Hypertension Research (2019) 42:779–789
https://doi.org/10.1038/s41440-019-0226-6

ARTICLE

Pathogenic role of angiotensin II and the NF-κB system in a model of malignant hypertensive nephrosclerosis

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Received: 12 July 2018 / Revised: 24 September 2018 / Accepted: 22 October 2018 / Published online: 26 February 2019

Abstract

We previously reported that rats treated with an NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), during lactation developed hypertension in adult life, without apparent functional or structural damage to kidneys, providing a new model of essential hypertension. Here, we investigated whether uninephrectomy associated with salt overload would unveil a latent renal dysfunction in this model, aggravating arterial hypertension and promoting renal injury. Male Munich-Wistar rat pups received PDTC from maternal milk ($PDTC_{Lact}$) from 0 to 20 days after birth. Another group received no treatment during lactation. All offspring underwent uninephrectomy (UNx) at 10 weeks of age and then were subdivided into NS, receiving a normal salt (0.5% Na^+) diet, $PDTC_{Lact}$ + NS, HS, receiving a high-salt diet (2% Na^+ chow + 0.5% saline to drink), and $PDTC_{Lact}$+HS. Twelve weeks later, HS rats were moderately hypertensive with mild albuminuria and renal injury. In contrast, severe hypertension, glomerulosclerosis, and cortical collagen deposition were prominent in $PDTC_{Lact}$ + HS animals, along with "onion-skin" arteriolar lesions, evidence of oxidative stress and intense renal infiltration by macrophages, and lymphocytes and angiotensin II-positive cells, contrasting with low circulating renin. The NF-κB pathway was also activated. In a separate set of $PDTC_{Lact}$+HS rats, Losartan treatment prevented NF-κB activation and strongly attenuated glomerular injury, cortical fibrosis, and renal inflammation. NF-κB activity during late nephrogenesis is essential for the kidneys to properly maintain sodium homeostasis in adult life. Paradoxically, this same system contributed to renal injury resembling that caused by malignant hypertension when renal dysfunction caused by its inhibition during lactation was unmasked by uninephrectomy associated with HS.

Keywords NF-kB system · nephrogenesis · renal injury · chronic kidney disease · malignant hypertension

Introduction

Hypertensive nephrosclerosis is the second leading cause of chronic kidney disease (CKD) and end-stage renal failure throughout the world. The mechanisms by which hypertension adversely affects kidneys remain incompletely understood. In most hypertensive patients, autoregulation prevents transmission of elevated blood pressure to glomerular microcirculation, thus preventing renal damage. Incomplete operation of these autoregulatory mechanisms can lead to nephron deterioration and loss of renal function, a process that proceeds faster and leads to malignant nephrosclerosis when autoregulation failure is more severe [1, 2].

Renal inflammation is a crucial component of either benign or malignant hypertension, exerting a dual role. On the one hand, it can impair the kidney's ability to excrete sodium, leading to hypertension based on Guyton's hypothesis. On the other hand, elevated blood pressure promotes renal infiltration by macrophages and lymphocytes, as described in several experimental models and in patients with hypertensive nephrosclerosis [3, 4]. Thus, a vicious cycle can be established, perpetuating both hypertension and renal damage.

Supplementary information The online version of this article (https://doi.org/10.1038/s41440-019-0226-6) contains supplementary material, which is available to authorized users.

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Several inflammatory pathways can mediate inflammation in chronic kidney disease. We showed previously that the NF-κB pathway is activated in rats subjected to 5/6 renal ablation [5] and to adenine overload [6] and that its inhibition with pyrrolidine dithiocarbamate (PDTC) exerts a protective effect. Similar findings have been reported by others [7, 8].

Despite its proinflammatory action, NF-κB may play an important role during nephrogenesis. We recently described a new murine model of hypertension by inhibition of the NF-κB system with PDTC during lactation [9], when rat nephrogenesis is completed. These animals become moderately hypertensive in adult life without reduction in nephron number or any sign of renal inflammation or structural injury until at least 10 months of age, indicating adequate functioning of protective autoregulatory mechanisms. These characteristics make this rat model a suitable model of essential hypertension.

In the present study, we sought to determine whether renal damage could be induced in this model by reducing renal mass in the face of increased salt intake. In this manner, we were able to unveil a latent hemodynamic derangement that predisposes these rats to renal NF-κB activation and structural damage when their kidneys are exposed to such challenges.

Methods

Experimental protocol

Adult male Munich-Wistar rats, obtained from a local facility, were utilized in this study. All experimental procedures were specifically approved by the local Research Ethics Committee (process no. 369/11) and performed in rigorous compliance with institutional and international standards for the handling and care of laboratory animals.

Pregnant rats were kept in individual polypropylene cages and received a diet containing 0.5% Na^+ and 22% protein (Nuvital, Curitiba, Brazil) and free access to water. From 1 to 21 days after birth, six male pups per litter were kept with their respective mothers. During this time, 75 dams received the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), in drinking water at 280 mg/kg/day, such that the pups received the compound with the breast milk [10]. Another 38 dams received no treatment. At the end of 21 days, PDTC administration was discontinued, and the offspring were maintained with their mothers until weaning (25 days after birth). At 10 weeks of age, the offspring underwent uninephrectomy (UNx) after ventral laparotomy under anesthesia with ketamine (50 mg/kg im) and xylazine (10 mg/kg im). After surgery, the animals were kept in heated cages for 24 h and medicated with tramadol hydrochloride (10 mg/kg).

In Protocol 1, UNx rats were distributed among four groups: NS ($N = 17$), rats receiving a normal salt (NS) diet, $PDTC_{Lact}$ + NS ($N = 18$), rats treated with PDTC during lactation and receiving NS, HS ($N = 21$), rats receiving a high-salt (HS) diet (2% Na^+ chow + 0.5% saline to drink), and $PDTC_{Lact}$ + HS ($N = 21$), rats treated with PDTC during lactation and receiving HS.

After an additional 12 weeks (24 weeks of age), tail-cuff systolic blood pressure (SBP) was determined in awake rats with an optoelectronic automated device (BP 2000 Blood Pressure Analysis System, Visitech Systems, EUA) after preconditioning to the procedure. The animals were subsequently kept for 24 h in metabolic cages for measurement of urinary albumin excretion. In addition, blood samples were collected from awake rats for assessment of plasma renin activity (PRA). Rats were then anesthetized as described earlier. Blood samples were taken from the abdominal aorta for biochemical analysis, and the kidney was retrogradely perfused in situ through the abdominal aorta with saline to wash blood out of the renal vessels. The kidneys of NS ($N = 8$), $PDTC_{Lact}$ + NS ($N = 7$), HS ($N = 7$), and $PDTC_{Lact}$ + HS ($N = 8$) rats were excised and rapidly frozen at −80 °C for later protein extraction. The remaining rats had their kidneys perfusion-fixed with Duboscq-Brazil solution after washout with saline. Subsequently, two midcoronal slices of the kidney were postfixed in buffered 10% formaldehyde solution and embedded in paraffin using conventional sequential techniques. Histomorphometric and immunohistochemical analyses of the renal tissue were performed in 4-mm-thick sections.

In Protocol 2, specifically designed to investigate the role of angiotensin II (AngII) in this setting, 36 UNx rats were divided into $PDTC_{Lact}$ + HS ($N = 18$), treated as described before, and $PDTC_{Lact}$ + HS + L ($N = 18$), which additionally received the AT1 receptor blocker, Losartan, in drinking water at 50 mg/kg for 12 weeks with the HS diet. The remaining procedures were identical to those adopted for Protocol 1.

Biochemical and enzymatic analyses

Plasma sodium and potassium concentrations were determined with an electrolyte analyzer (9140 model AVL Medical Instruments). Urinary albumin was assessed by radial immunodiffusion [11] using rat albumin antibody (MPBiomedical LLC, EUA). Serum and urinary creatinine were determined using a commercially available kit (Labtest Diagnostica, São Paulo, Brazil). Plasma renin activity was determined by a radioimmunoassay kit (GENESE—Pharmaceutical Products and Diagnostics).

Histomorphometric analysis

For assessment of glomerular injury, sections were stained with the periodic acid-Schiff reaction (PAS). The extent of glomerular damage was estimated by the frequency of glomeruli with sclerotic lesions (GS), as described previously [12]. The PAS stain was also used to evaluate the percentage of arteriolar "onion-skin" lesions (% OSL) by examining 30 arteriolar profiles per section under ×400 magnification.

Immunohistochemistry

For immunohistochemical analysis, renal sections were mounted on glass slides coated with 6% silane. The following primary antibodies were used: monoclonal mouse anti-rat ED-1 (Serotec, Oxford, UK) for macrophages, polyclonal rabbit anti-collagen I (Abcam, Cambridge, United Kingdom), anti-CD3 primary antibody (Dako, Carpinteria, USA) for lymphocytes, and anti-AngII polyclonal antibody (Peninsula Laboratories, San Carlos, CA). The details of the immunohistochemical techniques used in this study are given elsewhere [13]. The renal density of macrophage infiltration (cells/mm^2) was evaluated in a blinded manner at ×400 magnification. The percentage of cortical interstitial area occupied by collagen I was estimated by a point-counting technique [14] at ×400 magnification. For each section, 50 microscopic fields (corresponding to a total area of 1.6 mm^2) were examined.

Total protein extraction and isolation of nuclei

Proteins were extracted from renal tissue using lysis buffer (Thermo Scientific, Rockford, USA) with protease and phosphatase inhibitors (Roche, Mannheim, Germany). Protein concentration was determined with the bicinchoninic acid (BCA) method. For the isolation of nuclei, kidney tissues were homogenized in ice with a special glass pestle (Sigma Aldrich, Saint Louis, USA) in lysis buffer and centrifuged at 1000 G for 10 min at 4 °C to obtain a crude nuclear pellet. The supernatant (cytosolic fraction) was discarded. The pellet was reconstituted in high-sucrose Laemmli buffer and centrifuged at 1500 G for 10 min at 4 °C to obtain a purified nuclear supernatant.

Western blot

For Western blot analysis, 100 µg of protein aliquots were mixed with 2x Laemmli loading buffer and denatured at 96 °C for 5 min, except for analysis of the nuclear fraction. Protein fractionation was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to a nitrocellulose membrane and incubation with 5% nonfat milk or 5% BSA in TBS for 1 h at room temperature to block nonspecific binding. The membrane was then incubated overnight at 4 °C with primary antibodies for β-actin (Sigma Aldrich, Saint Louis, USA) at 1:5000, Toll-like receptor 4 (TLR4) (Santa Cruz Biotechnology®) at 1:250, interleukin 6 (IL-6) (Abcam, Cambridge, UK) at 1:500, manganese-dependent superoxide dismutase (MnSOD) (Cayman, Michigan, USA) at 1:10000, heme oxygenase 1 (HO-1) (Abcam, Cambridge, UK) at 1:500, phosphorylated NF-κB p65 fraction (Cell Signaling Technology, Danvers, USA) at 1:100, and histone H2B (Abcam, Cambridge, UK) at 1:1500. After rinsing with Tris-buffered saline Tween 20 (TBST) buffer, membranes were incubated with secondary antibodies labeled with HRP. Immunostained bands were detected using a chemiluminescence kit (Thermo Scientific, Rockford, USA) and were further analyzed by densitometry with a gel documentation system and Uvisoft-UvibandMax software (Uvitec Cambridge, UK).

ELISA

The serum concentration of MCP-1 and the renal content of IL-1β were determined using a commercial ELISA kit (R&D Systems, Minneapolis, MN). All analyses were performed strictly following the manufacturer's instructions.

Statistical analysis

The results are expressed as the means ± SEM. Statistical differences were assessed by one-way ANOVA with Newman–Keuls post hoc tests (Protocol 1) and Student's t tests (Protocol 2). Differences were considered significant at $p < 0.05$. All calculations were performed using GraphPad Prism 4.0, GraphPad Software Inc., San Diego, CA [15]. The significance of linear correlations was assessed using Pearson's correlation coefficient.

Results

UNx and HS aggravated hypertension, renal functional loss, albuminuria, and renal injury in rats that received PDTC during lactation

At 24 weeks of age, only one animal died in the $PDTC_{Lact}$ + HS Group, and body weight (BW) was similar in all groups (NS = 370 ± 6 g, $PDTC_{Lact}$ + NS = 360 ± 8 g, HS = 364 ± 8 g, and $PDTC_{Lact}$ + HS = 357 ± 4 g). Albuminuria was markedly increased in both groups receiving the HS diet

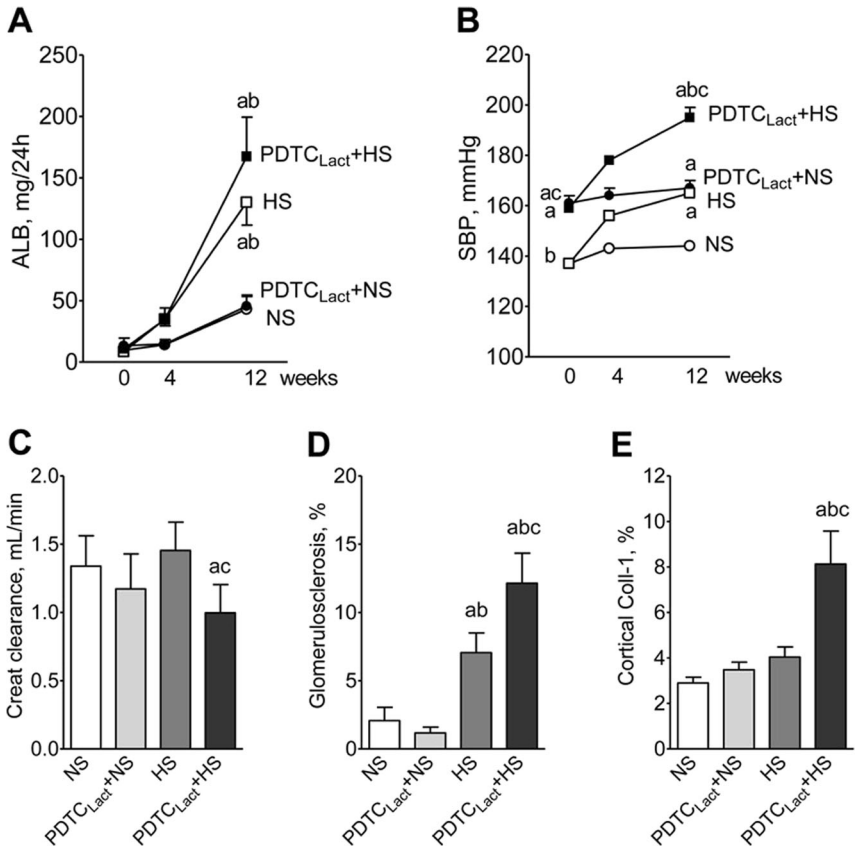

Fig. 1 Albuminuria (**a**) and systolic blood pressure (**b**) measured during HS treatment. After 12 weeks of HS, creatinine clearance (**c**), percentage of glomerulosclerosis (**d**), and cortical fraction occupied by collagen-1 (**e**) in NS, PDTC$_{Lact}$ + NS, HS, and PDTC$_{Lact}$ + HS Groups. The results are expressed as the means ± SE. [a]$p < 0.05$ NS, [b]$p < 0.05$ PDTC$_{Lact}$ + NS, [c]$p < 0.05$ HS

(Fig. 1a) compared with the groups that received the NS diet (NS and PDTC$_{Lact}$ + NS). In the PDTC$_{Lact}$ + NS Group, SBP was higher than in the NS Group. The association of UNx and HS diet further aggravated hypertension in the PDTC$_{Lact}$ + HS Group (Fig. 1b).

Creatinine clearance was similar among groups receiving NS but was decreased in the PDTC$_{Lact}$ + HS Group compared to the HS Group (Fig. 1c).

The association of UNx and HS promoted glomerulosclerosis (GS) in both the HS and PDTC$_{Lact}$ + HS Groups, but GS was more extensive in the latter group (Fig. 1d). Of note, the percentage of GS correlated positively with SBP in the PDTC$_{Lact}$ + HS Group (Supplementary Fig. 1A), stressing the pathogenic importance of hemodynamic factors. Other types of glomerular lesions, such as microaneurysms, microthrombosis, and glomerular atrophy, were observed exclusively in the PDTC$_{Lact}$ + HS Group (Supplementary Fig. 2).

Cortical collagen-1 deposition was similarly low in the NS and PDTC$_{Lact}$ + NS Groups and was not aggravated when UNx rats received HS. However, cortical collagen-1 deposition was markedly increased in the PDTC$_{Lact}$ + HS Group, indicating the establishment of renal fibrosis (Fig. 1e). The representative microphotographs of renal injury are shown in Fig. 2.

UNx and HS led to marked renal inflammation in PDTC$_{Lact}$ rats

The serum levels of MCP-1 (Fig. 3a), a key cytokine for the recruitment of macrophages, were markedly elevated in PDTC$_{Lact}$ + HS rats in association with intense renal interstitial infiltration by macrophages (Fig. 3b) and lymphocytes (Fig. 3c). A positive correlation between macrophage infiltration and SBP in the PDTC$_{Lact}$ + NS Group was also observed (Supplementary Fig. 1B). Although the HS diet drastically reduced PRA (Fig. 3d), AngII-positive cells were prominent in the renal interstitium in PDTC$_{Lact}$ + HS rats (Fig. 3e). The representative microphotographs of renal inflammation are shown in Fig. 2.

UNx and HS promoted severe arteriolar lesions ("onion-skin" arteriolar lesions) and inflammation in animals that received PDTC during lactation

Severe arteriolar lesions, with myointimal cell proliferation and fibrinoid necrosis, which are associated with severe hypertension, were seen in the PDTC$_{Lact}$ + HS Group only. These "onion-skin" arteriolar lesions were associated with intense infiltration of the arteriolar wall by macrophages and lymphocytes (Fig. 4). Although HS promotes an increase in

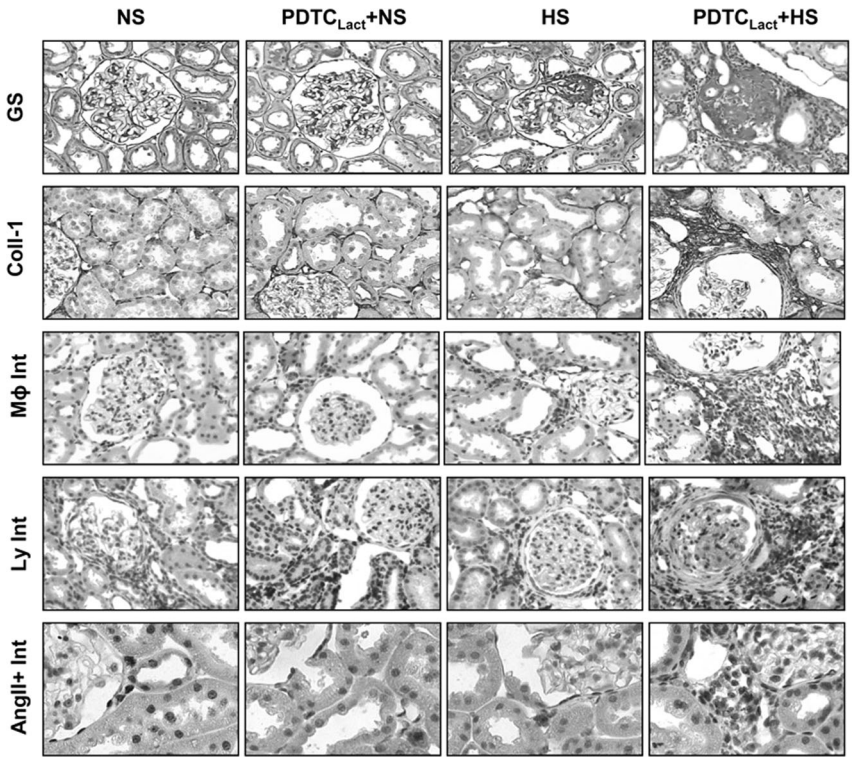

Fig. 2 Representative microphotographs of glomerulosclerosis (GS, 200×), PAS staining, and (in immunohistochemically stained sections) collagen-1 deposition (Coll-1, 200×), interstitial infiltration by macrophages (Mϕ, 200×), lymphocytes (Ly, 200×), and angiotensin II-positive cells (AngII, 400×) in NS, PDTC$_{Lact}$ + NS, HS, and PDTC$_{Lact}$ + HS Groups

albuminuria, only the animals in the PDTC + HS Group showed a positive correlation with OSL (Supplementary Fig. 1C).

UNx and HS treatment activated the TLR4/NF-κB pathway, increased the production of IL-6, and promoted oxidative stress in the PDTC$_{Lact}$ + HS Group only

The PDTC$_{Lact}$ + HS Group exhibited increased expression of TLR4 (Fig. 5a). Accordingly, a marked increase in the nuclear content of the p65 component of the NF-κB system was seen in this group (Fig. 5b), indicating the activation of this system. Consistently, these rats exhibited increased renal abundance of IL-6, one of the main final products of this pathway (Fig. 5c). The renal content of IL-1β was comparable between NS and PDTC$_{Lact}$ + NS Groups (1.75 ± 0.31 and 2.19 ± 0.43 pg/mg, respectively, $p > 0.05$). Both HS (3.23 ± 0.63 pg/mg) and PDTC$_{Lact}$ + HS (3.34 ± 0.57 pg/mg) Groups exhibited a slight numerical increase in the renal content of IL-1β ($p > 0.05$).

In the PDTC$_{Lact}$ + HS rats, renal HO-1 content was increased (Fig. 5d), suggesting the need for protection against cytotoxicity caused by reactive oxygen species. In agreement with these findings, the reduction in renal MnSOD in the PDTC$_{Lact}$ + HS animals suggests mitochondrial dysfunction and deficiency in the removal of reactive oxygen species (Fig. 5e). Together, these findings indicate that in this group, the renal tissue was exposed to severe oxidative stress.

Losartan attenuated hypertension, renal injury, and inflammation, and prevented loss of renal function in PDTC$_{Lact}$ + HS rats

Body growth was better preserved in animals that received Losartan (PDTC$_{Lact}$ + HS = 355 ± 6 g and PDTC$_{Lact}$ + HS + L = 367 ± 9 g). Losartan treatment strongly attenuated hypertension (Fig. 6a) and albuminuria (Fig. 6b) and kept the creatinine clearance at normal levels (Fig. 6c). In addition, Losartan significantly lowered the levels of GS (Fig. 6d) and collagen-1 deposition (Fig. 6e) and promoted a numerical decrease in the frequency of severe arteriolar lesions (Fig. 6f). Accordingly, Losartan treatment reduced the serum levels of MCP-1 (Fig. 7a) and reduced to control levels of the intensity of renal infiltration by macrophages (Fig. 7b) and lymphocytes (Fig. 7c). Although Losartan treatment slightly increased PRA (Fig. 7d), the expression of AngII-positive cells in renal tissue was markedly reduced (Fig. 7e). The representative microphotographs of renal injury and inflammation in all groups are shown in Fig. S3.

Losartan treatment prevented TLR4/NF-κB pathway activation and attenuated oxidative stress

As expected, AT1R inhibition decreased p65 nuclear translocation (Fig. 8b) and IL-6 generation (Fig. 8c),

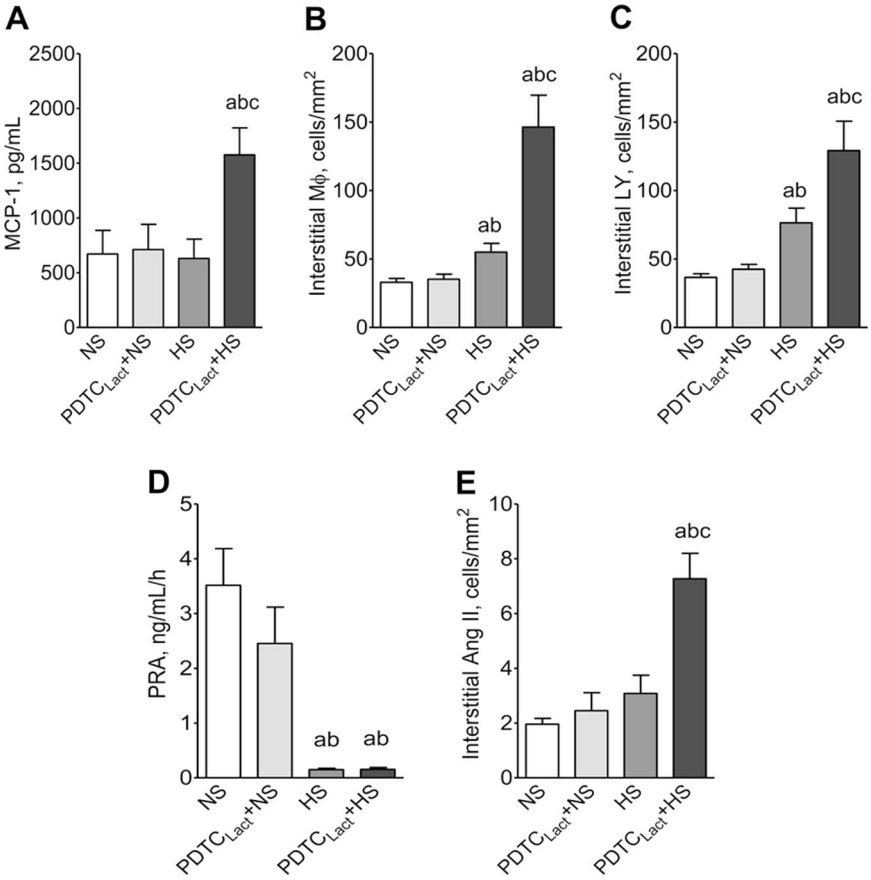

Fig. 3 Serum MCP-1 (**a**), interstitial macrophage infiltration (**b**), interstitial lymphocyte infiltration (**c**), PRA (**d**), and interstitial angiotensin II-positive cells (**e**) in Groups NS, $PDTC_{Lact}$ + NS, HS, and $PDTC_{Lact}$ + HS. The results are expressed as the means ± SE. $^{a}p < 0.05$ NS, $^{b}p < 0.05$ $PDTC_{Lact}$ + NS, $^{c}p < 0.05$ HS

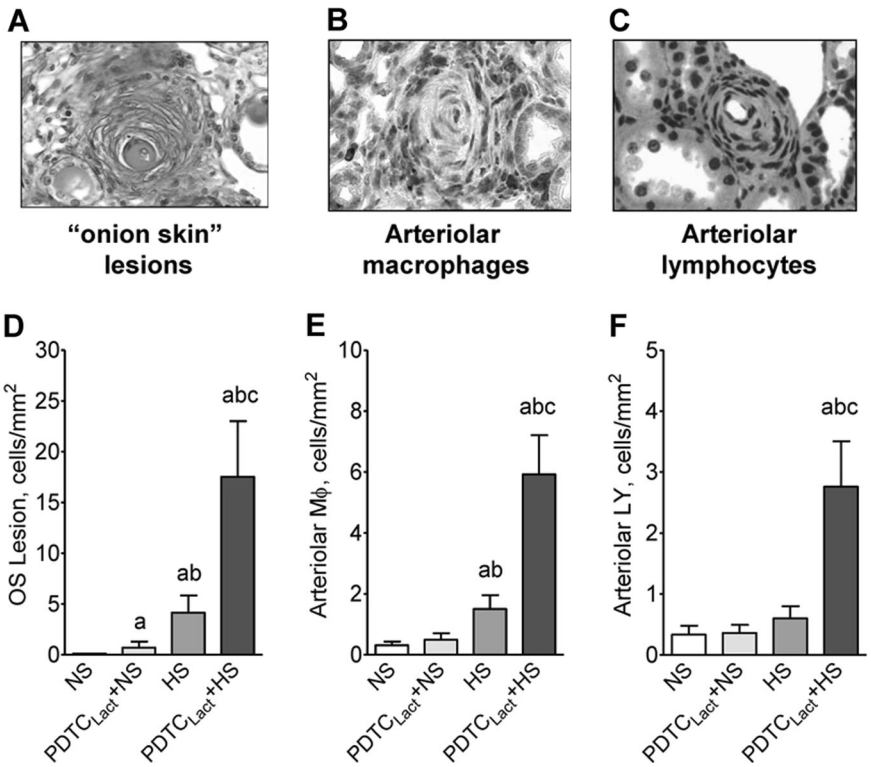

Fig. 4 Representative microphotographs (400×) of "onion-skin" arteriolar lesions (PAS-stained, **a**) and (in immunohistochemically stained sections) arteriolar infiltration by macrophages (**b**) and lymphocytes (**c**) in NS, $PDTC_{Lact}$ + NS, HS, and $PDTC_{Lact}$ + HS Groups. The respective quantitative analyses are shown in **d**, **e**, and **f**

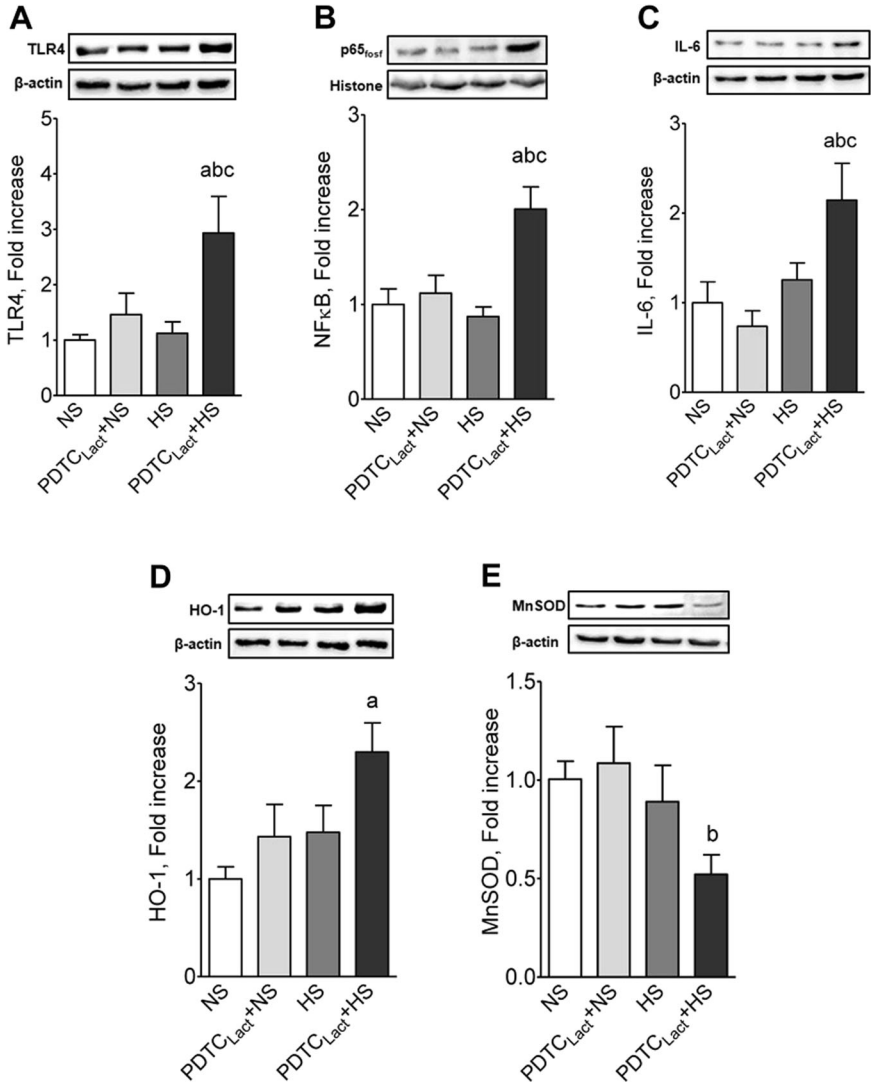

Fig. 5 Protein content of TLR4 (**a**), nuclear p65 (**b**), IL-6 (**c**), heme oxygenase 1 (HO-1) (**d**), and manganese-dependent superoxide dismutase (MnSOD) (**e**) in NS, $PDTC_{Lact}$ + NS, HS, and $PDTC_{Lact}$ + HS Groups. The results are expressed as the means ± SE. $^{a}p < 0.05$ NS, $^{b}p < 0.05$ $PDTC_{Lact}$ + NS, $^{c}p < 0.05$ HS

indicating that the NF-κB pathway was inhibited. In addition, TLR4 expression was reduced in $PDTC_{Lact}$ + HS + L rats (Fig. 8a), whereas the renal abundance of HO-1 was decreased (Fig. 8d), although the renal content of MnSOD was not restored (Fig. 8e).

Discussion

Unlike in humans, rat nephrogenesis is not completed at birth and continues during the first two postnatal weeks [16]. We previously reported that neonatal treatment of rats with Losartan limits nephron number and promotes hypertension and progressive renal disease in adult life [17], in agreement with earlier findings [16] and with the concept that a reduced nephron population at birth increases the risk of cardiovascular and renal disease [18]. More recently, we showed that NF-κB inhibition during lactation is followed by permanent hypertension and cardiac fibrosis without nephron number reduction or renal damage [9]. In the present study, rats in the $PDTC_{Lact}$ + NS Group receiving standard diet exhibited an elevation in blood pressure that was comparable to that shown in the original model. These rats exhibited no renal functional or morphological changes, indicating that even a 50% reduction in renal mass was insufficient to promote renal injury in this model of essential hypertension. However, it is noteworthy that although renal inflammation was not significantly increased compared to NS alone, the density of renal interstitial macrophages in the $PDTC_{Lact}$ + NS Group showed a significant positive correlation with blood pressure in line with previous observations in the SHR model [19]. This finding indicates a latent renal inflammatory effect of hypertension in this model, which might be exacerbated by additional insults.

Increased salt intake by itself is not expected to cause renal functional or structural impairment but may aggravate injury initiated by other mechanisms [20–22]. In the present study, the combination of two insults, UNx and HS diet,

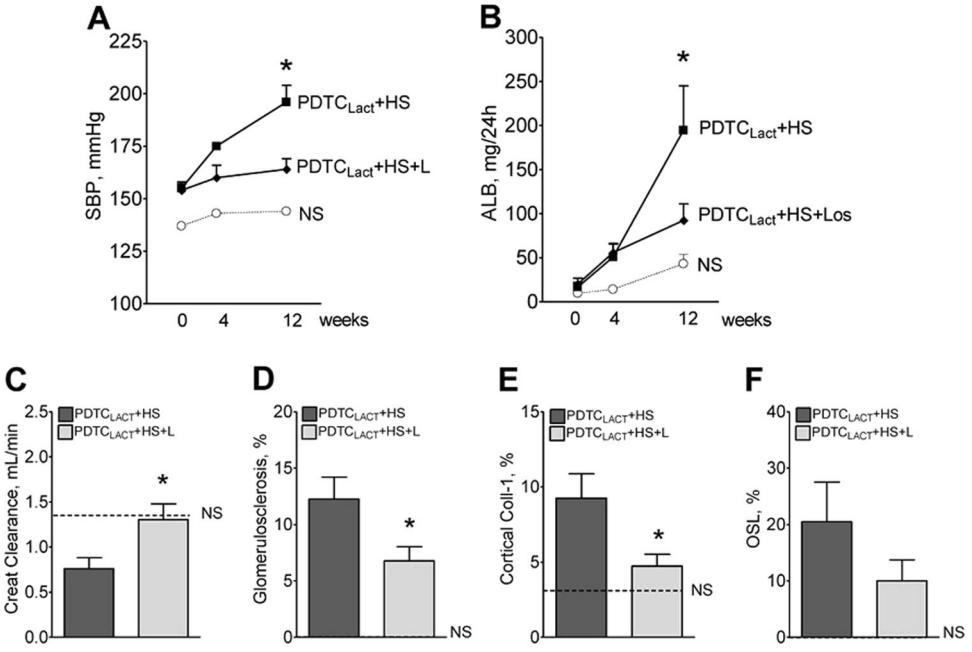

Fig. 6 Systolic blood pressure (**a**) and albuminuria (**b**) measured during HS plus Losartan treatment. After 12 weeks of HS + L, creatinine clearance (**c**), glomerulosclerosis (**d**), cortical collagen-1 deposition (**e**), and arterial "onion-skin" lesions (**f**) in PDTC$_{Lact}$ + HS and PDTC$_{Lact}$ + HS + L Groups. The results are expressed as the means ± SE. *$p < 0.05$ PDTC$_{Lact}$ + HS

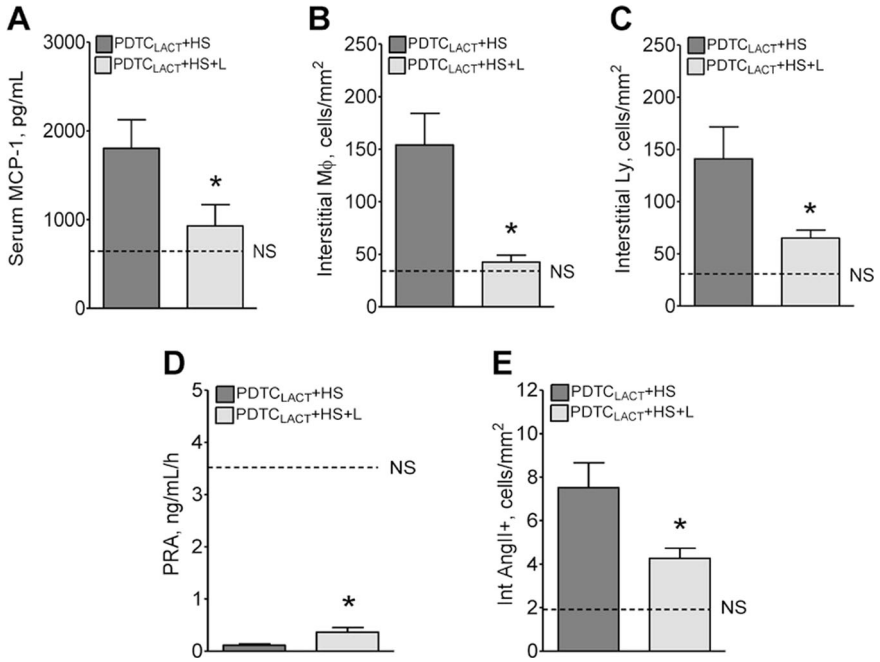

Fig. 7 Serum MCP-1 (**a**), interstitial macrophage infiltration (**b**), lymphocyte infiltration (**c**), PRA (**d**), and interstitial angiotensin II-positive cells (**e**) in PDTC$_{Lact}$ + HS and PDTC$_{Lact}$ + HS + L Groups. The results are expressed as the means ± SE. *$p < 0.05$ PDTC$_{Lact}$ + HS

resulted in moderate hypertension, albuminuria, and glomerulosclerosis, corroborating those previous observations. However, we did not observe renal interstitial fibrosis or inflammation in this group, suggesting the operation of a purely hemodynamic effect, possibly involving podocyte damage [23].

Renal and hemodynamic abnormalities were maximal in rats that had been treated with PDTC during lactation and subjected to both UNx and salt overload in the adult phase (PDTC$_{Lact}$ + HS Group). In these animals, severe hypertension was associated with exacerbated albuminuria and a wide range of glomerular, vascular, and interstitial lesions. As with HS diet alone, hemodynamic factors seem to have played an important role in this process, as indicated by a significant correlation between blood pressure and the percentage of sclerotic glomeruli. Extreme mechanical stress to the capillary walls, possibly reflecting insufficient afferent constriction, is suggested by the frequent finding of glomerular microaneurysms and partial tuft necrosis, similar to those shown in other CKD models associated with severe glomerular hypertension [20, 24]. Injury to the endothelial layer is indicated by the finding of glomerular

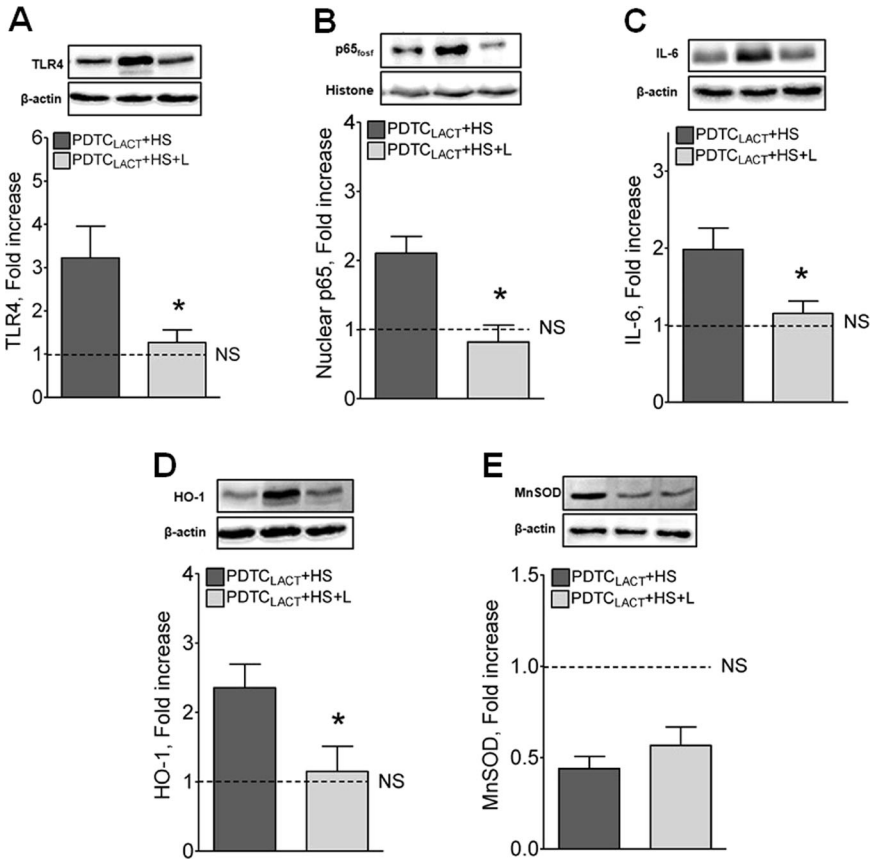

Fig. 8 Protein contents of TLR4 (**a**), nuclear p65 (**b**), IL-6 (**c**), heme oxygenase 1 (HO-1) (**d**), and manganese-dependent superoxide dismutase (MnSOD) (**e**) in $PDTC_{Lact}$ + HS and $PDTC_{Lact}$ + HS + L Groups. In each graph, the first band on the left (NS Group) is represented by the dotted line. The results are expressed as the means ± SE. *$p < 0.05$ $PDTC_{Lact}$ + HS

microthrombi, as shown previously in 5/6 renal ablation rats receiving a VEGF inhibitor [25]. Glomerular capillaries were not the only vessels affected. Severe arteriolar lesions, with fibrinoid necrosis and myointimal cell proliferation ("onion-skin" lesions), commonly seen in human malignant hypertension, were also abundant in the $PDTC_{Lact}$ + HS Group, as indicated by a significant correlation between albuminuria and the frequency of "onion-skin" lesions.

The subtle inflammatory trend observed in the $PDTC_{Lact}$ + UNx Group was also exacerbated in the $PDTC_{Lact}$ + UNx + HS Group; severe inflammation is likely to have contributed to the aggravation of renal and arteriolar lesions observed in these rats, as reported previously with other experimental models [6, 26, 27]. Intense renal infiltration by macrophages and lymphocytes, mainly in the interstitial compartment, was observed in this group, with a positive correlation between the density of interstitial lymphocyte infiltration and albuminuria. In addition, the positive correlation between the percentage of "onion-skin lesions" and the density of vessel lymphocyte infiltration indicates that inflammation was involved in vascular injury as well. Moreover, the finding of glomerular microthrombosis suggests that severe endothelial damage may also have contributed to vascular injury. Also noted in the $PDTC_{Lact}$ + HS Group was a marked elevation in circulating MCP-1, a key cytokine for the recruitment of macrophages, underscoring the systemic impact of the renal inflammatory process. Additionally, we observed marked renal collagen deposition in this group, indicating advanced CKD and renal fibrosis, thus explaining the pronounced fall in creatinine clearance.

As shown in several experimental models and clinical instances of CKD [5, 6, 28], the NF-κB complex exerts a key role in the pathogenesis of CKD by driving the transcription of a number of potent inflammatory mediators. In the present study, in an apparent paradox, adult animals that had this system inhibited during lactation exhibited enhanced nuclear translocation of the p65 moiety in response to UNx and HS, in concomitancy with renal injury and inflammation. Accordingly, the renal abundance of IL-6, a well-known NF-κB target and inflammatory mediator, was significantly increased in this group, along with a slight increase in the renal content of IL-1β.

Several stimuli may have contributed to activate the NF-κB system in the $PDTC_{Lact}$ + HS Group. Increased renal TLR-4 expression, possibly in association with the presence of cell debris and other damage-associated molecular patterns, may have favored p65 translocation, consistent with the finding of a positive correlation between TLR-4 abundance and blood pressure. Activation of the NF-κB

cascade may also have resulted from the development of renal oxidative stress, a pathogenic factor demonstrated in several models of hypertension associated with renal damage [29, 30]. In the $PDTC_{Lact}$ + HS Group, the occurrence of renal oxidative stress was indicated by the finding of increased renal abundance of HO-1 and, simultaneously, a reduction in renal MnSOD, two enzymes associated with the tissue response to oxidative stress in hypertension and CKD [31–33].

NF-κB activation may also have been triggered by local production of AngII [34–36], as one of the multiple pathogenic effects of this peptide in CKD. AngII-positive cells were detected mainly in the renal interstitium of $PDTC_{Lact}$ + HS rats. It is noteworthy that any pathogenic effect of AngII must have been due to renal local production of the peptide since plasma renin activity was brought to extremely low values in all rats receiving the HS diet, while the density of interstitial AngII-positive cells matched the parameters of renal injury and inflammation, clearly underscoring the difference between "classical" AngII, linked to sodium conservation, and "inflammatory" AngII, associated with leukocyte recruitment and fibrosis. The significant linear correlation between the density of AngII-positive cells and several parameters of renal injury/inflammation is consistent with a central role of AngII in the pathogenesis of renal damage in this group.

The importance of "inflammatory" AngII in the pathogenesis of hypertension and renal injury is further underscored by the effect of Losartan therapy. In treated $PDTC_{Lact}$ + HS rats, hypertension and renal structural injury were attenuated along with renal fibrosis/inflammation, while the loss of renal function was prevented. Of note, although PRA was expectedly elevated by Losartan treatment, the presence of AngII-positive cells, which parallels the intensity of renal inflammation, was diminished, thus underscoring the dissociation between the "classical" and "proinflammatory" roles of AngII. Of note, Losartan treatment decreased the renal content of TLR4 and, accordingly, limited the nuclear translocation of p65 and IL-6 production. However, Losartan treatment provided incomplete protection against renal arteriolar injury/inflammation, suggesting the participation of other pathogenic mechanisms in the renal vascular compartment.

In summary, after confirming our previous finding that rats that received an NF-κB inhibitor during lactation exhibit hypertension without renal injury in adult life, we showed that, after UNx, the kidneys of these rats exhibit signs of incipient inflammation. Additional treatment with salt overload unmasked the vulnerability of the renal tissue to mechanical stress, which translated into exuberant inflammation and severe glomerular, interstitial, and vascular injury, along with marked functional loss, mimicking the renal changes observed in malignant hypertension. In an apparent paradox, activation of the NF-κB complex, possibly driven by TLR4 stimulation, oxidative stress, and local production of AngII, may play a key role in the pathogenesis of renal injury in this new experimental model.

Acknowledgements These studies were supported by the São Paulo Research Foundation (FAPESP, grant no. 2012/10926-5), the National Council for Scientific and Technological Development (CNPq, grant no. 303684/2013-5), and the Coordination for the Improvement of Higher Education Personnel (CAPES, no. 40944413870). We thank Claudia R. Sena, Camilla Fanelli, Vivian L. Viana, and Janice G. P. Silva for expert technical assistance.

Funding These studies were supported by the São Paulo Research Foundation (FAPESP, grant no. 2012/10926-5), the National Council for Scientific and Technological Development (CNPq, grant no. 303684/2013-5), and the Coordination for the Improvement of Higher Education Personnel (CAPES, no. 40944413870).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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