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Gender-related differences in kidney of rats with chronic renal failure

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Summary. Chronic renal failure is characterized by adaptive mechanisms secondary to the loss of functioning nephrons. Clinical and experimental studies suggest participation of gender-related hormones on renal function and progression of chronic renal failure.

We evaluated the effect of castration on renal alterations in male and female Wistar control rats and after 30 days of chronic renal failure (CRF) induced by 5/6 nephrectomy.

The CRF male group showed higher proteinuria. Glomerular hypertrophy was similar amogroups. Podocyte morphology showed disorders of foot processes and thickening of the basement membrane in the CRF male group. The CRF female group showed fewer alterations compared to males. Castration changed the profile in CRF male animals and the filtration barrier was preserved. CRF males showed the presence of alfa-smooth muscle actin suggesting an early prefibrotic event in this group. After castration this phenomenon was not observed. Noteworthy, in females, castration exacerbated the presence of alfa-smooth muscle actin.

In summary, proteinuria was higher in males and appeared early in the course of CRF, probably contributing to fibrotic events. Data were influenced by gender suggesting that male sex hormones aggravate renal alterations.

Key Words: Alfa-smooth muscle actin, Castration, Chronic renal failure, Gender, Proteinuria

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Introduction

Chronic renal failure (CRF) is characterized by structural and functional responses of remnant nephrons, which ultimately lead to glomerulosclerosis and tubulo-interstitial injury (Remuzzi et al., 2006). The unbalance between synthesis and degradation of extracellular matrix (ECM) components, associated to loss of size and/or charge-selectivity of the glomerular barrier, have been indicated as important mechanisms in the process of mesangial expansion and glomerulosclerosis (Ruiz-Torres et al., 2005).

Alterations in glomerular permselectivity lead to proteinuria (PTN) and abnormally filtered proteins interact with the mesangium and tubular cells promoting progressive glomerular and interstitial injury (Hischberg and Wang, 2005). In this context, myofibroblasts appear to play an important role in the onset of early renal changes. Myofibroblasts are fibrogenic cells, which have similar characteristics to smooth muscle cells and fibroblasts, and are characterized by expressing alfa-smooth actin (alfa-SMA). Studies have shown that mesangial and interstitial cells can transdifferentiate into myofibroblasts in a step that precedes glomerulosclerosis (Klien et al., 1996; Zhang et al., 2005). The proliferation of myofibroblasts correlates significantly with glomerular and interstitial lesions, thereby perpetuating the disease, and is an excellent marker of renal disease progression (Ng et al., 1998; Yang et al., 1998). The correlations between glomerular and interstitial abnormalities are related to pathophysiological processes in the progression of CRF (Petrica et al., 2000).

Although the mechanisms of glomerular filtration are not completely elucidated, it is clear that podocytes

are an important component in this process. Podocytes stabilize glomerular architecture and the contractile structure of the pedicels is sensitive to vasoactive hormones, and therefore able to modulate the ultrafiltration coefficient (Miner, 2003; Shakland, 2006). Changes in podocyte morphology, including retraction of the pedicels, and detachment of podocytes have been implicated in early stages of the process of PTN and glomerulosclerosis (Pavenstãdt, 2000; Ichikawa et al., 2005). Injury that initially reached only the podocytes appears to spread to other resident cells by still unknown mechanisms (Hayden et al., 2005). Thus, in a parallel step to remodeling, abnormally filtered proteins interact adversely with the mesangium and cells lining the tubular space, causing interstitial changes similar to those observed in the glomerulus (Remuzzi et al., 2006). Nephrotoxicity caused by the accumulation of macromolecules, mainly in the mesangium, may trigger a local inflammatory process, leading to cell proliferation, changes in quantitative or qualitative components of the mesangial matrix (MM), glomerulosclerosis, and finally loss of glomerular filtration (Ruggenenti and Remuzzi, 2000). Actually, more than a marker of glomerular injury, PTN can be considered as an indicator of the severity of renal disease.

Several studies suggest that the impact of gender on renal disease progression reflects genetically determined differences between genders in renal structure and function, as well as direct effects of sex hormones on mesangial cells. Sex hormones probably interfere in cellular proliferation, matrix accumulation, synthesis and release of cytokines, vasoactive agents and growth factor actions (Silbiger and Neugarten, 1995; Ji et al., 2005). Progression of renal disease is faster in males, and some authors suggest that estrogens may contribute to the protective effect of the female gender in renal disease progression (Lemos et al., 2005; Sandberg, 2008; Silbiger and Neugarten, 2008). The present study evaluated early gender-related differences on chronic renal failure, studying castrated and non-castrated subtotally nephrectomized animals.

Material and methods

Experimental model

Randomly, male (M) and female (F) Wistar rats at 5 weeks old were divided into castrated (c) and non castrated groups, after castration, animals were separated into 4 groups: Castrated and non castrated control (C) groups (CMc, n=7; CFc, n=7; C: CM, n=7; CF, n= 7). At seven weeks old, animals randomly underwent 5/6 nephrectomy (CRF) and were separated into those with CRF with castration and without castration: CRFMc, n=10; CRFFc n=8; CRFM, n=8; CRFF, n=7. Surgical procedures were performed with animals anesthetized intraperitoneally with sodium thiopental (50 mg/Kg body weight). In male rats, castration was performed through the removal of the testicles; in female, the ovaries were removed through bilateral lumbar incision. Reduction of total renal mass was performed through abdominal incision, and the left renal artery was isolated and 2 or 3 branches were ligated; right nephrectomy was then performed through lumbar incision (Olson et al., 1982; Bregman et al., 1993). Nephrectomy 5/6 was performed on day 0, and the animals were maintained on a standard rat chow and water *ad libitum* until the end of the study (30 days). The animals received proper care according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication no 85-23, revised 1996). All experimental procedures were previously approved by the Ethics and Research Committee for the Care and Use of Experimental Animals of the Institute of Biology of State University of Rio de Janeiro (UERJ, Rio de Janeiro/RJ, Brazil).

General data

The general data were verified after 30 days post-5/6 nephrectomy. The systolic blood pressure (SBP) was measured in conscious rats, using the non-invasive method of the tail-cuff plethysmography (RTBP 1007, kent Scientific CO, Litchfield, USA). This procedure was performed in a quiet room on trained unasnesthetized animals. Measurement of creatinine and proteinuria (PTN) were performed in 24-hour urine samples, collected from metabolic cages (Nalgene), where animals were previously trained and received only water *ad libitum*. Urine volumes were measured, and urine stored at -20°C. PTN was measured by sulfossalicylic method (Schwartz and Bidani, 1991). After the collection of 24-hour urine samples, animals were anesthetized and a blood sample was taken from the aorta for creatinine determination. Urinary and serum creatinine were determined by kinetic modified Jaffé method in the Central Laboratory of Pedro Ernesto Hospital. Renal function was determined by creatinine clearance corrected by 100g of body weight (CreCl/100g) (Lombet et al., 1989). Kidneys were perfused with 4% ρ-formaldehyde in phosphate buffer, pH 7.4, were removed, weighed, fixed in formalin and embedded in paraffin for subsequent morphologic evaluation. Kidneys from 2 animals per group were perfused with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2 for transmission electron microscopy analysis.

Morphologic data

Morphological evaluations were made in Periodic Acid-Schiff (PAS) and Masson's trichrome stained sections. Glomerular volume (GV) was calculated from the formula $4/3\pi r^3$ (Weibel et al., 1966); the maximum and minimum individual glomerular diameters between

the internal edges of Bowman's capsule were measured and GV was estimated as the mean of 10 measurements.

Immunohistochemistry

Kidney tissue sections of 3-4 μm were evaluated for alfa-smooth muscle actin (alfa-SMA) tissue distribution. Antigen retrieval was performed with citrate buffer, pH 6.0, endogenous peroxidase was quenched with Methanol/hydrogen peroxide 10% and nonspecific binding was inhibited with phosphate-buffered saline/bovine serum albumin 3%. Sections were incubated with anti-alfa-SMA antibody (1:100; Dako, Denmark). The reaction was amplified with a biotin-streptavidin system (Universal DakoCytomation LSAB + kit, Peroxidade, Glostrup, Denmark). The reaction was visualized after incubation with 3,3' Diaminobenzidine tetrachloride (DakoCytomation) and sections were counterstained with Mayer's hematoxylin.

Transmission electron microscopy

Small pieces (~1 mm^3) of the kidney were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH7.2) and 0.25% tannic acid (Merck) for 4 hours and post-fixed with 1% osmium tetroxide (Sigma-Aldrich) and 0.8% ferrocyanide in 0.1 M cacodylate buffer (pH7.2). After, the specimens were dehydrated in ascending grades of alcohol and embedded in Epon. Later, semithin (1 μm) and ultrathin (100 nm) sections were cut in an ultramicrotome (Leica Ultracut-UCT, Viena), collected on copper grids, and contrasted with uranyl acetate and lead citrate. Ultrastructural analyses were performed with a transmission electron microscope (Zeiss EM 906; Carl Zeiss, OberKöchen, Germany) at 80 kV.

Statistical analysis

Data are expressed as mean ± standard error. The differences between groups were tested by one-way analysis of variance (ANOVA) and Duncan *post-hoc* test was used to compare the groups. Significance was assigned as $p<0.05$ (SPSS version 13.0, IBM, USA).

Results

All animals started the study at the same age (7 weeks). Body weight was 30% lower in females compared to male rats. Systolic blood pressure (SBP) was similar in control animals. Castrated and non-castrated CRF groups showed higher levels for SBP compared to controls ($p<0.05$). Castration reduced SBP in CRF males, but not in CRF females. Kidney weight (KW) corrected for 100g of body weight (BW) showed hypertrophy of the remaining renal tissue in CRF groups. Glomerular volume (GV) was not different among groups, although CRF groups showed a tendency to increase this parameter when compared to their controls. Creatinine clearance (CreCl) as expected was reduced in CRF groups (Table 1).

Fig. 1 shows that proteinuria (PTN) (mg/24h) was significantly higher in CRF groups compared to controls (CRFM=112±16 vs CM=39±3.6; CRFF=56.6±13.6 vs CF=9.6±1.8 and CFc=8.7±0.9 mg/24 h, $p<0.05$). Castration reduced PTN in control and CRF males

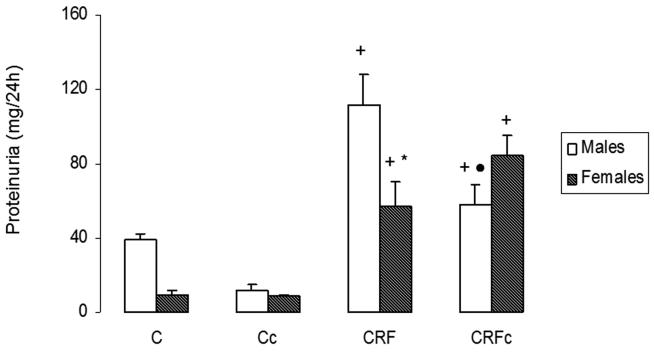

Fig. 1. Proteinuria after 30 days. Mean±SE; $p<0.05$: + vs respective controls; * vs CRFM; • vs CMc. C, control; c, castration; CRF, chronic renal failure.

Table 1. Data of systolic blood pressure, kidney weight, glomerular volume and creatinine clearance after 30 days.

GROUPS (n)	SBP (mmHg)	KW/BW (mg/100g BW)	GV (x 10^5 μm^3)	CreCl (mL/min/100g BW)
CM (7)	[a]119±2.2	0.35±0.01	8.9±0.9	0.51±0.04
CMc (7)	115±1.1	0.42±0.04	6.7±0.4	0.41±0.05
CRFM (8)	155±0.7	0.53±0.10[#]	10.0±1.8	0.26±0.06[#]
CRFMc (10)	147±3.2+*◊	0.47±0.01	10.7±0.9	0.30±0.03[#]
CF (7)	114±2.3	0.40±0.05	5.2±0.6	0.55±0.04
CFc (7)	116±2.2	0.41±0.04	5.3±0.5	0.41±0.05
CRFF (7)	151±1.2+[$]	0.59±0.02+◊	8.0±1.5	0.34±0.05+
CRFFc (8)	155±0.8+[$]	0.54±0.10	8.9±1.0	0.27±0.07+

[a]Mean±SE; $p<0.05$: [#] vs CM; ◊ vc CRFM; * vs CRFFc; + vs control M and F simultaneously; [$] vs CF and CFc. CRF, chronic renal failure; SBP, systolic blood pressure; KW/BW, kidney weight by 100g of body weight; GV, glomerular volume; CreCl, creatinine clearance.

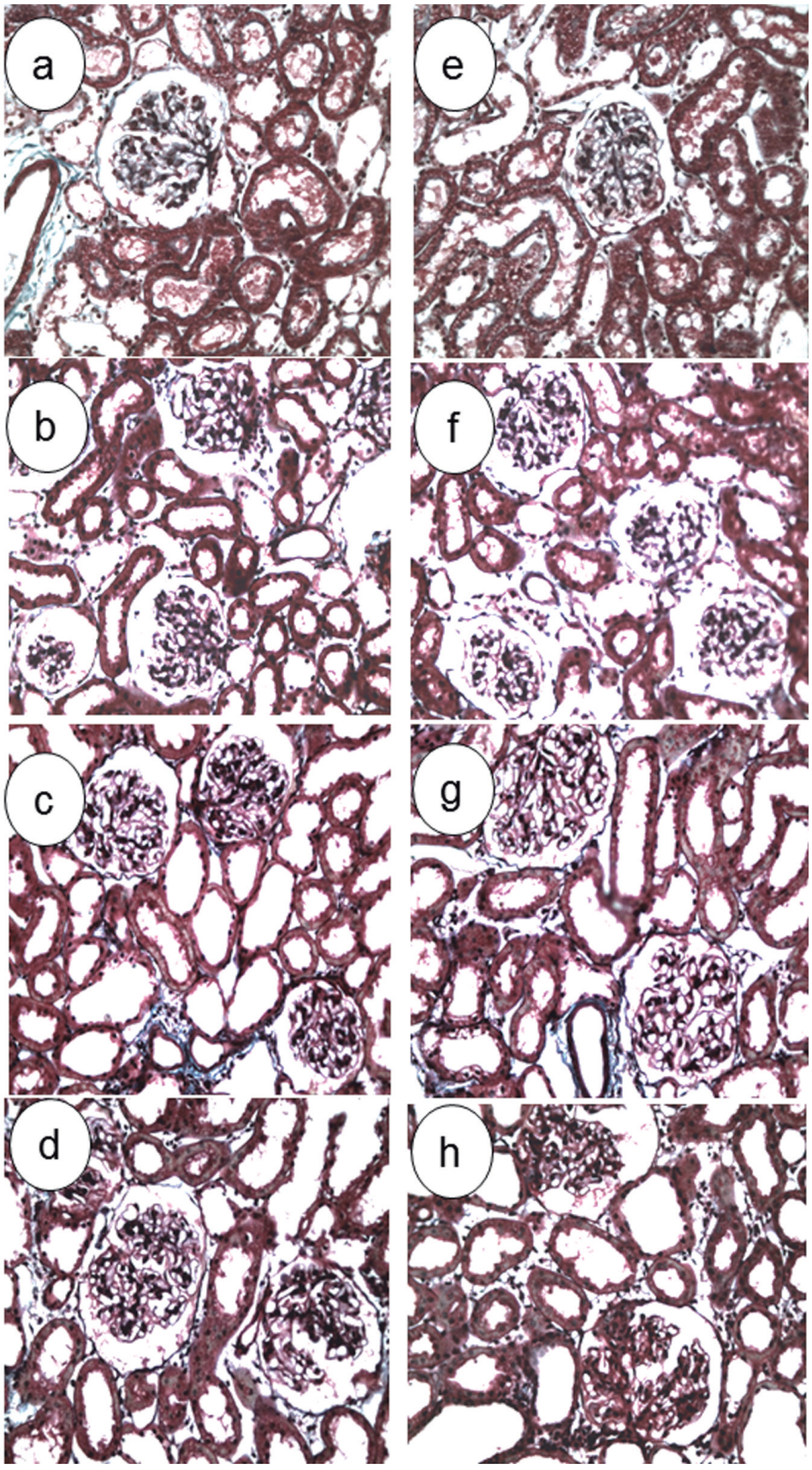

Fig. 2. Photomicrographs of Masson`s trichome stained sections: CM **(a)**, CMc **(b)**, CRFM **(c),** CRFMc **(d),** CF **(e),** CFc **(f)**, CRFF **(g),** CRFFc **(h)**. C, control; c, castration; M, male; F, female; CRF, chronic renal failure. x 200

(CMc=12±2.6 mg/24 h; CRFMc=58.1±10.5 mg/24 h) when compared to non-castrated ($p<0.05$). CRF females did not change PTN after castrations, (CFc=8.7±0.9 mg/24 h; CRFFc=83.9±10.5 mg/24h), although the CRFFc group showed a tendency to increase PTN.

Morphological evaluation did not present glomerular or tubulo-interstitial alterations, and the same was observed in castrated groups (Fig. 2).

Electron microscopy showed a preserved filtration barrier in control groups in both genders, with a normal relationship of the foot process with the endothelial cells and glomerular capillaries (Fig. 3). Pedicels anchored to

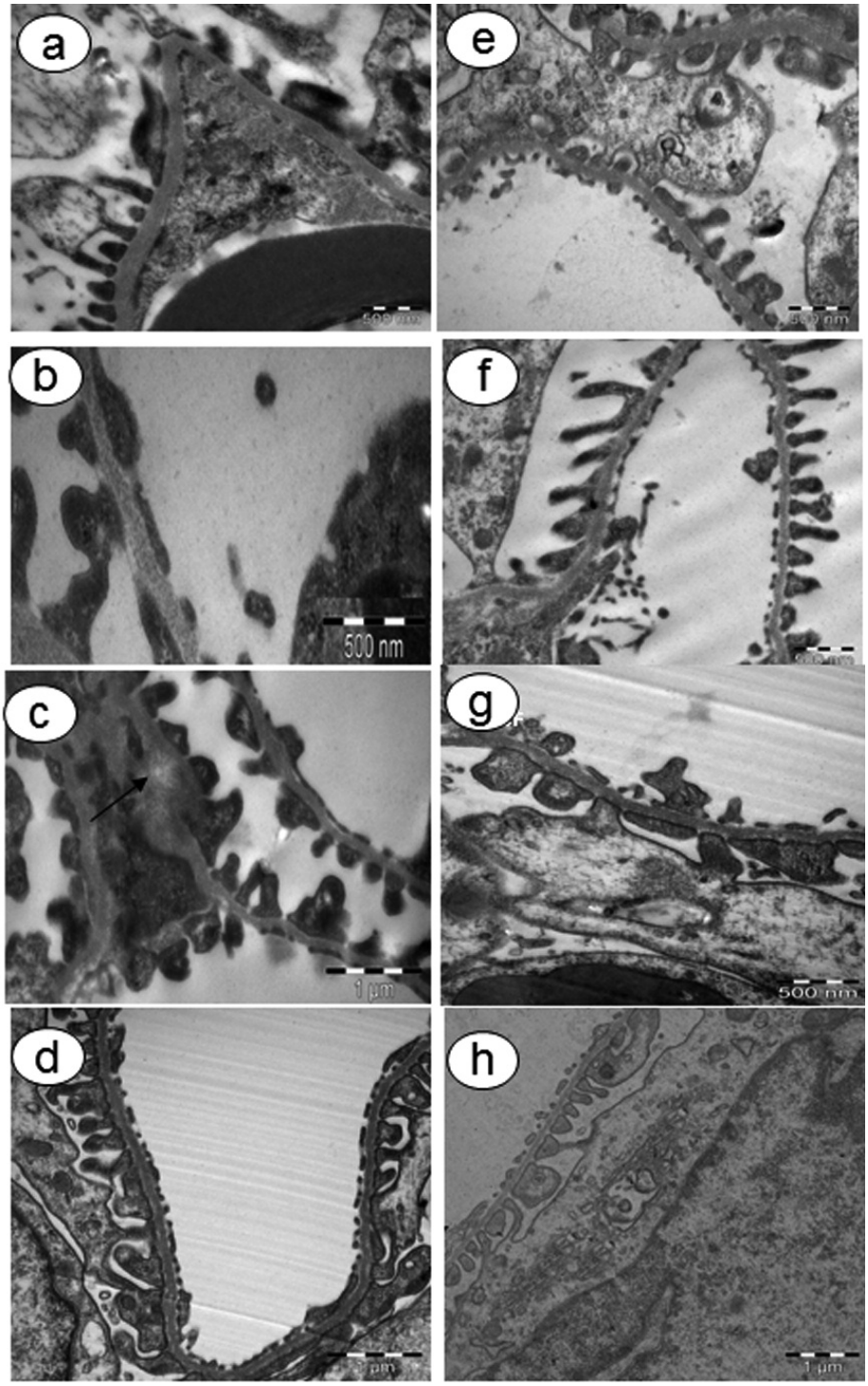

Fig. 3. Electron microscopy image of groups after 30 days: CM (**a**; 27800x), CMc (**b**; 27800x), CRFM (**c**; 21560x), CRFMc (**d**; 21560x), CF (**e**; 27800x), CFc (**f**; 27800x), CRFF (**g**; 27800x), CRFFc (**h**; 16700x). C, control; c, castration; M, male; F, female; CRF, chronic renal failure.

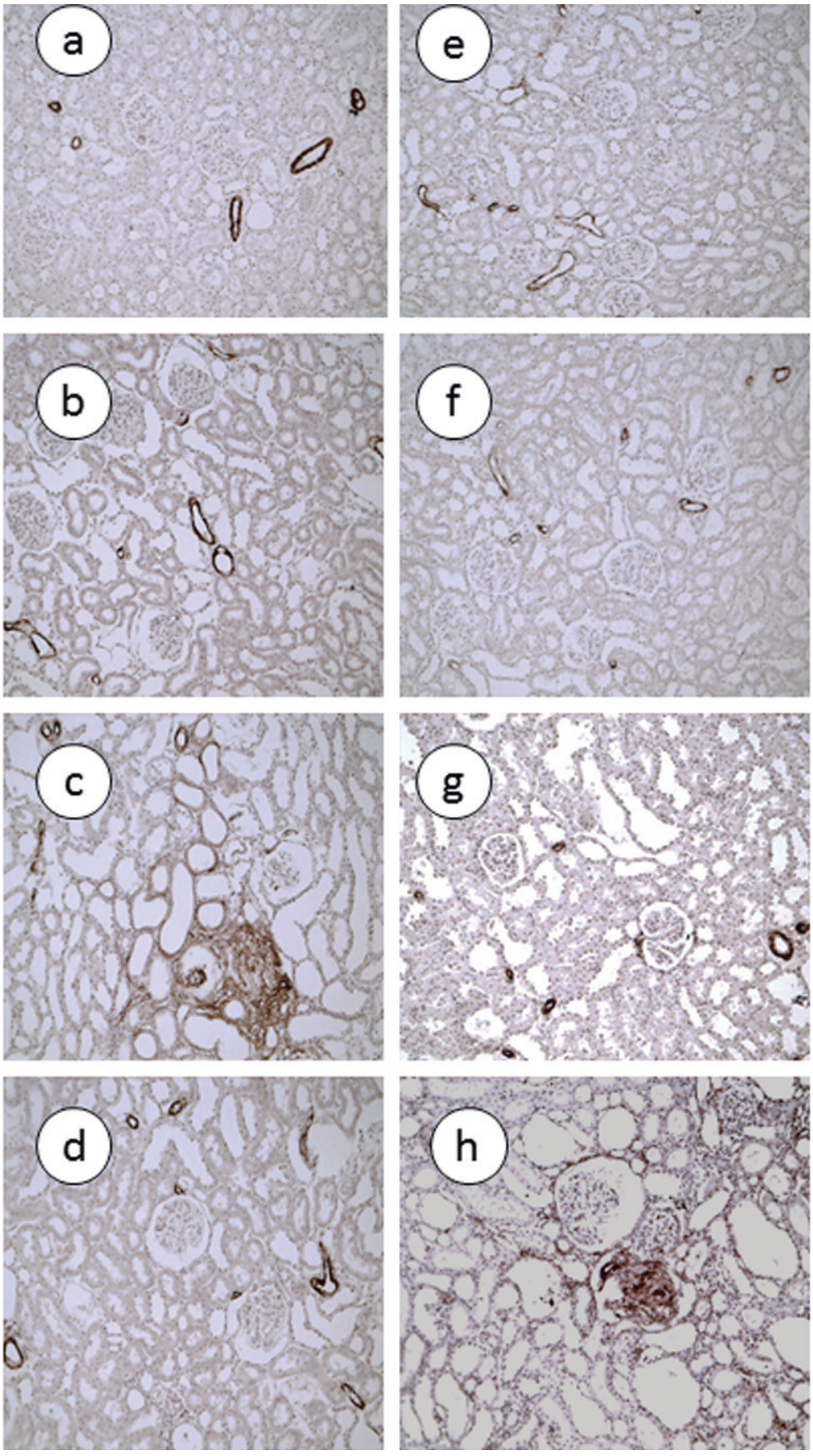

Fig. 4. Photomicrographs of immunohistochemical expression of alfa-smooth muscle actin: CM **(a),** CMc **(b),** CRFM **(c),** CRFMc **(d),** CF **(e),** CFc **(f),** CRFF **(g),** CRFFc **(h)**. C, control; c, castration; M, male; F, female; CRF, chronic renal failure. x 200

the basement membrane of glomerular capillary, with regular spacing between them and containing preserved "slit" diaphragm. The CRFM group showed a thickening of the basement membrane (arrow), while in CRFF glomerular filtration barrier was preserved, but presented a slight shortening of podocyte foot processes. Castration changed the profile in CRFM animals and the filtration barrier was better preserved.

Immunostaining for alfa-SMA in control groups (castrated or not) was present only in blood vessels (Fig. 4). CRF groups presented gender differences: CRFM showed immunostaining of tubular basement membranes, periglomerular region, Bowman's space and mesangial matrix, while in the CRFF group the presence of alfa-SMA was limited to blood vessels, similar to control groups. After castration, gender differences were also observed, as males did not show alterations while in females these were more prominent.

Discussion

It is suggested that gender differences exist with regard to the evolution of chronic kidney disease (CKD). On the other hand, the majority of CKD occurs in a population of postmenopausal women. However, there is some guidance that cellular differences exist regarding gender. The scarcity of information about the role of sex hormones after menopause and sex-specific tissue responses make this issue unclear.

The present study evaluated renal alterations in castrated and non castrated CRF male and female rats, in order to assess gender dependent renal changes. Castration was performed immediately prior to the onset of puberty to avoid the interference of sex hormones on renal function.

Compensatory hypertrophy of the remaining renal tissue is a well known phenomenon. Animals submitted to unilateral (Baylis and Wilson, 1989) and subtotal (Lemos et al., 2005) nephrectomy showed higher kidney weight compared to intact animals. We found renal hypertrophy 30 days after 5/6 nephrectomy in both genders, suggesting that renal hypertrophy is likely to occur early in the remnant kidney model independently of gender.

Blood pressure plays a role in the progression of renal failure (Hannedouche et al., 1993). Spontaneously hypertensive rats (SHR) exhibit gender differences in SBP, where males present higher levels compared to females (Lopes-Ruiz et al., 2008), while other studies showed that castration reduces SPB in SHR animals (Sartori-Valinotti et al., 2007; Toot et al., 2008). Our data showed similar SBP in CRF animals, except in the CRFMc group that presented a slight decrease in this parameter compared to CRFM.

PTN is considered by many as a major risk factor for progression of CRF (Strutz, 2009; Gagliardini et al., 2010) and gender differences are also implicated. One study shows that in men urinary albumin excretion was the strongest independent predictor of renal function decline (Halbesma et al., 2008). However, another study showed that differences in renal disease progression were no longer significant, after adjusting for baseline PTN and mean arterial pressure comparing males and females (Coggins et al., 1998).

We found a higher PTN in the CRFM group compared to its control and to CRFF. This finding is in accordance with Lombet et al. (1989), who found higher PTN in male rats 3 weeks after 5/6 nephrectomy compared to females. Castration of male rats was associated to PTN reduction in hyperlipidemic rats (Sakemi and Baba, 1993). Some experimental studies associate testosterone with the faster male progression of chronic renal failure (Lu et al., 2006; Elliot et al., 2007; Metcalfe et al., 2008). In the present study, after castration, PTN was reduced in CRF males while it increased in females. These data suggest gender participation in PTN.

PTN is also associated to changes in podocyte morphology, which in turn are secondary to changes in the expression of specific proteins such as nephrin, alfa-actin-4 and podocin (Tryggvason and Wartiovaara, 2001; Koop et al., 2008). These changes trigger abnormal interactions between podocytes and the glomerular basement membrane (GBM) due to actin cytoskeleton reorganization, especially in pedicels (Shakland, 2006). These alterations lead to foot process effacement, loss of the podocyte slit and, finally, total denudation of the glomerular capillary wall (Lahdenkari et al., 2004). Ultrastructural analysis showed morphological alterations in the glomerular filtration barrier of CRF groups. These alterations did not change with castration, suggesting therefore that sexual hormones did not participate in these processes early in the course of CRF.

Histopathological data showed neither glomerular nor tubulo-interstitial alteration, nor glomerular hypertrophy, in CRF groups after 30 days. The same was observed by Badid et al. (2000) after 4 weeks; and significant morphological alterations were present only after 8 weeks. However, Tapia et al. (2003) showed glomerular hypertrophy after 30 days. Schwartz and Bidani (1991) studying Wistar-Kyoto rats six weeks after 5/6 nephrectomy, observed compensatory renal hypertrophy, although it was not followed by injury. Castrated hyperlipidemic male rats presented glomerular hypertrophy after 24 weeks (Sakemi and Baba, 1993). These apparent discrepancies are likely to be due to strain differences. Thus, it seems that 30 days after nephrectomy, in Wistar rats, is a short period of time to detect significant histopathological alterations in both genders.

In normal kidney, interstitial fibroblasts are rare and myofibroblasts are not present (Hischberg and Wang, 2005). Myofibroblasts originating from the trans differentiation of the resident renal cells, which express alfa-SMA, are implied in the early process of CKD injury; some data corroborate the theory that the presence of myofibroblasts precedes glomerular and tubulointerstitial injury (Ng et al., 1998; Yang et al.,

1998; Strutz, 2009). After 30 days of 5/6 nephrectomy, CRF males showed the presence of alfa-SMA, both in periglomerular and tubulointerstitial regions, suggesting an early prefibrotic event in these animals.

It has been suggested that the impact of gender on renal disease progression may reflect direct receptor-mediated effects of sex hormones (Kwan et al., 1996), since estradiol and testosterone receptors are expressed on mesangial cells (Neugarten and Silbiger, 1995; Pawluczyk et al., 2009). The faster kidney function decline in males has been attributed to the specific proapoptotic and profibrotic properties of androgens (Elliot et al., 2007; Metcalfe et al., 2008). Besides, female sex hormones reduced inflammation and oxidative stress in kidney of SHR (Sullivan et al., 2007). Considering these hormones as protective for females, this could explain the absence of alfa-SMA in the female CRF group despite the increased PTN.

In summary, PTN was an early finding and was associated to the presence of alfa-SMA in males, while castration inhibited these alterations. Conversely, castrated females presented an increase of alfa-SMA. Although available data still do not allow a definitive conclusion, higher PTN, probably contributing to fibrotic events, seems to be influenced by gender, suggesting a link of renal alterations with male sex hormones.

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