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Hypertension-Induced Renal Damage in Rat Model – an Electron Microscopic Study

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Hypertensive nephrosclerosis is associated with ubiquitously pronounced structural changes in both renal parenchyma and interstitium. Although hypertension-induced renal damage has been well studied, changes appear to be non-specific and can be observed as a result of aging or other underlying pathological condition. The aim of the present study was to investigate the ultrastructural alterations during the initial and chronic phase of hypertension-induced kidney damage. For the present study, we used 6- and 12-month-old spontaneously hypertensive rats and age-matched Wistar rats as controls. A transmission electron microscopic study of the renal cortex was conducted according to a standard protocol. Hypertension-induced kidney damage was associated with prominent changes in the structure of the glomerular filtration barrier, severe podocyte injury, as well as pronounced tubular atrophy. The present work is a detailed ultrastructural study of the renal cortex in a model of hypertension-induced renal damage.

Key words: Kidney, ultrastructure, hypertension, rat

Introduction

Essential hypertension is recognized as a primary risk factor second only to diabetes for the development of chronic renal failure [9]. Hypertension-induced renal damage is associated with a progressive decrease in the functional capacity of the kidney due to numerous morphological alterations in the nephrons and renal interstitium. These changes include severe glomerulosclerosis, hyalinization and sclerosis of interlobular and afferent arterioles, tubular atrophy and an altered balance between collagen synthesis and degradation resulting in the development of renal interstitial fibrosis [2,16]. From an ultrastructural perspective, glomerular damage is characterized by thickening and wrinkling of the glomerular basement membrane (GBM), cytoplasmic vacuolization of the endothelial cells, as well as pronounced alterations in the visceral epithelial cells of Bowman's capsule – the podocytes – shortening and diffuse fusion

of foot processes and loss of podocytes resulting in denudation of the GBM [4, 17, 24]. In addition, podocyte injury has been implicated in the development of hypertensive nephrosclerosis [25]. The deposition of filtered proteins in the proximal tubular segments due to the altered selective permeability of the glomerular filtration barrier (GFB) has potential pro-inflammatory and cytotoxic effects, which contribute to the progression of the tubulointerstitial changes [1]. Tubular atrophy is accompanied by thickening of the tubular basement membranes, increased extracellular space between tubular epithelial cells, as well as substantial injury of the specialized cell membrane structures [7, 12]. However, it appears that renal damage secondary to hypertension is a nonspecific process, as all changes described above can be observed as a result of aging, as well as in the course of different pathological conditions [20, 23]. The lack of certain criteria for demarcation of the etiology of renal structural alterations suggests possible inaccuracy in the evaluation of the histological findings.

The spontaneously hypertensive rat (SHR) is a widely used experimental model of essential hypertension. In this strain, the prolonged and untreated elevated blood pressure provokes ubiquitously pronounced renal morphological changes, which correlate with the observed alterations in case of hypertensive nephrosclerosis in the human population [12].

The aim of the present study was to observe and describe the ultrastructural changes in the GFB and the tubular epithelial cells in the renal cortex in SHR during the initial and chronic phase of hypertensive kidney damage. We analyzed and compared the obtained results with those in age-matched controls.

Materials and Methods

Experimental animals

For the present study, we used two age groups of SHR – 6-month-old (established hypertension) and 12-month old (advanced or late stage hypertension) [12]. We also used two groups of age- and weight-matched control animals – normotensive Wistar rats (WR). Each group consisted of six male rats randomly selected from a large population of SHR and WR in the Laboratory of the Department of Anatomy, Histology and Embryology at the Medical University of Sofia, Bulgaria. The rats were housed in Macrolon cages with free access to food and tap water under controlled environmental conditions (12-h light–dark cycle, room temperature 22 ± 1 °C and humidity 55 ± 15%) in order to diminish the variation. All animal procedures conformed to the guidelines of Directive 2010/63/EU of the European Parliament concerning the protection of animals used for scientific purposes. All experiments were conducted with the approval of the University Committee on Animal Resources (No. 4866). All animals received humane care in compliance with the 'Principles of laboratory animal care' formulated by the National Society for Medical Research and the 'Guide for the care and use of laboratory animals' prepared by the National Institute of Health (NIH publication No. 86–23, revised 1996). Systolic and diastolic arterial blood pressure was measured through the tail-cuff method on a Model MK-2000ST (Muromachi Kikai Co., Ltd., Tokyo, Japan) and was recorded in all age groups of SHR

and control WR. The mean systolic and diastolic pressure was the average of three separate measurements on each animal (**Table 1**).

Table 1. Mean systolic and mean diastolic blood pressure of 6- and 12-month-old spontaneously hypertensive rats (SHR) and 6- and 12-month-old normotensive Wistar rats (WR). Each group consisted of six animals (n=6) (SD – standard deviation)

Age group	Mean systolic blood pressure (mm Hg) ± SD	Mean diastolic blood pressure (mm Hg) ± SD
6-month-old SHR	160 ± 1.0	100 ± 2.0
12-month-old SHR	200 ± 2.0	110 ± 1.0
6-month-old WR	110 ± 1.0	80 ± 2.0
12-month-old WR	120 ± 2.0	85 ± 1.0

Tissue preparation

The rats were anesthetized intraperitoneally with Thiopental (Sigma Aldrich Catalogue No. T1022, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) 40 mg/ kg b.w. The chest cavity was opened and transcardial perfusion was made with 4 % paraformaldehyde (Merck Catalogue No. 1040051000, Merck KGgA, Darmstadt, Germany) in 0.1 M phosphate buffer, pH 7.2 (Merck Catalogue No. 6505-4 L). The abdomen was open through an incision along the midline and the kidneys were rapidly removed and rinsed in oxygenated physiological saline (Merck Catalogue No. 1465690010) for a few minutes. Next, they were fixed in a 10% neutral phosphate buffered formalin solution, prepared under laboratory conditions from 37% formaldehyde solution (Merck Catalogue No. 1040031000) for at least 24 h. Their capsule was removed and the kidneys were sectioned parallel to their long axis. All sections were done in accordance with the standardized methods [14]. Samples were obtained from each kidney, which were then dehydrated in increasing concentrations of alcohol (70%, 80%, 95%, 100%) (Merck Catalogue No. 1009835000), cleared in xylene (Merck Catalogue No. 1082984000) and embedded in paraffin (Merck Catalogue No. 1071511000).

Electron microscopy

After deparaffinization with xylene (Merck Catalogue No. 1082984000), the samples were rehydrated with alcohol (100%, 95%, 80%, 70%) (Merck Catalogue No. 1009835000) and washed in 0.1M phosphate buffer (Merck Catalogue No. 1465920006), pH 7.4, at room temperature. They were briefly treated with 3% glutaraldehyde prepared under laboratory conditions from 25% glutaraldehyde (Merck Catalogue No. 354400) for 30 min. Next, they were post-fixed in 1% osmium tetroxide (Merck Catalogue No. 1245050500) at 4 °C for 2 h. After rinsing in distilled water, the samples were

dehydrated in alcohol (70%, 80%, 95%, 100%) (Merck Catalogue No. 1009835000) and treated for 30 min with propylene oxide (Merck Catalogue No. 8070270100). The samples were then embedded in Durcupan (Fluka Catalogue No. 14040, Fluka Chemie AG, Buchs, Switzerland). Afterwards, all samples were cut with a diamond knife on an ultramicrotome (LKB, Stockholm-Bromma, Sweden) to ultrathin sections (100 nm thick), transferred to copper grids (300 mesh) and contrasted with 2.5% uranyl acetate (Electron Microscopy Sciences Catalogue No. 102092-284, Electron Microscopy Sciences, Hatfield, Pennsylvania, United States), lead nitrate (Merck Catalogue No 1073980100) and sodium citrate (Merck Catalogue No. 1110371000). For the electron microscopic study we used a transmission electron microscope Hitachi model H-500 (Hitachi, Ltd., Tokyo, Japan), with an acceleration voltage of 100 kV. Micrographs were recorded on 3¼" × 4" Kodak electron image plates in accordance with the well-established protocol [5,15]. In order to ensure inter-observer reliability and reproducibility of results, all observations were made by two independent and experienced investigators.

Results

Ultrastructural findings in the GFB

In 6-month-old WR, the three-layered structure of the GFB was well demarcated. The foot processes and the slit diaphragms showed relatively preserved morphology. In a few podocytes, we noted cytoplasmic vacuolization and presence of electron dense inclusions. No significant changes were observed in the structure of the cell nuclei in the visceral layer of Bowman's capsule (**Fig. 1A**). In the older group of normotensive animals, the GFB showed altered morphology. The GBM appeared thickened. A higher number of podocytes were characterized by pronounced cytoplasmic vacuolization, condensation of the chromatin and reduced foot processes. We also observed regions of pathological contacts between the visceral and parietal layers of Bowman's capsule (**Fig. 1B**).

In 6-month-old SHR, the GBM was thicker compared to the age-matched WR. A large population of podocytes showed altered structure – shortening and reduction of foot processes and presence of cytoplasmic electron-dense granules and vacuoles of variable shape. The cell nuclei showed signs of condensation of the chromatin (**Fig. 1C**), which was not seen in 6-month-old normotensive WR. In 12-month-old SHR, the most severe structural alterations were observed in the mid-cortical and juxtamedullary nephrons. We observed prominent thickening of the GBM compared to the age-matched normotensive controls. Podocyte injury was associated with reduction and shortening of the foot processes; the borders of the slit diaphragms were not well demarcated. We also described regions of denudation of the GBM. Some podocytes exhibited features which suggested that they had undergone apoptosis, which was represented morphologically by prominent condensation of the chromatin in the nucleus (**Fig. 1D**).

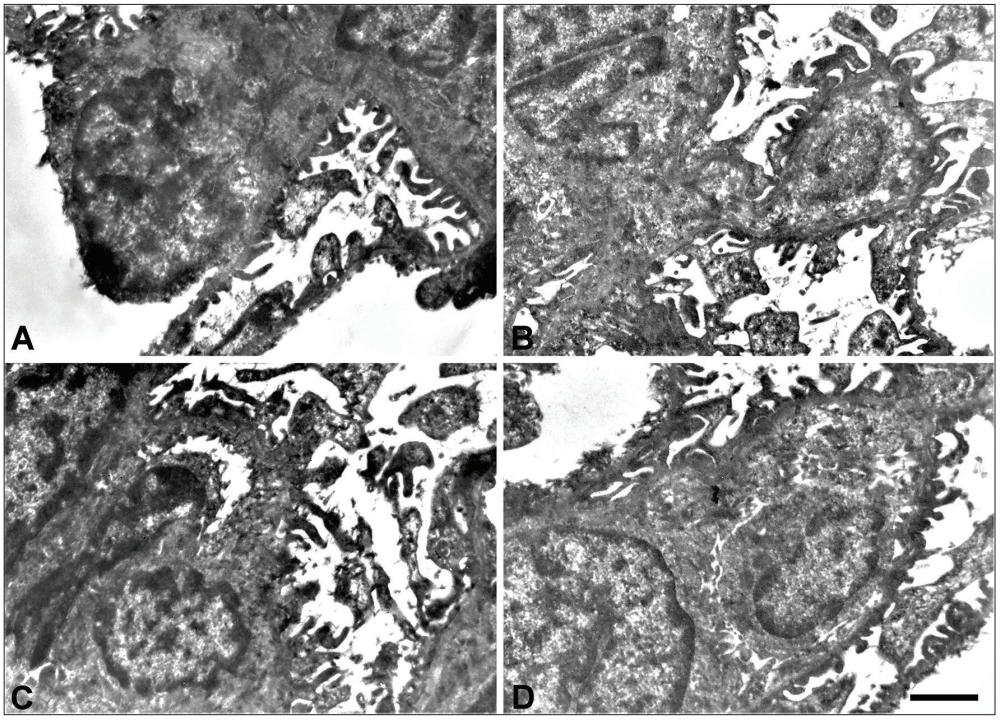

Fig. 1. Electron micrographs of the glomerular filtration barrier (GFB) in 6- and 12-month-old spontaneously hypertensive rats (SHR) and age-matched controls – Wistar rats (WR).

A. 6-month-old WR. Scale bar – 1.5 μm.
B. 12-month-old WR. Scale bar – 2.5 μm.
C. 6-month-old SHR. Scale bar – 1.5 μm.
D. 12-month-old SHR Scale bar – 2.5 μm.

Ultrastructural findings in the renal tubules

In 6-month-old WR, the basement membranes in the proximal and distal tubular segments were well preserved. We noted the presence of few electron dense granules in the cell cytoplasm. The ultrastructure of the cell nuclei was relatively well preserved, with several nucleoli and a smooth nuclear membrane. Mitochondria presented with well demarcated cristae. We found reduced apical microprojections, as well as presence of narrowed spaces between the interdigitations of the tubular epithelial cells (**Fig. 2A**). In the older group of normotensive animals, the basement membranes of the proximal and distal tubular segments were thickened. Some cells had lost their contact to the underlying basement membrane. The cytoplasm of the tubular cells contained numerous electron dense granules and vacuoles. Initial condensation of the chromatin in the cell nuclei was described, as well as a lower number of mitochondria. There was prominent reduction in the specialized cell membrane structures. The intercellular junctions appeared widened (**Fig. 2B**).

The basement membranes of the proximal and distal tubular segments in 6-month-old SHR were thickened compared to the age-matched WR. A higher incidence of cytoplasmic vacuolization and electron dense accumulations were described. Chromatin in the cell nuclei appeared much more condensed than in 6-month-old WR. Mitochondria with altered morphology were frequently observed. There was increased intercellular space as well as reduction in the number of microvilli compared to the age-matched normotensive group (**Fig. 2C**). In 12-month-old SHR, the proximal and distal tubules were characterized by more severe process of tubular atrophy compared to 12-month-old WR – significant number of electron dense cytoplasmic granules and vacuoles, condensation of the chromatin, indistinguishable morphology and lower number of the mitochondria in the basal infoldings, as well as reduction of the apical microprojections. In addition, the spaces between the interdigitations of the tubular epithelial cells were widest in 12-month-old SHR (**Fig. 2D**).

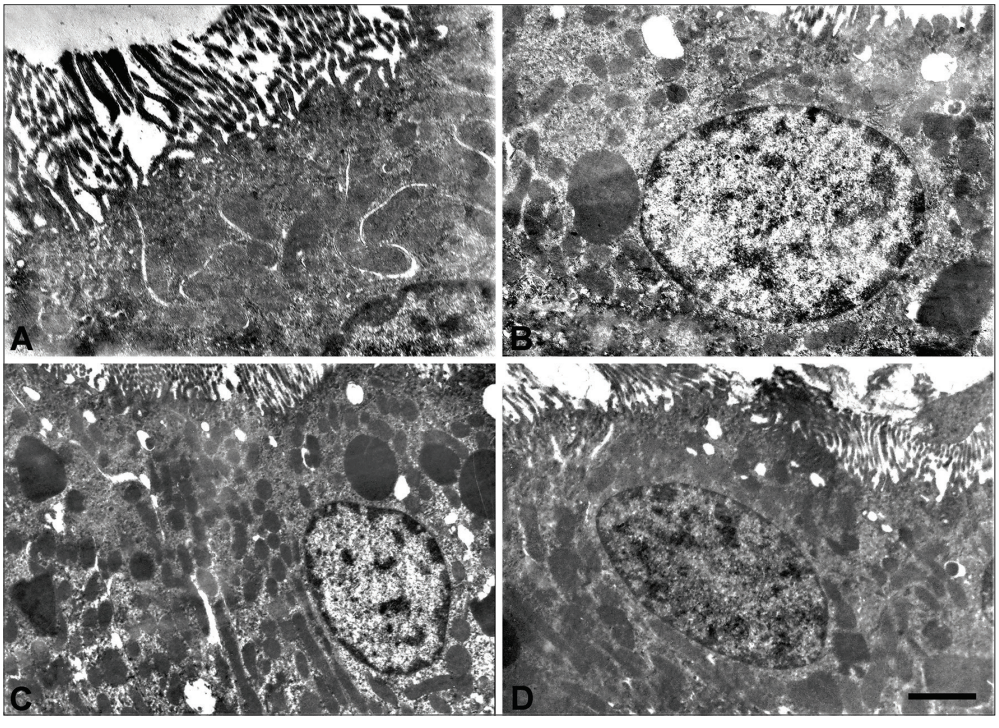

Fig. 2. Electron micrographs of the tubular epithelial cells in 6 and 12-month-old spontaneously hypertensive rats (SHR) and age-matched controls – Wistar rats (WR).

A. 6-month-old WR. Scale bar – 1.0 μm.
B. 12-month-old WR. Scale bar – 3.0 μm.
C. 6-month-old SHR. Scale bar – 3.0 μm.
D. 12-month-old SHR Scale bar – 3.0 μm.

Discussion

The present study focuses on the electron microscopic changes in the structural elements of nephrons occurring throughout the process of aging and as a result of the impact of essential hypertension. Our observations revealed that ultrastructural alterations were more severe in the inner rather than the outer cortex in SHR, while no significant differences were found in the renal cortex in WR.

Earlier, Garcia-Pinto et al., 2011 reported that podocyte foot processes and slit diaphragms were better preserved in WR compared to SHR, which is in accordance with the results from the present study. Based on scientific data, the glomerular capillary pressure in the SHR kidney is higher in the inner rather than the outer cortex even in the initial phase of essential hypertension and tends to increase with age [10]. The described hemodynamic difference may explain the more severe structural changes in the inner cortex in SHR, as well as their earlier onset. It is well known that the structural organization of the GFB includes the fenestrated endothelium, the GBM and the podocytes [21]. However, there appear to be morphological differences in the GFB between SHR and WR [6]. These authors reported a smaller diameter in the glomerular endothelial fenestrae in SHR, which were observed during the prehypertensive period. Haensly et al. [10] described an increased incidence of metaplasia of the epithelium lining the parietal layer of Bowman's capsule in SHR compared to WR. The presence of epithelial cells similar to those in the proximal tubule may be explained by the elevated blood pressure or may be a trigger mechanism for the development of hypertension. On the other hand, metaplasia may be considered an age-related alteration, as it was also observed in WR [22].

In the present study, we did not find any morphological changes in the epithelium of the parietal layer of Bowman's capsule in the studied normotensive and hypertensive groups. The main morphological changes in the podocytes in the initial phase of hypertension in SHR include shortened and widened foot processes, as well as accumulation of protein droplets in the cell body and major foot processes. As hypertension advances, some foot processes are effaced [18]. It seems that the trans-membrane protein podocalyxin contributes to the maintenance of the structural integrity of podocytes. The role of this protein has been discussed in the development of renal injury in SHR as it showed decreased expression compared to the normotensive groups [18]. The described fusion of podocyte foot processes containing membrane-limited electron dense inclusions, as well as the pronounced thickening of the GBM in the group of SHR with chronic hypertension are in accordance with the results of Christiansen et al. which also noted the presence of pseudocysts and condensation of the cytoplasm adjacent to the basement membrane [4]. In addition, we also described areas of denudation of the GBM. In case of progressive nephropathy, Fiori et al. described segmental effacement of the podocyte foot processes, which contained vacuoles and dense granules, as well as podocytes characterized by hypertrophy and karyokinesis. The authors also described the presence of subendothelial hyaline inclusions, as well as filamentous structures in the region of the glomerular capillary tufts [11].

The aspects of renal ultrastructural changes in cases of benign and malignant arterial hypertension in the human population have been discussed [19]. The glomerular structural alterations caused by benign hypertension include expansion of the mesangial matrix, as well as mesangial hyperplasia and the presence of lipofuscin inclusions and microvesicles in the cell cytoplasm [19]. Changes initiated by malignant hypertension included narrowed lumens and dense oxyphil basal membranes of the glomerular capillaries due to the accumulation of plasma proteins and fibrin [19]. According to our results, the ultrastructural changes observed in the chronic stage of hypertension in 12-month-old SHR were similar to those described in the setting of malignant hypertension. The described thickening of the GBM was most prominent in the juxtamedullary nephrons in the group of 12-month-old SHR.

In the present study, tubular atrophy in the proximal and distal tubular segments of nephrons was most severe in 12-month-old SHR. It has been reported that cells of the proximal tubules in SHR suffer higher oxidative stress compared to those in WR [28]. Moreover, it has been suggested that proximal tubular segments are more vulnerable and regarded as primary target of injury in the progression of kidney disease [3]. The thickening of the tubular basement membranes and increased space in the region of the interdigitations and basement membranes described by Garcia-Pinto et al. were confirmed by the present study [7]. In addition, we also described pronounced reduction in the specialized cell membrane structures of the tubular epithelial cells as hypertension-induced kidney injury progressed compared to the normotensive controls. One recent study has turned the focus on the ultrastructural changes in the mitochondria and endoplasmic reticulum of the tubular epithelial cells [13]. In addition, evidence in the literature suggests that the functional deficiency of these membrane-limited organelles may play a potential role in the development of arterial hypertension [8,27]. He et al. also described shortening of the mitochondria and widening of the endoplasmic reticulum as signs of injury under hypertensive conditions [26]. Our results supported these findings. Indeed, we noted that the ultrastructural morphology of the mitochondria in the basal infoldings of tubular epithelial cells was altered and hardly distinguishable in 12-month old SHR compared to age-matched WR.

Limitations of the present study existed and should be noted. First, with regard to the renal parenchyma, we only studied the ultrastructure of the renal cortex, but the presence of significant changes in the renal medulla under hypertensive conditions cannot be ruled out. Second, we only used male SHR and WR, in order to eliminate the impact of cyclical hormonal changes in the female organism. Nevertheless, the presence of sex-related differences in the ultrastructure of the kidney under hypertensive conditions as a result of estrogens synthesis in the female organism merits further studies. Last but not least, we did not conduct a quantitative analysis of specific indices of the ultrastructural changes in the GBM and tubular basement membranes, which could potentially provide further evidence of the significance of these findings.

Conclusion

The present work is a detailed study on the specific ultrastructural changes in the GFB and tubular epithelial cells in a rat model of hypertension-induced kidney injury. The obtained results indicate severe glomerulosclerosis represented with thickening of the

GBM and substantial podocyte injury, as well as more prominent tubular atrophy in the proximal and distal tubular segments in the renal cortex under hypertensive conditions as opposed to age-matched normotensive controls.

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