERK-ATF4-CHOP, induces apoptosis in some kidney diseases [78]. It is interesting to note that both diabetic animals (as in other publications [79]) and those treated with BPA showed high CHOP levels. In this way, the hypothesis that BPA can induce similar damage to diabetic nephropathy is reaffirmed.

In order to get inside into the molecular mechanisms involves in the renal changes observed, we first analyzed the proapoptotic and profibrogenic TGF-β system. As expected, diabetic and BPA groups display the upregulation of TGF-β, a cytokine involved in hypertrophy, apoptosis, and renal fibrosis. Interestingly, this upregulation was significantly higher in the D+BPA group, thus suggesting a molecular mechanism for the observed renal parameters impairment in the latter group.

We then analyzed the renal expression of MCP-1, TNF-α and IL-1β well-known pro-inflammatory mediators in DN [80]. As expected, these molecules were found to be upregulated in D mice. On the contrary, BPA mice did not show changes in the expression of these pro-inflammatory mediators. It is well stablished that BPA can promote an inflammatory response by upregulating inflammatory mediators such as IL-1β, TNF-α, or MCP-1 [76,81,82], it is also known that it can also trigger the expression of anti-inflammatory molecules like IL-10 or TGF-β [83,84]. Herein we observed a significant upregulation of IL-10 restricted to BPA-treated mice. This finding could account, at least in part, for the lack of activation of proinflammatory mediators in this animal group.

In any case, recent observations using a BPA concentration higher than renal NOAEL, demonstrate the upregulation of several proinflammatory mediators (including MCP-1, RANTES or IL-6) [76]. All data available strongly suggest that the level of BPA exposure might be critical to promote renal inflammation.

In conclusion, we observed that oral administration of BPA is capable of promoting in the kidney alterations that resemble early DN, such as increased in kidney damage biomarkers NGAL and KIM-1, UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli as well as TGF-β, CHOP and PCNA upregulation, albeit they did not develop changes in serum creatinine as observed in D mice. Moreover, UAE, collapsed glomeruli, PCNA staining, TGF-β (TIIR), and animal

survival, significantly impaired in diabetic animals receiving BPA. Furthermore, UAE, collapsed glomeruli and animal survival also displayed a sexual dimorphism pattern.

Collectively, these data show that further translational studies are needed to clarify the potential role of BPA in renal diseases, particularly in diabetic patients.

5. Funding

This work was supported in part by grants from Instituto de Salud Carlos III (PI15/02139) -Fondo Europeo de Desarrollo Regional (FEDER)-.

R. Moreno-Gómez-Toledano is recipient of a research contract from CAM (B2017-BMD-3686).

6. Acknowledgments

We want to thank M. Bosch for proofreading of the manuscript. We also want to thank Dr. Jordi Bover (Nephrology Service, Germans Trias i Pujol Hospital, Universitat Autònoma de Barcelona) for his suggestions and comments.

7. References

[1] B. Zhou, Y. Lu, K. Hajifathalian, J. Bentham, M. Di Cesare, G. Danaei, H. Bixby, M.J. Cowan, M.K. Ali, C. Taddei, W.C. Lo, B. Reis-Santos, G.A. Stevens, L.M. Riley, J.J. Miranda, P. Bjerregaard, J.A. Rivera, H.M. Fouad, G. Ma, J.Z. Cisneros, Worldwide trends in diabetes since 1980: A pooled analysis of 751 population-based studies with 4.4 million participants, Lancet. 387 (2016) 1513–1530. https://doi.org/10.1016/S0140-6736(16)00618-8.

[2] S. Wild, G. Roglic, A. Green, R. Sicree, H. King, Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030, Diabetes Care. 27 (2004) 1047–1053. https://doi.org/10.2337/diacare.27.5.1047.

[3] P. Saeedi, I. Petersohn, P. Salpea, B. Malanda, S. Karuranga, N. Unwin, S. Colagiuri, L. Guariguata, A.A. Motala, K. Ogurtsova, J.E. Shaw, D. Bright, R. Williams, Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition, Diabetes Res. Clin. Pract. 157 (2019). https://doi.org/10.1016/j.diabres.2019.107843.

[4] O. Gheith, N. Farouk, N. Nampoory, M.A. Halim, T. Al-Otaibi, Diabetic kidney disease: world wide difference of prevalence and risk factors., J. Nephropharmacology. 5 (2016) 49–56. http://www.ncbi.nlm.nih.gov/pubmed/28197499.

[5] M. Narres, H. Claessen, S. Droste, T. Kvitkina, M. Koch, O. Kuss, A. Icks, The incidence of end-stage renal disease in the diabetic (compared to the non-diabetic) population: A systematic review, PLoS One. 11 (2016). https://doi.org/10.1371/journal.pone.0147329.

[6] E. Ritz, I. Rychlik, F. Locatelli, S. Halimi, End-stage renal failure in type 2 diabetes: A medical catastrophe of worldwide dimensions, Am. J. Kidney Dis. 34 (1999) 795–808. https://doi.org/10.1016/S0272-6386(99)70035-1.

[7] E. Ribeiro, C. Ladeira, S. Viegas, Occupational Exposure to Bisphenol A (BPA): A Reality That Still Needs to Be Unveiled, Toxics. 5 (2017) 22. https://doi.org/10.3390/toxics5030022.

[8] P. Allard, M.P. Colaiácovo, Bisphenol A, in: R.C. Gupta (Ed.), Reproductive and Developmental Toxicology , Elsevier, Amsterdam, 2011: p. 1202. https://doi.org/10.1016/B978-0-12-382032-7.10050-5.

[9] J. Corrales, L.A. Kristofco, W. Baylor Steele, B.S. Yates, C.S. Breed, E. Spencer Williams,

B.W. Brooks, Global assessment of bisphenol a in the environment: Review and analysis of its occurrence and bioaccumulation, Dose-Response. 13 (2015) 1–29. https://doi.org/10.1177/1559325815598308.

[10] A. Bacle, S. Thevenot, C. Grignon, M. Belmouaz, M. Bauwens, B. Teychene, N. Venisse, V. Migeot, A. Dupuis, Determination of bisphenol A in water and the medical devices used in hemodialysis treatment, Int. J. Pharm. 505 (2016) 115–121. https://doi.org/10.1016/j.ijpharm.2016.03.003.

[11] M.K. Björnsdotter, J. de Boer, A. Ballesteros-Gómez, Bisphenol A and replacements in thermal paper: A review, Chemosphere. 182 (2017) 691–706. https://doi.org/10.1016/j.chemosphere.2017.05.070.

[12] C. Freire, J.-M. Molina-Molina, L.M. Iribarne-Duran, I. Jimenez-Diaz, F. Vela-Soria, V. Mustieles, J. Pedro Arrebola, M.F. Fernandez, F. Artacho-Cordon, N. Olea, Concentrations of bisphenol A and parabens in socks for infants and young children in Spain and their hormone-like activities, Environ. Int. 127 (2019) 592–600. https://doi.org/10.1016/j.envint.2019.04.013.

[13] A.M. Calafat, Z. Kuklenyik, J.A. Reidy, S.P. Caudill, J. Ekong, L.L. Needham, Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population, Environ. Health Perspect. 113 (2005) 391–395. https://doi.org/10.1289/ehp.7534.

[14] A. Covaci, E. Den Hond, T. Geens, E. Govarts, G. Koppen, H. Frederiksen, L.E. Knudsen, T.A. Morck, A.C. Gutleb, C. Guignard, E. Cocco, M. Horvat, E. Heath, T. Kosjek, D. Mazej, J.S. Tratnik, A. Castano, M. Esteban, F. Cutanda, J.J. Ramos, M. Berglund, K. Larsson, B.A.G. Jonsson, P. Biot, L. Casteleyn, R. Joas, A. Joas, L. Bloemen, O. Sepai, K. Exley, G. Schoeters, J. Angerer, M. Kolossa-Gehring, U. Fiddicke, D. Aerts, H.M. Koch, J. Jose Ramos, M. Berglund, K. Larsson, B.A.G. Jonsson, P. Biot, L. Casteleyn, R. Joas, A. Joas, L. Bloemen, O. Sepai, K. Exley, G. Schoeters, J. Angerer, M. Kolossa-Gehring, U. Fiddicke, D. Aerts, H.M. Koch, Urinary BPA measurements in children and mothers from six European member states: Overall results and determinants of exposure, Environ. Res. 141 (2015) 77–85. https://doi.org/10.1016/j.envres.2014.08.008.

[15] Z. Zhang, H. Alomirah, H.S. Cho, Y.F. Li, C. Liao, T.B. Minh, M.A. Mohd, H. Nakata, N. Ren, K. Kannan, Urinary bisphenol a concentrations and their implications for human exposure in several Asian countries, Environ. Sci. Technol. 45 (2011) 7044–7050. https://doi.org/10.1021/es200976k.

[16] X. Li, G.-G.G. Ying, J.-L.L. Zhao, Z.-F.F. Chen, H.-J.J. Lai, H.-C.C. Su, 4-Nonylphenol, bisphenol-A and triclosan levels in human urine of children and students in China, and the effects of drinking these bottled materials on the levels, Environ. Int. 52 (2013) 81–86. https://doi.org/10.1016/j.envint.2011.03.026.

[17] T. Bushnik, D. Haines, P. Levallois, J. Levesque, J. Van Oostdam, C. Viau, Lead and bisphenol A concentrations in the Canadian population, Heal. REPORTS. 21 (2010) 7–18.

[18] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J.R.R. Paumgartten, G. Schoenfelder, Urinary, Circulating, and Tissue Biomonitoring Studies Indicate Widespread Exposure to Bisphenol A, Environ. Health Perspect. 118 (2010) 1055–1070. https://doi.org/10.1289/ehp.0901716.

[19] A.C. Gore, V.A. Chappell, S.E. Fenton, J.A. Flaws, A. Nadal, G.S. Prins, J. Toppari, R.T. Zoeller, Executive Summary to EDC-2: The Endocrine Society's second Scientific Statement on endocrine-disrupting chemicals, Endocr. Rev. 36 (2015) 593–602. https://doi.org/10.1210/er.2015-1093.

[20] E. Testai, P. Hartemann, E. Rodriguez-Farre, S.C. Rastogi, J. Bustos, U. Gundert-Remy, A. Hensten, H.M. Kopperud, N.N. Olea, A. Piersma, W. De Jong, The safety of the use of bisphenol A in medical devices., Regul. Toxicol. Pharmacol. 79 (2016) 106–107. https://doi.org/10.1016/j.yrtph.2016.01.014.

[21] J.R. Rochester, Bisphenol A and human health: A review of the literature, Reprod. Toxicol. 42 (2013) 132–155. https://doi.org/10.1016/j.reprotox.2013.08.008.

[22] M. Saura, S. Marquez, P. Reventun, N. Olea-Herrero, M.I. Arenas, R. Moreno-Gómez-Toledano, M. Gómez-Parrizas, C. Muñóz-Moreno, M. González-Santander, C. Zaragoza, R.J. Bosch, Oral administration of bisphenol A induces high blood pressure through angiotensin II/CaMKII-dependent uncoupling of eNOS, FASEB J. 28 (2014) 4719–4728. https://doi.org/10.1096/fj.14-252460.

[23] Y. Ilagan, R. Mamillapalli, L.G. Goetz, J. Kayani, H.S. Taylor, Bisphenol-A exposure in utero programs a sexually dimorphic estrogenic state of hepatic metabolic gene expression, Reprod. Toxicol. 71 (2017) 84–94.

[24] B. Debenedictis, H. Guan, K. Yang, Prenatal Exposure to Bisphenol A Disrupts Mouse Fetal Liver Maturation in a Sex-Specific Manner, J. Cell. Biochem. 117 (2016) 344–350. https://doi.org/10.1002/jcb.25276.

[25] Y. Pu, J.D. Gingrich, J.P. Steibel, A. Veiga-Lopez, Sex-Specific Modulation of Fetal Adipogenesis by Gestational Bisphenol A and Bisphenol S Exposure, Endocrinology. 158 (2017) 3844–3858. https://doi.org/10.1210/en.2017-00615.

[26] V. Mustieles, R. Pérez-Lobato, N. Olea, M.F. Fernández, Bisphenol A: Human exposure and neurobehavior, Neurotoxicology. 49 (2015) 174–184. https://doi.org/10.1016/j.neuro.2015.06.002.

[27] K.A. McCaffrey, B. Jones, N. Mabrey, B. Weiss, S.H. Swan, H.B. Patisaul, Sex specific impact of perinatal bisphenol A (BPA) exposure over a range of orally administered doses on rat hypothalamic sexual differentiation, Neurotoxicology. 36 (2013) 55–62. https://doi.org/10.1016/j.neuro.2013.03.001.

[28] S. Mhaouty-Kodja, L.P. Belzunces, M.C. Canivenc, H. Schroeder, C. Chevrier, E. Pasquier, Impairment of learning and memory performances induced by BPA: Evidences from the literature of a MoA mediated through an ED, Mol. Cell. Endocrinol. 475 (2018) 54–73. https://doi.org/10.1016/j.mce.2018.03.017.

[29] S.E. Arambula, D. Jima, H.B. Patisaul, Prenatal bisphenol A (BPA) exposure alters the transcriptome of the neonate rat amygdala in a sex-specific manner: a CLARITY-BPA consortium study, Neurotoxicology. 65 (2018) 207–220. https://doi.org/10.1016/j.neuro.2017.10.005.

[30] R.B. Gear, S.M. Belcher, Impacts of Bisphenol A and Ethinyl Estradiol on Male and Female CD-1 Mouse Spleen, Sci. Rep. 7 (2017) 856. https://doi.org/10.1038/s41598-017-00961-8.

[31] P. Nuñez, T. Fernandez, M. García-Arévalo, P. Alonso-Magdalena, A. Nadal, C. Perillan, J. Arguelles, Effects of bisphenol A treatment during pregnancy on kidney development in mice: a stereological and histopathological study., J. Dev. Orig. Health Dis. 9 (2018) 208–214. https://doi.org/10.1017/S2040174417000939.

[32] T. Pollock, D. deCatanzaro, Presence and bioavailability of bisphenol A in the uterus of rats and mice following single and repeated dietary administration at low doses, Reprod.

Toxicol. 49 (2014) 145–154. https://doi.org/10.1016/j.reprotox.2014.08.005.

[33] M. Li, Y. Bi, L. Qi, T. Wang, M. Xu, Y. Huang, Y. Xu, Y. Chen, J. Lu, W. Wang, G. Ning, Exposure to bisphenol A is associated with low-grade albuminuria in Chinese adults, Kidney Int. 81 (2012) 1131–1139. https://doi.org/10.1038/ki.2012.6.

[34] L. Trasande, T.M. Attina, H. Trachtman, Bisphenol A exposure is associated with low-grade urinary albumin excretion in children of the United States, Kidney Int. 83 (2013) 741–748. https://doi.org/10.1038/ki.2012.422.

[35] A. Shankar, S. Teppala, Urinary bisphenol A and hypertension in a multiethnic sample of US adults., J. Environ. Public Health. 2012 (2012) 481641. https://doi.org/10.1155/2012/481641.

[36] S. Bae, Y.-C.C. Hong, Exposure to bisphenol a from drinking canned beverages increases blood pressure: Randomized crossover trial, Hypertension. 65 (2015) 313–319. https://doi.org/10.1161/HYPERTENSIONAHA.114.04261.

[37] N. Olea-Herrero, M.I. Arenas, C. Munoz-Moreno, R. Moreno-Gómez-Toledano, M. Gonzalez-Santander, I. Arribas, R.J. Bosch, Bisphenol-A Induces Podocytopathy With Proteinuria in Mice, J. Cell. Physiol. 229 (2014) 2057–2066. https://doi.org/10.1002/jcp.24665.

[38] M. Kitada, Y. Ogura, D. Koya, Rodent models of diabetic nephropathy: Their utility and limitations, Int. J. Nephrol. Renovasc. Dis. 9 (2016) 279–290. https://doi.org/10.2147/IJNRD.S103784.

[39] A. Izquierdo, P. López-Luna, A. Ortega, M. Romero, M.A. Gutiérrez-Tarrés, I. Arribas, M.J.R. Álvarez, P. Esbrit, R.J. Bosch, The parathyroid hormone-related protein system and diabetic nephropathy outcome in streptozotocin-induced diabetes, Kidney Int. 69 (2006) 2171–2178. https://doi.org/10.1038/sj.ki.5000195.

[40] M.W. Pfaffl, A. Tichopad, C. Prgomet, T.P. Neuvians, Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations, Biotechnol. Lett. 26 (2004) 509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47.

[41] C. Jean-Faucher, M. Berger, C. Gallon, M. De Turckheim, G. Veyssiere, C. Jean, Sex-related differences in renal size in mice: Ontogeny and influence of neonatal androgens, J. Endocrinol. 115 (1987) 241–246. https://doi.org/10.1677/joe.0.1150241.

[42] A. Yabuki, S. Tanaka, M. Matsumoto, S. Suzuki, Morphometric study of gender differences with regard to age-related changes in the C57BL/6 mouse kidney, Exp. Anim. 55 (2006) 399–404. https://doi.org/10.1538/expanim.55.399.

[43] L. Huang, A. Scarpellini, M. Funck, E.A.M. Verderio, T.S. Johnson, Development of a Chronic Kidney Disease Model in C57BL/6 Mice with Relevance to Human Pathology, Nephron Extra. 3 (2013) 12–29. https://doi.org/10.1159/000346180.

[44] A. Yoldas, M.O. Dayan, Morphological characteristics of renal artery and kidney in rats, Sci. World J. 2014 (2014). https://doi.org/10.1155/2014/468982.

[45] R.P. Amin, A.E. Vickers, F. Sistare, K.L. Thompson, R.J. Roman, M. Lawton, J. Kramer, H.K. Hamadeh, J. Collins, S. Grissom, L. Bennett, C.J. Tucker, S. Wild, C. Kind, V. Oreffo, J.W. Davis, S. Curtiss, J.M. Naciff, M. Cunningham, R. Tennant, J. Stevens, B. Car, T.A. Bertram, C.A. Afshari, Identification of putative gene-based markers of renal toxicity, Environ. Health Perspect. 112 (2004) 465–479. https://doi.org/10.1289/ehp.6683.

[46] W.C. Prozialeck, V.S. Vaidya, J. Liu, M.P. Waalkes, J.R. Edwards, P.C. Lamar, A.M. Bernard, X. Dumont, J. V. Bonventre, Kidney injury molecule-1 is an early biomarker of cadmium nephrotoxicity, Kidney Int. 72 (2007) 985–993. https://doi.org/10.1038/sj.ki.5002467.

[47] J. Hu, Y. Wang, X. Xiang, C. Peng, R. Gao, R. Goswami, H. Zhou, Y. Zhang, Q. Zhen, Q. Cheng, S. Yang, Q. Li, Serum bisphenol A as a predictor of chronic kidney disease progresion in primary hypertension: A 6-year prospective study, J. Hypertens. 34 (2016) 332–337. https://doi.org/10.1097/HJH.0000000000000780.

[48] J. Hu, S. Yang, Y. Wang, R. Goswami, C. Peng, R. Gao, H. Zhou, Y. Zhang, Q. Cheng, Q. Zhen, Q. Li, Serum bisphenol A and progression of type 2 diabetic nephropathy: a 6-year prospective study, ACTA Diabetol. 52 (2015) 1135–1141. https://doi.org/10.1007/s00592-015-0801-5.

[49] M.L. Gross, E. Ritz, A. Schoof, M. Adamczak, A. Koch, O. Tulp, A. Parkman, A. El-Shakmak, A. Szabo, K. Amann, Comparison of renal morphology in the Streptozotocin and the SHR/N-cp models of diabetes, Lab. Investig. 84 (2004) 452–464. https://doi.org/10.1038/labinvest.3700052.

[50] M.P. O'Donnell, B.L. Kasiske, W.F. Keane, Glomerular hemodynamic and structural alterations in experimental diabetes mellitus, FASEB J. 2 (1988) 2339–2347. https://doi.org/10.1096/fasebj.2.8.3282959.

[51] M. Romero, A. Ortega, A. Izquierdo, P. López-Luna, R.J. Bosch, Parathyroid hormone-related protein induces hypertrophy in podocytes via TGF-β1 and p27Kip1: Implications for diabetic nephropathy, Nephrol. Dial. Transplant. 25 (2010) 2447–2457. https://doi.org/10.1093/ndt/gfq104.

[52] Tisher C.C., T.H. Hostetter, Diabetic Nephropathy, in: T. CC, B. BM (Eds.), Renal Pathology (vol.2), JB Lippincott Co. Ed, Philadelphia, 1994: p. 1387.

[53] M.E. Cooper, D. Vranes, S. Youssef, S.A. Stacker, A.J. Cox, B. Rizkalla, D.J. Casley, L.A. Bach, D.J. Kelly, R.E. Gilbert, Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes, Diabetes. 48 (1999) 2229–2239. https://doi.org/10.2337/diabetes.48.11.2229.

[54] M. Katoh, Y. Ohmachi, Y. Kurosawa, H. Yoneda, N. Tanaka, H. Narita, Effects of imidapril and captopril on streptozotocin-induced diabetic nephropathy in mice, Eur. J. Pharmacol. 398 (2000) 381–387. https://doi.org/10.1016/S0014-2999(00)00320-4.

[55] T. Yotsumoto, T. Naitoh, K.I. Shikada, S. Tanaka, Effects of specific antagonists of angiotensin II receptors and captopril on diabetic nephropathy in mice, Jpn. J. Pharmacol. 75 (1997) 59–64. https://doi.org/10.1254/jjp.75.59.

[56] R.W. Tyl, C.B. Myers, M.C. Marr, C.S. Sloan, N.P. Castillo, M.M. Veselica, J.C. Seely, S.S. Dimond, J.P. Van Miller, R.N. Shiotsuka, D. Beyer, S.G. Hentges, J.M. Waechter Jr., J.M.J. Waechter, Two-generation reproductive toxicity study of dietary bisphenol a in CD-1 (Swiss) mice, Toxicol. Sci. 104 (2008) 362–384. https://doi.org/10.1093/toxsci/kfn084.

[57] EFSA, Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA J. 13 (2016) 3978. https://doi.org/10.2903/j.efsa.2015.3978.

[58] EFSA, What potential health effects of BPA has EFSA identified? EFSA's risk assessment in more detail How did EFSA's experts calculate the new TDI?, (n.d.). https://doi.org/10.2805/075460.

[59] K. Ćwiek-Ludwicka, Bisphenol A (BPA) in food contact materials - new scientific opinion

from EFSA regarding public health risk, Rocz. Panstw. Zakl. Hig. 66 (2015) 299–307. https://doi.org/10.1016/j.reprotox.2016.06.020.

[60] G. Giebisch, E.E. Windhager, P.S. Aronson, Glomerular filtration and renal blood flow, in: W.F. Boron, E.L. Boulpaep (Eds.), Medical Physiology, 3rd ed., Elsevier, Amsterdam, 2016: p. 1312.

[61] A. Ortega, M. Romero, A. Izquierdo, N. Troyano, Y. Arce, J.A. Ardura, M.I. Arenas, J. Bover, P. Esbrit, R.J. Bosch, Parathyroid hormone-related protein is a hypertrophy factor for human mesangial cells: Implications for diabetic nephropathy, J. Cell. Physiol. 227 (2012) 1980–1987. https://doi.org/10.1002/jcp.22926.

[62] L. Caporossi, B. Papaleo, Exposure to Bisphenol a and Gender Differences: From Rodents to Humans Evidences and Hypothesis about the Health Effects, J. Xenobiotics. 5 (2015) 5264. https://doi.org/10.4081/xeno.2015.5264.

[63] C. Teng, B. Goodwin, K. Shockley, M. Xia, R. Huang, J. Norris, B.A. Merrick, A.M. Jetten, C.P. Austin, R.R. Tice, Bisphenol A affects androgen receptor function via multiple mechanisms, Chem. Biol. Interact. 203 (2013) 556–564. https://doi.org/10.1016/j.cbi.2013.03.013.

[64] V. Müller, G. Losonczy, U. Heemann, Á. Vannay, A. Fekete, G. Reusz, T. Tulassay, A.J. Szabó, Sexual dimorphism in renal ischemia-reperfusion injury in rats: Possible role of endothelin, Kidney Int. 62 (2002) 1364–1371. https://doi.org/10.1046/j.1523-1755.2002.00590.x.

[65] M.K.O. Grant, D.M. Seelig, L.C. Sharkey, W.S.V. Choi, I.Y. Abdelgawad, B.N. Zordoky, Sexual dimorphism of acute doxorubicin-induced nephrotoxicity in C57Bl/6 mice, PLoS One. 14 (2019) 1–19. https://doi.org/10.1371/journal.pone.0212486.

[66] M. Shi, K.L. McMillan, J. Wu, N. Gillings, B. Flores, O.W. Moe, M.C. Hu, Cisplatin nephrotoxicity as a model of chronic kidney disease, Lab. Investig. 98 (2018) 1105–1121. https://doi.org/10.1038/s41374-018-0063-2.

[67] J. Neugarten, A. Acharya, S.R. Silbiger, Effect of Gender on the Progression of Nondiabetic Renal Disease.pdf, J Am Soc Nephrol. 11 (2000) 319–329.

[68] J. Neugarten, L. Golestaneh, Gender and the prevalence and progression of renal disease, Adv. Chronic Kidney Dis. 20 (2013) 390–395. https://doi.org/10.1053/j.ackd.2013.05.004.

[69] F. de Álvaro Moreno, A. Aguilera Peralta, E. Valdés Chiong, Nefropatía diabética, Med. - Programa Form. Médica Contin. Acreditado. 8 (2000) 1001–1008. https://doi.org/10.1016/S0304-5412(00)70188-0.

[70] A. Martínez-Castelao, Nefropatía diabética, in: M. Ed (Ed.), Manual de Nefrología clínica, díalisis y transporte renal, 1998: pp. 1043–1059.

[71] M.D. Breyer, E. Böttinger, F.C. Brosius, T.M. Coffman, R.C. Harris, C.W. Heilig, K. Sharma, AMDCC, Mouse Models of Diabetic Nephropathy, J. Am. Soc. Nephrol. 16 (2005) 27–45. https://doi.org/10.1681/ASN.2004080648.

[72] G. D'Amico, C. Bazzi, Pathophysiology of proteinuria, Kidney Int. 63 (2003) 809–825. https://doi.org/10.1046/j.1523-1755.2003.00840.x.

[73] T.W. Meyer, P.H. Bennett, R.G. Nelson, Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria,

Diabetologia. 42 (1999) 1341–1344. https://doi.org/10.1007/s001250051447.

[74] H. V. Alderson, J.P. Ritchie, S. Pagano, R.J. Middleton, M. Pruijm, N. Vuilleumier, P.A. Kalra, The associations of blood kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin with progression from CKD to ESRD, Clin. J. Am. Soc. Nephrol. 11 (2016) 2141–2149. https://doi.org/10.2215/CJN.02670316.

[75] K.G. Garlo, W.B. White, G.L. Bakris, F. Zannad, C.A. Wilson, S. Kupfer, M. Vaduganathan, D.A. Morrow, C.P. Cannon, D.M. Charytan, Kidney biomarkers and decline in eGFR in patients with type 2 diabetes, Clin. J. Am. Soc. Nephrol. 13 (2018) 398–405. https://doi.org/10.2215/CJN.05280517.

[76] A.R. Priego, E.G. Parra, S. Mas, J.L. Morgado-Pascual, M. Ruiz-Ortega, S. Rayego-Mateos, Bisphenol a modulates autophagy and exacerbates chronic kidney damage in mice, Int. J. Mol. Sci. 22 (2021). https://doi.org/10.3390/ijms22137189.

[77] A. Zhuang, J.M. Forbes, Stress in the kidney is the road to pERdition: Is endoplasmic reticulum stress a pathogenic mediator of diabetic nephropathy?, J. Endocrinol. 222 (2014) R97–R111. https://doi.org/10.1530/JOE-13-0517.

[78] M. Taniguchi, H. Yoshida, Endoplasmic reticulum stress in kidney function and disease, Curr. Opin. Nephrol. Hypertens. 24 (2015) 345–350. https://doi.org/10.1097/MNH.0000000000000141.

[79] H. Xie, L. Huang, Y. Li, H. Zhang, H. Liu, Endoplasmic reticulum stress and renal lesion in mice with combination of high-fat diet and streptozotocin-induced diabetes, Acta Cir. Bras. 31 (2016) 150–155. https://doi.org/10.1590/s0102-865020160030000001.

[80] J.F. Navarro, C. Mora, M. Macía, J. García, Inflammatory parameters are independently associated with urinary albumin in type 2 diabetes mellitus, Am. J. Kidney Dis. 42 (2003) 53–61. https://doi.org/10.1016/S0272-6386(03)00408-6.

[81] M. Murata, J.H. Kang, Bisphenol A (BPA) and cell signaling pathways, Biotechnol. Adv. 36 (2018) 311–327. https://doi.org/10.1016/j.biotechadv.2017.12.002.

[82] G.J. Alekhya Sita, M. Gowthami, G. Srikanth, M.M. Krishna, K. Rama Sireesha, M. Sajjarao, K. Nagarjuna, M. Nagarjuna, G.K. Chinnaboina, A. Mishra, N. SreeHarsha, Protective role of luteolin against bisphenol A-induced renal toxicity through suppressing oxidative stress, inflammation, and upregulating Nrf2/ARE/ HO-1 pathway, IUBMB Life. 71 (2019) 1041–1047. https://doi.org/10.1002/iub.2066.

[83] J. Xu, G. Huang, T.L. Guo, Developmental bisphenol a exposure modulates immune-related diseases, Toxics. 4 (2016). https://doi.org/10.3390/toxics4040023.

[84] X. Tian, M. Takamoto, K. Sugane, Bisphenol A promotes IL-4 production by Th2 cells, Int. Arch. Allergy Immunol. 132 (2003) 240–247. https://doi.org/10.1159/000074305.

Supplementary Table 1. SYBR Green primers and TaqMan probes.

RT-PCR primers		
TNF-α	Forward	5´- AGGCACTCCCCCAAAAGATG-3´
	Reverse	5´-TGAGGGTCTGGGCCATAGAA
TGF-β1	Forward	5´-GCAACATGTGGAACTCTACCAG-3’
	Reverse	5´-CAGCCACTCAGGCGTATCA-3’
TIIR	Forward	5’- CCTACTCTGTCTGTGGATGACCT - 3’
	Reverse	5´- ACTTCCGGGGCCATGTAT - 3’
MCP-1	Forward	5’- CACTCACTCCACAACCCAAGA - 3’
	Reverse	5’- CAAAGACCCTCAAAACATCCC - 3’
IL-10	Forward	5’- AGGGTGTCTCCTTCCTCACA - 3’
	Reverse	5’- TGTTACACTCGCCCCCTTTG - 3’
IL-1β	Forward	5’- TGGGCCTCAAAGGAAAGAAT - 3’
	Reverse	5’- CAGGCTTGTGCTCTGCTTGT - 3’
KIM-1	Forward	5’- ATGAATCAGATTCAAGTCTTC - 3’
	Reverse	5’- TCTGGTTTGTGAGTCCATGTG - 3’
NGAL	Forward	5’- CACCACGGACTACAACCAGTTCGC - 3’
	Reverse	5’- TCAGTTGTCAATGCATTGGTCGGTG - 3’
TaqMan probes		
β-Actin	Forward	5´- GCTCTGGCTCCTAGCACCAT- 3´
	Reverse	5´- GCCACCGATCCACACAGAGT- 3´
	Probe	5´- ATCAAGATCATTGCTCCTCCTGAGCGC- 3´