Brief Report

Alterations in Kidney Structures Caused by Age Vary According to Sex and Dehydration Condition

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Abstract: Aging is a complex biological process, with gradual and progressive decline in structure and function in many organ systems. Our objective is to determine if structural changes produced by aging, vary with sex, in a stressful situation such as dehydration. The expression of *Slc12a3* mRNA in renal cortex, α-smooth muscle actin (α-SMA) and fibronectin, was evaluated in male and female rats aged 3 and 18 months submitted or not to water deprivation (WD) for 48 h. When comparing ages, 18-month-old males showed lower expression of *Slc12a3* mRNA than 3-month-old males, and control and WD 18-moth-old male and female rats exhibited higher expression of α-SMA than respective 3-month-old rats. Fibronectin was higher in both control and WD 18-month-old males than respective 3-month-old males. In females, only control 18-month-old rats showed higher fibronectin than control 3-month-old rats. When we compared sex, control and WD 3-month-old female rats had lower expression of *Slc12a3* mRNA than respective males. WD 18-month-old male rats presented higher expression of fibronectin and α-SMA than WD 18-month-old female rats. When we compared hydric condition, WD 18-month-old males displayed lower relative expression of *Slc12a3* mRNA and higher α-SMA expression than control 18-month-old males. Aging, sex, and dehydration lead to alteration in kidney structure.

Keywords: aging; body composition; kidney; sex differences

1. Introduction

The kidneys are primarily organs that regulate volume and composition of the internal fluid environment through the specialized sequence of functions in the different segments of nephron. The number and length of the loops of Henle and the arrangement of the capillary circulation, as well as the feed-back interactions between different parts of nephron, and the extrarenal control through nervous innervation and humoral agents are important factors that operate throughout the entire kidney [1].

Aging is a complex biological process, with gradual and progressive decline in structure and function in many organ systems. In the kidney, morphological changes occur in the glomeruli, in the glomerular basement membrane, tubulointerstitium, and renal vasculature with aging [2]. The structural changes in the kidney associated with hemodynamic changes include, the loss of renal mass; hyalinization of afferent arterioles; and development of aglomerular arterioles, sclerotic glomeruli, and tubulointerstitial fibrosis [3].

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Changes in the chemical composition of the glomerular basement membrane, related to increases in non-enzymatic glycosylation of proteins; increased expression of collagen, laminin, fibronectin, and thrombospondin; and changes in the degree of sulfation of glycosaminoglycans are also observed [6].

In the injured kidney, interstitial infiltrate macrophages synthesize fibronectin and tubular cells change their phenotype to myofibroblasts, which are able to produce extracellular matrix components, such α-smooth muscle actin (α-SMA), a protein that is expressed normally in renal cortex only by vascular smooth muscle cells [7].

Also, renal aging is characterized by progressive tubular disfunction, such as decreased sodium reabsorption; potassium excretion; and renal concentrating and diluting abilities [8]. The lowered ability to concentrate urine in age rats may be related to the reduced level of sodium transporter (7). However, the among sodium transports, the thiazide sensitive Na+/Cl- cotransporter (NCC), encoded by the Slc12a3 gene, was not reduced by age in male rats and in the water deprivation condition resulted in either no increase, or a reduced increase in the protein abundance of NCC (7–9).

As aging alters kidney structure and older people are more at risk of dehydration because by the reduced thirst perception and the renal capacity to concentrate urine (10,11). Also, changes in hydromineral homeostasis in the elderly are also related to sex [11]. Moreover, the effects of estrogens on fluid regulation in older women are mediated in the kidney [12]. Our goal is to determine if structural changes produced by aging, vary with sex, in a stressful situation such as dehydration. We hypothesized that alterations produced in the kidney by aging show sexual differences and change with the dehydration.

2. Results

2.1. Kidney and body weight

Table 1 provides the information about the left kidney and body weight of the animals. Both hydrated and dehydrated 3-month-old male and female rats had higher left kidney weight than 18-month-old male and female rats (Age: Male: $F_{1,32} = 18.9$, $p<0.001$; Female: $F_{1,33} = 63.6$, $p<0.00001$). Both dehydrated 3- and 18-month-old male rats presented higher kidney weight than hydrated rats (Hydration: $F_{1,32} = 22.0$, $p<0.0001$). In both ages studied, the dehydrated male rats exhibited heavier left kidney than dehydrated female rats (Sex: 3-month-old: $F_{1,40} = 8.7$, $p<0.01$; 18-month-old: $F_{1,25} = 29.7$, $p<0.0001$).

Table 1. Left kidney and body weight of normal (Ctrl) and dehydrated (WD) 3 and 18-month-old male (M) and female (F) rats.

Parameters	Sex	Age (months)			
		3		18	
		Ctrl	WD	Ctrl	WD
Number of animals	M	10	10	8	8
	F	12	12	7	6
Kidney weight (g/100g bw)	M	0.35 (0.02) ^{ac}	0.38 (0.03) ^{bcg}	0.31 (0.02) ^{ad}	0.35 (0.02) ^{bdh}
	F	0.33 (0.02) ^{e}	0.35 (0.02) ^{fg}	0.29 (0.03) ^{e}	0.28 (0.02) ^{fh}
Body weight (g)	M	561.5 (39.5) ^{iot}	476 (49.4) ^{kom}	810.1 (147.9) ^{iv}	733 (123) ^{ky}
	F	383.7 (19) ^{pjt}	334.2 (20.3) ^{qjm}	550 (98.6) ^{pv}	466.8 (59.4) ^{qy}

Values are expressed as means (SD). Averages with the same superscript differ significantly $p<0.05$. ANOVA factorial followed by the Newman-Keuls post-test or Games-Howel.

Male and female rats at 18 months old showed heavier body weight than 3-month-old rats (Age: Male: $F_{1,31} = 57.5$, $p<0.0001$; Female: $F_{1,31} = 86.1$, $p<0.0001$). Dehydrated 3-month-old male and female rats had lighter body weight than hydrated 3-month-old male and female rats (Hydration: Male: $F_{1,31} = 6.5$, $p<0.05$; Female: $F_{1,31} = 13.6$, $p<0.01$). Both, 3-

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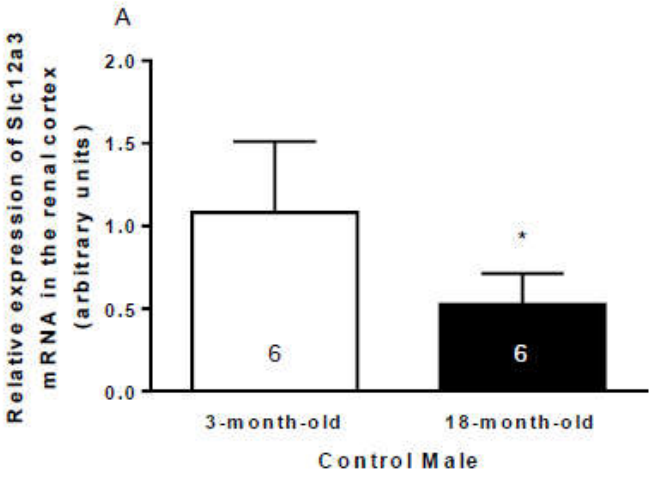
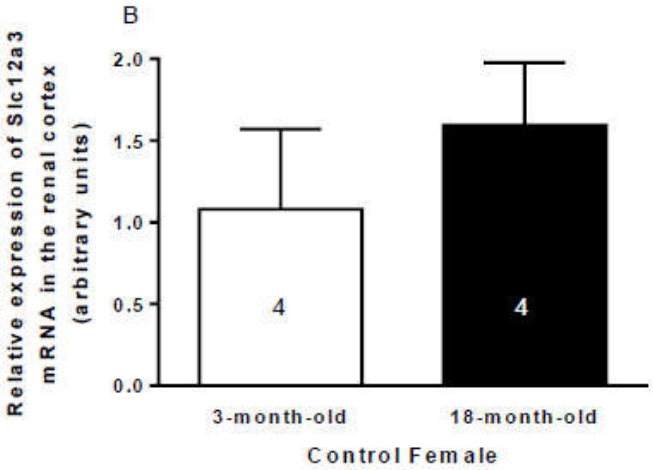
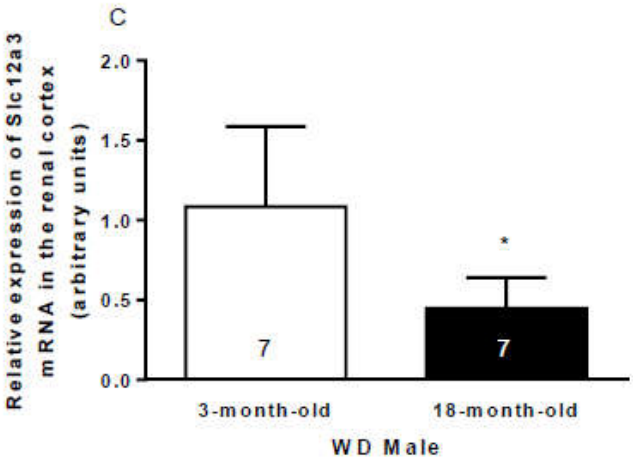
and 18-month-old male rats presented more body weight than 3- and 18-month-old female rats (Sex: 3-month-old: $F_{1,40} = 250.2$, $p<0.0001$; 18-month-old: $F_{1,25} = 37,0$ $p<0.0001$).

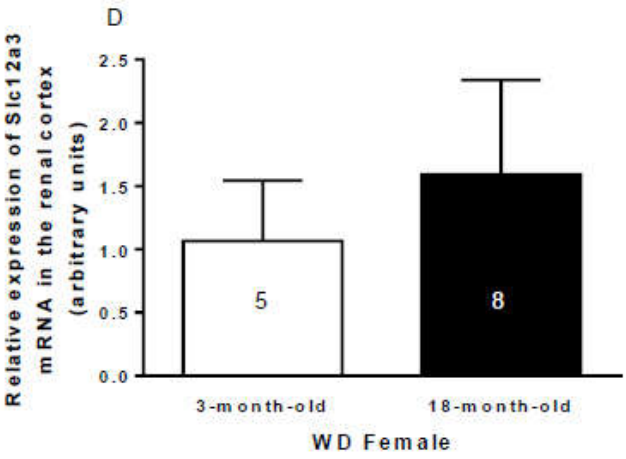
2.2. The relative expression of Slc12a3 mRNA in the renal cortex

Both, control and WD 18-month-old males had lower expression of Slc12a3 mRNA than 3-month-old males (Age: Control: $t_{10} = 2.90$, $p<0.05$; WD: $t_{12} = 3.12$, $p<0.01$; fig 1A-D). Control and WD 3-month-old female rats both had lower expression of Slc12a3 mRNA than respective males (Sex: Control: $t_8 = -4.10$, $p<0.01$; WD: $t_{10} = -3.67$, $p<0.01$; fig 1E-H). Dehydrated 18-month-old males presented lower relative expression of Slc12a3 mRNA than hydrated old males (Hydration: $t_{24} = 2.29$, $p<0.05$; fig 1I-L).









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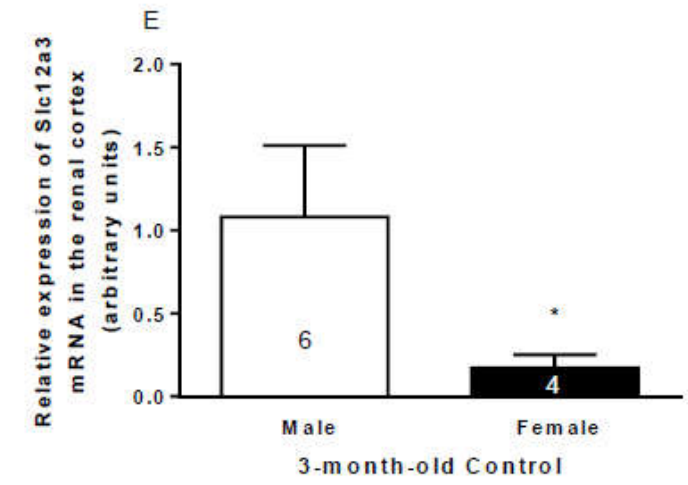

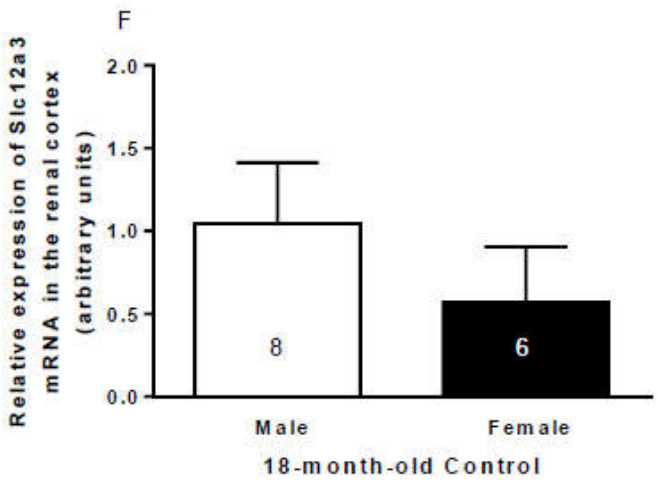

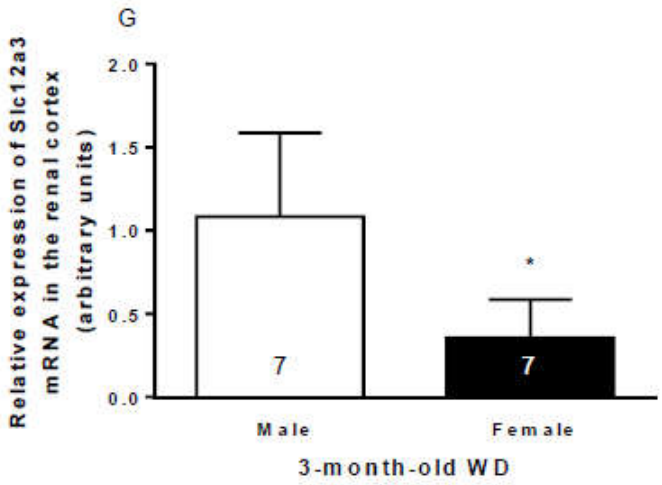

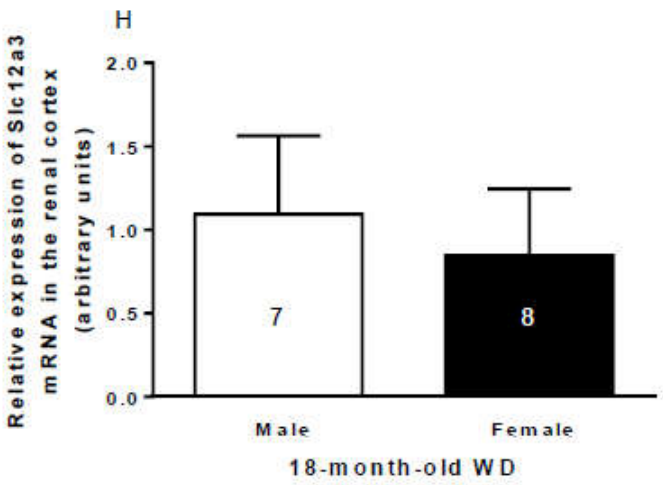

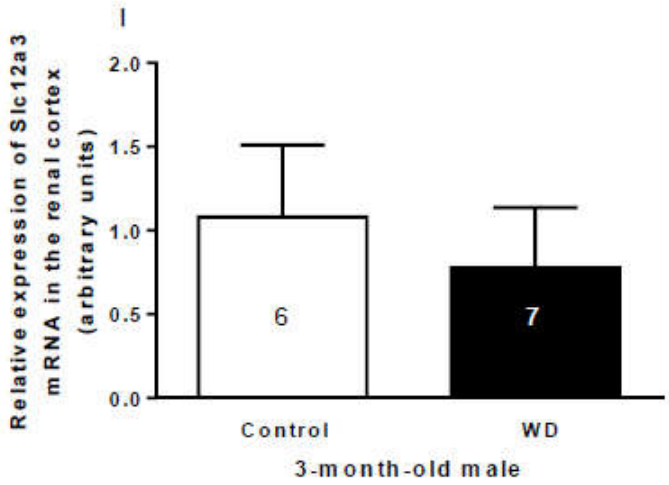

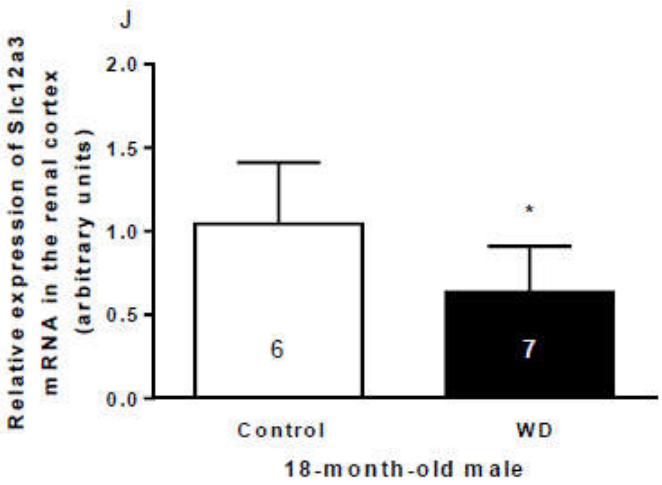

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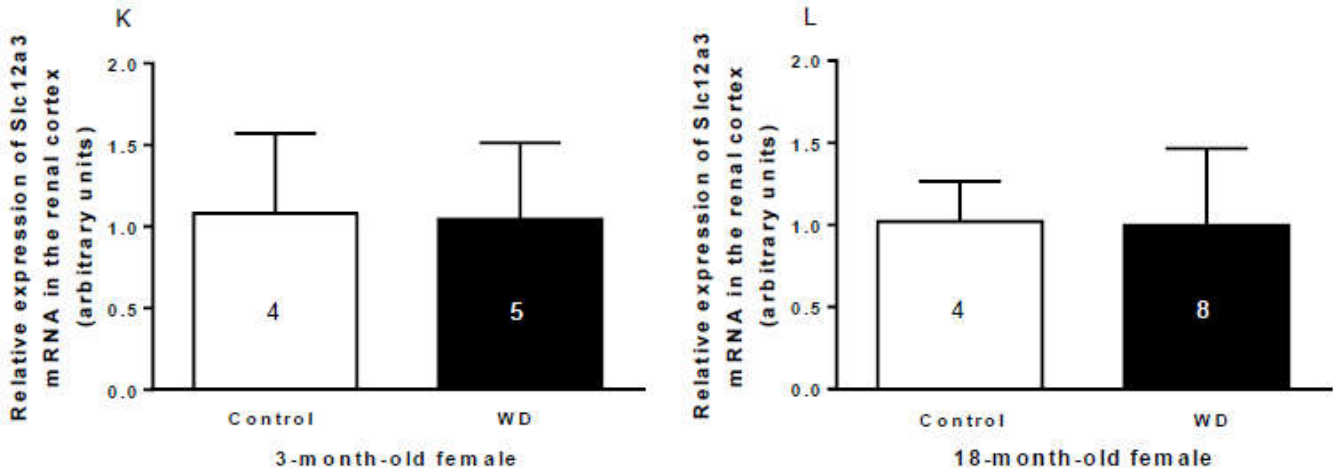

Figure 1. Relative *Slc12a3* mRNA expression in the renal cortex of 3- and 18-month-old control and water deprived (WD) male and female rats. Comparing by age in the control male (A) and female rats (B) and WD male (C) and WD female rats (D). Comparing by sex in the 3-month-old (E) and 18-moth-old control rats (F) and 3-month-old (G) and 18-month-old WD rats (H). Comparing by hydric condition in the 3-month-old (I) and 18-month-old male rats (J) and 3-month-old (K) and 18-month-old female rats (L). Data are presented as means (SD), * $p<0.05$ relative to the reference group. Student's unpaired t-test. Number (n) of the sample is indicated inside the column.

2.3. Immunohistochemical analysis

The immunohistochemical studies found a higher score for α-SMA in the cortical tubulointerstitium in 18-month-old animals than 3-month-old animals (Age: $F_{1,43} = 111.05$, $p<0.00001$, fig 2). WD 18-month-old males exhibited higher renal expression of α-SMA than control 18-month-old male rats (Hydric condition: $F_{1,43} = 7.70$, $p<0.01$) and WD 18-month-old female rats (Sex: $F_{1,43} = 5.74$, $p<0.05$, fig 2).

Immunohistochemical analysis also revealed that cortical tubulointerstitium expression of fibronectin was higher in control 18-month-old rats than control 3-month-old rats as well as WD 18-month-old male rats than WD 3-month-old male rats (Age: $F_{1,42} = 68.34$, $p<0.0001$, fig 3). WD 18-month-old male rats presented higher expression of fibronectin than WD 18-month-old female rats (Sex: $F_{1,42} = 11.74$, $p<0.001$, fig 3).

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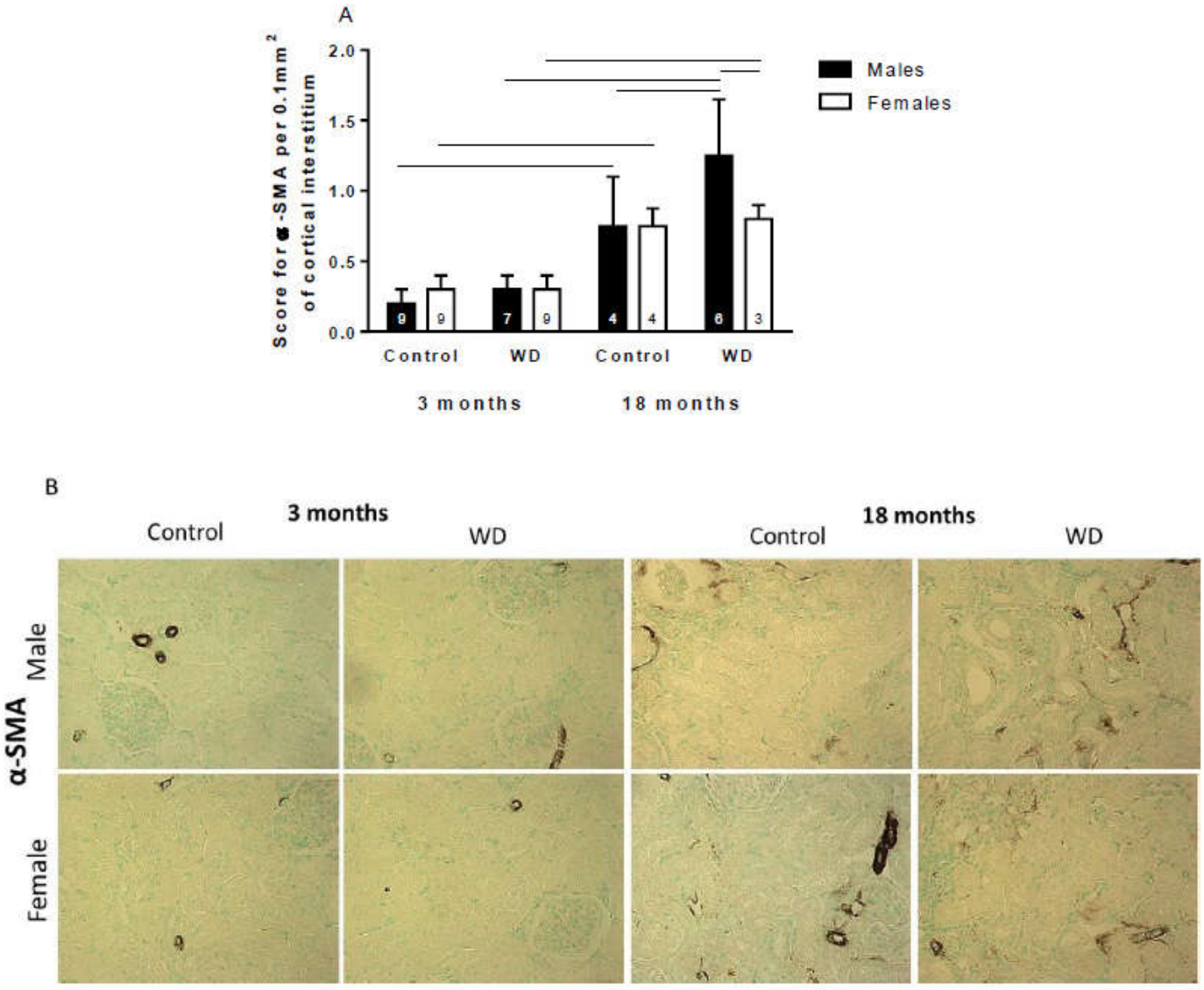

Figure 2. Score (A) and representative images (B) for α-SMA per 0.1 mm^2 of cortical interstitium in control and water deprived (WD) 3- and 18-month-old male and female rats. Data are expressed as median and interquartile range (25th—75th) _____ $p<0.05$ among the indicated groups. Factorial ANOVA followed by Newman-Keuls post-test. Number (n) of animals is indicated inside the column.

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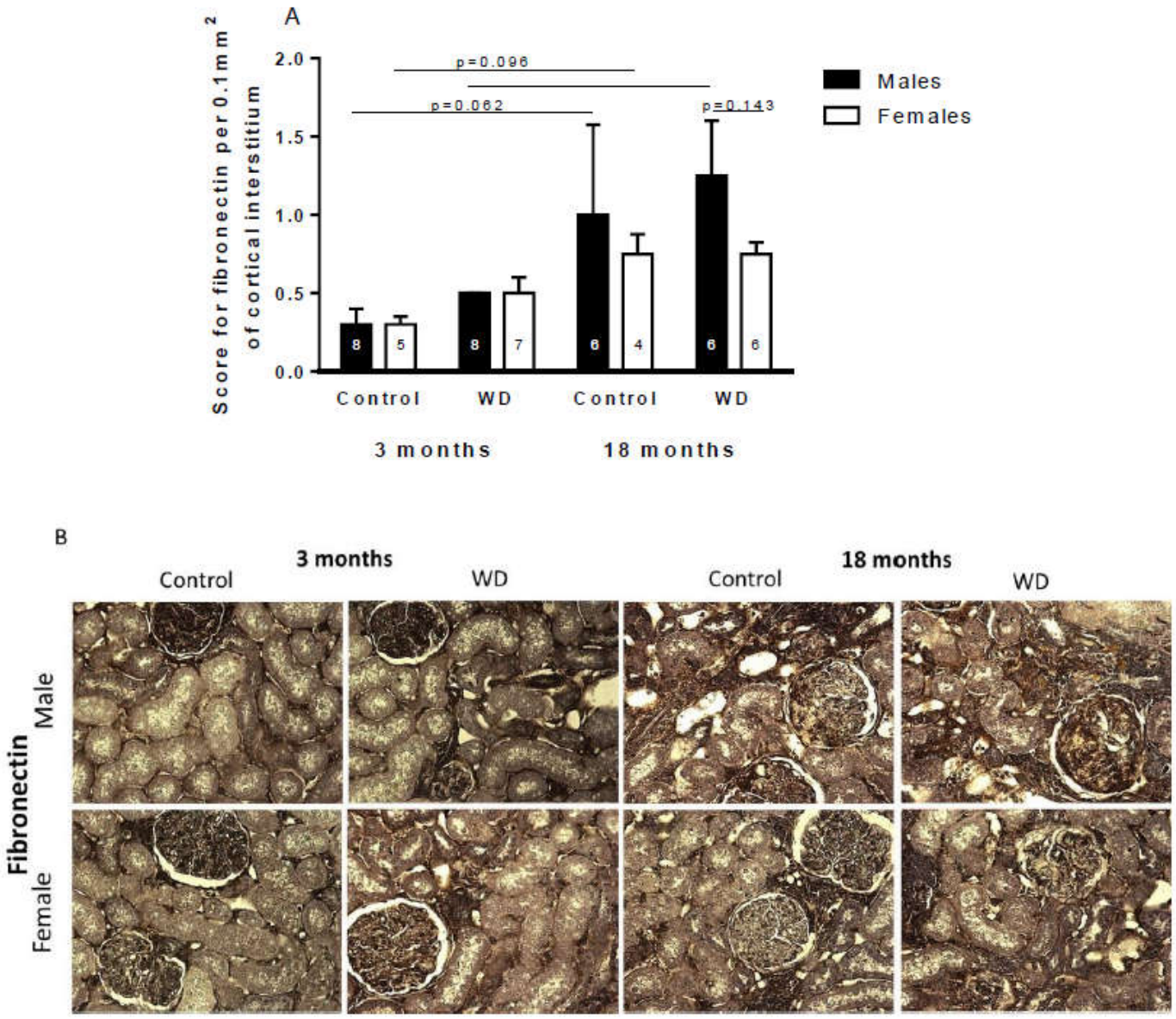

Figure 3. Score (A) and representative images (B) for fibronectin per 0.1 mm² of cortical interstitium in control and water deprived (WD) 3- and 18-month-old male and female rats. Data are expressed as median and interquartile range (25th—75th) _____ $p<0.05$ among the indicated groups. Factorial ANOVA followed by Duncan post-test. Number (n) of animals is indicated inside the column.

3. Discussion

As is already known and we confirm in our results, renal mass declines with aging and the sex also influence the kidney weight (14,15). The increase in renal weight observed in dehydrated 3-month-old animals and 18-month-old males confirms the results reported by Bankir *et al.*, who indicated that weight gain is influenced by urine concentration mechanism, and the outer medulla suffered changes, such as the increased volume of epithelium of thick ascending limb of Henle's loops and collecting duct (16). Despite this, we found that 18-month-old females did not show changes in kidney weight with dehydration, but more research is required to understand the mechanism that explain that interesting finding.

The 3-month-old female rats presented lower relative expression of Slc12a3 mRNA in the renal cortex than 3-month-old males, as expected, since estradiol decreases expression of NCC in the membrane of the distal convoluted tubule (17). The absence of sexual

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difference in relative expression of Slc12a3 mRNA in the renal cortex observed in 18-month-old animals may be because the female rats were in reproductive senescence.

The expression of NCC decreased in the renal cortex of 18-month-old male Wistar rats, possibly because testosterone is decreased in these animals. This hormone increases the plasma levels of fibroblast growth factor-23, which increases the expression of NCC in the kidney (18–20).

Interestingly, water deprivation produced decreased expression of NCC in the renal cortex of dehydrated 18-month-old males, but not any change in the expression of NCC of 3-month-old rats or 18-month-old females. These results may reflect a balance between gonadal hormones and arginine vasopressin, which increases plasma level in dehydration and increases the NCC expression (19,21). Therefore, in 18-month-old male rats, the decrease in testosterone possibly exerts a greater effect on the expression of NCC in relation to the increase in plasma vasopressin.

According our immunohistochemical results, aging is characterized by renal structural changes, such as alterations in the extracellular matrix because of increased fibronectin and α-SMA (22,23). Interestingly and described for the first time, the dehydrated 18-month-old male rats showed higher renal expression of α-SMA than control 18-month-old male rats. Similar results were observed in the lizard *Uromastix acanthinura*, with the expression of α-SMA surrounding the collecting duct, indicating that this renal structural characteristic is involved in body water economy and may be considered as an adaptive mechanism to resist dehydration in an arid environment (24).

The sexual dimorphism observed in the immunohistochemical label of α-SMA and fibronectin in the animals submitted to dehydration could be explained by the fact that estrogen stimulates Angiotensin II receptor types, and this receptor is associated with increased nitric oxide production, whose chronic inhibition is related with elevated expressions of fibronectin and α-SMA (25–28).

In conclusion, alterations in the kidney structure confirm the effect of accumulation of injury with aging. Also, dehydration alters the kidney structure of 18-month-old male rats. Furthermore, altered renal structures in 18-month-old animals show sexual dimorphism when the animals were subjected to dehydration. However, sexual differences in relative Slc12a3 mRNA expression observed in 3-month-old rats disappear in 18-month-old rats.

4. Materials and Methods

4.1. Animal model and experimental design

Female and male Wistar rats were acquired from the bioterium located at the University of Sao Paulo, Ribeirao Preto Campus, SP, Brazil. The animals were kept under a controlled temperature of 23 ± 2 °C and exposed to a 12:12 h light-dark cycle (light on: 6 am to 6 pm) with tap water and standard pelleted food (QuimtiaNuvilab®) *ad libitum*. Experimental methods were performed in the morning (8:00–11:00 h). All procedures were approved by the Ethic Committee for Animal Use of the School of Medicine of Ribeirao Preto, University of Sao Paulo (protocol # 014/2014-1) and conducted according to the "Guide for the Care and Use of Laboratory Animals" (NIH; Publication No. 85-23, revised 1996).

The lifespan of the Wistar rat colony in our animal facility is ~ 2 years, which correlated with the human life expectancy would be ~ 80 years. Therefore, we infer that the age of 18 months corresponds to the sixth decade of human life. At 18 months, the female rats are in a permanent diestrus.

4.2. Experimental methods

Male and female Wistar rats at the age of 3 and 18 months were submitted or not to WD for 48h, they had free access to food. After that, the animals were weighted and sacrificed by decapitation, by an experienced technician, quickly and with care not to cause stress.

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4.3. Tissue collection

After euthanasia, both kidneys were collected and cleaned of connective tissue. The left kidneys were weighted and fixed for immunohistochemical analysis, and the right kidneys were used for relative gene expression of mRNA.

4.4. Microdissection, RNA isolation, and semiquantitative real-time PCR

After dissected the right kidney, it was sagittal cut and with the aid of a sterile microdissection needle (5 mm internal diameter), a sample of renal cortex was dissected and placed in sterile tubes, and stored at -70 °C.

Total RNA was extracted using the RNeasy Mini Kit-Qiagen® and treated with DNase using the DNA-free TM kit (Ambion®, now Life Technologies). The RNA purity and concentration were verified in a spectrophotometer (SpectraMax® i3x Multi-Mode Microplate Reader).

The cDNA synthesis was made from 250 ng of RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems®). Reverse transcription was made in a thermocycler (GeneAmp PCR System 9600, Applied Biosystems) at 25 °C for 10 min and at 37 °C for 120 min. After that, the samples were stored at -20 °C.

The QT-PCR was performed in the 7500 QT-PCR System (Applied Biosystems®) using Taqman® assays (Applied Biosystems®). Each sample was run in triplicate. *Rat ACTB* (actin, beta; Rn00667869_m1) was the internal control gene and *Slc12a3* (Rn01531762_m1) was the target gene. The relative expression of target gene was determined based on the threshold cycle (Ct). The results were analyzed conformity with the ΔΔCt method.

4.5. Immunohistochemical studies

Kidneys were fixed for 24 h in methacarn solution (60% v/v methanol, 30% v/v chloroform and 10% v/v acetic acid) followed by washes in 70% v/v alcohol and embedding in paraffin, sectioned into 4 μm slices, deparaffinized, and incubated overnight at 4 °C with the following antibodies: 1/1000 anti-α-SMA (Dako Corporation, Glostrup, Denmark) or 1/500 anti-rat fibronectin (Chemicon International Inc., Temecula, CA, USA). The reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The color reaction was developed with 3,3-diamino-benzidine (Sigma Chemical Company, St. Louis, MO, USA). Counterstaining was performed with methyl green, and after that, the material was dehydrated, and mounted.

For the evaluation of the immunoperoxidase staining for α-SMA and fibronectin, cortical 30-grid field (measuring 0.100 mm^2 each) was semiquantitatively graded, and the mean score per kidney was calculated. The scores mainly reflect changes in the extent rather than in the intensity of staining, and depend on the percentage of grid field showing positive staining: 0=absent or <5% staining; 1, 5—25%; 2, 25—50%; 3, 50—75%; and 4, >75% staining [13].

The analyses in the cortical tubulointerstitium were identified at 40x magnification (Zeiss microscope supplied with a DC 200 Leica digital camera affixed to a contrast enhancement device) using a double-blind method. Counts were performed randomly throughout all fields.

4.6. Statistical analyses

Results are presented as the means (Standard Deviation (SD)). For immunolocalization as median and interquartile range (25^{th}—75^{th}). Data were plotted using GraphPad Prism (GraphPad Software, USA), and analyzed using Statistica (StatSoft, USA) and SPSS software (IBM, USA). The analysis Shapiro-Wilk's W test was carried out to confirm the assumption of the normality of the distribution. The ROUT test, with Q=10%, was used to identify and remove outliers in all data. The statistical significance of the difference between the means of the studied groups was assessed by the t-student for independent groups for relative expression of *Slc12a3* mRNA in the renal cortex, and for the immunohistochemical analysis and kidney and body weight by the analysis of variance (ANOVA)

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factorial followed by the Newman-Keuls or Duncan post-test, where appropriate. The independent variables were age, sex, and water deprivation condition. The significance level of $p<0.05$ (two-tailed) was adopted.

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Conflicts of Interest: None.

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