Renal fibrosis in diabetic and aortic-constricted hypertensive rats

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1Departamento de Fisiología y Farmacología, Instituto Reina Sofía de Investigación Nefrológica, and 2Departamento de Anatomía e Histología Humanas, Universidad de Salamanca, 37007 Salamanca, Spain

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Gallego, Belén, Miguel A. Arévalo, Olga Flores, José M. López-Novoa, and Fernando Pérez-Barrioncal. Renal fibrosis in diabetic and aortic-constricted hypertensive rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R1823–R1829, 2001.—To assess if the renal damage observed in rats with diabetes and hypertension is due to hemodynamic or metabolic changes, a progressive aortic constriction between the two renal arteries has been done in streptozotocin-induced diabetic rats (constriction + diabetes group) and in nondiabetic rats (constriction group). This model allows us to study two kidneys subjected to different perfusion pressure (PP) in the same metabolic environment. One-month-old rats (100–120 g body wt) were subjected to the aortic constriction procedure. Three months after constriction, glomerular filtration rate and renal plasma flow were similar in both kidneys of the two groups. PP was greater in the kidney placed over the ligature (constriction + diabetes high-pressure kidney (DH) or constriction + diabetic high-pressure kidney (DH)) than in the one placed below the ligature (constriction low pressure (CL) or constriction + diabetic low pressure (DL)). Proteinuria was higher in the CH than in the CL kidneys (512 ± 61 vs. 361 ± 38 μg/30 min, respectively) and much higher in the DH kidney (770 ± 106 μg/30 min). Renal fibrosis was measured in tissue sections stained with Syrius red using a computer-assisted image analysis system. DH and DL kidneys showed higher corpuscular cross-sectional and capillary tuft areas than the CH and CL ones. The DH kidney showed slight mesangial expansion and thickening of the capillary walls, which were more pronounced in the former. Most renal corpuscles from CH and DH groups were nearly normal in morphology appearance, and only in some instances a slight increment in mesangium was observed. Transforming growth factor-β1 (TGF-β1) immunostaining revealed that DH kidneys showed the highest glomerular expression. We concluded that 1) diabetic animals develop glomerular but not interstitial fibrosis to a greater extent than nondiabetic animals and that this lesion principally occurs in the hypertensive kidney (DH), and 2) increased TGF-β expression is associated with diabetic renal damage.

diabetes mellitus; renovascular hypertension

DIABETIC NEPHROPATHY is a major cause of chronic renal failure. More than 40% of the patients starting dialysis treatment in United States in the period 1993–1997 were diabetic, and the prevalence of diabetics in dialysis in 1999 was ~40% (20). Although the prevalence of diabetes in chronic renal failure is lower in other industrialized countries, it is continuously increasing and, in most of them, is the major cause of chronic renal failure (21). There is a frequent association of diabetes and hypertension (27). The patients with this association have a higher risk of developing chronic renal failure and a poorer prognosis than patients with diabetes alone (6, 19). The precise mechanisms of worsening of renal function in diabetes + hypertension are not fully understood. Some studies on this topic have been performed by inducing one-clip, two-kidney Goldblatt hypertension in diabetic rats (15, 23). However, this model of renovascular hypertension induces an abrupt fall in blood pressure to the ligated kidney, a fact that does not occur in spontaneous renovascular hypertension, where renal perfusion pressure decreases progressively. Here we used a model of experimental renovascular hypertension induced by restricting aortic growth between the renal arteries in small rats. The characteristic feature of this model is a slow and progressive increase in constriction, thus avoiding abrupt changes in renal perfusion pressure (12).

We combined streptozotocin (STZ)-induced diabetes with the model described above and attempted to distinguish between the renal functional and structural alterations due to hypertension and those due to diabetes, since the model allowed us to have two kidneys in the same metabolic milieu: one exposed to hypertension and the other not. In addition, the model allows the measurement of perfusion pressure and renal function in each kidney. Because diabetic nephropathy is characterized by glomerular volume increase (2, 16) and mesangial expansion (9, 28), we have studied the effects of diabetes and hypertension on glomerular cross-sectional area and mesangial expansion in kidneys with diabetes and high perfusion pressure (DH) and diabetes and low perfusion pressure (DL), compared with kidneys without diabetes and high perfusion pressure (CH) or low perfusion pressure (CL).

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Transforming growth factor-β1 (TGF-β1) plays a central role in both glomerular hypertrophy and mesangial expansion (3, 25, 26), and its increased expression has been demonstrated in experimental diabetes (24, 25). We have studied TGF-β1 expression in the kidneys with or without diabetes subjected to high or low perfusion pressure.

MATERIALS AND METHODS

Animals, experimental procedure, and groups. The study was performed in male Wistar rats (Charles River, Barcelona, Spain), 1 mo old, weighing 100–120 g, with a mean arterial pressure (MAP) of 122 ± 3 mmHg measured in awake animals with a tail cuff method (Electrophigmomanometer, LE 5100; Letica, Barcelona, Spain), a blood glucose level of 1.38 ± 0.06 g/l, and a packed cell volume (PCV) of 46 ± 3%. Rats were fed standard rat chow (Panlab, Madrid, Spain) and water ad libitum. Diet composition was salt: 5.5%, protein: 16.1%, carbohydrate: 58.5%, and fat: 2.65%. Lighting and temperature were controlled by a timer that permitted light between 0800 and 2000 and a temperature of 20 ± 1°C. Animals were handled was performed according to the Guide for the Care and Use of Laboratory Animals (30).

The animals were subjected to surgery as previously described (12). In brief, rats were anesthetized with 2,2,2-trifluoroethanol (25 mg·ml⁻1·100 g body wt⁻¹), Sigma Chemical, Madrid, Spain), a median laparotomy was performed, and a silk ligature (4/0) was placed around the aorta below the right and above the left renal artery. A polyethylene tube (PE-50; OD: 1.0 mm) was placed next to the aorta, and the ligature was tied tightly around the tube and the aorta. Then, the tube was removed, allowing the aorta to reexpand inside the ligature. Because the diameter of the tube was slightly greater than that of the aorta, no initial constriction of the aorta was produced with this procedure. However, as rats grew, the aortic constriction increased progressively. The abdominal wall was carefully closed, and the rats were allowed to recover in the animal facilities of Salamanca University in the same conditions as previously described. The surgical time was ~10 min.

Two weeks later, rat body weight was 151 ± 4 g, MAP was 121 ± 4 mmHg, and blood glucose level was 1.40 ± 0.06 g/l. Then, in one-half of animals randomly chosen, insulin-dependent diabetes was induced by a single intraperitoneal injection of STZ (Sigma Chemical, St. Louis, MO), 60 mg/kg body weight was 453 ± 8 and 372 ± 7 g (P < 0.05) in constriction and constriction + diabetes groups, respectively; their blood glucose levels were 1.37 ± 0.06 and 3.48 ± 0.04 g/l (P < 0.05) in constriction and constriction + diabetes groups, respectively; and PCV was 51 ± 0.2 and 51 ± 0.4% in constriction and constriction + diabetes groups, respectively.

Rats were surgically prepared for clearance studies as previously reported (11). Briefly, under pentobarbital sodium anesthesia (50 mg/kg body wt), PE-50 catheters were placed in the carotid and femoral arteries and femoral vein and in both ureters. Carotid and femoral arteries were connected to a pressure transducer for the continuous recording of perfusion pressure of both kidneys. Carotid arterial pressure (CAP) represents the perfusion pressure of the right kidney placed above the ligature, whereas femoral arterial pressure (FAP) represents the perfusion pressure of the left kidney placed below the ligature. [3H]inulin and p-[14C]aminohippuric acid (PAH) were infused continuously intravenously (3 ml/h) in both groups (constricted and constricted + diabetes) to determine the glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively. After a 45-min period for equilibration and hemodynamic stabilization (when the values of blood pressure and heart rate were within a 5% variation), four clearance periods, of 30 min each, were studied.

Urine was collected in preweighed vials containing 0.5 ml of water-stabilized mineral oil. Blood samples (150 μl) were taken at the beginning of each clearance period and at the end of the experiment. The PCV was determined by the microcapillary method. 3H and 14C activities were measured using a two-channel liquid scintillation counter that corrects cross-talk between isotopes (WALLAC 1409 DSA, Turku, Finland). GFR and RPF were measured by the clearance of inulin and PAH, respectively. Renal blood flow (RBF) was calculated from the RPF and PCV. Renal vascular resistance (RVR) was calculated as the CAP-to-RBF ratio for the right kidney and the FAP-to-RBF ratio for the left kidney.

Urine protein concentrations were measured colorimetrically by the Bradford method (4). Sodium and potassium concentrations were assayed by flame photometry (NAK II, Meteor, Madrid, Spain).

Histology studies. After clearance studies, the animals were perfused through the abdominal aorta with isotonic saline to wash the blood out. Then, the kidneys were removed and weighed. A median sagittal cut of each entire kidney was trimmed down to divide the organ in two symmetrical pieces that were then fixed by immersion in 4% buffered formalin for 24 h. The blocks were dehydrated in a graded series of ethanol and embedded in paraffin. For descriptive studies, 3-μm sections were cut by using a paraffin microtome with stainless steel knives. The sections thus obtained were mounted on glass slides, deparaffined with xylene, dehydrated through a graded series of ethanol, and stained with Masson’s trichrome. A slide per each animal was prepared.

For morphometric studies, 5-μm sections were cut and stained with Syrirus red (13). Images were captured by a high-resolution videocamera (SONY ccd-iris) connected to a light microscope (Leitz Laborlux S) using a 20× objective and a green optical filter (IF 550). Evaluation and image analysis procedures were performed with specific software (5, 13). Briefly, Syrirus red mainly stains collagen fibers, and the program automatically transforms the red-colored areas into specific gray levels and quantifies the area of these elements in the image previously isolated from the background. In the case of glomerular images, it was necessary to split the corpuscular area by indicating on the monitor where the glomerulus was located. Then, the program automatically discriminates the area of renal corpuscles, which is usually surrounded by a Syrirus red-stained pericapsular coat of fibers. To further increase the precision of the method, the same person in a “blind” fashion always captured images.

From 10 rats per group, a total of 50 corpuscular (25 outer and 25 inner) and 15 interstitial images were captured from each kidney and processed. The parameters measured were...
I) corpuscular area, 2) capillary tuft area, 3) glomerular fibrosed area, and 4) area of interstitial fibrosis.

TGF-β1 expression. Three-micrometer-thick sections were deparaffinized and rehydrated. Sections were pretreated with 3% H₂O₂ to block endogenous peroxidase. In addition, sections were digested with pepsin at 37°C for 20 min. Sections were next incubated in protein-blocking serum for 60 min at room temperature. This was followed by incubation with anti-rabbit TGF-β1 antiserum (Santa Cruz Biotecnology, Heidelberg, Germany) for 60 min at room temperature. This was followed by incubation with anti-rabbit TGF-β1 antiserum (Santa Cruz Biotecnology, Heidelberg, Germany) for 60 min at room temperature washed in phosphate-buffered saline and by incubation with biotinylated immunoglobulin and avidin-biotin complex. Peroxidase conjugates were localized using diaminobenzidine as a chromogen. Sections were then counterstained with hematoxylin and examined by two independent observers blinded to the disease status of the kidney. Immunostaining was scored in 50 glomeruli and 25 interstitial fields (20×) for slides on a scale of 0–4, where 0 = no staining and 4 = maximum staining. Negative controls were included omitting the primary antibody and replacing it with normal IgG at an equivalent protein concentration.

Statistical analysis. The Kolmogorov-Smirnov test was used to assess the normality of data distribution. Results are expressed as means ± SE for parametric data and as medians and ranges for nonparametric data. Statistical analysis was performed by one-way (ANOVA 1) or two-way (ANOVA 2) analysis of variance for group comparisons followed by Scheffe’s test when the data were normally distributed and the Kruskal-Wallis test when they were not normally distributed. A P value <0.05 and a z value >1.96 were considered significant.

RESULTS

The data on kidney weights at the end of the study are shown in Fig. 1A. Both kidneys had a similar weight in the two groups, but in the diabetic group both kidneys weighed more than in the control group.

Perfusion pressures are shown in Fig. 1B. CAP was higher than FAP in both groups, and the gradient of arterial pressure (CAP-FAP) did not differ significantly in the two groups.

Diuresis (Fig. 1C) and urinary sodium excretion were higher in the CH and DH kidneys (0.056 ± 0.012 and 0.068 ± 0.009 meq/30 min, respectively) than in the CL and DL ones (0.022 ± 0.006 and 0.038 ± 0.008 meq/30 min, respectively, P < 0.05). Urinary potassium excretion was also higher in CH and DH kidneys (0.042 ± 0.006 and 0.043 ± 0.003 meq/30 min, respectively) than in the CL and DL ones (0.029 ± 0.003 and 0.028 ± 0.003 meq/30 min, respectively, P < 0.05).

Table 1 shows GFR and RPF in the kidneys studied. GFR was similar in the CH and CL and in the DH and DL kidneys. Although GFR was higher in both kidneys of diabetic rats than in those of control rats, these differences did not reach statistical significance. No differences in RPF between kidneys or between groups were observed. The RVR values were higher in CH kidneys than in CL, but the difference did not reach statistical significance. In the constriction + diabetes group, the RVR values were similar in both kidneys.

Urinary protein excretion (Fig. 1D) was higher in CH than in CL. Proteinuria in DH was much higher than in CL. Proteinuria in DH was much higher than in CL. Proteinuria in DH was much higher than in CL. Proteinuria in DH was much higher than in CL. Proteinuria in DH was much higher than in CL. Proteinuria in DH was much higher than in CL. Proteinuria in DH was much higher than in CL.

Table 1. Renal function parameters for both kidneys in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>RPF, ml/min</th>
<th>GFR, ml/min</th>
<th>RVR, mmHg·min⁻¹·ml</th>
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<tbody>
<tr>
<td>Control (n = 23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>5.0 ± 0.6</td>
<td>1.33 ± 0.08</td>
<td>28.3 ± 3.9</td>
</tr>
<tr>
<td>Low</td>
<td>4.9 ± 0.4</td>
<td>1.18 ± 0.08</td>
<td>21.7 ± 3.2</td>
</tr>
<tr>
<td>Diabetic (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>5.5 ± 0.6</td>
<td>1.62 ± 0.14</td>
<td>20.1 ± 3.7</td>
</tr>
<tr>
<td>Low</td>
<td>5.0 ± 0.5</td>
<td>1.51 ± 0.17</td>
<td>19.3 ± 3.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. RPF, renal plasma flow; GFR, glomerular filtration rate; RVR, renal vascular resistance.
Histology studies. Light microscopy study in tissue sections stained with Masson’s trichrome revealed no dramatic alterations in any group; however, slight differences were seen as the groups were compared. Thus DH and CH kidneys showed slight mesangial expansion and thickening of capillary walls, which were more pronounced in the former (Fig. 2B); in this group, dilated capillary lumens were also observed in some instances. Most renal corpuscles from CH and DH groups were nearly normal in morphology appearance (Fig. 2A), and only in some instances a slight increment in mesangium was observed.

DH and CH groups showed, in general, dilated tubular lumens and more flattened epithelial cells than those in DL and CL tubular walls. Tubular basement membrane thickening was observed in all groups, but it was more remarkable in DH and DL groups.

Morphometric results. Because glomerulosclerosis is a focal phenomenon and because it is possible to observe glomeruli with different degrees of lesion, the results of the image analysis study were expressed in frequency distribution diagrams to obtain information on data dispersion. The frequency distribution diagram of the corpuscular cross-sectional area for outer (Fig. 3A) and inner (Fig. 3B) nephrons revealed that corpuscular size was larger in the DH than in the DL kidney of constriction + diabetes group and larger in both kidneys of this last group than in the kidneys of constriction group. The frequency distribution diagram of the capillary tuft area for outer nephrons (Fig. 4A) showed that the DH kidney in the diabetic group had the highest number of glomeruli with larger capillary tuft areas. For the inner nephrons (Fig. 4B), both kidneys of the diabetic group showed more glomeruli with larger capillary tuft areas than the constriction group. Glomerular fibrosed area of outer and inner glomeruli is represented in Fig. 5, A and B. It was higher in the two kidneys of the constriction + diabetes group than in the constriction group, and no differences were found between the two kidneys in each group.

Figure 6 represents interstitial fibrosis area. The number of fields with larger fibrosis areas was higher in the CL and DL kidneys, especially in the DL kidney of the constriction + diabetes group.
Table 2 shows a review of morphometric results expressed as means ± SE.

**TGF-β1 expression.** Glomerular TGF-β1 immunoreactivity was found in podocytes and mesangial cells in all the kidneys studied, but the highest expression was found in the DH kidney (Fig. 2C). No interstitial expression of TGF-β1 was observed in the kidneys of any group. No staining was observed in negative control (Fig. 2D).

**DISCUSSION**

This study was designed to assess whether the functional and structural alterations observed in diabetic and hypertensive rat kidneys are due to the metabolic alterations of diabetes or to the increase in renal perfusion pressure, because the model used in the present study presents in each animal two kidneys in the same metabolic environment but with different perfusion pressures. In addition, perfusion pressure for each kidney can be measured.

Urinary protein excretion can be used as an index of the renal lesion associated with diabetes and glomerular sclerosis (18). When we studied urinary protein excretion in each kidney, we found it to be higher in the hypertensive (right) kidney in both groups (CH, DH), although it was much higher in the DH kidneys than in the corresponding controls (CH).

Quantitative measurement of glomerular and interstitial fibrosis reveals certain differences that do not emerge from classical semiquantitative assessments of renal damage. In addition, the quantitative method of measuring the degree of fibrosis gives a good idea of the focal nature of the damage, because frequency distribution reveals the variability in the parameter investigated.

Because diabetic nephropathy is characterized by glomerular volume increase (2, 16) and mesangial expansion (9, 28), we quantified the corpuscular and capillary tuft areas, mesangial expansion, and interstitial fibrosis in the diabetic and control kidneys subjected to high or low blood pressure. Our data reveal that the corpuscular and capillary tuft areas were higher in DH kidneys than in the other kidneys. In the diabetic group, glomeruli and capillary tufts were larger than those of the constriction group. In this group, the CH kidneys also showed higher values of glomerular fibrosis than CL kidneys but only in the inner glomeruli.

The mechanism responsible for the higher degree of glomerular damage in the kidneys of the diabetic rats subjected to high perfusion pressure could involve hemodynamic, metabolic, or hypertrophic changes. The predominance of hemodynamic or metabolic changes in renal disease associated with diabetes has long been
controversial (10, 32). Our data suggest a defective autorregulatory mechanism in the diabetic group, because although RPF and GFR were similar in both kidneys in both groups of animals, the RVR was higher in the CH than in the DH kidney. This defective autorregulatory mechanism in the diabetic group allows the systemic arterial pressure to be easily transmitted to the glomeruli. This lack of autorregulation in the diabetic kidney has been already described (7, 32), and it leads to increases in intraglomerular pressure. Increased intraglomerular pressure is one of the most important factors in the development of progressive glomerular lesion (29) and tubulo-interstitial fibrosis (14). Our experiments also demonstrate that the largest glomeruli and glomerular tufts were observed in kidneys subjected to both hypertension and diabetes (DH). It has been suggested that enlargement of the glomerulus is a major cause of glomerular sclerosis (1). Thus glomerular hypertrophy can play a role in the higher damage rate observed in these kidneys.

Another important result of this study is that interstitial fibrosis was higher in DL than in DH kidneys. This can be explained because a decrease in perfusion pressure resulting from stenosis may cause the hypoperfusion of some segments of renal vasculature, as renal ischemia is a stimuli for tubulo-interstitial lesions. A recent study (17) combining ischemia and diabetes concludes that ischemia causes rapidly progressive kidney damage in diabetic rats, characterized by tubulo-interstitial inflammation and fibrosis, while glomerular atrophy seems to be a subsequent feature. It has been demonstrated that TGF-β1 plays a major role in glomerular extracellular matrix accumulation in diabetic nephropathy (24). In this study, the major glomerular expression of TGF-β1 was observed in the DH kidney, the one with higher proteinuria, glomerular volume, and mesangial expansion. It should be noted that in cultured mesangial cells, high glucose concentrations stimulate mesangial cell production of both TGF-β1 and collagen, whereas neutralization of the cytokine reduces glucose-induced secretion of collagen (33). TGF-β1 also plays a pathogenic role in the increased matrix production induced by mesangial cells’ mechanical strain (22). However, Riser et al. (22) showed that the increase in TGF-β1 activity participated in the mesangial cells metabolic response to the stretching only when glucose concentration was elevated, suggesting that net extracellular matrix accumulation occurs only when glomerular hypertension and hyperglycemia are present. In addition, DH kidneys seem to have a poor autorregulatory response to high pressure, which would cause intraglomerular hypertension, as previously described, and it has been recently reported that increased intraglomerular pressure is associated with increases in TGF-β1 expression (8, 31).

We conclude that diabetic animals develop glomerular fibrosis to a greater extent than nondiabetic animals and that the major lesion has been found in the hypertensive kidneys of diabetic rats. Our experiments suggest that the combination diabetes and hypertension accelerates the development of glomerulosclerosis and that TGF-β1 expression is associated with diabetic and hypertensive glomerular damage.

**Perspectives**

Diabetic nephropathy is now the most common cause of end-stage renal disease in North America and Europe. New evidence suggests that the majority of diabetic patients, whether insulin dependent or not, are developing fibrotic changes in glomeruli that, if they live long enough, will manifest as overt nephropathy. In the case of diabetic nephropathy concurrent with hypertensive disease, the process occurs more rapidly and with increased severity. Potentially, treatment of both diseases together could offer significant protection against the observed fibrotic process. One of the common mechanisms in kidney injury secondary to either diabetes or hypertension is overexpression of the cytokine TGF-β1. Due to this finding, we propose that suppression of TGF-β1 activity may represent a potentially important therapeutic strategy acting as a blocker of the fibrotic process induced by both diabetes and hypertension.

### Table 2. Morphological parameters for both kidneys in each group

<table>
<thead>
<tr>
<th></th>
<th>CH</th>
<th>CL</th>
<th>DH</th>
<th>DL</th>
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<tbody>
<tr>
<td><strong>Outer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpuscular area</td>
<td>9,905 ± 114</td>
<td>9,913 ± 115</td>
<td>11,081 ± 119</td>
<td>10,193 ± 97</td>
</tr>
<tr>
<td>Capillary tuft area</td>
<td>8,386 ± 93</td>
<td>8,422 ± 91</td>
<td>9,614 ± 109</td>
<td>8,915 ± 87</td>
</tr>
<tr>
<td>Glomerular fibrosed area</td>
<td>2,676 ± 52</td>
<td>2,657 ± 52</td>
<td>2,896 ± 53</td>
<td>2,855 ± 55</td>
</tr>
<tr>
<td><strong>Inner</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpuscular area</td>
<td>12,193 ± 165</td>
<td>11,733 ± 164</td>
<td>13,255 ± 172</td>
<td>12,594 ± 151</td>
</tr>
<tr>
<td>Capillary tuft area</td>
<td>10,666 ± 150</td>
<td>9,886 ± 128</td>
<td>11,401 ± 148</td>
<td>10,991 ± 132</td>
</tr>
<tr>
<td>Glomerular fibrosed area</td>
<td>2,684 ± 67</td>
<td>2,401 ± 65</td>
<td>2,987 ± 63</td>
<td>2,916 ± 68</td>
</tr>
<tr>
<td><strong>Interstitial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial fibrosis area</td>
<td>12,357 ± 354</td>
<td>13,726 ± 443</td>
<td>12,707 ± 344</td>
<td>14,188 ± 382</td>
</tr>
</tbody>
</table>

Values are means ± SE. Morphological parameters for constriction high-pressure kidney (CH), constriction low-pressure kidney (CL), constriction + diabetes high-pressure kidney (DH), and constriction + diabetes low-pressure kidney (DL). *P < 0.05 vs. CH kidney in constriction group. †P < 0.05 vs. CL or DL kidney in each group.
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REFERENCES