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Original Paper

Exposure of Pregnant Rats to Cigarette-Smoke Condensate Causes Glomerular Abnormalities in Offspring

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Key Words
Fetal programming • Glomerular volume • Kidney development • Nicotine • Podocytes • Smoking

Abstract
Background: Higher blood pressure and albuminuria are found in offspring of mothers who smoke during pregnancy. Whether or not kidney development is affected by maternal smoking is unknown. ***Methods:*** Sprague-Dawley rats were randomly allocated to twice-daily cigarette-smoke and nicotine condensate (1 mg/kg) or vehicle at day 10 of pregnancy until delivery. ***Results:*** Exposed offspring did not differ from control offspring with respect to body weight, kidney weight, albuminuria, and creatinine clearance. Both male and female offspring had higher tail-plethysmographic blood pressures and lower mean glomerular volume, podocyte, mesangial-cell, and endothelial-cell number, compared to control offspring. ***Conclusions:*** The data document that prenatal exposure to cigarette-smoke condensate containing nicotine influences normal kidney development and could predispose to higher blood pressures later in life.

Introduction

Maternal smoking during pregnancy is associated with complications such as spontaneous abortion, placental abruption, and *placenta previa* [1]. Smoking also adversely influences fetal development. The average birth weight is 150-300 g lower compared to

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non-exposed offspring and the relative risk of "small for gestational age" (SGA), preterm birth, or neonatal mortality is significantly increased [1-3]. The epidemiological magnitude of maternal smoking is illustrated by the data from the UK-Millennium cohort [4]. This study showed that 36% of British infants had been exposed to maternal smoking before birth. In the US, one out of five pregnant women below age 20 years smokes during pregnancy [5]. Maternal smoking influences offspring organ development. Animal experiments documented that prenatal tobacco smoke or nicotine exposure adversely affects fetal development of lungs, pancreas, and central nervous system [6-8]. Children of smoking mothers more commonly have attention deficits or a decreased intelligence quotient [9]. Furthermore, an association between prenatal exposure to smoke and increased blood pressure in early childhood and adult life has also been reported, although the data are not completely consistent [10-13]. We reasoned that some of the adverse cardiovascular effects could be related to abnormal development, including possible nephron under-dosage effects [14]. To address this hypothesis, we established an animal model.

Subjects and Methods

Experimental Protocol

The animal experiment board of the Medical University of Silesia in Katowice, Poland approved the study along American Physiological Society animal-care guidelines. Ten female Sprague Dawley rats aged 3 months were mated according to the harem protocol with 1 male rat [15]. Pregnancy was diagnosed when semen was detected in the vaginal smear on day 1 after mating. The animals were then randomly allocated to two arms. One treatment consisted of solvent vehicle (n=5 pregnant rats). The alternative treatment (n= 5 pregnant rats) consisted of cigarette-smoke condensate 10 mg containing nicotine 1 mg (CSC). Cigarette smoke (Voivodship Sanitary and Epidemiological Station; Lodz, Poland) was dissolved in acetone containing nicotine (Sigma-Aldrich; Poznan, Poland) and applied to the oral mucosa twice-daily using cotton flocks. These amounts on a weight basis roughly correspond to the daily load of cigarette smoke and nicotine by pregnant women who are moderate smokers [6]. The treatments were begun on day 10 of pregnancy and were continued until delivery. The pregnant dams were housed singly and were kept at a constant room temperature (21C°) and humidity (50%) with exposure to a 12 h light on, 12 h light off cycle. The rats were fed standard pellets (Feed Lab., Poland) according to a pair-feeding protocol. One day before the expected delivery, pregnant rats were placed in metabolic cages for 24 h urine collection. Subsequently, urine cotinine and albumin concentrations were measured.

The pups were weighed 48 h after delivery. After delivery the pups were kept with their mothers until weaning at 4 weeks. Thereafter, male and female offspring were kept in separate cages and weighed weekly. Rat chow and water were provided ad libitum. Twelve weeks after delivery the experiment was terminated. At that time, blood and urine measurements were carried out and systolic blood pressure was measured by the tail plethysmography under light halothane anesthesia. Hematocrit and serum creatinine concentration were measured by standard methods. Urine albumin excretion was measured using a rat specific ELISA (ICL Inc. Newberg, USA). Urine cotinine excretion was determined by mass spectroscopy (Gemeinschaftspraxis Limbach, Heidelberg, Germany). At sacrifice, we performed retrograde aortic perfusion with 3% glutaraldehyde for morphometric and stereological investigations. The kidneys were weighed and dissected in a plane perpendicular to the interpolar axis, yielding slices of 1 mm width. Ten small pieces of one kidney were selected by area weighted sampling for embedding in Epon-Araldite. Tissue slices were also embedded in paraffin; 4 μm sections were prepared and stained with periodic acid Schiff (PAS). Five of the resin blocks were randomly chosen, from which semithin sections (0.5 μm) were prepared and stained with PAS.

Quantifications

Area (A_A) and volume density (Vv) of the renal cortex and medulla as well as the number of glomeruli per area (N_A) were measured using a Zeiss eyepiece (Integrationsplatte II; Zeiss Co., Oberkochen, Germany) and the point counting method ($P_P=A_A=V_V$) at a magnification of X400. All glomeruli of one PAS section were counted to calculate glomerular volume density, area density, and tuft volume. This PAS section represents

several equidistant slices of the kidney and gives thus a representative sample of all kidney areas. The number of glomeruli per area (N_A) was then corrected for tissue shrinkage (1.08^2). Total cortex volume (V_{cortex}) was derived from kidney mass divided by specific weight of the kidney (1.04 g/cm^3). Glomerular geometry was analyzed as follows: volume density (V_V) of glomeruli and tubule interstitium as well as the area density of the glomerular tuft (A_{AT}) were measured by point counting according to $P_P=A_A=V_V$ at a magnification of X400 on the entire PAS sections. The total area of the glomerular tuft (A_T) was then determined as $A_T = A_{AT} X A_{cortex}$. The number of glomeruli per volume (N_V) was derived from the glomerular area density (N_A) and the volume density (V_V) of glomeruli using the formula: $N_V = k/\beta \times N_A (1.5)/V_V (0.5)$ with k = 1.1 (size distribution coefficient) and β = 1.382 (shape coefficient for spheres). The total number of glomeruli was calculated from the total volume of the renal cortex and the number of glomeruli per cortex volume: $N_{glom} = N_V \times V_{Cortex}$. The mean glomerular tuft volume was determined according to $v = \beta/k \times A_T (1.5)$ with β =1.382 and k = 1.1 [16]

Tubulo-interstitial and vascular damage was assessed on PAS stained paraffin sections at a magnification of X100 using a semi-quantitative scoring system [17]. For determination of the tubulo-interstitial damage score, ten fields per kidney were randomly sampled and the changes (0–4) were graded as follows: grade 0, no change; grade 1, lesions involving less than 25% of the area; grade 2, lesions affecting 25 to 50%; grade 3, lesions involving more than 50%; grade 4, involving (almost) the entire area. Similarly, for the vascular damage score, the following scheme was adopted: grade 0, no wall thickening; grade 1, mild wall thickening; grade 2, moderate wall thickening; grade 3, severe wall thickening; grade 4, fibrinoid necrosis of the vascular wall.

On five semithin sections per animal, glomerular capillarization and cellularity were analyzed using the point counting method and a 100 point eyepiece (Integrationsplatte II; Zeiss Co.) at a magnification of X1000 (oil immersion) as described previously [16]. Briefly, the length density (L_V) of glomerular capillaries was determined according to the standard stereological formula, $L_V = 2Q_A$ (with Q_A being the number of capillary transects per area of the capillary tuft) [16]. The total length of glomerular capillaries per one kidney (L_{total}) was then derived from LV and the total glomerular volume (V_{glom}) with $V_{glom} = V_{Vglom} \times Vcortex$ [16]. Glomerular cells (podocytes, cells within the mesangium, and endothelial cells) were assessed by stereological techniques in at least 30 randomly selected glomeruli per animal from cell density per volume (Nc_V) and volume density of the cell type (Vc_V) according to this equation: $Nc_V = k/\beta \times Nc_A (1.5)/Vc_V (0.5)$ with β for podocytes = 1.5 and for cells within the mesangium and endothelial cells = 1.4 and k = 1 [16]. However, it should be mentioned that in this study based on morphologic criteria, we were not able to distinguish between resident mesangial cells and infiltrating mononuclear cells. We used a micropublisher 3.3 RTV camera (Qimagine Inc. Surrey, Canada) with Image-Pro Plus version 5.1 software (Media Cybernetics, Inc; Bethesda, USA). Tissue blocks from 2 male and 2 female offspring in either group (saline controls and cigarette smoke) exposed were analyzed. Three slices from each kidney were randomly selected and the diameters of all detected glomeruli were measured. In total 2920 and 2843 glomeruli were measured in the exposed and in control groups, respectively.

Statistical Analysis

Results are given as mean ± SD. Normally distributed data were analyzed using T-test, non-normally distributed data were analyzed using Mann-Whitney U test. Sex distribution analyses in offspring were done with chi^2 test. Differences were considered significant when the probability of error (p) was < 0.05.

Results

There were no significant differences between dams in either group (Table 1). Food intake during the entire pregnancy was stable and because of pair feeding was not different between groups. Cotinine excretion in the urine of cigarette smoke exposed pregnant rats was 753±239 vs 31±25 μg/l in control dams. Pregnancy duration, litter size, offspring sex distribution, and body weight at 48 h did not differ between the groups (Table 2). Body weight gain during the subsequent 12 weeks was similar in both male and female offspring. At 12 weeks, there were no significant differences with respect to body weight, kidney weight, kidney/body-weight ratio, and heart weight. Systolic blood pressure was higher in

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Table 1: Characteristic of mother rats exposed to cigarette smoke extract and controls. Body weight and systolic blood pressure were measured before conception. Urinary albumin excretion rate was measured day before expected delivery. Serum creatinine concentration as well as morphometric parameters assessed 12 weeks after delivery (mean ±SD)

	Exposed group	Control group
	n=5	n=5
Body weight [g]	311±14	317±15
Systolic blood pressure [mmHg]	108±3	107±9
Serum creatinine concentration [µmol/l]	42.4±10.2	42.1±4.3
Creatinine clearance [µl/min]	1510±410	1488±450
Urinary albumin excretion rate [mg/24h]	0.94±0.23	1.01±0.31
Kidney weight [g] of left kidney	1.19±0.22	1.14±0.21
Number of glomeruli [n] per kidney	44466±5612	46235±6321
Glomerular volume [10^6 µm^3]	1.41±0.15	1.34±0.17

Table 2: Pregnancy outcomes in rat exposed to cigarette smoke extract and controls (mean ±SD)

	Exposed group	Control group
	n=5	n=5
Pregnancy duration [days]	22.8	22.8
Offspring number per litter [n]	10.8±3.7	10.2±2.9
Male offspring litter [n]	4.4±2.2	5.2±2.0
Female offspring litter [n]	6.4±2.3	5.0±1.9
Male newborn offspring body weight [g]	8.4±1.3	8.7±1.3
Female newborn offspring body weight [g]	8.5±1.6	8.4±1.5

Table 3: Findings 12 weeks after delivery in male and female offspring of pregnant rats exposed to cigarette smoke extract and controls (mean±SD)

	Offspring			
	Exposed group		Control group	
	Male	Female	Male	Female
	n=22	n=32	n=26	n=25
Body weight [g]	363±43	257±24	361±40	255±22
Kidney weight[(g]	1.51±0.20	0.94±0.14	1.60±0.30	1.00±0.16
Kidney weight/body weight ratio	0.004±0.0004	0.004±0.0005	0.004±0,0005	0.004±0.0005
Heart weight [g]	1.13±0.16	0.84±0.11	1.14±0.15	0.86±0.11
Systolic blood pressure [mmHg]	125±7*	121±7*	118±7	113±9
Urinary albumin excretion rate [mg/24h]	0.90±0.97	0.65±0.74	0.90±0.70	0.65±0.34
Sodium excretion rate [µmol/24h]	531±237†	531±251†	877±422	812±344
Potassium excretion rate [µmol/24h]	2509±765	710±1095	3033±1152	2527±2942
Calcium excretion rate [µmol/24h]	27±10	22±18	20±14	25±14
Serum creatinine concentration [µmol/l]	42.2±5.4	49.8±4.5	46.9±9.3	46.9±7.9
Creatinine clearance [µl/min]	1601±490	1050±201	1554±591	1139±379
Haematocrit value [%]	44.3±2.9	44.4±6.6†	45.4±3.3	39.5±3.1

† $p < 0.01$; *$p < 0.001$, exposed vs control (male vs male; female vs female)

male and female offspring of mothers exposed to CSC compared to control ones (Table 3). There was no significant difference in 24 h urinary-albumin excretion rate, serum creatinine concentration, or creatinine clearance. Female offspring of CSC-exposed dams had higher hematocrits than controls, which was not the case in male offspring (Table 3).

Table 4: Morphometric parameters in male and female offspring of pregnant rats exposed to cigarette smoke extract and controls – findings at 12 weeks of age (mean±SD)

	Offspring			
	Exposed group		Control group	
	Male	Female	Male	Female
	n=22	n=32	n=26	n=25
Glomeruli number (per left kidney)	53323±7313*	45523±9325	47107±5056	45083±5301
Glomerular volume [10^6 μm^3]	1.33±0.28†	1.03±0.18*	1.50±0.30	1.33±0.19
Total glomerular volume [mm^3]	6.94±1.4	4.65±1.14*	7.21±1.76	6.04±1.36
Tubulointerstitial damage index	0.06±0.03	0.05±0.04	0.06±0.04	0.06±0.05
Vascular damage index	0.04±0.03	0.04±0.04	0.04±0.05	0.04±0.04
	n=6	n=9	n=6	n=10
Podocyte number per glomerulus [n]	106±10†	96±14†	144±21	147±16
Mean podocyte volume [μm^3]	771±99	778±88	722±148	723±94
Number of cells within mesangium per glomerulus [n]	337±50†	320±41*	451±116	445±100
Endothelial cells number per glomerulus [n]	192±48†	186±41†	242±52	222±47
Mean endothelial cells volume [μm^3]	223±140	179±80	195±63	211±98
Capillary length density [mm/mm^3]	5824±430	6392±553	6119±737	6457±855

† p<0.01; *p<0.001, exposed vs control (male vs male; female vs female)

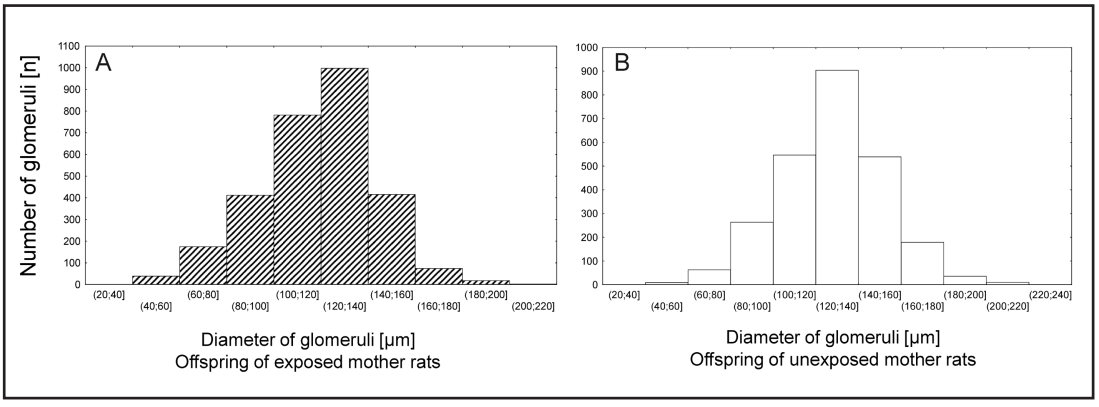

Fig. 1. Distribution of glomerular diameter in 12 week-old offspring of pregnant rats exposed to cigarette smoke extract (n=2920) (A) and controls (n=2843) (B).

Kidney morphometry

Male offspring from the CSC group had significantly more glomeruli than male offspring from the control group. In female offspring, the numbers were not different. Male and female offspring from the CSC group had smaller glomerular diameters and volumes than controls (Fig. 1-3). A representative cortical section is shown (Fig. 2). A highly significant reduction in podocyte number per glomerulus was seen without any change in mean podocyte volume in both male and female offspring in the CSC group, compared to controls. Furthermore, a significant reduction in mesangial cell and endothelial cell number per glomerulus was found, while the mean endothelial cell volume and the capillary length density were unchanged (Table 4). There were no signs of interstitial fibrosis in any group and the vascular damage indexes were not different.

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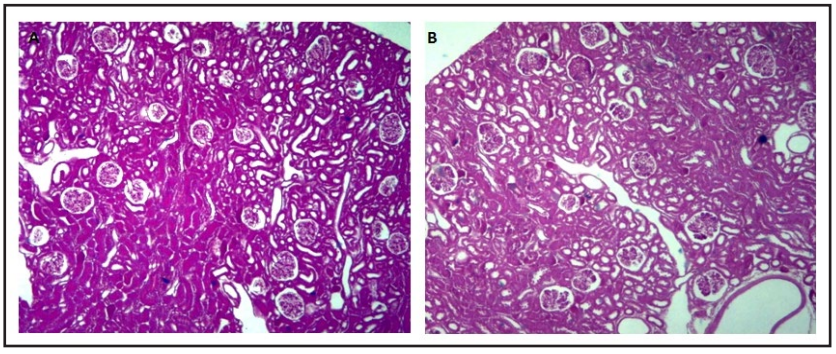

Fig. 2. Representative section of cortex in 12 weeks old offspring of pregnant rats exposed to cigarette smoke extract (A) and controls (B) (PAS stained, magnification 40x).

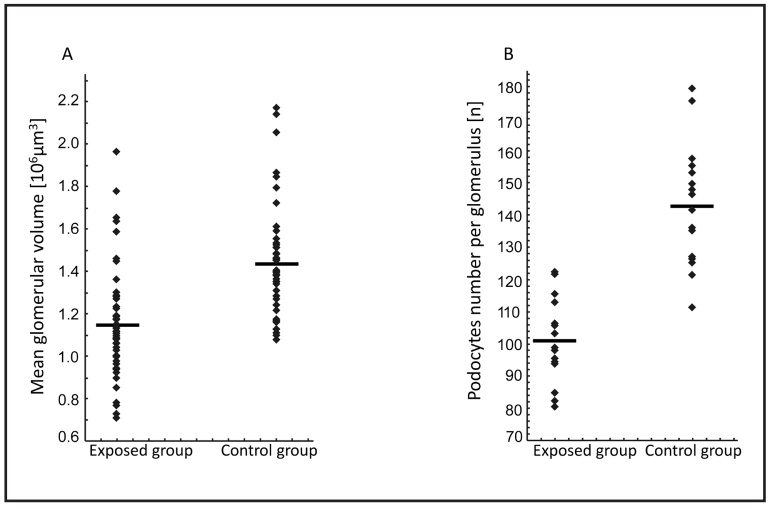

Fig. 3. Distribution of mean glomerular volume (A) and podocytes per glomerulus (B) in 12 weeks old offspring of pregnant rats exposed to cigarette smoke extract and controls. Black bars show a mean value. p<0.001 exposed vs control.

Discussion

The important findings in this study are that cigarette-smoke extract containing nicotine interferes with kidney development in the offspring. Furthermore, the developmental abnormalities could foster the development of high blood pressure at a later age. Our study design addressed important confounders, including differences of food intake, differences in litter size, and subsequent growth rate of the offspring. We found no differences in albuminuria or glomerular filtration rate. However, we observed our offspring only at a single time point, age 12 weeks. Rats live >100 weeks and subsequent changes dictated by differences in glomerular size and cell number could very well have occurred at later time points.

Unlike what had been seen in other models of impaired fetal programming, such as protein malnutrition [18], hyperglycemia in dams [19], and others models, the number of glomeruli per kidney was not reduced. However, we found marked abnormalities of glomerular volume and numbers of glomerular cells. Although the capillary length and density were not changed, the number of endothelial cells was reduced. We noticed sex differences in number of glomeruli per kidney in offspring exposed *in utero* to CSC. Sex-dependent differences in fetal programming were also reported previously [20]. For example, Woods at al found that modest maternal protein restriction fails to influence glomeruli number per kidney and blood pressure in female rats but was sufficient to evoke a decreased number of glomeruli as well as hypertension in male rats. This effect was also observed in female rats but only after a more severe protein restriction in the dams. Thus, in female offspring a stronger nutritional insult may be required to generate the same change in nephrogenesis.

The renin-angiotensin system plays pivotal role in kidney development. If blocked by angiotensin receptor antagonist [21] nephron under-dosing is observed. Smoking is a potent activator of the renin angiotensin system. Fetal and neonatal exposure change also pattern of expression of angiotensin receptors, increasing AT_1R/AT_2R ratio [22]. Xiao at al. found that prenatal nicotine significantly increased angiotensin II–stimulated blood pressure in male but not female offspring [23]. Possibly, exaggerated activation of renin angiotensin system by CSC may have been responsible for the changes in glomerular number we observed. Further studies would be necessary to support such an interpretation since we measured no renin-angiotensin-system related variables here and did not address that hypothesis directly.

In the offspring of mother rats exposed to CSC, we measured higher systolic blood pressures by tail plethysmography at 12 weeks. We interpret these findings with caution, since we do not have repetitive measurements and critical observers would expect measurements with radiotelemetry. However, if confirmed by invasive methods the finding would be of considerable interest in the context of the recent human epidemiological data [24]. We found decreased numbers of endothelial cells per glomerulus and the glomeruli were smaller. Thus, the glomerular filtration coefficient may have been reduced, thereby shifting pressure-natriuresis rightward. Furthermore, the regulation of sodium homeostasis via nitric oxide signaling from endothelial cells is well established [25]. Other potential mechanisms that could have contributed to the blood pressure increase observed in our experiment are increased plasma concentration of reactive oxygen species and increase activity of sympathetic nervous system, perhaps as influenced by renalase.

In humans, the evidence that smoking is deleterious to renal health is overwhelming, particularly in diabetic patients [26]. Furthermore, smoking accelerates the rate of chronic kidney disease progression [27]. Finally, in diabetic patients cigarette smoking adversely affects glomerular structure [28]. Even idiopathic nodular glomerulosclerotic lesions resembling Kimmelstiel-Wilson glomerulosclerosis have been found in nondiabetic smokers [2, 29]. In renal-damage models of adult rats, renal injury is aggravated by exposure to nicotine [30] or cigarette smoke extract, irrespective of the insult [31]. We believe that our precise morphometric quantitation is a strong point in our study. Observations on the effects of maternal cigarette smoking during pregnancy were commonly qualitative in nature [32]. In this context, the observations of Mañalich et al. are interesting [33]. They studied premature infants that died of hyaline-membrane disease. These infants had fewer glomeruli (by coronal sections) and half their mothers were heavy smokers.

We suggest that the fewer numbers of cells in the glomeruli of our CSC offspring could have resulted from decreased cell proliferation or increased apoptosis. Nelson et al. [32] found that newborn rats from dams exposed to cigarette smoke during pregnancy had increased apoptosis of renal cells. Others have also observed increased apoptosis in various tissues [34]. There are human data supporting hypothesis. Lampl et al. used longitudinal ultrasound observations of fetal kidney growth after cigarette smoke exposure *in utero*. The smoke-exposed fetal kidney was wider and thicker compared to unexposed kidneys during the second and early third trimester. However, the ratio of kidney volume to fetal weight was lower, compared to unexposed fetuses [35]. Taal et al. recently reported similar observations [36].

Past studies showed that nicotine has adverse effects on progression of renal injury [37] and that nicotine influences kidney weight [22, 38]. Adverse effects on blood pressure have also been described in spontaneously-hypertensive rats [38]. We found no effects on birth weight or kidney weight; however, we modeled “moderate” smoking and the doses in these studies were substantially higher than in our study [22, 38]. Furthermore, we are unable to attribute our findings solely to nicotine, since other components in our extract may have been responsible. This distinction is important, since nicotine patches are offered to young women during pregnancy clinically [39].

Reduced renal mass from a lesser nephron number or presumably a smaller glomerular size could contribute to a higher likelihood of chronic kidney disease. For instance, Argueso et al. studied 157 patients, aged mean 37 years, who were born with unilateral agenesis and

a normal contra-lateral kidney. Renal failure and death developed in 6%, while 13% had reduced renal function, 19% had proteinuria >150 mg/day, and 47% had high blood pressure [42]. These observations contrast with the experience that kidney loss in adulthood does not seem to cause progressive renal dysfunction. Narkon-Burgess followed 62 American servicemen who had lost one kidney during World War II. Twenty-four years later, they had completely normal renal function [43]. A similar group of kidney donors observed for 20 years failed to show evidence of progressive renal damage [44]. To answer the question whether or not glomerular changes caused by exposure to cigarette smoking in fetal life have an impact on subsequent kidney function, long-term observations are necessary. Another important question is whether or not offspring of smokers are more susceptible to superimposed kidney damage, remains unanswered.

There are limitations to our study. We fixed the kidneys with glutaraldehyde perfusion, which is ideally suited to quantification. However, we were not able to conduct stains for surface cell markers or apoptosis determinations. We conducted a single-endpoint observation in our offspring at a juvenile age and are solely able to speculate what may have developed later. Serial blood pressure measurements, preferably with radiotelemetry should be performed. We were not able to address important mechanism in our study, including the behavior of renal growth regulators, the renin-angiotensin-aldosterone axis, reactive oxygen-related mechanisms, or nitric oxide. Nonetheless, we believe there are important perspectives that should be followed on the basis of our findings, particularly since this solely environmental cause of renal and cardiovascular injury is entirely preventable. We will pursue the limitations in our study with future studies of this nature.

Conclusion

This study provides data that kidney development could be affected by maternal smoking. Rat offspring exposed to smoke condensate *in utero* had decreased mean glomerular volume, fewer glomerular cells, and higher systolic blood pressures, compared to control offspring.

Conflict of Interests

No conflicts of interest declared.

Acknowledgments

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