

Tributyltin chloride induces renal dysfunction by inflammation and oxidative stress in female rats



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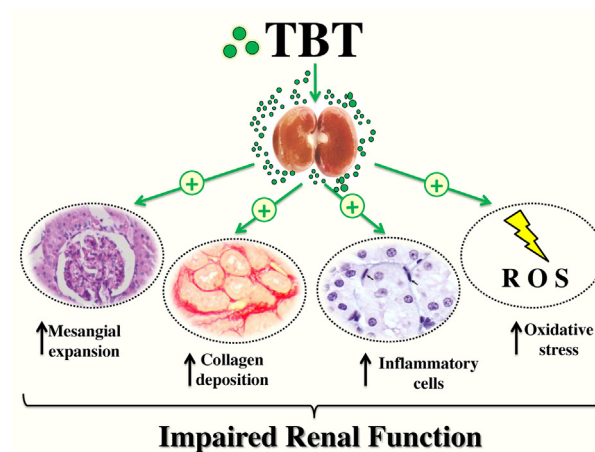
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HIGHLIGHTS

- TBT reduced GFR and increased proteinuria levels in female rats.
- TBT increased renal mesangial expansion and collagen deposition.
- TBT increased inflammation and oxidative stress in renal tissue.

GRAPHICAL ABSTRACT



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ABSTRACT

Tributyltin chloride (TBT) is an organometallic pollutant that is used as a biocide in antifouling paints. TBT induces several toxic and endocrine-disrupting effects. However, studies evaluating the effects of TBT on renal function are rare. This study demonstrates that TBT exposure is responsible for improper renal function as well as the development of abnormal morphophysiology in mammalian kidneys. Female rats were treated with TBT, and their renal morphophysiology was assessed. Morphophysiological

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abnormalities such as decreased glomerular filtration rate and increased proteinuria levels were observed in TBT rats. In addition, increases in inflammation, collagen deposition and α -smooth muscle actin (α -SMA) protein expression were observed in TBT kidneys. A disrupted cellular redox balance and apoptosis in kidney tissue were also observed in TBT rats. TBT rats demonstrated reduced serum estrogen levels and estrogen receptor- α (ER α) protein expression in renal cortex. Together, these data provide *in vivo* evidence that TBT is toxic to normal renal function and that these effects may be associated with renal histopathology complications, such as inflammation and fibrosis.

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1. Introduction

Xenobiotic exposure leads to improper renal function (Robertson et al., 1987; Crean et al., 2014). The kidneys are especially vulnerable to environmental exposure because approximately 20–25% of the cardiac output goes to the kidneys, a fraction of which is then filtered (Stegmayr, 2003; Ximenes et al., 2015). Furthermore, pollutants can accumulate in the kidney, leading to pathological complications such as impaired renal function, inflammation, oxidative stress, and fibrosis (Lin et al., 2003; Hernandez and Rodriguez, 2014; Van der Hauwaert et al., 2014). Robertson et al. (1987) reported that the trimethyltin chloride (TMT) induced nephrotoxicity in the rat kidney, as demonstrated by cortical tubular dilation, inflammation, brush border attenuation and basolateral vacuolization in the proximal tubule cells. Organotin (OTs) such as tributyltin chloride (TBT) are a class of organometallic pollutants used for various industrial purposes, such as antifouling agents (USEPA, 1997; Lyssimachou et al., 2015). Although TBT use in paints has been banned by the IMO (2001) and European Union since 2003, it is still found at high levels that exceed toxicity levels in certain ecosystems and seafoods (Antizar-Ladislao, 2008; Podratz et al., 2015). TBT is a highly persistent chemical that promotes immune neural, metabolic or reproductive toxicity in various mammalian models (Whalen et al., 1999; Grün et al., 2006; Grote et al., 2009; Mitra et al., 2013).

Previous studies performed for us reported the cardiovascular abnormalities associated with a reduction of estrogen (E_2) levels after exposure of TBT in the female rats (dos Santos et al., 2012; Rodrigues et al., 2014; Ribeiro et al., 2016). In addition, several studies have suggested that E_2 reduces the risk of renal disease, as result of a decrease in the collagen deposition and oxidative stress effects in the glomerular mesangial cells (Silbiger et al., 1999; Grodstein et al., 2000). Thus, E_2 may limit the progression of renal disease (Neugarten et al., 1999; Silbiger and Neugarten, 2008).

Despite these discoveries of TBT toxicity, few studies evaluating the relationship between TBT and renal function and its effects on kidney role remain unclear (Robertson et al., 1987; Mitra et al., 2014). In the present study, we investigated the effects of TBT on renal function in female rats. We assessed rat renal function exposed to TBT by analyzing key indicators of kidney competence, including glomerular filtration rate (GFR), proteinuria, kidney histopathology, inflammation, fibrosis, oxidative stress and apoptosis. The identification of the toxic signaling mechanisms affected by TBT in the kidney greatly contributes to our continuously involving understanding of the renal function.

2. Materials and methods

2.1. Chemicals

Tributyltin chloride (TBT, 96%, Sigma, St. Louis, MO, USA) was dissolved in 0.4% ethanol based on protocols from previous studies performed in our laboratory (Podratz et al., 2012; Bertuloso et al., 2015). The reagent used was of analytical grade.

2.2. Experimental animals

Adult female Wistar rats (12-week-old) were maintained under controlled temperature between 23 and 25 °C with a 12:12 h light/dark cycle. Rat chow and filtered tap water were provided *ad libitum*. All protocols were approved by the Ethics Committee of Animals of the Federal University of Espirito Santo (UFES, N° 05/2016). Additionally, all experiments were performed in accordance with the Biomedical Research Guidelines for the Care and Use of Laboratory Animals available online and followed the recommendations of the American Veterinary Medical Association Guidelines (2007). The rats were divided into three groups. Control (CON, n = 12) group rats were treated daily with vehicle (0.4% ethanol). TBT100 (TBT100, n = 12) and TBT500 (TBT500, n = 12) group rats were treated daily with 100 and 500 ng/kg/day TBT, respectively, by gavage for 15 days. All animals were anesthetized using sodium thiopental (50 mg/kg, ip., Fontoveter, Brazil) before euthanasia, and wet organs were weighed. Doses and routes of exposure were chosen based on protocols from previous studies by us (Podratz et al., 2012; Bertuloso et al., 2015; Ribeiro et al., 2016) and others (Zhou et al., 2013) to induce an increase in serum tin levels (Bertuloso et al., 2015). Whole body composition of rodents could be changed with TBT exposure (Grün et al., 2006; Penza et al., 2011). Hence, the weights of control and TBT groups were assessed at the beginning and end of the study.

2.3. Tin assessment

To assess serum tin levels, blood samples were collected, and tin levels were measured using an inductively coupled plasma mass spectrometry (ICP-MS, Nexlon 300-D, Perkin Elmer, Germany) (Podratz et al., 2015). Briefly, blood samples were diluted 1:49 with a diluent solution containing 0.5% (v/v) double-distilled ultra-pure HNO_3 (Elga – Purelab, Marlow, UK) (Palmer et al., 2006). Kidney tin levels were also assessed. Kidney samples were collected, and tin levels were measured using an ICP-MS. Briefly, kidney samples were digested with 30% H_2O_2 (m/m) and ultra-pure HNO_3 (Elga – Purelab, Marlow, UK) using a microwave oven equipped with PTFE vessels (Multiwave 3000 microwave, Anton Paar, Graz, Austria).

The blood and kidney analyses were carried out with an ICP-MS, and argon gas (99.999% Air Liquid, RJ, Brazil) was used in ICP-MS determinations for plasma generation, nebulization and auxiliary gas. The sample introduction system was composed of a cyclonic spray chamber and a Meinhard[®] nebulizer. The protocol quality control section requirements were closely followed to demonstrate accurate quantification of tin in the blood and kidney samples used in this study. The mass-to-charge ratio (m/z) was monitored with isotope $118Sn^+$. The ICP-MS operating conditions were performed with generator frequency of 40.0 MHz, plasma gas flow rate of 16.01 min^{-1} , auxiliary gas flow rate of 1.201 min^{-1} , nebulizer gas flow rate of 1.021 min^{-1} , and RF power of 1.55 kW. The accuracy of the procedure was checked using a reference sample (Seronom Urine, Sero AS, Norway). The level of detection (LOD) for tin determined by ICP-MS was 4.0 ng g^{-1} . LOD levels were determined for each analysis performed.

2.4. Estrogen assessment

To measure the serum estrogen (E_2) levels, blood samples were collected from female rats after euthanasia. Serum E_2 levels were measured using an ELISA assay (#42K034 DRG Instruments GmbH, Germany) on an ELx808™ Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). A standard curve was generated using 6-fold serial dilutions of the hormone reference provided by Diagnostic Systems Laboratories. Low- and high-quality controls were run on each assay to assess the coefficient of variation values. The assay detection limit for E_2 was 9.71 pg/ml. The intra-assay coefficient of variation for each assay was between 4% and 7%. The inter-assay coefficient of variation for each assay was between 6% and 9%. This E_2 assay has previously been validated for use in rats (Podratz et al., 2015; Rossi et al., 2016).

2.5. Glomerular filtration rate (GFR) assessment

The animals were anesthetized with sodium thiopental. A tracheotomy was performed to facilitate breathing, followed by insertion of polyethylene catheters into the femoral artery to collect blood samples and into the femoral vein to infuse saline containing 3% mannitol (Rocco et al., 2008). A catheter was inserted into the bladder for urine collection. The arterial catheter was connected to a pressure transducer (Cobe Laboratories, USA), which was plugged into a pressure-processor amplifier and data acquisition system (MP100, Biopac Systems, USA) for continuous monitoring of arterial pressure and heart rate. A 30 min infusion with saline containing 3% mannitol at a rate of 0.1 ml/min was performed, followed by a priming injection of 1 ml of saline containing inulin (300 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). After that, an infusion of saline, inulin (15 mg/ml), and 3% mannitol at a rate of 0.1 ml/min was started. Four urine and blood samples were collected at 30 min intervals starting 30 min after injection of the priming solution. Plasma and urinary inulin levels were measured using a colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). Inulin is freely filtered into the glomeruli and not reabsorbed or secreted in tubules. Hence, inulin clearance is considered the gold standard to determine the GFR.

2.6. Proteinuria assessment

During the GFR protocol, urine samples were collected to evaluate the proteinuria levels as described in Landgraf et al. (2014). Samples were centrifuged before the urinary analysis, and the supernatant was separated and stored at -20°C until the assays. The urinary protein levels were determined by the modified method of Lowry et al. (1951). The proteinuria assessment was normalized by the urinary volume collected over 2.5 h. In addition, urine samples were resolved on 10% SDS-PAGE gels (50 μg). As a control, we also resolved bovine serum albumin (BSA) on the SDS-PAGE gel (5 μg). Gels containing urine samples were stained with Coomassie Blue, and the proteinuria analysis was based on the protein band intensity of Coomassie Blue staining.

2.7. Morphological analysis

The animals were anaesthetized to collect various organs, and wet weights were obtained. In addition, the renal index (organ weight/body weight) was calculated to determine the extent of organ hypertrophy using the kidney wet and dry weights (Lowe et al., 2012; Dias et al., 2014). For to obtain the kidney dry weight, other set of control, TBT100 and TBT500 rats were performed ($n=5$). The kidneys were removed, sectioned at 2 mm thickness and fixed in paraformaldehyde diluted in phosphate-buffered saline (4% PF-PBS) pH 7.4 for 24–48 h at room temperature. After

fixation, the tissues were embedded in paraffin at 60°C and subsequently sectioned into 5 μm slices. The sections were stained with hematoxylin and eosin (H&E) to visualize renal regions (Trujillo et al., 2015), Alcian blue to visualize the presence of mast cells (Bertuloso et al., 2015), and Periodic acid-Schiff (PAS) (Sigma-Aldrich, St. Louis, MA) to visualize the basement membrane and mesangial expansion (Castiglione et al., 2013). The superficial collagen deposition in the cortical interstitial area and glomerular tuft was determined using Picrosirius Red (PS) (Sigma-Aldrich, St. Louis, MO) (Landgraf et al., 2014).

2.8. Histomorphometry

For histomorphometry, 25 glomeruli and cortical interstitial areas per animal were captured with a 40 $\times$ objective and then analyzed. High-quality images (2048 $\times$ 1536 pixels) were obtained with an Olympus microscope (AX70; Olympus, Center Valley, PA), photographed with an AxioCamICc1 camera and exported to Axio-Vision Software (AxioVision Rel. 4.8 software). The images were measured by Image J software (version 1.33, Public Domain).

2.9. Mast cell assessment

Renal sections were stained with Alcian blue according to a standard protocol (Sigma-Aldrich, St. Louis, MO). Each 5 μm section was used to obtain 20 photomicrographs (40 $\times$ objective). The number of positively stained cells (i.e., cells containing purple cytoplasmic granules) within the tissue was evaluated. The areas to be analyzed were randomly selected, with the exception that fields containing medium-sized blood vessels were carefully avoided. The number of positively stained cells was then expressed as units per area (mm^2) as described previously (Arzi et al., 2010; Bertuloso et al., 2015).

2.10. Glomerular histological analysis

PAS-stained sections were used to capture 25 photomicrographs of randomly chosen glomeruli. Glomerular area and glomerular tuft area were measured in each image using Image-Pro Plus Software version 4.5.1 for Windows (Media Cybernetics, Silver Spring, MD, USA). The fields for basement membrane and mesangial expansion were chosen randomly, and the results represent the percentage of total expansion per tissue area (Castiglione et al., 2013).

2.11. Collagen density surface assessment

PS staining was used to quantify the total collagen fiber deposition area in glomeruli (PSG) and the tubulointerstitial (PST) region (25 photomicrographs each). The fields were chosen randomly, and the results represent the percentage of total collagen fibers per tissue area (Castiglione et al., 2013).

2.12. Immunohistochemistry in kidney

The renal tissues were fixed in PF4%-PBS and embedded in paraffin. The 4 μm tissue sections were deparaffinised as described previously (Castiglione et al., 2013) and then subjected to antigen retrieval for 40 min using 10 mM citrate buffer, pH 6.0 at 95°C . Thereafter, the slides were rinsed in PBS (pH 7.4). The endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 30 min. The sections were incubated with a blocking solution containing 1% BSA for 1 h at room temperature. The sections were incubated with the caspase-3 primary antibody (1:200, sc-7148, SCBT, Inc., CA) overnight at 4°C . After washing with PBS, the antibody was visualized with the Dako LSAB 2 System HRP kit

(Dako, Carpinteria, CA, USA) using 3, 3'-diaminobenzidine tetrahydrochloride as chromogenic substrate (Liquid DAB, Dako) and counterstained with Mayer's haematoxylin. To provide a negative control for the caspase-3 immunostaining, the primary antibody was omitted, and the positive control was provided by the use of kidney tissue. During the microscopic analysis, an overview was performed to qualify the slides, and then photomicrographs of the stained cells were captured at 400 $\times$ magnification using a system that incorporated a light microscope (AX70; Olympus, Center Valley, PA) and a digital camera (AxioCamICc1).

2.13. Tissue extraction and assessment of myeloperoxidase and *n*-acetyl- β -D-glucosaminidase activity

The infiltration of mononuclear cells into the kidneys was quantified by measuring the level of the lysosomal enzyme *n*-acetyl- β -D-glucosaminidase (NAG), which is present at high levels in activated macrophages (Merlo et al., 2016). The kidney previously stored at -80°C were homogenized in 0.9% saline solution containing 0.1% v/v Triton X-100 (Promega) and then centrifuged. A sample of the resulting supernatant was incubated for 10 min with 100 μl of *p*-nitrophenyl-*n*-acetyl- β -D-glucosaminide (Sigma-Aldrich Co., LLC) that was prepared in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4 ; pH 4.5) to yield a final concentration of 2.24 mM. The reaction was terminated by adding 100 μl of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring absorption at 400 nm. The results are expressed as OD/mg wet tissue.

The number of neutrophils in the kidney was measured by assaying myeloperoxidase (MPO) activity, as previously described (Merlo et al., 2016). Kidney was weighed, homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M NaPO_4 , 0.015 M NaEDTA), and centrifuged. The pellets were then re-suspended in 0.05 M NaPO_4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide, followed by three freeze-thaw cycles using $\text{N}_2(1)$. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by adding 50 ml of H_2SO_4 (4 M). The results are expressed as a change in OD/mg wet tissue.

2.14. Protein extraction and immunoblotting

The renal cortex and medulla were dissected using a Stadie-Rigg microtome as previously described in Gracelli et al. (2012). The renal cortex and medulla were homogenized in lysis buffer, and total protein was obtained (Bertuloso et al., 2015). The protein concentration was determined using the Lowry assay (1951). Briefly, proteins were transferred to nitrocellulose membranes in Tris-glycine buffer (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated overnight with 5% blotting-grade block containing non-fat dry milk in Tris-buffered saline plus 0.1% Tween 20 solution and specific antibodies (Bio-Rad Laboratories). The primary antibodies anti-ER α (1:500, sc-542, SCBT, CA, USA), anti- α -SMA (1:500, sc-53142, SCBT, CA, USA) and anti-GAPDH (1:1000, sc-25778, SCBT, CA, USA) were incubated in blocking solution overnight at 4°C . Goat anti-rabbit IgG-alkaline phosphatase conjugate (A3687, 1:1000, Sigma-Aldrich, St. Louis, MO, USA) was used as a secondary antibody for the ER α and GAPDH blotting assays unless otherwise noted. The α -SMA proteins were detected using a secondary anti-mouse IgG alkaline phosphatase conjugate (NA931V, 1:1000, GE Healthcare Life Sciences). The ER α , α -SMA and GAPDH bands for each sample were visualized by a color development reaction using nitroblue tetrazolium chloride (NBT) and 50 mg/mL of 5-bromo-4-chloro-3-indolylphosphate p-

toluidine salt (BCIP) (all from Life Technologies, Rockville, MD, USA). The ER α , α -SMA and GAPDH bands were analyzed by densitometry using Image J software. The relative expression was normalized by dividing the ER α and α -SMA values by the corresponding internal control values (GAPDH).

2.15. Cell samples and viability assay for flow cytometry

Cell-enriched fractions from kidneys were prepared based on previous studies (Folkman et al., 2007; Swain and Subba, 2011). The renal cortex and medulla from left kidney were dissected and grossly triturated using surgical scissors and incubated with an extraction solution containing proteinase K (Sigma-Aldrich, St. Louis, MO, USA) and collagenase type II (Gibco Life Technologies, Sao Paulo, SP, Brazil) to dissociate the cells. The cell extracts were filtered through a nylon screen (BD Biosciences, San Diego, CA, USA, Falcon, 70 μm) to remove cell debris. The samples were washed twice in PBS and stored at -80°C until further analysis. Cell viability was assessed by propidium iodide (PI) (Sigma-Aldrich) exclusion. The cells were incubated with 2 μl propidium iodide for 5 min in the dark at room temperature. Then, cells were washed with PBS and analyzed with a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were acquired using the FACSDiva software (BD Biosciences, San Diego, CA, USA), and overlay histograms were analyzed using a FCS Express software trial (De Novo).

2.16. Renal oxidative stress assessment

The cellular oxidative stress analysis was performed by flow cytometry (FC, Becton Dickinson, San Jose, CA, USA) using dihydroethidium (DHE), 2',7'-dichlorofluorescein diacetate (DCF), diamino fluorescein (DAF) and hydroxyphenyl fluorescein (HPF) to detect intracellular $\cdot\text{O}_2^-$, H_2O_2 , NO and $\cdot\text{ONOO}^-$, respectively, as previously described (Campagnaro et al., 2013). Briefly, 160 mM DHE, 10 μM of HPF or 2 μM of DAF was added to the cell suspension (10^6 cells) and incubated at 37°C for 30 min (DHE and HPF) or 180 min (DAF) in the dark. For positive controls, samples were treated for 5 min with 50 mM H_2O_2 and/or 100 μM of sodium nitroprusside to create oxidative stress without being cell toxicity. For negative controls, the cells were incubated with ethanol. DCF is a cell-permeable indicator of H_2O_2 production that is non-fluorescent until oxidation occurs within the cell, which converts DCF into the fluorescent form that remains trapped in the cell. To determine the intracellular H_2O_2 levels, DCF (20 mM) was added to cell suspensions (10^6 cells), which were then incubated at 37°C for 30 min in the dark (Dias et al., 2014). Cells were then washed, resuspended in PBS and kept on ice for immediate detection by FC (BD Biosciences, San Diego, CA, USA). To quantify DHE, DCF, DAF and HPF fluorescence, samples were acquired in duplicate, and 10,000 events were used for each measurement. The cells were excited at 488 nm. DHE fluorescence was detected using a 585/42 bandpass filter, and DCF/DAF/HPF fluorescence was detected using a 530/30 bandpass filter. FC data are expressed as the geometric mean (GeomMean) fluorescence intensity or the percentage of stained cells (Dias et al., 2014).

2.17. Renal apoptosis analysis

Renal cortex and medulla apoptotic cells were quantified using the annexin V-FITC apoptosis detection kit (BD Biosciences, San Diego, CA, USA). Briefly, annexin V-FITC and propidium iodide were added to cell suspensions, incubated for 15 min at room temperature (25°C) in the dark and analyzed by FC using a FACSCanto II (BD Biosciences, San Diego, CA, USA). Apoptotic cells were defined as annexin V $^+$ cells.

2.18. Renal cellular senescence assessment

Quantification of cellular senescence was performed using the lysosomal mass method with FC (Porto et al., 2015). The cells were stained with acridine orange (AO, Invitrogen, Eugene, OR, USA). Briefly, 0.2 ml of cell suspensions was gently mixed with a solution containing 1 mg/ml AO, 1 mM EDTA-Na, and 0.15 M NaCl in phosphate-citric acid buffer pH 7.4 for 10 min. The photomultiplier settings on the FC were adjusted to detect the green fluorescence signal of AO (mostly due to nucleic acid staining) on the 530/30 nm bandpass filter and the orange fluorescence signal (due to lysosomal staining) on the 585/42 nm bandpass filter.

2.19. Superoxide anion assessment

To detect tubular superoxide anion (O_2^-) levels, cryosections (8 μ m) of kidney tissues previously stored at -80°C from control, TBT100 and TBT500 rats ($n = 5$) embedded in OCT were allowed to thaw and were then incubated with the O_2^- -sensitive fluorescent dye dihydroethidium (DHE) at 37°C for 30 min in the dark (Rodrigues et al., 2014). Images were obtained using a Leica microscope with fluorescence detected at 585 nm (DM 2500). The signal intensity, indicating O_2^- production, was analyzed in the area of interest in 20 sections through the organ by a blinded researcher. Processing and microscopic analyses were performed at the Laboratory of Molecular Histology and Immunohistochemistry (LHMI), UFES, Brazil. The O_2^- production level indicates oxidative stress.

2.20. TBARS assessment

Lipid peroxidation was assessed using TBARS assay as described previously (Pires et al., 2014). Briefly, the renal cortex and medulla samples previously stored at -80°C were mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid; subsequently, the samples were heated in a boiling water bath for 15 min. TBARS were determined by absorbance at 530 nm and 600 nm and expressed as nmol/g calculated from a standard curve using standard dilutions.

2.21. Reduced glutathione assessment

The renal cortex and medulla samples were collected and prepared for reduced glutathione quantification assay. Briefly, the glutathione levels were measured spectrophotometrically by

reaction with Ellman's reagent DTNB (5, 50 –dithiobis-2-nitrobenzoic acid, Sigma, USA), as described elsewhere (Pires et al., 2014).

2.22. Statistical analysis

All data are reported as the mean $\pm$ SEM. To identify possible outlier data, the two-sided Grubbs' test was used to identify whether at least one outlier was present in each data set. When Grubbs' test identified one outlier, we used an adapted ROUT method to detect any outliers from that column of data and removed them according to the Q setting at 1% ($\alpha = 0.01$). For each data set, a D'Agostino-Pearson omnibus normality test was also performed. If D'Agostino-Pearson omnibus normality test revealed non-Gaussian data, then Kruskal-Wallis followed by Dunn's multiple comparisons test was used. If the data passed the normality test, then one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons was applied. To evaluate the relationship between the assessed parameters, Spearman's or Pearson's correlation was used if non-Gaussian or Gaussian distribution, respectively, was detected. All correlations were obtained from the same animal paired values. Finally, when statistical significance was identified, we tested the better adjustment for linear or nonlinear regression fitting. The statistical analyses were performed using Prism software (Prism 6.04, GraphPad Software, Inc., San Diego, CA, USA). The differences were considered significant when $p \leq 0.05$.

3. Results

3.1. TBT rats have high serum and kidney tin levels

Serum samples were obtained from CON, TBT100 and TBT500 rats, and serum tin levels were determined using an ICP-MS. TBT exposure led to increased serum tin levels in TBT100 ($p < 0.05$) and TBT 500 rats ($p < 0.001$) compared with control rats (CON: 3.51 ± 0.65 ; TBT100: 56.22 ± 9.21 ; TBT500: $262.00 \pm 15.93 \text{ ng g}^{-1}$, $n = 5$, Fig. 1A). Additionally, the higher serum tin levels observed in TBT500 rats were significant differently than those of the TBT100 rats ($p < 0.001$), indicating a dose-dependent effect. Similarly, increased kidney tin levels were observed in TBT100 ($p < 0.05$) and TBT500 ($p < 0.001$) rats compared with control rats (CON: 0.27 ± 0.02 ; TBT100: 7.38 ± 0.42 ; TBT500: $36.47 \pm 6.99 \mu\text{g g}^{-1}$, $n = 5$, Fig. 1B). In addition, increased kidney tin levels were observed in TBT500 rats compared to TBT100 rats ($p \leq 0.001$),

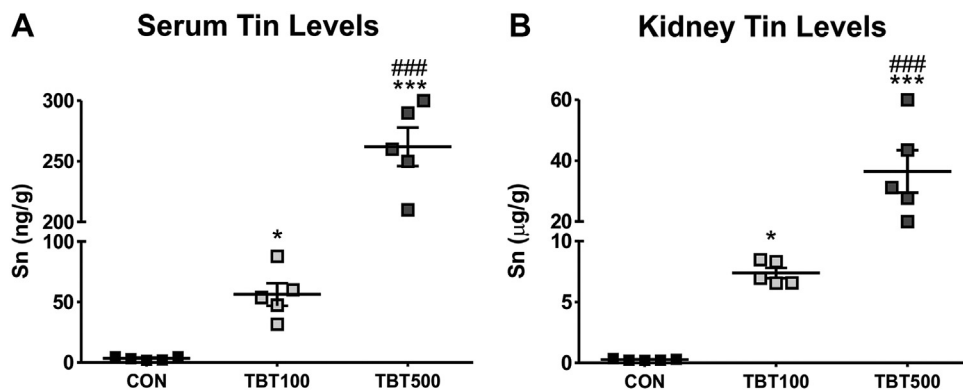


Fig. 1. Tin level assessment in sera and kidneys of CON, TBT100 and TBT500 rats. A) TBT100 and TBT500 rats exhibited increased serum tin levels. B) TBT100 and TBT500 rats exhibit increased kidney tin levels. Values are expressed as the mean $\pm$ SEM ($n = 5$). * $p \leq 0.05$, *** $p \leq 0.001$ vs CON. ### $p \leq 0.001$ vs TBT100 (one-way ANOVA followed by Tukey's test).

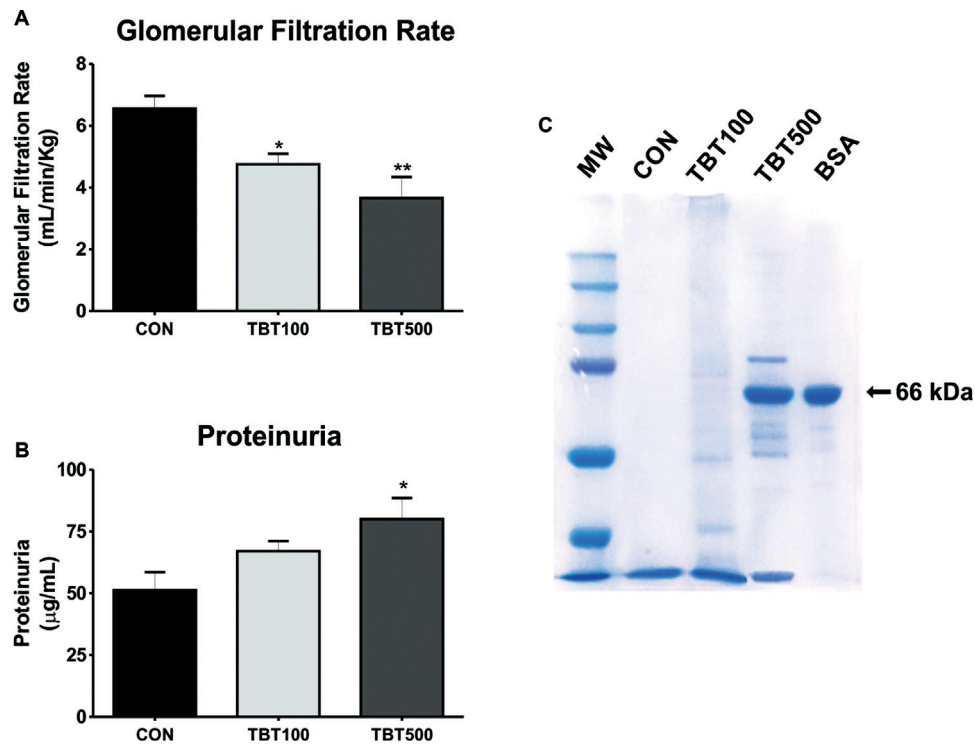


Fig. 2. GFR and proteinuria level assessment in CON, TBT100 and TBT500 rats. A) GFR was reduced in both TBT treatment groups. B) TBT500 rats had higher proteinuria levels. C) Urine samples were resolved on SDS-PAGE gels, and protein analysis was based on the intensity of Coomassie Blue staining. Values are expressed as the mean $\pm$ SEM (n = 8). * $p \leq 0.05$, ** $p \leq 0.01$ vs CON (one-way ANOVA followed by Tukey's test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggesting a dose-dependent effect. This renal tin accumulation could be attributed to the high serum tin levels from both TBT treatments.

3.2. TBT rats have low GFR and high proteinuria levels

To characterize the effects of TBT exposure on the renal function, GRF was assessed (Fig. 2). There were significantly reduced GFR levels in TBT100 ($p \leq 0.05$) and TBT500 ($p \leq 0.01$) rats compared with control rats (CON: 6.56 ± 0.40 ; TBT100: 4.75 ± 0.33 ; TBT500: 3.66 ± 0.67 , $n = 8$, Fig. 2A). However, TBT500 treatment did not significantly change GFR levels compared with TBT100 treatment ($p \geq 0.05$).

The amount of protein excreted in 2.5 h was assessed in the urine of control and both TBT rats as a marker of renal injury. TBT500 rats exhibited higher urinary protein levels only compared with control rats (CON: 51.22 ± 7.30 ; TBT100: 67.05 ± 4.10 ; TBT500: 80.07 ± 8.47 $\mu\text{g}/2.5$ h, $p \leq 0.05$, $n = 8$, Fig. 2B). Proteinuria levels were also measured in the urine samples resolved on SDS-PAGE gel showing higher protein band intensity of TBT500 rats compared with control and TBT100 rats (Fig. 2C).

3.3. TBT rats have biometric abnormalities

To determine the effect of TBT on the biometry, the body, organ and tissue weights were assessed (Table 1, $n = 10$). The initial body

Table 1
Body, organ and tissue weight assessment.

Body/organ/tissue weight (n = 10)	CON	TBT100	TBT500
Initial body weight (g)	208.71 $\pm$ 1.87	220.00 $\pm$ 4.26	209.33 $\pm$ 5.74
Final body weight (g)	223.50 $\pm$ 3.27	238.42 $\pm$ 5.45	242.12 $\pm$ 4.86*
Pituitary gland (mg)	8.00 $\pm$ 0.90	9.00 $\pm$ 0.70	11.00 $\pm$ 1.70
Heart (g)	0.67 $\pm$ 0.01	0.68 $\pm$ 0.02	0.68 $\pm$ 0.02
Liver (g)	8.07 $\pm$ 0.38	7.34 $\pm$ 0.17	8.46 $\pm$ 0.58
Kidneys (g)	1.40 $\pm$ 0.02	1.46 $\pm$ 0.05	1.79 $\pm$ 0.09**,#
Adrenal gland (mg)	55.00 $\pm$ 4.00	57.00 $\pm$ 3.00	64.00 $\pm$ 6.00
Ovaries (mg)	80.00 $\pm$ 8.00	78.00 $\pm$ 6.00	79.00 $\pm$ 6.00
Uterus (g)	0.48 $\pm$ 0.02	0.44 $\pm$ 0.06	0.43 $\pm$ 0.02
Perirenal fat (g)	0.94 $\pm$ 0.14	1.21 $\pm$ 0.19	1.19 $\pm$ 0.14
Parametrial fat (g)	2.07 $\pm$ 0.09	3.38 $\pm$ 0.27†	3.51 $\pm$ 0.46*

Values are expressed as the mean $\pm$ SEM.

* $p \leq 0.05$.

** $p \leq 0.01$ vs CON.

$p \leq 0.05$ vs TBT100 (one-way ANOVA followed by Tukey's test).

weight was similar among the control and both TBT groups ($p \geq 0.05$). However, both TBT100 and TBT500 treatments increased the final body weight compared with control ($p \leq 0.05$). However, no significant changes were observed in the pituitary and adrenal glands, hearts, livers, ovaries, uteri and perirenal fat of control rats compared with both TBT rats ($p \geq 0.05$). However, both TBT rat groups exhibited an increase in the parametrial fat weight compared with control rats ($p \leq 0.05$). In addition, TBT500 rats

exhibited higher wet kidney weight compared with control rats ($p \leq 0.01$) and TBT100 rats ($p \leq 0.05$) (CON: 1.40 ± 0.02 ; TBT100: 1.46 ± 0.05 ; TBT500: 1.79 ± 0.09 g).

3.4. Abnormal renal morphology in TBT rats

To characterize the effects of TBT exposure, kidney anatomy and histology were evaluated (Fig. 3). A small increase in kidney size

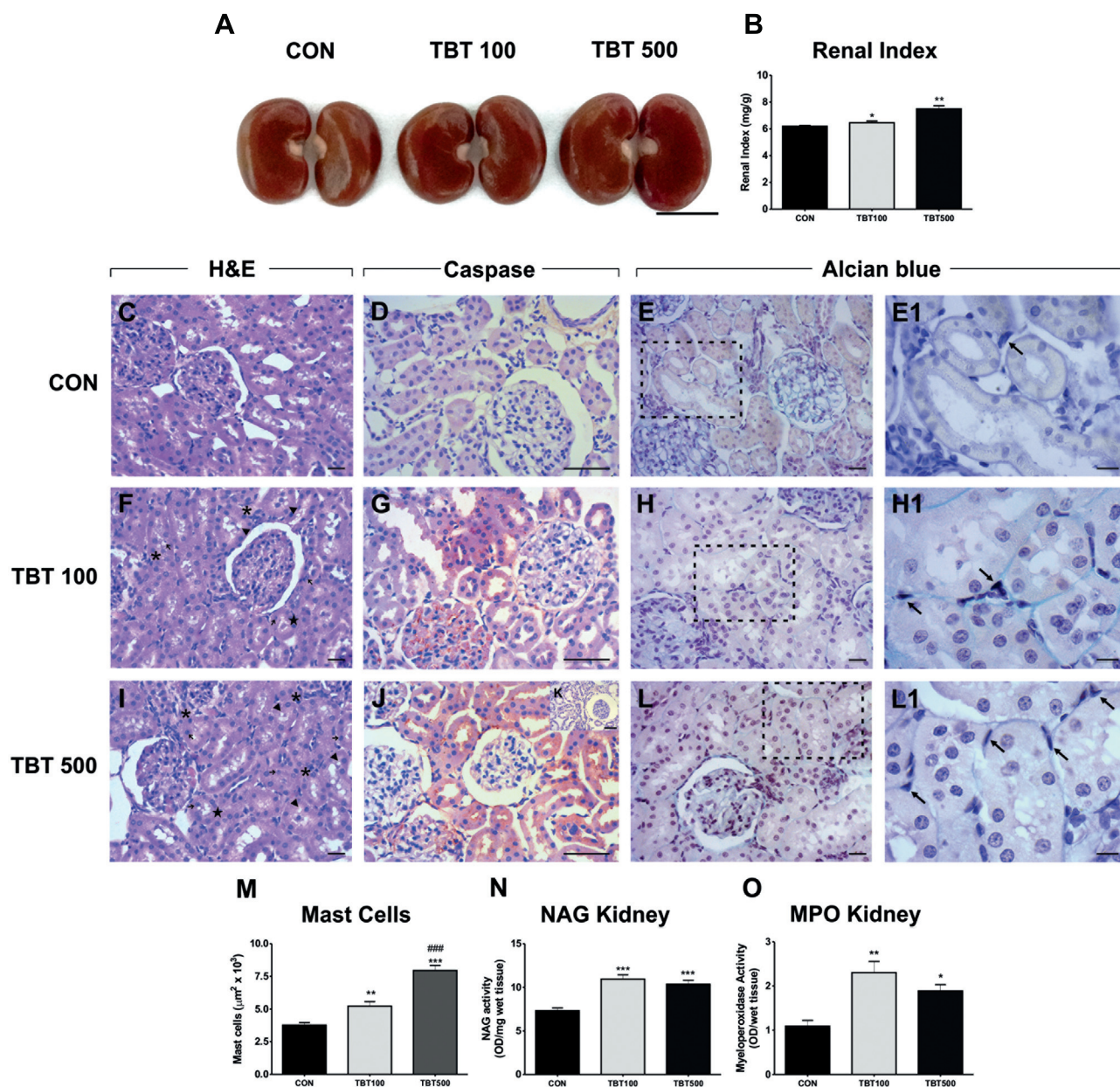


Fig. 3. Kidney anatomy and histomorphology of CON, TBT100 and TBT500 rats. A) Kidneys from CON, TBT100 and TBT500 rats showing a small increase in kidney size in TBT500 rats. B) An increase in the renal index of TBT500 rats. Representative H&E sections from (C) CON, (F) TBT100 and (I) TBT500 kidneys are showing the presence of disorganization at the apical border in PT cells (star), vacuolar degeneration (arrowhead), apoptosis (arrow) and inflammatory cells (asterisks) surrounding renal tubules. TBT leads to the positive staining for caspase-3 in the kidney by the immunohistochemical assay. (G) and (J) Reported the representative immunostaining of renal cortex from TBT 100 and TBT500 animals showing positive staining in cytoplasm of renal tubule cells, as proximal tubules cells (n = 3). No positive staining in cytoplasm of renal tubule cells in (D) control and (K) negative control was observed. Images D, G, J (scale bar: 50 μm) Representative mast cells (arrow) in sections from (E and E1) CON, (H and H1) TBT100 and (L and L1) TBT500 kidneys stained with Alcian Blue. Images C, E, F, H, J, K, L (scale bar: 20 μm). Images E1, H1, L1 (scale bar: 10 μm). (M) Graphic representation showing a dose-dependent increase in kidney mast cell numbers in TBT100 and TBT500 rats. (N) Increased NAG activity in a kidney from a TBT100 and TBT 500 rats. (O) Increased MPO activity in a kidney from a TBT100 and TBT 500 rats. Disorganization: abnormal morphology. Vacuolar degeneration: an increase in cell size with an increase in the number and size of vacuoles. Apoptosis: an active form of cell death characterized by biochemical and morphological processes, such as chromatin condensation. Inflammatory cells: cells that play important action in the inflammation process (the complex biological response of body tissue to harmful stimuli). NAG: N-Acetyl- β -D-Glucosaminidase. MPO: Myeloperoxidase. Values are expressed as the mean $\pm$ SEM (n = 5). * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$ vs CON. #### $p \leq 0.001$ vs TBT100 (one-way ANOVA followed by Tukey's test).

was observed in both groups of TBT-exposed rats (Fig. 3A). A renal index (wet organ weight/body weight mg/g) increase of between 4% and 20% was observed in TBT100 and TBT500 rats, respectively. The renal index of TBT500 rats was significantly higher than in control ($p \leq 0.01$) and TBT100 ($p \leq 0.05$) rats (CON: 6.19 ± 0.06 ; TBT100: 6.45 ± 0.13 ; TBT500: 7.50 ± 0.23 mg/g, $n = 10$, Fig. 3B). No significant change in renal index was observed in TBT100 rats compared with control rats ($p \geq 0.05$). Similarly, the dry kidney weight/body weight values were higher in TBT500 rats only compared with control rats (CON: 1.62 ± 0.067 ; TBT100: 1.71 ± 0.048 ; TBT500: 1.87 ± 0.056 mg/g, $n = 5$, $p < 0.05$). No significant change in dry kidney weight/body weight (m/g) was observed in TBT100 rats compared with control rats ($p \geq 0.05$).

The TBT kidneys (Fig. 3F and I) exhibited histological abnormalities, with an impaired renal cortex organization in contrast to the normal aspect in control kidneys (Fig. 3C). Both TBT kidneys exhibited apical border disorganization (star), vacuolar degeneration (arrowhead), apoptosis (arrow) and inflammatory cells (asterisks) surrounding renal tubules (Fig. 3F and I).

3.5. Caspase-3 protein expression was present in the kidney of TBT rats

To analyze the caspase-3 protein expression in the kidney, the immunohistochemical (IHC) assay was performed. Both TBT kidney tissues were shown to express caspase-3 protein expression with predominant brown positive staining observed in the cytoplasm of the renal tubules, as tubule proximal cells (Fig. 3G and J). No brown staining was observed in control kidneys (Fig. 3D). The negative controls performed by omitting the primary antibodies demonstrated no brown staining: only negative (blue) staining for kidney tissue sites was observed (Fig. 3K).

3.6. Inflammation was higher in the kidney of TBT rats

Mast cell assessment was performed using Alcian blue staining. Increased numbers of mast cells were observed in TBT100 ($p < 0.01$, Fig. 3H and H1) and TBT500 ($p < 0.001$, Fig. 3L and L1) kidneys compared to control kidneys (CON: 3.76 ± 0.20 ; TBT100: 5.22 ± 0.33 ; TBT500: 7.95 ± 0.38 mast cells/ $\mu\text{m}^2 \times 10^3$, $n = 5$, Fig. 3M). In addition, increased numbers of mast cells were observed in TBT500 kidneys compared to TBT100 kidneys ($p \leq 0.001$), suggesting a dose-dependent effect. In addition, kidney NAG activity was higher in TBT100 and TBT500 rats compared to control rats (CON: 7.33 ± 0.29 ; TBT100: 10.94 ± 0.48 ; TBT500: 10.36 ± 0.45 OD/mg, $n = 5$, $p \leq 0.001$, Fig. 3N). In addition, an increase in renal MPO activity was observed in TBT100 ($p \leq 0.01$) and TBT500 ($p \leq 0.05$) rats compared to control rats (CON: 1.09 ± 0.13 ; TBT100: 2.30 ± 0.25 ; TBT500: 1.90 ± 0.14 OD/g, $n = 5$, Fig. 3O). Furthermore, there was no difference into both NAG and MPO activity between TBT100 and TBT500 rats ($p \geq 0.05$).

3.7. Glomerular tuft area was higher in the kidneys of TBT rats

In kidney histology, control rats displayed normal renal glomeruli, Bowman's capsules and Bowman's spaces (Fig. 4A). However, the kidneys in both TBT rats exhibited Bowman's space abnormalities, leading to glomerular tuft area increases in TBT100 (Fig. 4D) and 500 (Fig. 4G) rats with compared control rats (CON: 8.25 ± 0.99 ; TBT100: 14.75 ± 3.62 ; TBT500: 25.75 ± 3.18 , $p \leq 0.01$, $n = 5$, Fig. 4J). Additionally, TBT500 rats had significantly increased glomerular tuft areas compared with TBT100 rats ($p \leq 0.05$), indicating a dose-dependent effect. However, TBT100 treatment did not lead to a significant change in glomerular tuft area compared with control rats ($p \geq 0.05$).

3.8. Collagen deposition was higher in the kidneys of TBT rats

The development of renal fibrosis was assessed in CON, TBT100 and TBT500 rats. Increased glomerular collagen deposition (PSG) was only observed in TBT500 kidneys with compared control kidneys (CON: 9.88 ± 0.82 ; TBT100: 11.49 ± 0.69 ; TBT500: 15.04 ± 2.12 , $n = 5$, $p \leq 0.05$, Fig. 4K). No significant change was observed in the PSG in TBT100 kidneys compared with control kidneys ($p > 0.05$). In addition, increased tubulointerstitial collagen deposition (PST) was observed in TBT500 kidneys compared to control ($p \leq 0.001$) and TBT100 ($p \leq 0.01$) kidneys, suggesting a dose-dependent effect (CON: 3.77 ± 0.86 ; TBT100: 7.92 ± 2.21 ; TBT500: 17.22 ± 1.71 , $n = 5$, Fig. 4L). However, no significant change was observed in the PST in TBT100 kidneys with compared control kidneys ($p > 0.05$).

3.9. α -SMA was higher in the kidney of TBT rats

Western blotting analysis was performed, and α -SMA protein expression was evaluated in the renal cortex (Fig. 4). Increased α -SMA protein expression was observed only in the renal cortices of TBT500 rats compared with control rats (CON: 1.00 ± 0.00 ; TBT100: 1.12 ± 0.06 ; TBT500: 1.31 ± 0.04 , $n = 5$, $p \leq 0.05$, Fig. 4M). No significant change in α -SMA protein expression was observed in the renal cortices of TBT100 rats compared with control rats ($p \geq 0.05$). In addition, a significant positive correlation with a linear association was observed between the tubulointerstitial collagen deposition and α -SMA protein expression (Pearson $r = 0.66$; $p < 0.001$, Fig. 4N).

3.10. TBT rats have low serum estrogen levels and ER α protein expression

To determine the effect of TBT exposure on serum estrogen (E_2) levels, an ELISA assay was performed (Fig. 5). A reduction in the serum E_2 levels was observed in both TBT rats compared with the control rats (CON: 35.10 ± 4.48 ; TBT100: 25.72 ± 2.48 ; TBT500: 20.56 ± 1.88 pg/mL, $n = 5$, $p \leq 0.05$, Fig. 5A). No significant changes in serum E_2 levels were observed in TBT100 and TBT500 rats ($p \geq 0.05$).

The ER α protein expression in the kidneys was determined using Western blot analysis. Similarly, a reduction in ER α protein expression was only observed in the renal cortices of TBT500 rats compared with the control ($p \leq 0.01$) and TBT100 ($p \leq 0.05$) rats (CON: 1.00 ± 0.01 ; TBT100: 0.90 ± 0.04 ; TBT500: 0.76 ± 0.03 , $n = 5$, Fig. 5B). No significant changes in ER α protein expression were observed in the renal cortices of TBT100 and control rats ($p \geq 0.05$). In addition, ER α protein expression in the renal medulla was not significantly different among all three groups (CON: 1.00 ± 0.00 ; TBT100: 1.09 ± 0.01 ; TBT500: 0.99 ± 0.08 , $n = 5$, $p \geq 0.05$, Fig. 5C).

In addition, the serum E_2 levels were negatively correlated with the oxidative stress (Spearman $r = -0.79$; $p < 0.001$, Fig. 6D) and inflammation (Pearson $r = -0.58$; $p < 0.05$, Fig. 6F). No significant correlation was observed between interstitial collagen deposition and serum E_2 levels (Pearson $r = -0.29$; $p = 0.29$, Fig. 6E). The cortical ER α protein expression was also positively correlated with the GFR (Pearson $r = 0.79$; $p < 0.001$, Fig. 6G) and it was negatively correlated with the inflammation (Pearson $r = -0.57$; $p < 0.05$, Fig. 6H) and interstitial collagen deposition (Pearson $r = -0.91$; $p < 0.0001$, Fig. 6I).

3.11. Oxidative stress was higher in the kidneys of TBT rats

The renal oxidative stress status was determined using flow cytometry (CF) (Fig. 6). Using the DHE marker, both TBT kidneys exhibited increased renal cortex $\bullet\text{O}_2^-$ levels compared with

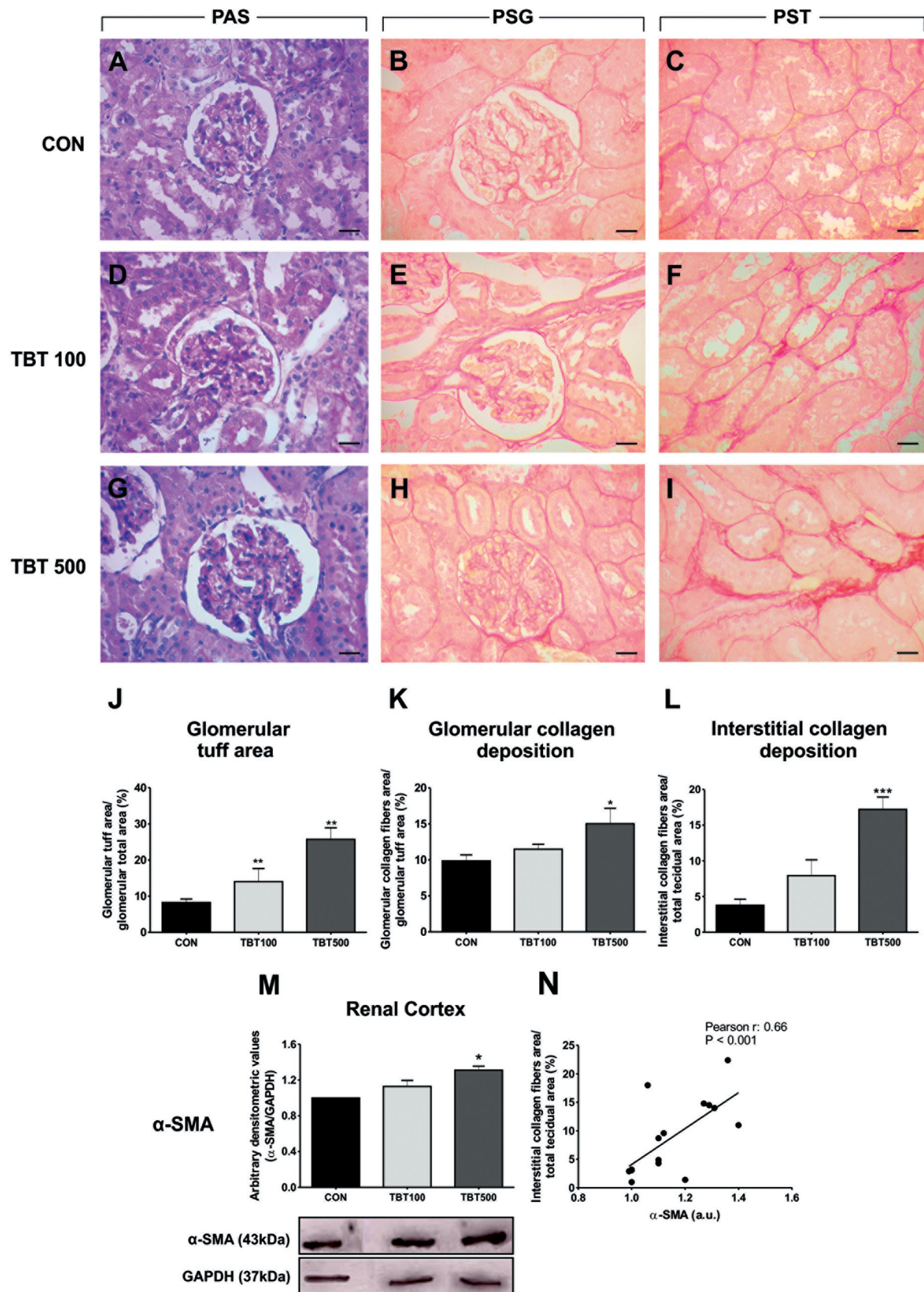


Fig. 4. Glomerular structure and renal collagen fiber assessment in CON, TBT100 and TBT500 rats. Representative kidney sections stained with Periodic-Acid-Schiff (PAS) emphasizing the glomeruli of (A) CON, (D) TBT100 and (G) TBT500 rats. (J) Graphic representation of the dose-dependent increase in glomerular tuft area and glomerular total area from the TBT100 and TBT500 rats. Representative glomeruli stained with Picrosirius Red (PSG) from (B) CON, (E) TBT100 and (H) TBT500 rats. (K) Graphic representation of increased collagen surface density only in TBT500 glomeruli. Representative tubulointerstitial area stained with Picrosirius Red (PST) from (C) CON, (F) TBT100 and (I) TBT500 rats. Images A–I (scale bar: 20 μm). (L) Graphic representation of the dose-dependent increase in collagen surface density in the TBT100 and TBT500 tubulointerstitial area. (M) Increased α-SMA protein expression in the renal cortex from TBT500 rats. (N) Tubulointerstitial collagen deposition was plotted against the α-SMA protein

control kidneys (CON: 1920.68 ± 44.97 ; TBT100: 2191.74 ± 55.44 ; TBT500: 2173.50 ± 35.09 , $n=8$, $p \leq 0.01$, Fig. 6A). Similarly, increased renal medulla O_2^- levels were observed in TBT100 ($p \leq 0.05$) and TBT500 ($p \leq 0.01$) kidneys compared to control kidneys (CON: 1920.24 ± 52.46 ; TBT100: 2115.82 ± 64.89 ; TBT500: 2228.86 ± 42.87 , $n=8$, Fig. 6B). No significant change was observed in renal O_2^- levels in TBT100 kidneys compared with TBT500 kidneys ($p \geq 0.05$). Using the DCF marker, higher H_2O_2 levels were observed in the renal cortices of TBT100 and TBT500 rats compared with the renal cortices of control rats (CON: 1631.80 ± 378.6 ; TBT100: 2021.76 ± 112.64 ; TBT500: 2131.64 ± 161.97 , $n=8$, $p \leq 0.05$, Fig. 6C). Similarly, higher renal medulla H_2O_2 levels were observed in TBT100 and TBT500 kidneys compared with control kidneys (CON: 1600.10 ± 59.31 ; TBT100: 2063.24 ± 177.31 ; TBT500: 2256.22 ± 185.49 , $n=8$, $p \leq 0.05$, Fig. 6D). No significant change was observed in renal cortex H_2O_2 levels in TBT100 kidneys compared with TBT500 kidneys ($p \geq 0.05$). Increased ONOO^- levels were observed in the renal cortices of TBT100 and TBT500 kidneys compared with control kidneys (CON: 1388.29 ± 22.17 ; TBT100: 1602.03 ± 50.62 ; TBT500: 1602.81 ± 72.49 , $n=8$, $p \leq 0.05$, Fig. 6E). Similarly, higher ONOO^- levels were observed in the renal medullas of TBT100 ($p < 0.01$) and TBT500 ($p < 0.05$) kidneys compared with control kidneys (CON: 1387.07 ± 29.40 ; TBT100: 1593.83 ± 51.42 ; TBT500: 1532.81 ± 33.60 , $n=8$, Fig. 6F).

3.12. Annexin V⁺ cells were higher in the kidneys of TBT rats

In addition, the renal apoptosis assessment was evaluated using an annexin V⁺ assay (ANX). Increased apoptosis was only observed in the renal cortices of TBT500 kidneys compared with control kidneys (CON: 2.53 ± 0.63 ; TBT100: 3.31 ± 0.34 ; TBT500: 4.50 ± 0.44 , $n=8$, $p \leq 0.05$, Fig. 6G). However, no significant changes in apoptosis were observed in the renal medullas of TBT100 and TBT500 kidneys compared with control kidneys (CON: 3.52 ± 0.32 ; TBT100: 3.96 ± 1.1 ; TBT500: 4.79 ± 0.65 , $n=8$, $p \leq 0.05$, Fig. 6H). No significant changes were observed in renal NO and senescence levels among the all three groups (data not shown, $p \geq 0.05$).

3.13. O_2^- levels are higher in the renal tubules of TBT rats

To analyze the production of O_2^- in the renal tubules, DHE marking was performed (Fig. 7). In the renal cortex, we detected an increase in O_2^- levels in both TBT100 ($p < 0.05$, Fig. 7B) and TBT500 ($p < 0.01$, Fig. 7C) rats compared to the CON rats (Fig. 7A) (CON: 84.24 ± 10.54 ; TBT100: 116.19 ± 9.86 ; TBT500: 131.39 ± 7.21 , $n=5$, $p \leq .001$, Fig. 7G). No significant difference in O_2^- levels was observed in the renal cortex between TBT100 and TBT500 rats ($p \geq 0.05$). Similarly, an increase in O_2^- levels in the renal medulla from TBT100 ($p < 0.01$, Fig. 7E) and TBT500 rats ($p < 0.05$, Fig. 7F) was observed when compared to the control rats (Fig. 7D) (CON: 55.34 ± 10.03 ; TBT100: 136.25 ± 10.36 ; TBT500: 114.59 ± 19.60 , $n=5$, $p \leq 0.01$, Fig. 7H). No significant difference in O_2^- levels was observed in the renal medulla between TBT100 and TBT500 rats ($p \geq 0.05$).

3.14. TBARS levels are higher and reduced glutathione levels are lower in the kidney of TBT rats

The lipid peroxidation assessment was evaluated using a TBARS assay. Increased TBARS levels were only observed in the renal

cortices of TBT500 kidneys compared with control and TBT 100 kidneys (CON: 214.13 ± 10.22 ; TBT100: 240.40 ± 13.25 ; TBT500: 281.41 ± 17.40 nmol/g, $n=5$, $p \leq 0.05$, Fig. 7I). No significant difference in TBARS levels were observed in the renal medulla between control, TBT100 and TBT500 rats ($p \geq 0.05$, Fig. 7J).

The reduced glutathione levels were evaluated in the renal cortex and medulla. A reduction in the glutathione levels were only observed in the renal cortices of TBT500 kidneys compared with control and TBT 100 kidneys (CON: 543.31 ± 41.91 ; TBT100: 337.76 ± 17.22 ; TBT500: 286.75 ± 53.89 mM/mg, $n=5$, $p \leq 0.05$, Fig. 7K). No significant difference in the glutathione levels were observed in the renal cortex between control and TBT100 rats ($p \geq 0.05$). In the renal medulla, we detected a reduction in the glutathione levels in both TBT100 and TBT500 rats compared to the control rats (CON: 285.87 ± 21.27 ; TBT100: 178.78 ± 19.10 ; TBT500: 150.86 ± 38.26 mM/mg, $n=5$, $p \leq 0.05$, Fig. 7L). No significant difference in the glutathione levels was observed in the renal medulla between TBT100 and TBT500 rats ($p \geq 0.05$).

3.15. Correlation among TBT exposure, GFR, inflammation, collagen deposition and oxidative stress development

Correlation analysis among different parameters was performed, and a linear or a non-linear fitting was plotted when appropriate (Fig. 8). The serum and kidney tin levels, GFR, inflammation, interstitial collagen deposition, apoptosis and proteinuria levels data exhibited a Gaussian distribution, and parametric Person's correlation tests were performed when these parameters were analyzed (D'Agostino & Pearson normality test, $p > 0.10$, $p > 0.10$, $p > 0.10$, $p > 0.10$, $p > 0.10$, $p > 0.10$ and $p > 0.10$, respectively, $n=9-15$). The oxidative stress data did not exhibit a Gaussian distribution, and nonparametric Spearman's correlation tests were performed when these parameters were analyzed (D'Agostino & Pearson normality test, $p=0.0077$ $n=9-15$). A significant positive correlation with a linear association was observed between the serum tin and kidney levels (Pearson $r=0.88$; $p < 0.0001$, Fig. 8A). The serum tin levels presented a negative correlation with serum E_2 levels (Pearson $r=-0.62$; $p < 0.05$, Fig. 8B). The kidney tin levels were negatively correlated with the GFR (Pearson $r=-0.72$; $p < 0.01$, Fig. 8C). A positive correlation between GFR and inflammation was observed (Pearson $r=0.67$; $p < 0.05$, Fig. 8D). A negative correlation between GFR and oxidative stress was observed (Spearman $r=-0.52$; $p < 0.05$, Fig. 8E). The inflammation was also positively correlated with the kidney tin levels (Pearson $r=0.74$; $p < 0.01$, Fig. 8F), interstitial collagen deposition (Pearson $r=0.62$; $p < 0.05$, Fig. 8G) and apoptosis (Pearson $r=0.62$; $p < 0.05$, Fig. 8H). A positive correlation between the interstitial collagen deposition and kidney tin levels (Pearson $r=0.77$; $p < 0.0001$, Fig. 8I) and oxidative stress (Spearman $r=0.81$; $p < 0.01$, Fig. 8J) were observed. The oxidative stress was also positively correlated with the kidney tin levels (Spearman $r=0.77$; $p < 0.01$, Fig. 8K), proteinuria levels (Spearman $r=0.71$; $p < 0.05$, Fig. 8L) and apoptosis (Spearman $r=0.66$; $p < 0.05$, Fig. 8M). In addition, a positive correlation between the proteinuria levels and apoptosis was observed (Pearson $r=0.67$; $p < 0.05$, Fig. 8N).

4. Discussion

In this study, we provide evidence that TBT impairs renal function in female rats, leading to a reduced GFR and increased proteinuria levels. These findings were related with renal

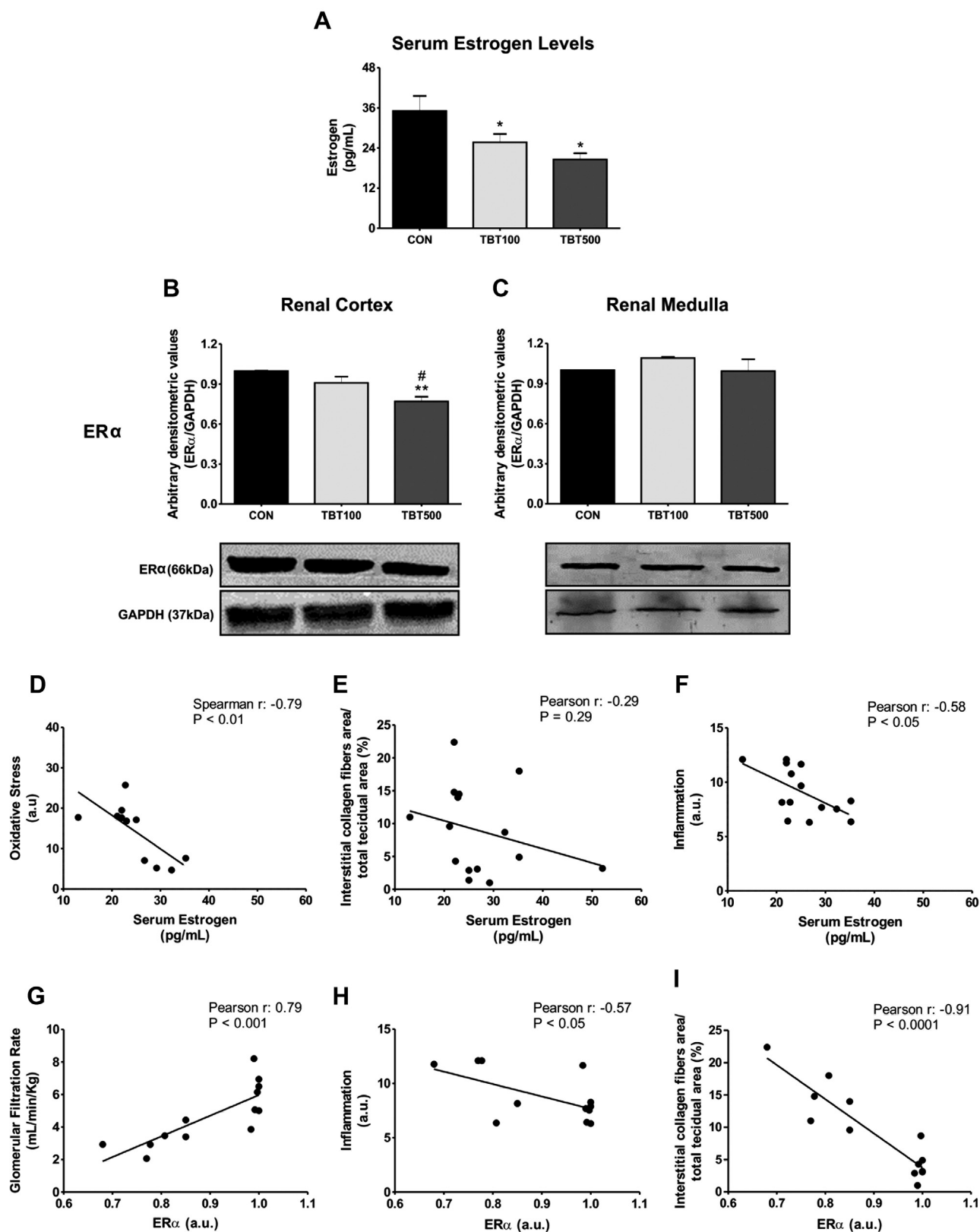


Fig. 5. Serum E_2 levels and ER α protein expression were assessed in CON, TBT100 and TBT500 rats. (A) A reduction in the serum E_2 levels was observed in TBT100 and TBT500 rats. (B) A reduction in ER α protein expression was observed only in the renal cortices of TBT500 rats. (C) ER α protein expression was observed in the renal medulla of CON, TBT100 and TBT500 rats. Serum E_2 levels were plotted against the respective (D) oxidative stress, (E) interstitial collagen deposition and (F) inflammation. ER α protein expression was plotted against the respective (G) GFR, (H) inflammation and (I) interstitial collagen deposition. Values are expressed as the mean $\pm$ SEM ($n = 5$). * $p \leq 0.05$, ** $p \leq 0.01$, vs CON. # $p \leq 0.05$, # $p \leq 0.05$ vs TBT100 (one-way ANOVA followed by Tukey's test). Statistic significance ($p \leq 0.05$) was tested using the Spearman's or Pearson's test if non-Gaussian or Gaussian data distribution, respectively, was detected.

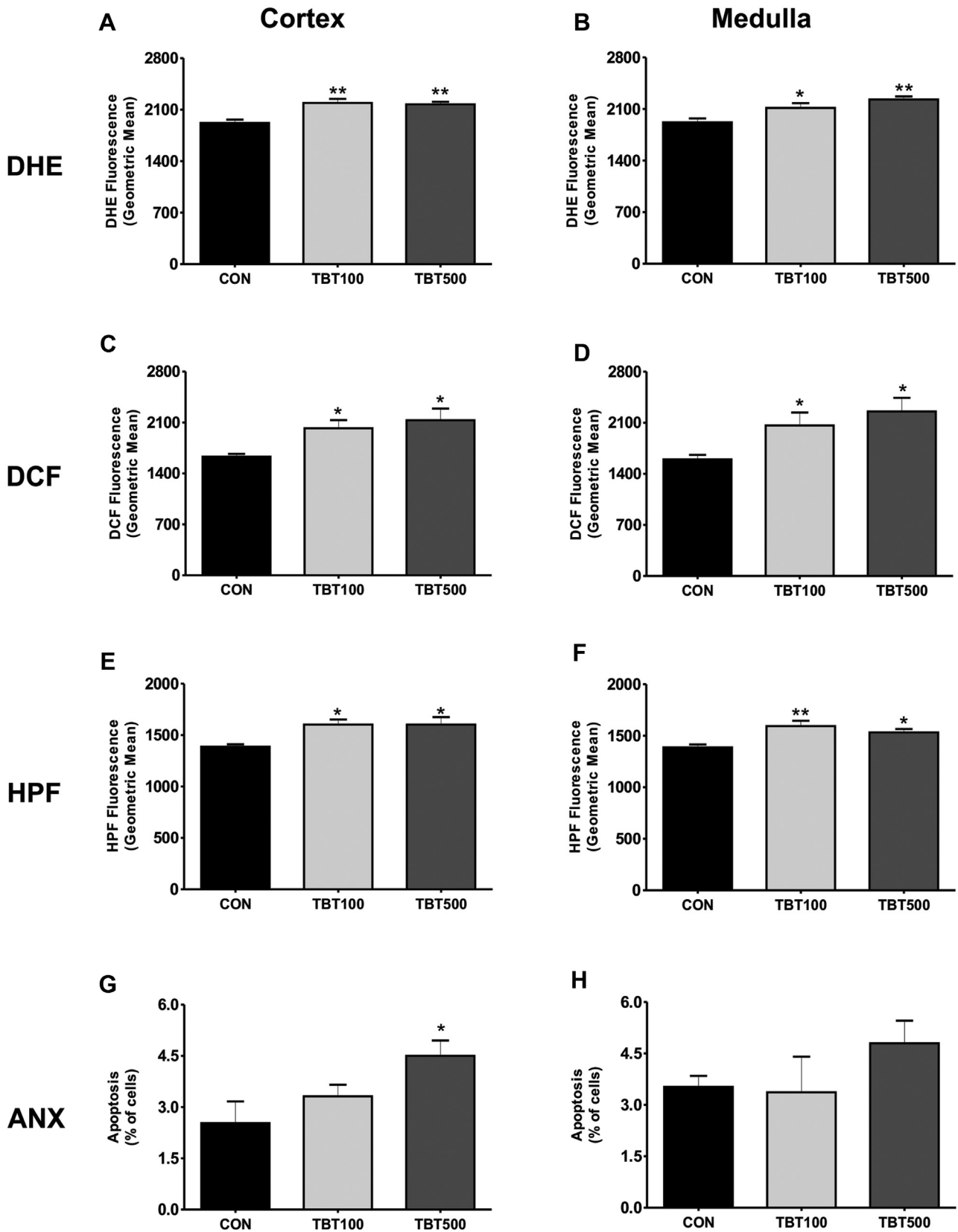


Fig. 6. Renal oxidative stress and apoptosis were assessed in CON, TBT100 and TBT500 rats. Higher O_2 levels were observed in both the (A) renal cortex and (B) medulla from TBT100 and TBT500 rats using the DHE assay. Higher H_2O_2 levels were observed in both the (C) renal cortex and (D) medulla from TBT100 and TBT500 rats using the DCF assay. Higher ONOO levels were observed in both the (E) renal cortex and (F) medulla from TBT100 and TBT500 rats using HPF assay. Renal apoptosis was evaluated using the annexin V⁺ assay (ANX). (G) Increased annexin V⁺ cells were observed in the renal cortices of TBT500 rats. (H) Annexin V⁺ cells (ANX) in renal medullas from CON, TBT100 and TBT500 rats. Values are expressed as the mean $\pm$ SEM ($n=8$). * $p \leq 0.05$, ** $p \leq 0.01$ vs CON (one-way ANOVA followed by Tukey's test).

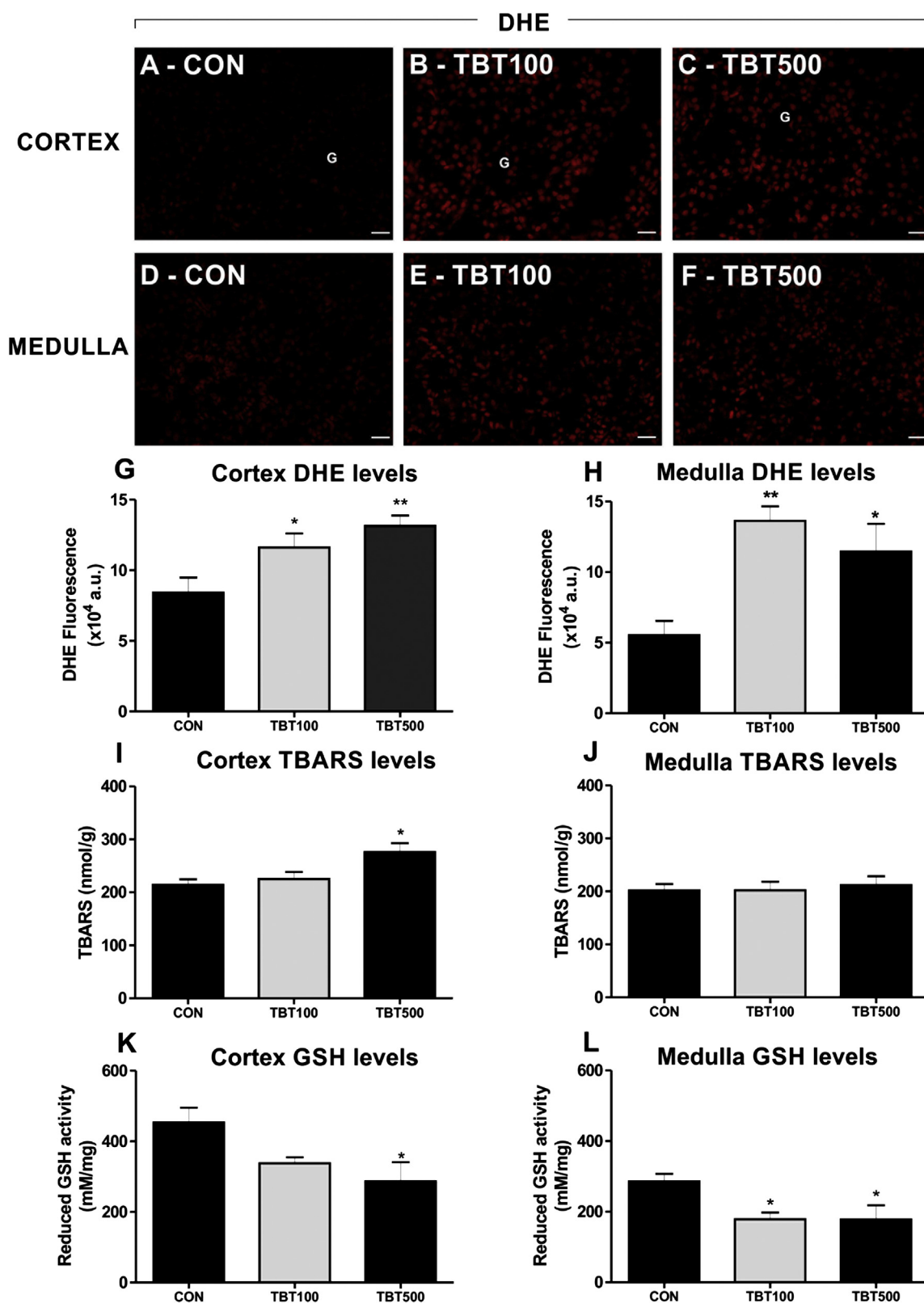


Fig. 7. Renal tubular O_2^- , TBARS and glutathione levels were assessed in CON, TBT100 and TBT500 rats. Fluorescence micrographs stained with the O_2^- -sensitive dye DHE (red fluorescence) were obtained from (A) control, (B) TBT100 and (C) TBT500 renal cortex and from (D) control, (E) TBT100 and (F) TBT500 renal medulla. Increased production of O_2^- level was observed in (G) the renal cortex and (H) medulla of TBT100 and TBT500 rats. (I) Higher TBARS levels were observed only in the renal cortex of TBT 500 rats. (J) TBARS levels in the renal medulla from control, TBT100 and TBT 500 rats. (K) Lower reduced glutathione levels were observed in renal cortex from TBT100 and TBT500 rats using the GSH assay. (L) Lower reduced glutathione levels were observed in renal medulla from TBT100 and TBT500 rats using the GSH assay. G: renal glomeruli. Values are expressed as the mean $\pm$ SEM ($n = 5$). * $p \leq 0.05$, ** $p \leq 0.01$ vs CON (one-way ANOVA followed by Tukey's test).

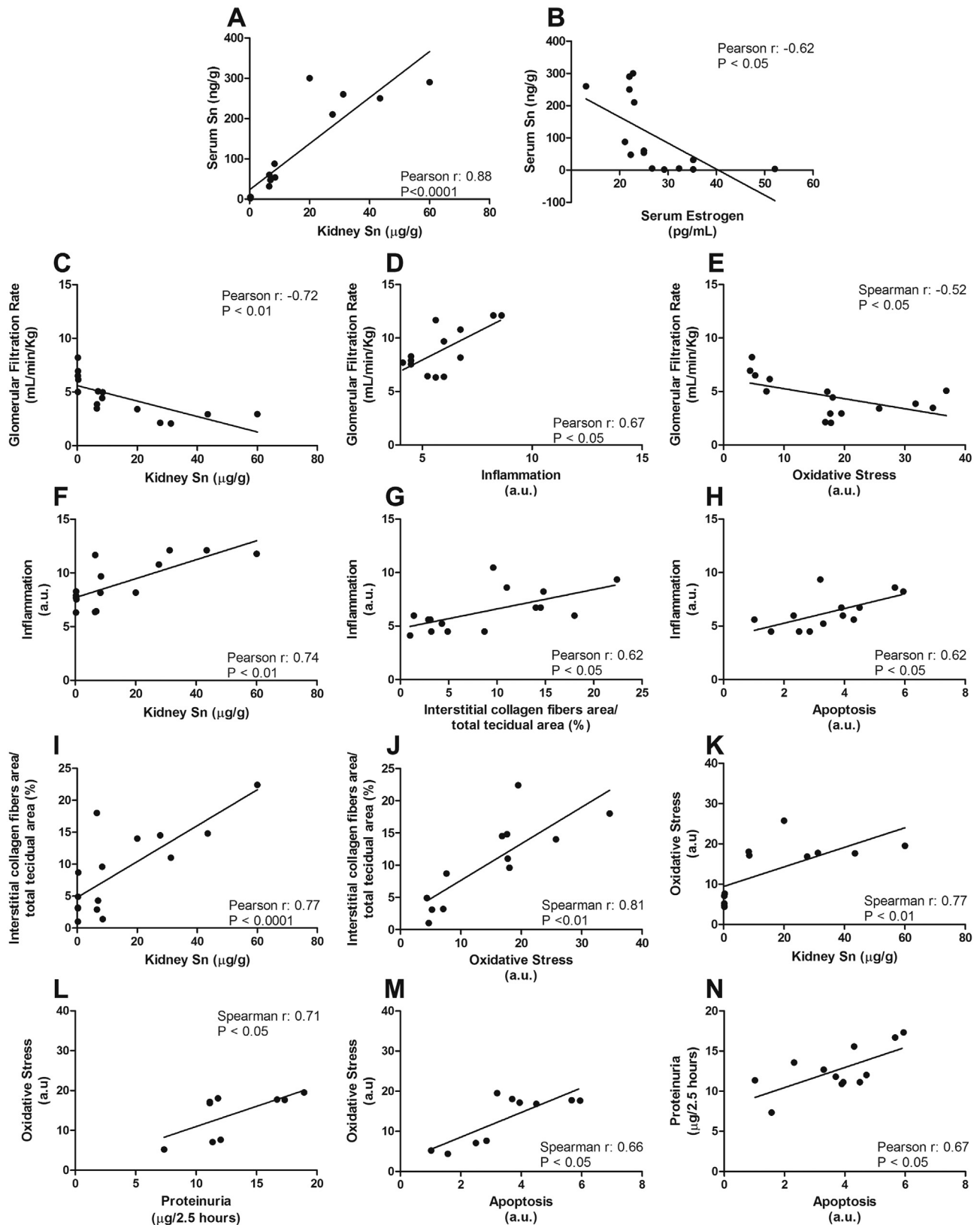


Fig. 8. Correlations between serum tin levels, kidney tin levels, GFR, inflammation, collagen deposition and oxidative stress were performed. Serum tin levels were plotted against the respective (A) kidney tin levels and (B) serum estrogen levels of the 11–15 rats from control, TBT 100 and TBT500 groups. Correlations between GFR levels and (C) kidney tin levels, (D) inflammation, (E) oxidative stress were plotted from 11 to 15 rats from all groups, respectively. Correlation between inflammation and (F) kidney tin levels, (G) interstitial collagen deposition and (H) apoptosis were plotted from 12 to 15 rats from all groups. Collagen deposition was also plotted against the respective (I) the kidney tin levels and (J) renal oxidative stress from 12 to 13 rats from all groups. Oxidative stress were plotted against the respective (K) kidney tin levels, (L) proteinuria levels and (M) apoptosis of the 13–14 rats from control, TBT 100 and TBT500 groups. (N) Proteinuria levels were also plotted against the apoptosis ($n = 12$). Statistic significance ($p \leq 0.05$) was tested using the Spearman's or Pearson's test if non-Gaussian or Gaussian data distribution, respectively, was detected.

structural abnormalities such as inflammation, increased glomerular tuft area and fibrosis. Additionally, TBT led to higher renal oxidative stress and apoptosis levels, as well as reduced serum E₂ levels and ER α protein expression.

OTs exposure leads to higher OTs accumulation in the blood and tissues in animal models (Krajnc et al., 1984; Whalen et al., 1999). Mitra et al. (2014) demonstrated that TBT exposure at 1 and 5 mg/kg for 30 days increased blood and kidney tin levels in rats. Our results agree with these previous findings. We show that both TBT100 and TBT500 exposure increased serum and kidney tin levels in a dose-dependent manner. Similarly, the tin levels present in the serum were the result of TBT exposure, and the tin kidney resulted from the serum tin accumulation in both TBT rat groups, as shown for us in a positive correlation (Fig. 8A).

OTs play a direct role in the renal dysfunction (Robertson et al., 1987; Nebbia et al., 1997; Kobayashi-Hattori et al., 2006; Tang et al., 2010; Mitra et al., 2014) (Table 2). Tang et al. (2012) reported that low occupational trimethyltin (TMT) level exposure results in the increase of blood TMT levels and the prevalence of kidney stones (nephrolithiasis) in healthy workers from Guangdong Province (China). Our results agree with these previous findings, showing a reduction in the GFR levels in both TBT rats. Surprisingly, Mitra et al. (2014) found no direct changes in rat renal functional parameters such as blood urea and creatinine levels from TBT-exposure at 1 and 5 mg/kg for 30 days, in contrast to our study. Thus, the reduction in the GFR could be associated with kidney tin accumulation, as shown in a positive correlation in our model (Fig. 8C).

In addition, there is a strict correlation between proteinuria, renal disease and abnormal protein reabsorption in proximal tubule (PT) cells (Pouidel et al., 2012; Gorriz and Martinez-Castelao, 2012). Our results agree with these findings, showing an increase in proteinuria levels in TBT500 rats. Additionally, proteinuria is associated with proinflammatory and profibrotic effects contributing directly to the development of tubulointerstitial injury (Gorriz and Martinez-Castelao, 2012). Thus, the proteinuria levels

could be associated with the consequences of kidney tin accumulation, such as oxidative stress and apoptosis, as shown in a positive correlation (Fig. 8L and N).

Exposure to TBT results in renal abnormalities (Mitra et al., 2014). Wester et al. (1990) reported that exposure to TBTO at 50 mg/kg diet for 1 year increased the rat kidney weights. Our results agree with these previous findings. We observed a small increase in kidney size and an increase in the renal index in the TBT500 rats using both the wet and dry kidney weight/body weight. However, Mitra et al. (2014) showed a reduction in renal index after TBT (5 mg/kg) for 30 days. Surprisingly, Silva de Assis et al. (2005) found no change in rat kidney weights after exposure to 2 and 6 μ g/kg TBT for 30 and 60 days. Other studies have shown that TBT exposure in rodents resulted in contradictory anatomical data, and several studies showed renal histopathology as common feature (Wester et al., 1990; Mitra et al., 2014). Robertson et al. (1987) reported that a single dose of TMT (12.25 mg/kg) resulted in hyaline droplet inclusions, attenuated brush borders and vacuolization in the rat PT cells as early as 2 days post-treatment. Mitra et al. (2014) reported that rat swollen glomeruli with distinct capsular space after TBT (5 mg/kg) for 30 days. Our results agree with these previous findings, showing vacuolization, apoptosis and disorganization of the brush border in cortical epithelial cells and an increase in the number of inflammatory cells in both TBT groups. In our previous study, Bertuloso et al. (2015) showed the presence of apoptosis in the TBT100 livers. Our results agree with these findings, showing a caspase-3 positive staining to in the cytoplasm of the renal tubules, as tubule proximal cells in TBT kidneys. The adverse effects of endocrine disruptors may depend on susceptibility, timing of the exposure, genetic differences, doses, type of xenobiotics and/or the association with different signaling pathways, as shown other endocrine disruptors effects of TBT (Penza et al., 2011; Bertuloso et al., 2015). The dramatic nature of these findings is therefore not fully understood.

A common feature of progressive renal disease is the presence of inflammatory cells associated with tubular abnormalities (Eddy,

Table 2
Summary of renal toxicity induced by OTs.

Renal parameter	Animal/OTs/Dose					
	Rat TMTc (12.25 mg/kg)	Rabbit TPTA (15,75, 150 mg/l)	Mice TBT (0.015, 0.05 mg/ml)	Rat TMT (10 mg/kg)	Rat TBT (1, 5 mg/kg)	Rat TBT (1 and 5 $\times 10^{-4}$ mg/kg)
Time of exposure	Acute	Chronic	Subchronic/Chronic	Acute	Subchronic	Subchronic
Body weight	↓/↑	NR	↔	NR	↓	↑
Serum tin levels	NR	NR	NA	NR	↑	↑
Kidney tin levels	NR	↑	↑	NR	↑	↑
Glomerular filtrate rate	NR	NR	NA	NA	NA	↓
Renal index	NR	NR	NA	NA	↓	↑
Kidney morphology	Impaired	NR	NA	NA	Impaired	Impaired
Expansion mesangial	NR	NR	NA	NA	NR	↑
Collagen deposition	NR	NR	NA	NA	NA	↑
α -SMA expression	NR	NR	NA	NA	NA	↑
Inflammation	NR	NR	NA	NA	NR	↑
Proteinuria	↑/↓	NR	NA	NR	NA	↑
Oxidative stress	NR	NR	NA	NA	↑/↔	↑
Apoptosis	NA	NR	NA	NR	NR	↑
ER α expression	NA	NA	NA	NA	NA	↓
Serum estrogen levels	NA	NA	NA	NA	NA	↓
Reference	Robertson et al. (1987)	Nebbia et al. (1997)	Kobayashi-Hattori et al. (2006)	Tang et al. (2010)	Mitra et al. (2014)	This study

OTs: Organotin; TMTc: Trimethyltin chloride; TPTA: Triphenyltin acetate; TET: Triethyltin; TBT: Tributyltin chloride; α -SMA: Smooth Muscle Actin; ER α : Estrogen receptor α ; MDCK: Mardin-Darby Canine Kidney; NR: not reported; NA: not applicable; ↑: increased; ↓: decreased; ↔: unchanged or similar to control.

2001; El-Koraie et al., 2001). Several studies suggested that mast cells play important roles in nephropathies such as glomerulonephritis and fibrosis (Hiromura et al., 1998; El-Koraie et al., 2001). Our results agree with these previous findings, showing renal inflammation with increased numbers of mast cells in both TBT100 and TBT500 rats a dose-dependent manner. Mast cells are pluripotent bone marrow derived cells found in the kidney (Roberts and Brenchley, 2000; Balakumar et al., 2009), associated with renal diseases such as diabetic nephropathy (Jones et al., 2003; Navarro-González et al., 2011). In addition, Jeong et al. (2016) reported the key role of macrophage in the mouse diabetic kidney injury model. Similarly, Wang et al. (2016) demonstrated a higher neutrophil accumulation and role in mouse glomerulonephritis induced by lipopolysaccharide. In our study, the TBT100 and TBT500 kidneys showed higher NAG and MPO activity, suggesting a role for macrophages and neutrophils in our kidney inflammation. Thus, the kidney inflammation could be associated with kidney tin accumulation and reduction in GFR levels, as shown in a positive correlation (Fig. 8F and D).

Clear evidence indicates that renal pathogenesis is multifactorial. Both genetic and environmental factors are responsible for triggering pathophysiological events (Cooper, 2001; Said and Hernandez, 2015). Our results agree with these previous findings, showing increased glomerular tuft area in both TBT100 and TBT500 rats in a dose-dependent manner. There is a close correlation in diabetic nephropathy between mesangial expansion and clinical manifestations such as proteinuria and decreased GFR (Mauer et al., 1984). We obtained similar data from both TBT rats. Other studies showed an association between inflammatory cells, such as mast cell and macrophage and renal fibrosis (Balakumar et al., 2009; Jeong et al., 2016). Our results agree with these previous findings, showing higher glomerular and interstitial collagen deposition in kidneys from both TBT groups. In addition, Scanduzzi et al. (2010) demonstrated that similar to chymase, mast cell protease-4 led to renal fibrosis in mast cell protease-4-deficient mice. In addition, the renal fibrosis is positively correlated with the inflammation in our model (Fig. 8G). Additionally, we saw an increase in α -SMA protein expression ($\sim 30\%$) in the renal cortices of TBT500 rats and a positive correlation between interstitial collagen deposition and α -SMA protein expression (Fig. 4N). We learned that α -SMA is the actin isoform present in myofibroblasts and fibroblasts, playing a key role in fibrogenesis in renal disease models (Kawasaki et al., 2008; Nakatani et al., 2008). Barreira et al. (2009) reported that α -SMA protein expression was approximately 7.0% higher in renal tissue from a unilateral ureteral obstruction rats compared to tissue from sham rats. Usually, renal diseases that are followed by the loss of urinary protein lead to kidney fibrosis (Abbate et al., 2006; Landgraf et al., 2014), as we saw in TBT rats.

E_2 plays an important role in the kidney function, including reduced renal extracellular matrix synthesis and fibrosis (Krepinsky et al., 2002; Mankhey et al., 2007). These renal beneficial effects of E_2 were not only attributable to E_2 levels but also to renal ER expression (Couse et al., 1997; Xu et al., 2014). Jelinsky et al. (2003) suggested that the kidney is the organ most influenced by ER α outside of the reproductive neuroendocrine axis. In diabetic nephropathy, there is an association between decreased serum E_2 levels and reduced renal ER protein expression in rodents (Wells et al., 2005; Karl et al., 2005). Our results agree with these previous findings, showing lower serum E_2 levels and cortical ER α protein expression in both TBT100 and TBT500 kidneys in a dose-dependent manner. In our previous studies, we reported a reduction in serum E_2 levels using TBT100 rats (Rodrigues et al., 2014; Bertuloso et al., 2015). Ribeiro et al. (2016) reported a reduction in serum E_2 levels in TBT500 rats. Similarly, Carneiro et al. (2014) observed a reduction in ER α protein expression in the

renal cortex that could be associated with oxidative stress and no alteration in the renal medulla in ovariectomized mice. Thus, these renal abnormalities found in both TBT kidneys could be associated with a reduction in beneficial E_2 role, as shown in the negative correlation between E_2 levels with renal oxidative stress (Fig. 5D) and inflammation (Fig. 5F) or the negative correlation between ER α protein expression with inflammation (Fig. 5H) and interstitial collagen deposition (Fig. 5I).

TBT is associated with metabolic, reproductive and neural dysfunctions (Grün et al., 2006; Grote et al., 2009). Although the exact mechanism of TBT-induced dysfunction remains to be elucidated, TBT toxicity is attributed to its ability to increase cellular oxidative stress in different tissues (Grondin et al., 2007; Merlo et al., 2016). Mitra et al. (2013) reported an increase in oxidative damage with marked decline in cerebral cortex antioxidant status on day 7 after a single dose of TBT (30 mg/kg). Mitra et al. (2014) demonstrated a 1.4-fold increase in the reactive oxygen species levels in rats kidneys exposed to 1 mg/kg TBT for 30 days. Our results agree with these findings, showing higher oxidative stress levels in the renal cortex ($\sim 13.5\%$ DHE; $\sim 27.5\%$ DCF and $\sim 15.0\%$ HPF) and medulla ($\sim 13.0\%$ DHE; $\sim 32.5\%$ DCF and $\sim 12.5\%$ HPF) in both TBT100 and TBT500 kidneys. In addition, TBT led to apoptosis in different tissues (Grondin et al., 2007; Podratz et al., 2012). Mitra et al. (2013) reported increased apoptosis in the rat cerebral cortex on day 7 after a single dose of TBT (30 mg/kg). Our results agree with these findings, showing increased apoptosis in the renal cortices of TBT500 rats using CF or IHC assay that could be associated with a positive correlation between apoptosis and proteinuria levels (Fig. 8N). Lowe et al. (2012) associated oxidative stress in renal tissue with collagen deposition and other renal abnormalities 24 h after exposure to unique single doses of cynotoxin microcystins (55 mg/kg) in rats. Our results agree with these previous findings by showing higher O_2^- levels in the renal cortex and medulla tubules in both groups of TBT rats using DHE stain. An et al. (2009) reported higher oxidative stress in the rat chronic renal failure model. An increase in the renal cortex TBARS levels were observed in the TBT 500 rats. In addition, we observed a reduction in the reduced glutathione levels in the renal cortex and medulla from TBT100 and TBT500 rats. Thus, renal oxidative stress could also contribute to the pathophysiological complications, including impaired renal function and collagen deposition in TBT rats, as shown with a positive correlation between a renal oxidative stress and GFR (Fig. 8E), proteinuria (Fig. 8L) and apoptosis (Fig. 8M).

In conclusion, our findings provide evidence that TBT leads to abnormal kidney function, associated with a reduction in GFR and increase in proteinuria levels. Additionally, renal inflammation, fibrosis and oxidative stress were associated with lower beneficial E_2 effects observed in TBT rats. This work clarifies our understanding of the TBT pathophysiological effects in the development of renal disorders.

Disclosure statement

The authors have nothing to disclose.

Conflict of interest statement

The authors declare no conflicts of interest related to this work.

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