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Glomerular profile numerical density per area and mean glomerular volume in rats submitted to nitric oxide synthase blockade

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Summary. Rats submitted to chronic inhibition of nitric oxide synthase (NOS) have developed systemic hypertension and consequent renal injury. The present study aims to determine glomerular quantitative changes due to NOS inhibition in rats. Adults and normotensive Wistar rats were separated into control and L-NAME groups (each group n=10). The animals received water and food *ad libitum*, while L-NAME rats received N^G-Nitro-L-Arginine methyl Ester hydrochloride to inhibit NOS (50mg/kg/day) in drinking water during 40 days. After that period the rats were sacrificed, the kidneys were removed, measured, and prepared for histological and stereological analyses. The glomerular density per area [N_A(glom)] and the mean glomerular volume [$\bar{v}$] were determined per animal in 15 random fields. In L-NAME rat the blood pressure was 76% higher than the respective control group with the same age. Glomeruli had global or segmental glomerular sclerosis; some glomeruli only presented an atrophic structure. The renal volume was not different between control and L-NAME rats ($p>0.05$). However, L-NAME rats had the N_A(glom) 33% smaller than the control rats ($p=0.0001$) and, concomitantly, L-NAME rats had the $\bar{v}$ (glom) 33% higher than the control ones ($p=0.004$). These results demonstrate morphological renal alterations caused by NOS inhibition and hypertension.

Key words: Nitric oxide, Hypertension, Renal glomerulus, Stereology

Introduction

Nitric oxide (NO) is an endothelial endogenous vasodilator synthesized by constitutive NO synthase (NOS) that has at least three distinct isoforms (Nathan and Xie, 1994). NOS catalyses the oxidation of the amino acid L-arginine to give rise to citruline and NO. Three or more NOS isoforms are expressed in the normal kidney; the brain-type NOS (bNOS) is greatly expressed in the macula densa cells and in some adjacent thick ascendant limb cells, cells of Bowman's capsule, some intrarenal nerves, and tubular structures (Bachmann et al., 1995; Zatz and Baylis, 1998).

A model of systemic hypertension has been developed in rats by chronic administration of L-Arginine analogues (like N^G-Nitro-L-Arginine Methyl Ester or simple L-NAME). The sustained hypertension induced by L-NAME involves inhibition of endothelial NOS activity. NO may also participate in the control of renin secretion by the adjacent juxtaglomerular cells (Reid and Chou, 1995) considering that the macula densa contains a high concentration of this enzyme (Mundel et al., 1992) and that arginine analogues which inhibit NOS alter the rate of renin secretrion (Kurtz et al., 1991; Johnson and Freeman, 1992). Renal endothelial, mesangial and macula densa cells release NO, which is involved in the regulation of glomerular and medullary microcirculation (Bachmann and Mundel, 1994). The NO produced within the kidney controls the following: glomerular filtration rate, total renal and medullary blood flow, pressure natriuresis, epithelial sodium transport; and the production of various vasoactive factors including renin, indicating that NO is one of the most important systems controlling renal function (Kone and Balys, 1997; Madrid et al., 1997).

The kidney is extremely sensitive to the inhibition of NO. Very low doses of L-Arginine analogs, which do not affect blood pressure, diminish diuresis, natriuresis and renal plasma flow (Nava and Lüscher, 1995). A deficiency in the synthesis of NO may contribute to the development of systemic hypertension because NO is thought to mediate the renal responses to volume expansion and in experimental hypertension NO-mediated dysfunction has been reported as partly responsible for the impaired pressure natriuresis (Ikenaga et al., 1993).

In conjunction with chronic NOS inhibition, the kidney structure shows pathological alterations like classic glomerulosclerosis with glomerular accumulation

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of hyaline material, collapse of capillary loops, and adhesion to the parietal layer of Bowman's capsule. The glomerular ischemia consists of uniform collapse of the glomerular tuft and the capillary loops with pronounced thickening and even duplication of the basement membrane. Glomerular segmental necrosis appears as sharply delimited areas, where lysis of necrotic material may result in the formation of microaneurysms. Microvascular lesions range between arteriolar wall thickening to "onion skin" proliferation with complete luminal occlusion, fibrinoid necrosis of the vascular wall, and periarteriolar fibrosis. All these renal injury types are associated with progressive albuminuria (Zatz and Baylis, 1998).

The chronic inhibition of the NOS in rat induces arterial hypertension and renal pathological changes. A previous study has shown that chronic inhibition of NOS also causes the decrease in intracellular cGMP levels (the second messenger of NO) in vascular smooth muscle, inducing structural changes of renal microvessels (Numaguchi et al., 1995). Otherwise, it is speculated that a reduced renal NO synthetic capacity may contribute to start or maintain intraglomerular hypertension (Baylis et al., 1992).

The NO-deficient hypertension is associated with the attenuation of vascular relaxation and hypercontractility in different parts of the vascular tree. The particular interest is the reduced relaxation in the renal artery. It might result in increased renin release and activation of the renin angiotensin system (Bernátová et al., 1996), which is recognized as a factor involved in the progression of chronic renal failure and in the development of hypertension in renal diseases (Anderson et al., 1986; Lai et al., 1988).

Acute and chronic impairment of NOS or endothelial dysfunction may contribute to the initiation or perpetuation of hypertension and renal damage in rats (Ribeiro et al., 1992; Ono et al., 1995). Tissue injury associated with chronic NOS inhibition is not confined to the kidney; rats receiving chronic L-NAME treatment exhibit focal areas of myocardial necrosis along with focal areas of fibrosis that may reflect organization of necrotic tissue (Pereira and Mandarim-de-Lacerda, 1998; Pereira et al., 1998). Stereology of cardiac microvessels revealed remodeling of these vessels in rats under NOS inhibition (Pereira and Mandarim-de-Lacerda, 1999).

Quantification of glomerular structure is considered important information to aid the identification of the glomerular structural change (Cahill et al., 1996). The present study intends to determine stereological glomerular parameters in rats submitted to NOS inhibition.

Materials and methods

Twenty adult male Wistar-strain rats were used in this study. Animals weighing 260.8 ± 20.1g (mean ± SD) were kept separate during 40 days in two groups of ten animals each. The arterial blood pressure (BP) was weekly verified with tail-cuff plethysmography. In the control group the animals received daily food (Nuvilab®) and fresh water *ad libitum*. In the L-NAME group animals received L-NAME, 50mg/kg/day (N^G-Nitro-L-Arginine Methyl Ester Hydrochloride, Sigma chemical Co., St Louis, Lot 44H0102) dissolved in drinking water.

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985), and protocols were approved by the animal subjects committee of the State University of Rio de Janeiro.

On the 41st day the animals were anaesthetized and sacrificed. Kidneys were removed and weighed according to Scherle's method (immersed in physiological saline solution) (Scherle, 1970). Since the renal tissue is anisotropic, the kidney was cut according to the "Orthrip" method (Mattfeldt et al., 1985), i.e. the kidney was initially cut at random; the organ was then placed on this cut and, again at random, the specimen was cut with a perpendicular section to the first plane. The specimen was again placed on the new cut surface and a new random orientation was defined cutting the organ in a perpendicular section to the plane. The last cut was considered to be uniformly isotropic: this means that without reference to the position of the specimen in the first cut the last surface has an orientation which varies from all possible ones.

Cortical renal fragments were then immersed in a buffered solution of 4% formaldehyde at pH 7.2 for 48-72 hours and embedded in Paraplast® (Sigma). After that, they were sectioned at 5μm thick and stained with hematoxylin-eosin, Masson's trichrome, PAS (Schiff's reagent) and Gomori's method for reticular fibers (silver impregnation).

The analysis used a video-microscopic system composed of a Leica DMRBE microscope coupled to a Kappa CF 15/5 video camera and a Sony trinitron monitor. The numerical density in the plane (glomerular profile numerical density per area) [N_A(glom)] was determined by observing 15 microscopic fields per animal, i.e. 150 fields per group (Mandarim-de-Lacerda, 1998). Glomeruli were counted in a frame of $1.31 \times 10^6 \mu m^2$, only considering well-preserved structures and not crossing the forbidden line (Gundersen, 1977). Maximum and minimum glomerular diameters were measured on 25 profiles per animal, since the glomerulus had been cut near the equatorial region. These diameters were measured using final magnification x800. The mean glomerular volume [$\bar{v}(glom)$] was determined by assuming volume of the sphere.

Differences comparing the groups were tested with non-parametric test of Mann-Whitney with significant level of 0.05 (Zar, 1999).

Results

BP was significantly increased in NOS blockade,

attaining 206.8 ± 21.7mmHg (mean ± SD) after 40 days of L-NAME administration, which was 73% higher than BP at the beginning of the experiment, and 76% higher than BP of the respective control group with the same age. The statistical difference in BP between control and experimental groups went up from the second week of L-NAME administration, continuously increasing until the end of the experiment (Fig. 1).

Figure 2 shows the glomerular morphology in control and L-NAME rats. Glomeruli had considerable and general alterations in L-NAME rats, characterized by global or segmental glomerular sclerosis. Renal parenchyma showed some glomeruli, only presenting an atrophic structure, tubular atrophy and extensive fibrosis (Fig. 2B). Furthermore, in L-NAME rats hypertrophied glomeruli had capsular thickening (Figs. 2D,F).

Quantitative results are summarized in Table 1. The renal volume was not different in control and L-NAME animals ($p>0.05$). However, L-NAME rats had the N_A(glom) 33% smaller than the control rats ($p=0.0001$) and, concomitantly, L-NAME rats had the $\bar{v}$ (glom) 33% higher than the control ones ($p=0.004$).

Discussion

This work has induced hypertension in rats using 50mg/kg/day of L-NAME. After only one week of experimentation the differences in blood pressure were significant when control and L-NAME rats were compared, and these differences were progressively accentuated in the weeks that followed. NO is certainly released in homeostatic mechanisms to regulate renal effects of angiotensin II in normotensive rats, and under hypertension conditions (Sánchez-Mendoza et al., 1998) this mechanism does not function any longer.

Previous studies using orally active NOS inhibitor in different schedules of time and dosage had shown the development of stable hypertension and glomerulosclerosis (Baylis et al., 1992) and severe and progressive form of hypertension associated with glomerular ischemia, glomerulosclerosis and renal interstitial expansion (Ribeiro et al., 1992).

The present study demonstrated that biometrical difference (renal volume) does not exist between control and L-NAME animals, agreeing with previous studies that confirmed no statistical difference in the renal weight in spontaneously hypertensive rats (SHR) (Ono et al., 1995, 1996) and in Sprague-Dawley rats (D'Amours et al., 1999) with and without L-NAME administration.

Renal lesions in rats submitted to NOS inhibition have been previously described in the literature: Wistar (Ribeiro et al., 1992; Bouriquet and Casellas, 1995) and SHR-Wistar rats (Ono et al., 1995). The main findings are increased mesangial matrix, capillary collapse, hyaline deposition, presence of segmental fibrinoid necrosis of the glomerular tuft, and adhesion of the glomerular tuft to Bowman's capsule. The arteries and arterioles revealed moderate to marked hypertrophy in the media with hyaline deposition.

Agreeing with these previous studies the present quantitative approach detected injury of the entire nephron population caused by L-NAME administration with severe morphological lesions of glomeruli and simultaneous increase of the glomerular volume in the reminiscent glomeruli. Present results agree with previous descriptions reporting that the diminished renal NO formation could play a role in the hemodynamic and non-hemodynamic abnormalities reported in the model of extreme renal ablation that precedes and eventually leads to the development of glomerulosclerosis and renal scarring (Aiello et al., 1997; Ichihara et al., 1998).

The responses of glomerular cells to injury and resulting architectural changes of the glomerulus such as mesangial hypertrophy, mesangial hyperplasia, and increased mesangial cell matrix production are often due

Table 1. Results of the quantitative study of the left kidney and the glomerular parameters: numerical density per area (NA(glom)) and mean glomerular volume ($\bar{v}$ (glom)).

GROUPS (1/mm²)	N_A(glom) (mm²)	$\bar{v}$ glom (mm³)	VOLUME (left kidney) (mm³)
Control			
Median	9.0	0.00036	794.0
CI 95%	8.4 to 9.9	0.0003 to 0.0004	753.5 to 891.1
CV %	11.2	20.9	11.7
CE %	3.5	6.6	3.7
L-NAME			
Median	6.0	0.00048	847.0
CI 95%	5.0 to 6.3	0.0004 to 0.0005	796.5 to 931.7
CV %	16.6	11.5	10.9
CE %	5.3	3.6	3.5
p	0.0001	0.004	NS

CE: coefficient of error; CI: confidence interval at 95%; CV: coefficient of variation; NS: not significant; p: statistical probability (Mann-Whitney test).

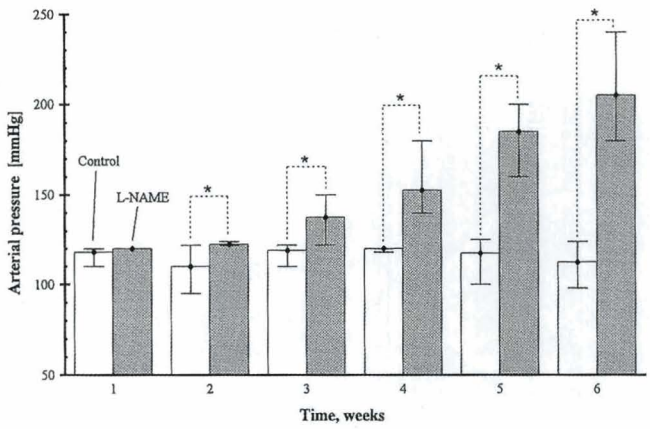

Fig. 1. Bar graph of the variation of the arterial tail pressure comparing Control and L-NAME groups regarding time in weeks of L-NAME administration. Asterisks show statistically significant differences ($p<0.05$).

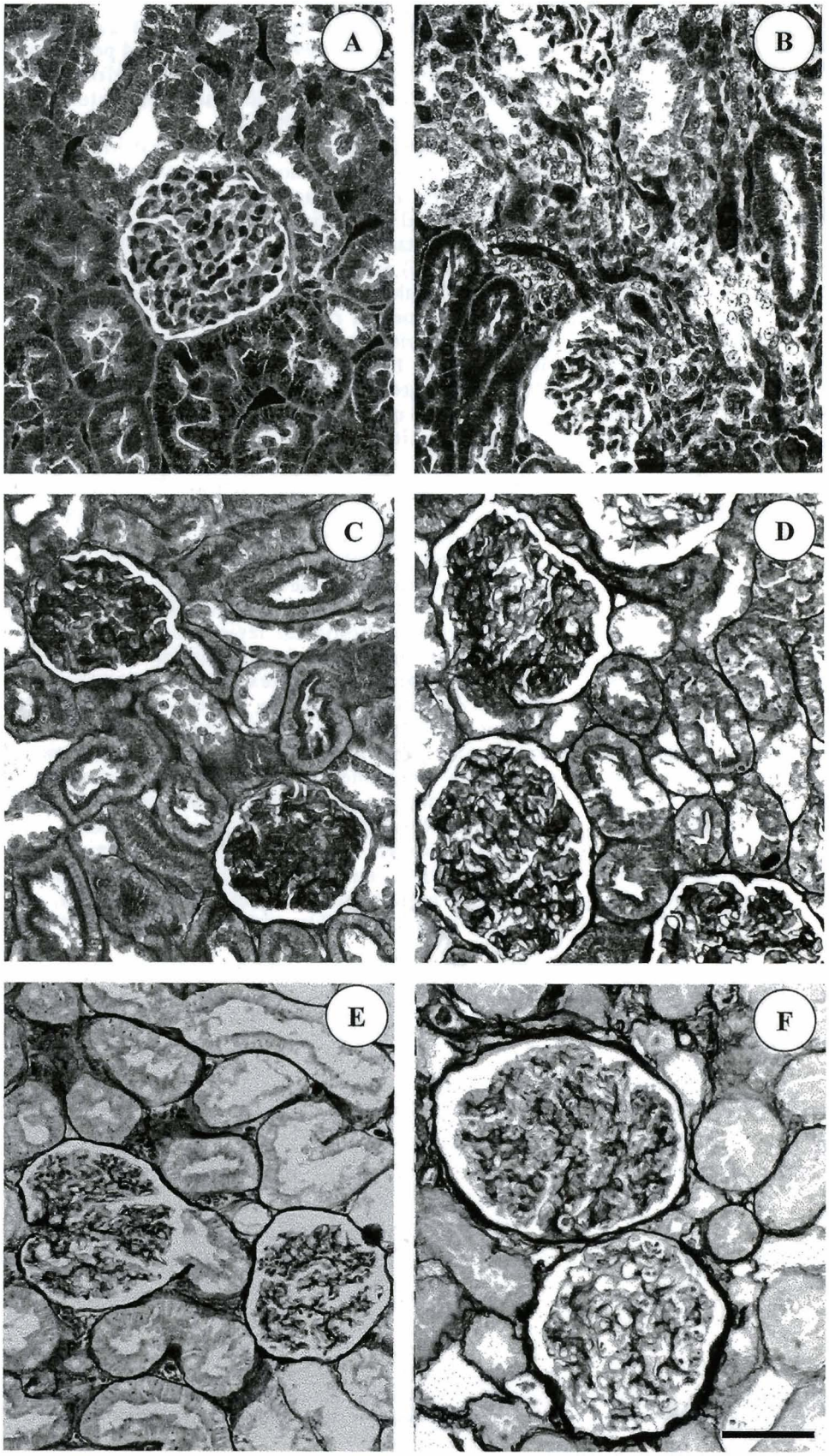

Fig. 2. Photomicrographs of the renal cortex in rats of the Control and L-NAME groups, same magnification (in Fig F, bar: 50μm). **A.** Control group, normal glomerulus, Masson stain. **B.** L-NAME group, disorganized architecture showing a deteriorated glomerulus, dilated tubules and extensive fibrosis, Masson stain. **C.** Control group, two normal glomeruli and tubules, PAS stain. **D.** L-NAME group, hypertrophied glomeruli showing hypercellularity, thrombi in glomerular capillaries characteristic of microangiopathic disorder, thickening of the capillary walls, mesangial proliferation and matrix increase, PAS stain. **E.** Control group, two normal glomeruli, silver impregnation. **F.** L-NAME group, hypertrophied glomeruli with glomerular capsule thickening, silver impregnation.

to the added effects of hemodynamic (glomerular hypertension) and nonhemodynamic actions of the vasoactive agents, much as occurred in systemic vascular beds (Gibbons and Dzau, 1994; Raij, 1998).

However, D'Amours and co-workers (1999) studying the effects of the NOS inhibition in Sprague-Dawley rats, found no difference in the body, heart and kidney weights comparing control and L-NAME animals, no difference was either found in the number of glomeruli between these two groups of animals. They concluded, even recognizing that their findings are in contrast to other studies reporting that L-NAME-induced hypertension causes renal damage, that NOS inhibitor caused severe hypertension without renal damage. This discrepancy is difficult to explain, but D'Amours and co-workers (1999) suggest that it may be due to a number of experimental factors, including rat strain, age, and the duration of the treatment.

Most studies on glomerular size consider glomerulus a sphere. Even so a more accurate approximation would be to consider it a slightly prolate or oblate ellipsoid. However, the first assumption is reasonable because the relationship between volume and mean axial values does not vary considerably between the latter solids and spheres (Pesce, 1998). In the present study the appearance of the glomeruli was relatively uniform among the animals of the same group allowing expecting that the biases are similar in both control and L-NAME groups. Furthermore, the sampling of only well preserved glomerular profiles in control and L-NAME groups may cause a biased determination, which is, however, comparable in both groups. If clearly injured structures had been included in the analysis it would have been impossible to compare normal kidneys with the ones having alteration due to NO inhibition, but that was not the case of the present study.

Apoptosis has been observed in the glomerular cells of experimental nephritis (Shimizu et al., 1995) and human proliferative glomerulonephritis (Sugiyama et al., 1996). Apoptosis has been considered a major mechanism for the resolution of glomerular hypercellularity in the recovery of glomerulonephritis, controlling the size of the glomerular population and clearing excess cells (Baker et al., 1994; Shimizu et al., 1995; Davis and Ryan, 1998) and it is responsible for the progressive cell loss occurring in the pathogenesis of glomerular sclerosis (Davis and Ryan, 1998). In L-NAME animals of the present study we observed glomerular sclerosis and some glomeruli presenting an atrophic structure, tubular atrophy and extensive fibrosis. We have considered the small, round nuclei in the glomeruli as morphological evidence of apoptosis, but the use of an immunolabelling technique is still necessary to confirm this evidence. According to Hattori and co-workers (1998) an increase in the extracellular matrix, which accumulates in parallel with a decrease in the number of glomerular cells, is related to apoptosis that subsequently causes the decrease in renal function. However, these authors have considered the role of apoptosis in the depletion of glomerular cells still controversial and that new studies are required.

In summary, the present study submitted Wistar rats to a chronic NOS inhibition that provoked arterial hypertension and renal morphological lesions. The quantitative information about glomerular changes in experimental animals showed alterations in the number and in the volume of the glomeruli but not in the entire renal volume. Further investigation is required to clarify the detailed mechanisms underlying the progression of the renal lesions in this experimental model.

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