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# Exploring the Impact of Continuous Ethinylestradiol and Drospirenone Administration on Kidney Morphofunction in Female Mice with or Without a High-Fat Diet

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## ABSTRACT

**Introduction:** Combined oral contraceptives (COCs) are composed of estrogen and progestin and are among the most used contraceptive methods worldwide. Being overweight may increase the risk of kidney diseases, especially in women. However, whether the continuous use of COCs can affect renal health remains unclear. **Objective:** To investigate the effects of continuous administration of a COC containing ethinylestradiol (EE) and drospirenone (DRSP) on the renal morphofunction of female mice, with or without a high-fat diet (HFD). **Methods:** For 65 days, adult *Swiss* female mice were fed a standard diet (SD) or a high-fat diet (HFD) and received a daily oral gavage of 200 µL distilled water [control SD (CTL-SD) and CTL-HFD groups, respectively], containing or not 0.6 µg EE plus 60 µg DRSP (COC-SD and COC-HFD, respectively). **Results:** COC-SD females exhibited increased water intake and urine excretion, reduced fasting glycemia and urine creatinine levels, but higher $Na^+$ and $K^+$ urinary excretion. In contrast, the COC-HFD group displayed increased glycemia and reductions in water intake, urinary volume, and $Na^+$ and $K^+$ excretion. COC administration caused a reduction in Bowman's space of renal corpuscles of COC-SD females, and tubular injury in the renal cortex and medulla. Nephrons of COC-HFD females showed reductions in glomerular area and an enlargement of Bowman's space, but tubular injury like that observed for COC-SD. **Conclusion:** Continuous administration of EE and DRSP can cause morphological kidney damage, which is a concerning finding, as these modifications occur before any apparent changes in renal function parameters in plasma and urine.

**Keywords:** contraceptives, oral, combined; kidney; obesity; acute kidney injury.

## INTRODUCTION

Oral contraceptives are the contraceptive method used by around 16% of women aged 15 to 49 years worldwide[1]. Among oral contraceptive formulations, combined oral contraceptives (COCs) stand out, as they are composed of an estrogen and a progestin. The estrogen component can be natural or synthetic, with ethinylestradiol (EE) being the most used synthetic estrogen in COCs. The progestin component, in turn, includes synthetic progestins, which are classified by generation based on when the group of progestins was developed and commercialized[2]. Drospirenone (DRSP) is a fourth-generation progestin structurally related to spironolactone, which shows binding affinity to the progesterone receptor, like that promoted by progesterone. In addition, DRSP exhibits high anti-mineralocorticoid and anti-androgenic activities, when compared to spironolactone and progesterone, respectively, and weak mineralocorticoid action[3,4]. The COC composed of EE and DRSP emerged as an alternative for contraception with reduced adverse effects compared to prior formulations, addressing issues such as salt/water retention and androgenic activity[5]. On the other hand, it is important to highlight that sex steroids, besides their significant role in reproduction, also regulate other organic systems, including renal function[6-8], which should be taken into account when prescribed to women with an increased risk of kidney diseases.

Overweight and obesity are public health problems worldwide that predispose individuals to various chronic diseases, including kidney diseases such as kidney stones, renal carcinoma, chronic kidney disease, and end-stage renal disease[9,10]. Notably, obesity-related kidney diseases are the second leading cause of disability-adjusted life years[10]. A retrospective study evidenced that overweight and obesity pose a greater risk of leading to kidney diseases in women than in men[9]. In contrast, the association between overweight, kidney function, and oral contraceptive use is understudied.

Another important point to consider is that many users of COCs choose not to follow the usual cycle of taking them for 3 weeks followed by 4 or 7 days without the COC, instead using the COC continuously for many years. Prolonged use of COCs is debated, with some studies suggesting it may reduce the risk of certain diseases while increasing the risk of others[11,12].

Herein, we aimed to investigate the effects of the continuous administration of a COC composed of EE and DRSP on the renal morphofunction of adult female mice, with or without an obesogenic diet.

## METHODS

### Experimental groups

All experimental procedures were approved by the *Centro Multidisciplinar* UFRJ-Macaé's Animal Care and Use Committee with certificate number MAC039. Twenty-one-day-old female *Swiss* mice were kept under controlled conditions of temperature (22±2°C), humidity, and luminosity (lights on: 7 am to 7 pm) with standard diet (SD; Nuvilab CR1, Quimtia SA., Colombo, PR, BRA) and filtered water *ad libitum*. From 80 to 145 days old, the female mice were fed on an SD (providing 3.8 kcal/g, which 9.9% of the total kcal from fat) or a high-fat diet (HFD; providing 6.2 kcal/g, which 60% of the total kcal from fat) and received a daily oral gavage of 200 µL distilled water [vehicle; control SD (CTL-SD) and CTL-HFD groups, respectively], containing or not 0.6 µg EE plus 60 µg DRSP (COC-SD and COC-HFD, respectively).

The doses of the COC, used in this study, as reported by Oliveira et al.[13], were like those of the EE and DRSP concentrations consumed by users of the commercial COC formulation containing 30 µg EE and 3 mg DRSP (EMS Pharma, Hortolândia, SP, BRA); and the mouse doses were obtained through allometric conversion from the human dose to mice dose[14]. The 65 experimental period was based on previous studies that showed kidney damage induced by HFD after 8 weeks of consuming this obesogenic diet[15,16].

The high-fat diet (HFD) was prepared in the laboratory by blending lard (*Aurora*, Chapecó, SC, Brazil) into the standard diet (SD), along with the addition of soybean oil (*Soya*, São Paulo, SP, Brazil) and casein (*Prag Soluções Biociências*, Jaú, SP, Brazil) to ensure adequate levels of essential fatty acids and protein, respectively. The final macronutrient composition of the HFD per 100 g was 25.8 g of protein, 30 g of carbohydrates, and 37.2 g of lipids. In comparison, the SD contained 22 g of protein, 60 g of carbohydrates, and 4 g of lipids per 100 g.

### Urine and plasma biochemical and general biometric parameters evaluation

In the last week of the experimental period, each female mouse was weighed and kept individually for 24 h in metabolic cages (Tecniplast S.p.a., Buguggiate, VA, Italy) with free access to water and diet. The urine was collected into vials that contained mineral oil to minimize evaporation. The 24-hour urinary volume was recorded for each mouse and expressed as mL/g body weight (BW). The urine collected was centrifuged at 2800 *g* for 5 min to discard residual oil or fragments and stored at -20°C.

At 65 days of the treatment, all female mice after 12 h of fasting were weighed, their nasoanal length was measured to obtain the Lee index ($BW^{1/3}$/nasoanal length x 1000), and they were euthanized by decapitation. Total blood was collected, centrifuged at 12,600 *g* for 15 min, and the plasma was stored at -20 °C. A laparotomy was performed to access the left and right kidneys, which were removed and weighed.

Plasma and urinary concentrations of glucose (cat. K082), plasma albumin (cat. K040), and total proteins (cat. K031; Bioclin-Quibasa Ltda, Belo Horizonte, MG, BRA), and plasma and urinary creatinine, urea, uric acid, and urinary proteins (Interkit,

*Katal Biotecnológica*, Belo Horizonte, MG, BRA) were measured. Urinary $Na^+$ and $K^+$ levels were measured using a flame spectrophotometer (Analyzer 910 MS, Analyzer, São Paulo, BRA) and were multiplied by the urinary volume in mL/24h, respectively, to obtain urinary $Na^+$ and $K^+$ excretion. The clearance of creatinine was calculated using the urinary creatinine divided by the plasma creatinine multiplied by the urinary volume in mL/24h. All urinary parameters and creatinine clearance per female mouse were divided by her respective BW.

### Kidney histomorphometry and histopathology

The left kidney of all females was fixed for 24 h in a sodium-phosphate buffer containing 4% paraformaldehyde. The kidney was transversally sectioned, dehydrated in alcohol, cleared in xylene, and embedded in paraffin. For each kidney, 5 µm semi-serial sections were obtained, stained with periodic acid Schiff (PAS) or picrosirius red, and photographed using a digital camera (Tucsen USB 2.0 H series, Tucsen Photonics Co. Ltd., Fuzhou, Fujian, China) coupled to an optical microscope (Novel BM 2100, Nanjing Jiangnan Novel Optics. Co. Ltd., Nanjing, Jiangsu, China).

In the PAS-stained section, thirty renal corpuscles were photographed per mouse at 1000x magnification, and the renal corpuscle and glomeruli areas were manually measured using the freehand tool of the Image J software (https://imagej.net/ij/). The Bowman's space area was obtained by the subtraction of each renal corpuscle area from the respective glomerular area. For analysis of the positive (+) areas stained for PAS or picrosirius, eight random images per kidney section were registered at 400x magnification. The area stained positively for PAS or picrosirius red was calculated by determining the specific threshold for magenta/purple or pink/red color, respectively[17], using the Image J Software.

For renal histopathological analysis, all kidney sections stained with PAS were doubled blind evaluated (by Chaves JO and Gonzalez SR). The percentage of glomeruli with dilation or reduction of the Bowman's space per section was calculated[18]. Tubular injury was defined as tubular dilation, tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells or loss of the brush border, and thickening of the tubular basement membrane using the following scoring system: score 0, no tubular injury; score 1, <10% of tubules injured; score 2, 10–25% of tubules injured; score 3, 25–50% of tubules injured; score 4, 50–74% of tubules injured; and score 5, >75% of tubules injured[19].

### Statistical analysis

Results are presented as means ± SEM. The study had two independent variables: COC administration and diet treatment. The data were analyzed by two-way ANOVA followed by Tukey's post-test using GraphPad Prism® version 9.00 (GraphPad Software, Boston, MA, USA). The level of significance was set at $p<0.05$.

## RESULTS

### Effects of COC and diet treatments on obesity parameters and general renal parameters

Figure 1 presents the obesity and general renal parameters at the end of the experimental period in CTL and COC females. Significant effects of both COC administration and diet treatment were observed on BW ($p<0.002$ and $p<0.0001$), Lee index ($p<0.05$ and $p<0.05$), kidney weight ($p<0.0005$ and $p<0.001$), water intake ($p<0.0001$ and $p<0.0001$) and urinary volume ($p<0.001$ and $p<0.0001$), respectively. However, significant interaction among these factors was found only for BW ($p<0.0001$) and urinary volume ($p<0.03$). Multiple comparison analysis revealed that COC administration did not alter BW (Figure 1A), nasoanal length (Figure 1B), Lee index (Figure 1C), or kidney weight (Figure 1D) in COC-SD females compared to CTL-SD. In contrast, COC treatment significantly increased daily water intake and urinary volume by 48% and 231%, respectively, in COC-SD females compared to CTL-SD ($p<0.01$ and $p<0.0001$; Figures 1E and 1F). HFD intake increased BW (Figure 1A) and Lee index (Figure 1C) in CTL-HFD females compared to CTL-SD ($p<0.05$) and resulted in reductions in kidney weight ($p<0.01$; Figure 1D) and water intake ($p<0.01$; Figure 1E), without modification in urinary volume (Figure 1F). COC-HFD females showed reduced daily water intake ($p<0.001$; Figure 1E) and urinary volume ($p<0.001$; Figure 1F), compared to COC-SD. No significant differences were observed between COC-HFD and COC-SD females in BW (Figure 1A), nasoanal-length (Figure 1B), Lee index (Figure 1C), and in kidney weight (Figure 1D).

### Effects of COC and diet treatments on plasma and urinary biochemical parameters

Table 1 summarizes the nutritional and renal biochemical parameters of COC and CTL females. Two-way ANOVA revealed significant effects of COC administration ($p<0.03$) and diet treatment ($p<0.02$) on fasting glycemia. Multiple comparison analysis showed that COC reduced fasting glycemia in COC-SD females ($p<0.05$; Table 1). HFD increased fasting glycemia in COC-HFD compared to COC-SD females ($p<0.02$), but no significant differences were observed among COC-HFD and CTL-HFD groups (Table 1). Additionally, a significant effect of COC treatment on plasma creatinine levels was observed ($p<0.04$), though multiple comparisons did not reveal any significant differences between groups. Blood urea nitrogen, uric acid, total proteins, and albumin plasma levels did not differ significantly among COC and CTL females, regardless of diet (Table 1).

Regarding urinary biochemical parameters, a significant interaction between COC and diet treatments was observed for urinary creatinine ($p<0.002$) and urea levels ($p<0.05$). Post-test analysis showed that COC significantly reduced urinary creatinine in

COC-SD compared to CTL-SD females (p<0.02; Table 1). HFD increased creatinuria in COC-HFD compared to COC-SD females (p<0.002), but not in CTL-HFD compared to CTL-SD (Table 1). Two-way ANOVA also showed significant effects of COC (p<0.0001 and p<0.001), diet (p<0.0001 and p<0.0001), and their interaction (p<0.03 and p<0.03) on urinary excretion of $Na^+$ and $K^+$, respectively. Post-test analysis revealed that $Na^+$ and $K^+$ excretion were 127% and 121% higher, respectively, in

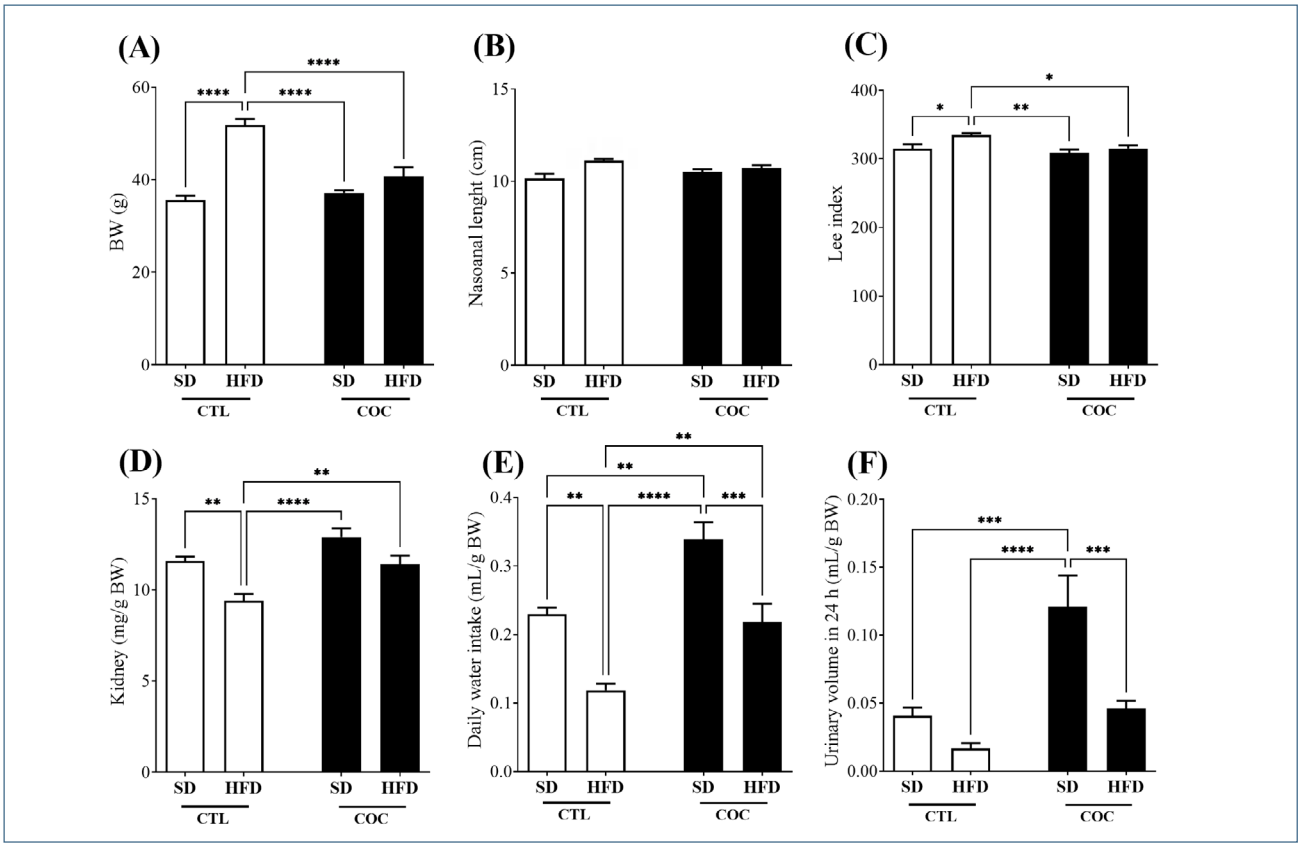


**Figure 1:** *COC treatment increased daily water intake and urinary volume in female rats fed an SD. In contrast, in the COC-HFD group, COC attenuated the HFD-induced increases in body weight (BW) and Lee index, as well as the reductions in kidney weight and water intake*. Means ± SEM of the BW (A), nasoanal length (B), Lee index (C), and weight of the kidneys (D), and the daily water intake (E) and urinary volume (F) at the end of the experimental period in CTL-SD (n=6), CTL-HFD (n=6), COC-SD (n=8) and COC-HFD (n=8) female mice. Data analyzed by 2-way ANOVA followed by Tukey post-test. The lines over the bars demonstrate the differences between the indicated groups. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

**Table 1:** Plasma and urinary nutritional and renal biochemical parameters of COC and CTL females that were fed on an SD or an HFD.

| | CTL-SD (n=6) | CTL-HFD (n=6) | COC-SD (n=8) | COC-HFD (n=8) |
|---|---|---|---|---|
| Plasma parameters | | | | |
| Glucose (mg/dL) | 132 ± 9.06 | 163 ± 2.60 | 87 ± 4.76* | 133 ± 14.20 |
| Creatinine (mg/dL) | 0.30 ± 0.61 | 0.30 ± 0.04 | 0.19 ± 0.02 | 0.11 ± 0.01 |
| Blood urea nitrogen (mg/dL) | 37.92 ± 2.75 | 35.60 ± 1.18 | 38.32 ± 1.89 | 33.67 ± 1.80 |
| Uric acid (mg/dL) | 5.66 ± 0.34 | 5.23 ± 0.85 | 5.17 ± 0.95 | 4.82 ± 0.71 |
| Total proteins (g/dL) | 4.89 ± 0.12 | 4.74 ± 0.19 | 4.53 ± 0.14 | 4.59 ± 0.18 |
| Albumin (g/dL) | 2.03 ± 0.08 | 2.09 ± 0.03 | 1.97 ± 0.07 | 1.92 ±0.16 |
| Urinary parameters | | | | |
| Glucose (µg/mL/BW) | 3.77 ± 1.19 | 5.38 ± 1.74 | 2.65 ± 0.57 | 3.33 ± 0.33 |
| Creatinine (µg/mL/BW) | 7.17 ± 0.63 | 5.83 ± 0.79 | 4.08 ± 0.55* | 7.73 ± 0.67 |
| Urea (µg/mL/BW) | 1251.00 ± 279.30 | 801.20 ± 140.10 | 702.70 ± 106.80 | 935.90 ± 97.06 |
| Uric acid (µg/mL/BW) | 0.87 ± 0.10 | 0.51 ± 0.14 | 0.70 ± 0.10 | 0.95 ± 0.12 |
| Protein (µg/mL/BW) | 38.31 ± 5.40 | 30.30 ± 1.23 | 38.69 ± 2.37 | 40.23 ± 2.66 |
| $Na^+$ (µmol/24h/BW) | 4.14 ± 0.48 | 1.18 ± 0.12 | 9.38 ± 1.53* | 2.99 ± 0.28 |
| $K^+$ (µmol/24h/BW) | 6.64 ± 1.44 | 2.02 ± 0.15 | 14.65 ± 2.60* | 3.90 ± 0.32 |

Data are means ± SEM * indicates that the COC-SD group is different from other groups for the same parameter evaluated (2-way ANOVA followed by Tukey post-test.

COC-SD females compared to CTL-SD ($p<0.0001$; Table 1). HFD decreased urinary $Na^+$ excretion in CTL-HFD compared to CTL-SD ($p<0.05$; Table 1). In COC-HFD females, urinary $Na^+$ and $K^+$ excreted were lower than in COC-SD ($p<0.0001$; Table 1). Urinary concentrations of glucose, urea, uric acid, and proteins did not differ significantly between COC- and CTL-treated females fed either an SD or an HFD (Table 1).

Creatinine clearance is shown in Figure 2. Two-way ANOVA revealed a significant effect of COC treatment ($p<0.02$) on creatinine clearance. Although a trend toward reduction was observed among CTL-HFD and CTL-SD groups, multiple comparison analysis showed that COC increased creatinine clearance in COC-HFD compared to CTL-HFD females (Figure 2).

## Effects of COC and diet treatments on renal histomorphometry and histopathology

Figure 3 displays representative 5 µm-thick kidney cortex sections and morphometric data from COC and CTL females. COC administration significantly affected renal corpuscle ($p<0.02$) and Bowman's space areas ($p<0.0001$). Diet had significant effects on renal corpuscle ($p<0.003$) and glomerular areas ($p<0.0001$), while interactions between COC and diet were observed only for Bowman's space area ($p<0.0001$).

Post-test analysis showed a 34% reduction in the Bowman's space area in COC-SD females compared to CTL-SD ($p<0.0001$; Figure 3A and Figure 3E). HFD significantly reduced the renal corpuscle ($p<0.0005$; Figure 3A and Figure 3C), glomerular

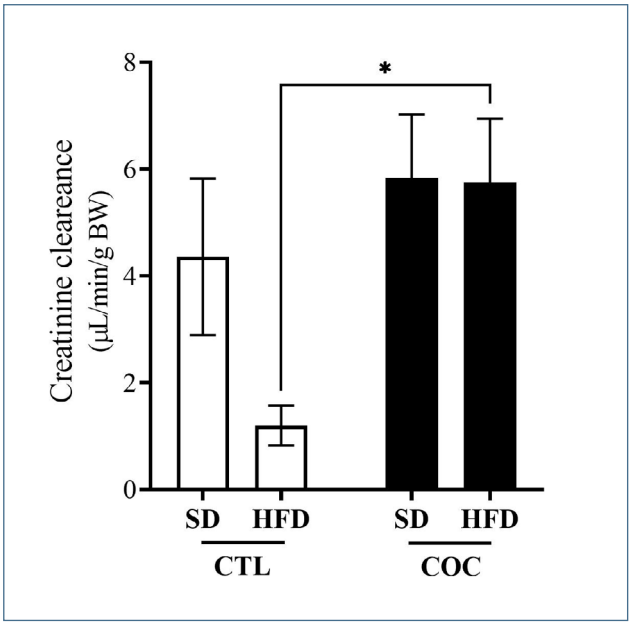


**Figure 2:** *COC treatment and diet effects on creatinine clearance in female mice.* Means ± SEM of creatinine clearance in CTL-SD (n=6), CTL-HFD (n=6), COC-SD (n=8), and COC-HFD (n=8) female mice. Data analyzed by 2-way ANOVA followed by Tukey post-test. *COC-HFD is different from CTL-HFD ($p<0.05$).

($p<0.03$; Figure 3A and Figure 3D), and Bowman's space areas ($p<0.0005$; Figure 3E) in CTL-HFD females compared with CTL-SD. In COC-HFD females, the glomerular area was reduced ($p<0.03$; Figure 3D), while the Bowman's space area increased by 32% compared to COC-SD ($p<0.01$; Figure 3E).

For PAS and picrosirius red staining, which indicate carbohydrate residue content and collagen deposition in the renal cortex, respectively, COC affected only the PAS-positive (+) area in the tubular region ($p<0.05$), while diet influenced the PAS + areas in glomerular ($p<0.05$) and tubular regions ($p<0.05$). Post-test analysis revealed a reduction in the percentage of PAS + area in the glomerular region of the COC-HFD group compared to COC-SD ($p<0.05$; Figure 3F). No significant differences in collagen deposition were found in the renal cortex between CTL and COC groups (Figure 3B, 3H, and 3I).

Figure 4 shows representative histological sections of the renal cortex and medulla from COC and CTL females either on an SD or an HFD. CTL-SD females displayed typical nephron structures, with PAS-positive proximal tubules showing intact brush borders, wide-lumen distal tubules, and well-defined renal corpuscles (Figure 4A). Their outer medulla exhibited collecting ducts with cuboidal cells lacking a brush border (Figure 4B). Conversely, COC-SD females exhibited tubular injury, including detached necrotic tubular cells, granular casts, denuded basement membrane, tubular dilatation, and flattened epithelium, along with reduced Bowman's space (Figure 4A). The outer medulla showed dilated collecting ducts with flattened epithelium and protein casts (Figure 4B), resulting in significantly higher cortical ($p<0.0001$; Figure 4C) and medullary injury scores ($p<0.0005$; Figure 4D) compared to CTL-SD.

HFD induced renal injury in CTL-HFD females, evidenced by necrotic tubular cells, granular and protein casts, dilated tubules, flattened epithelium, and enlarged intertubular space in both cortex and medulla (Figure 4A and Figure 4B). Injury scores were higher in CTL-HFD females compared with CTL-SD ($p<0.0001$ and $p<0.02$; Figure 4C and Figure 4D). COC-HFD females presented renal injury patterns like those in the cortex of COC-SD and CTL-HFD groups, including detached necrotic cells, granular and protein casts, denuded basement membrane, dilated tubules, and debris-filled lumens (Figure 4A). The outer medulla showed dilated tubules with enlarged intertubular spaces (Figure 4B). Although overall injury scores were comparable to COC-SD (Figure 4C and Figure 4D), the medullary injury score in COC-HFD was lower than in CTL-HFD ($p<0.05$; Figure 4D).

## DISCUSSION

This study demonstrated that the COC composed of EE and DRSP, when administered to adult female mice, led to modifications in water balance, as COC-SD females displayed enhanced

urinary volume and increased water intake. Partially, such effects may have occurred due to EE, as this synthetic steroid is a ligand of estrogen receptors (ER), presenting binding affinity to ER-α two times higher than that evidenced by estradiol[20]. In the nephron, estrogens binding to ER-α seem to be involved in the regulation of diuresis, as bilateral ovariectomy led to a reduction in diuresis, whereas estradiol treatment increased urinary volume due to reductions in the gene and protein expression of aquaporin-2 (AQP2) in the renal cortex.

The incubation of a murine principal kidney cortical collecting duct cell line with estradiol led to a reduction in AQP2. Furthermore, in the kidneys of ER-α knockout mice, higher AQP2 protein expression was observed in the cortex, outer medulla, and inner medulla[21]. Thus, these findings indicate that estrogens via ER-α can reduce renal AQP2, increasing diuresis, an effect that could be mediated by EE, contributing to the higher urine volume in the COC-SD group. In addition, the DRSP present in the COC used in this study may also, in part, have contributed to the higher diuresis in COC-SD females, since it is a spironolactone-derived progestin, a molecule that has natriuretic and diuretic effects. Possibly, the increased water intake in COC-SD females might represent a compensatory renoprotective effect to prevent dehydration caused by the increased urine excretion.

COC-treated females that were fed a standard diet (SD) also displayed enhanced Na+ and $K^+$ excretion. Natriuresis is an expected effect induced by DRSP, as it is a progestin with higher

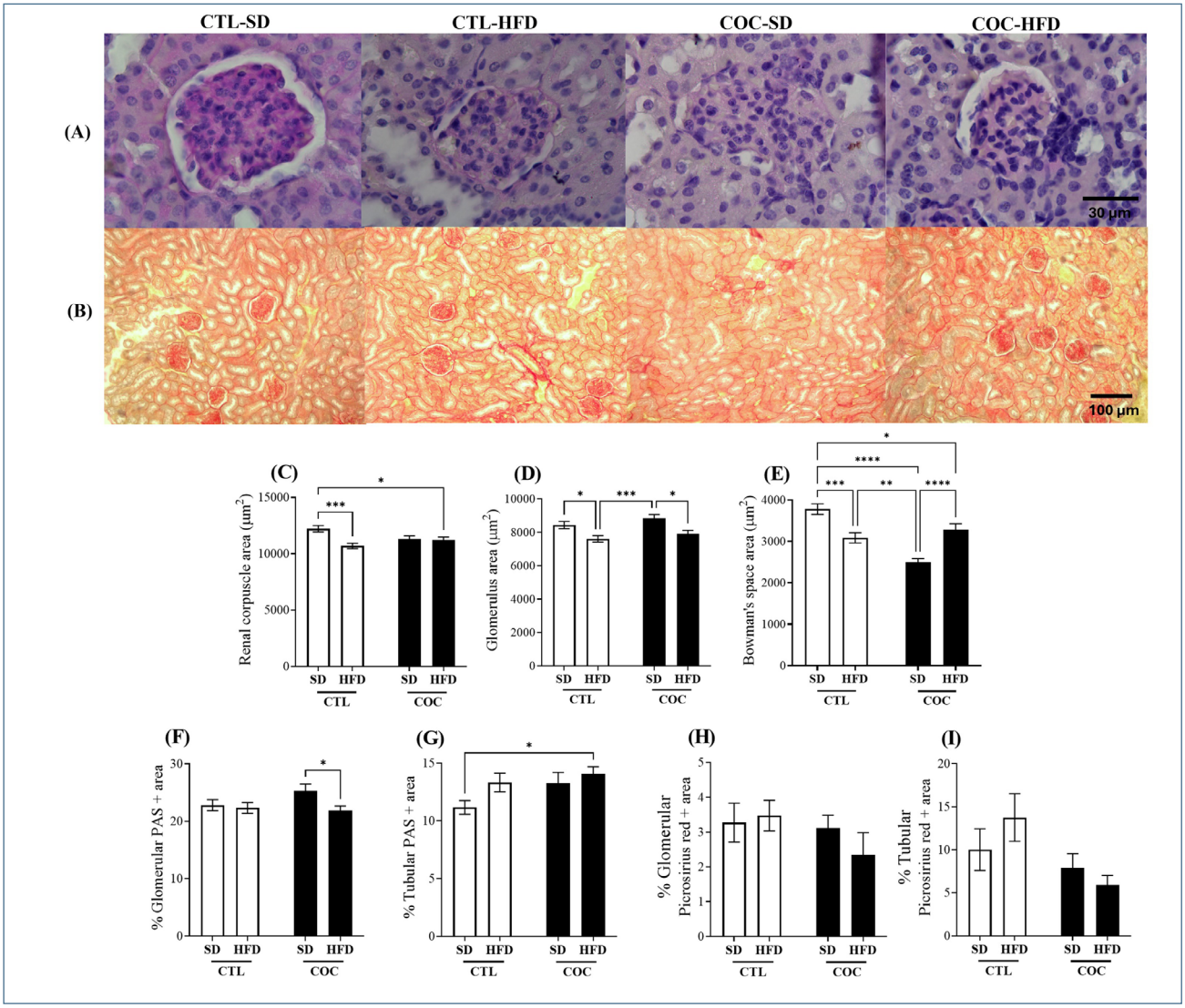


**Figure 3:** *COC treatment and HFD caused structural alterations in the renal corpuscle of female mice.* Representative 5 µm-thick section of the kidneys of CTL and COC female mice fed on or not a HFD, stained with PAS (A) or picrosirius red (B). Scale bars = 30 and 100 µm. Means ± SEM of renal corpuscle (C), glomerulus (D) and Bowman's space (E) areas, and the percentage area + for PAS and picrosirius red stain in the glomerular (F and H) and tubular (G and I) regions of the renal cortex of CTL-SD (n=6), CTL-HFD (= 6), COC-SD (n=8) and COC-HFD (n=8) female mice. Data analyzed by 2-way ANOVA followed by Tukey post-test. The lines over the bars demonstrate the differences between the indicated groups. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

affinity for the mineralocorticoid receptor[3], demonstrating itself to be a potent aldosterone antagonist, with an affinity approximately 8 times higher than that of spironolactone[22,23], which in part contributed to the increased natriuresis observed in COC-SD females. Conversely, the unexpectedly higher urinary $K^+$ excretion in the COC-SD group might be due to the weak mineralocorticoid action of DRSP[24].

HFD intake by rodents has been reported to lead to reductions in urinary volume[15,25,26], and water ingestion[27]. However, here, CTL-HFD only showed a statistically significant reduction in water intake, despite urine volume showing a decrease of 48.7% in comparison to CTL-SD. Notably, the HFD regimen seems to counteract COC's effects on such parameters, since COC-HFD females displayed lower water intake and urinary volume than those observed for COC-SD, but with values similar to those of CTL-SD. Furthermore, HFD also counteracts COC action on renal $Na^+$ handling, as COC-HFD females displayed reduced $Na^+$ and $K^+$ excretion. At least for the decreased $Na^+$ excretion, HFD has been shown to increase renal sympathetic nerve activity and tubular $Na^+$ reabsorption through mechanisms both dependent and independent of the epithelial $Na^+$ channel[26,28,29].

The literature lacks information about the potential mechanism by which HFD can decrease urinary volume. However, since renal sympathetic innervation augments tubular aquaporin expression[30], the increased sympathetic activity to the kidneys in obesity induced by HFD[28] may lead to changes in water excretion in COC-HFD females. Furthermore, as it has been demonstrated in *Sprague-Dawley* rats that renal sympathetic nerve activity also regulates urinary $K^+$ excretion[31], the reductions in $K^+$ excretion in COC-HFD females might be linked to the HFD-induced changes in renal nerve activity.

It is important to highlight that, although COC treatment in SD females did not lead to significant changes in renal biochemical parameters (as described above), it did result in renal structural alterations, including a reduction in Bowman's space and evidence of tubular damage. In accordance, a previous study reported that

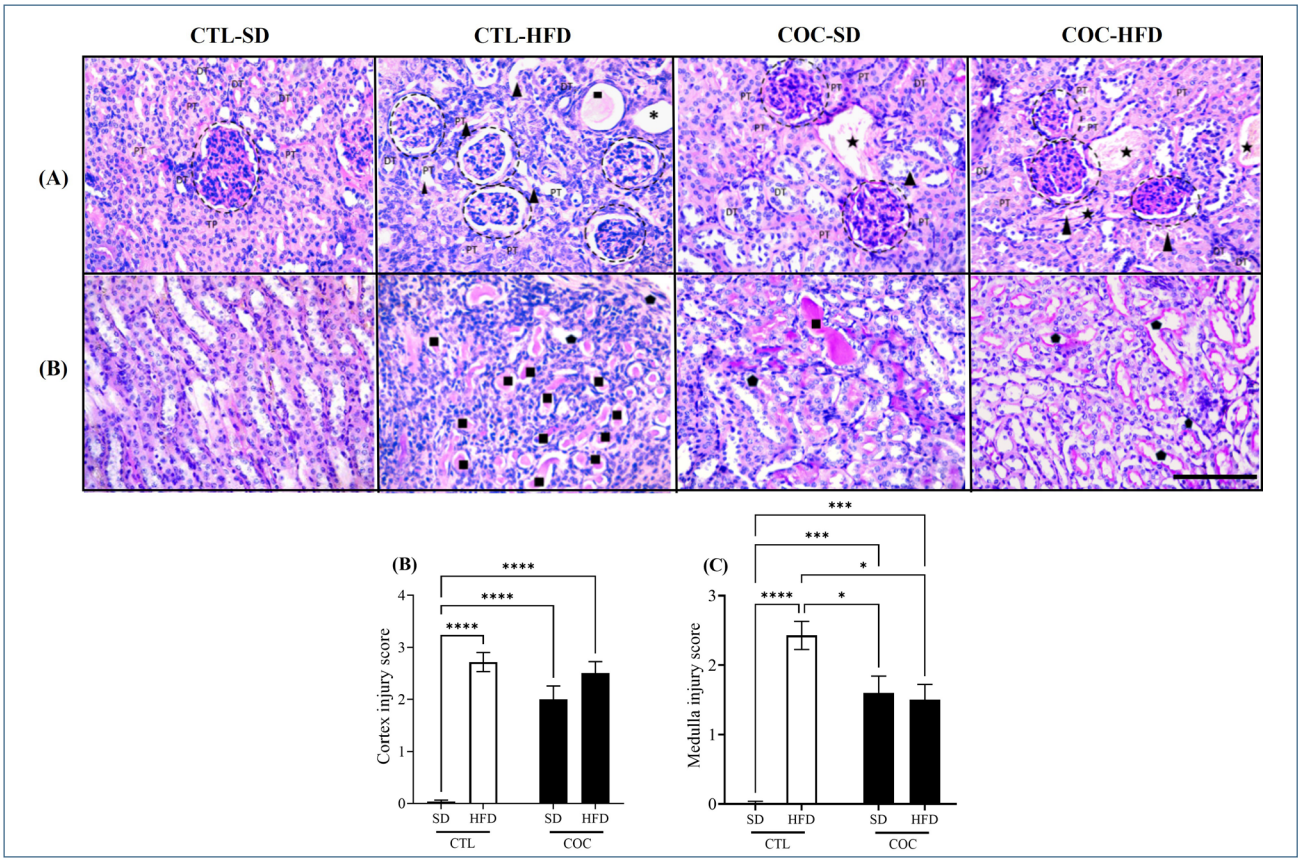


**Figure 4:** *COC treatment and HFD caused tubular injury in the kidneys of female mice.* Representative 5 µm-thick section of the renal cortex (A) and medulla (B) of CTL and COC female mice fed on or not a HFD, stained with PAS. Scale bar = 100 µm. **PT:** proximal tubule; **DT:** distal tubule; **circle:** glomeruli; **arrowhead:** represent detached necrotic tubular cells and granular casts with necrotic cellular debris, denuded basement membrane, tubular dilatation, and flattened tubular epithelium; **square:** protein casts; **asterisk:** enlarged space between the tubules; **star:** necrotic cell debris in some tubules; **black pentagon:** tubular dilatation and flattened tubular epithelium. Means ± SEM of the cortex (C) and medulla (D) injury scores in the kidneys of CTL-SD (n=6), CTL-HFD (n=6), COC-SD (n=8), and COC-HFD (n=8) female mice. Data analyzed by 2-way ANOVA followed by Tukey post-test. The lines over the bars demonstrate the differences between the indicated groups. *p<0.05, ***p<0.001 and ****p<0.0001.

both when EE and DRSP were administered alone to *Balb/c* female mice for 35 days, or when EE and DRSP were combined, they caused histopathological tubular modifications, especially in proximal tubules[32]. Importantly, our study differs from the former, as we carried out a longer treatment with COC and quantitative morphological analyses that support histopathological changes in the kidney and provide information that the combination of EE and DRSP, given to *Swiss* female mice for 65 days, results in damage to the renal corpuscles and tubules.

Notably, COC treatment combined with the HFD regimen caused similar renal morphological damage to the renal corpuscle and tubular cortical structures in the COC-HFD group, compared to those observed in CTL-HFD. Although it is important to emphasize that when the COC-HFD group was compared to the COC-SD, an increased Bowman's space and a reduced percentage of glomerular PAS+ area were observed. The increase in Bowman's space in COC-HFD can be linked to glomerular tuft retraction, a morphological glomerular damage induced by HFD intake, observed here and in other studies[15,33]. Since glomerular PAS+ area indicates basement membrane integrity[34], our results suggest that COC administration, when combined with HFD, may lead to lesions in the filtration membrane of COC-HFD females.

However, these morphological indicators of glomerular injury have not yet been associated with impairments in renal function in the COC-HFD group, since renal creatinine excretion was higher, and creatinine clearance was similar to that observed in COC-SD. But, when compared to CTL-HFD females, COC-HFD females displayed enhanced creatinine clearance, a condition that leads to hyperfiltration. Glomerular hyperfiltration, observed by increased glomerular filtration rate, contributes to accelerated renal function loss in the general population, particularly in obese patients with diabetes or chronic kidney disease[35]. Thus, further investigations are necessary to demonstrate the mechanisms by which EE and DRSP could change this parameter in obesity induced by HFD.

Regarding the COC effects on glycemia, the prolonged administration of 65 days of DRSP and EE in COC-SD females may disrupt insulin secretion and clearance, as previously demonstrated by our study group[13]. Interestingly, COC treatment seems to attenuate body weight (BW) increase induced by HFD in COC-HFD females, but it led to a significant increase in glycemia. A previous study reported that the administration of 6 mg DRSP/kg/day to C57Bl/6 female mice prevented HFD-induced obesity and glucose homeostasis disruptions[36]. Additionally, estradiol administration alone to female mice also prevented obesity[37]. In this way, both EE and DRSP may have modified body energy expenditure in COC-HFD females, resulting in attenuation of the HFD effects on BW gain. Interestingly, despite this, this group presented increased glycemia, indicating that the combination of EE and DRSP does not prevent comorbidities related to an obesogenic diet.

In summary, COC administration for 65 days to adult *Swiss* female mice fed an SD caused enhanced water ingestion, urine excretion, and increased $Na^+$ and $K^+$ urinary excretion. Also, the nephrons of COC-SD females exhibited reduced Bowman's space and various signs of tubular injury in the renal cortex and medulla. COC treatment combined with the HFD led to lower water ingestion and urinary volume, increased glycemia, urine creatinine levels, and reduced $Na^+$ and $K^+$ excretion. The findings serve as an alert that the COC composed of EE and DRSP can lead to morphological kidney damage that may not be detected through routine renal biochemical plasma and urinary parameters.

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