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Urodynamic and Histological Changes in a Sterile Rabbit Vesicoureteral Reflux Model

Minki Baek[1], Sung Hyun Paick[2], Seong Jin Jeong[3], Sung Kyu Hong[3], Soo Woong Kim[4], and Hwang Choi[4]

Department of Urology[1], Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul; Department of Urology[2], Konkuk University Medical Center, Seoul; Department of Urology[3], Seoul National University Budang Hospital, Seoul National University College of Medicine, Seongnam; Department of Urology[4], Seoul National University College of Medicine and Clinical Research Institute, Seoul, Korea

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Address for Correspondence:
Hwang Choi, M.D.
Department of Urology, Seoul National University Hospital, 101 Daehang-no, Jongno-gu, Seoul 110-744, Korea
Tel: +82.2-2072-3644, Fax: +82.2-742-4665
E-mail: hchoi@snu.ac.kr

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This study aimed to investigate pressure changes of renal pelvis and histological change of kidneys in a surgically induced sterile rabbit vesicoureteral reflux (VUR) model. Five rabbits served as a control group, 7 as the sham-operated group, and 8 served as the VUR group. Three weeks later, urodynamic studies were performed, and histological examinations evaluated degree of inflammation, fibrosis, and tubular damage in the kidneys. At a low infusion rate, renal pelvic pressure in the VUR group was stable until late filling phase and then increased slightly. At a high infusion rate, the renal pelvic pressures of the sham-operated and control groups were stable until late filling phase and then increased slightly, whereas the renal pelvic pressure in the VUR group steadily increased from mid filling phase. Focal thinning of the tubular epithelium and interstitial widening were observed in certain cortical areas of refluxing kidneys, without inflammatory cell infiltration. Obvious changes in the mean diameters of distal tubules and extracellular matrix volume fractions were observed in two highly refluxing kidneys. High pressure reflux with bladder instability may result in renal cortical changes.

Key Words: Vesico-Ureteral Reflux; Urodynamics; Histology

INTRODUCTION

Vesicoureteral reflux (VUR) is a common congenital urologic anomaly characterized by the retrograde transfer of urine from the bladder to the kidney. In the presence of VUR, hydrostatic pressure transmitted from the bladder elevates renal pelvic pressure. Although numerous studies have shown that reflux may cause renal scarring in the presence of urinary tract infections (1-3), the issue of whether sterile reflux causes renal damage has not been definitively resolved. Although the effect of elevated renal pelvic pressure on renal damage in VUR has been the subject of many investigations, to our knowledge, little research has been conducted on the quantitative estimation of elevated renal pelvic pressure in VUR (4).

We sought to investigate changes in cystometric findings and to analyze the interrelationship of bladder and renal pelvic pressures, at different infusion rates, with different levels of bladder fullness, in a surgically induced sterile rabbit VUR model. We also examined histological changes in the kidneys and correlated these with the presence and severity of reflux.

MATERIALS AND METHODS

Animals

Eight-week-old New Zealand female rabbits, 2.5–2.9 kg, were maintained under a dark-light cycle (12 hr dark) with pellet feed ad libitum for 1 week. Ten rabbits served as a control group, 15 underwent opening and closure of the bladder (the sham-operated group), and 15 served as the VUR group (n=40). Among them, adequate animals for data collection were included in the final analysis (n=20). All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee (approval number 07-0101). Korean National Research Council guidelines for the care and use of laboratory animals were observed.

Creation of VUR

Fifteen animals served as the VUR group, 15 rabbits underwent opening and closure of the bladder to form the sham-operated group, and 10 animals served as a control group. In the VUR group, animals underwent surgery for creation of reflux. The

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rabbits were anesthetized with an intramuscular injection of Zoletil (10 mg/kg). After a lower midline incision was made to expose the bladder, the anterior wall of the bladder was longitudinally opened through a 1 cm incision in the midline above the bladder neck. The right ureteral orifice was cannulated with polyethylene tube (PE-10) and the roof of the right intravesical ureter was incised (5). The bladder neck was untouched. The bladder was closed using a locked running stitch of 6-0 Vicryl and the abdominal wall was closed with 4-0 chromic catgut. In the sham-operated group, anesthesia and bladder incision were done as described above but the intravesical ureter was not unroofed. In the control group, rabbits were anesthetized only. After surgery, the animals were given an intramuscular injection of cephalosporin (50 mg/kg), and maintained under a dark-light cycle (12 hr dark) with pellet feed ad libitum for 3 weeks. To maintain sterile urine during the experimental period, sulfamethoxazole/trimethoprim (0.5 mg/mL) was given, mixed in drinking water. Urine cultures were performed at the end of the experimental period to confirm urine sterility.

Urodynamic assessment

Three weeks after surgery, urodynamic studies were performed on the three groups. The experimental technique was as described previously by Smyth and colleagues, with modifications (6). The rabbits were anesthetized with an intramuscular injection of Zoletil (10 mg/kg). To prevent dehydration during the urodynamic study, normal saline was delivered at a rate of 10 mL/h via a superficial vein of the ear. A midline laparotomy incision, with lateral extension from the superior aspect of the midaxillary line, exposed the right kidney, ureter and bladder. A 24-gauge angiocatheter was placed through the lateral renal parenchyma to the renal pelvis, as a nephrostomy tube, and two 24-gauge angiocatheters were placed directly through the anterior bladder wall. The angiocatheters were tied up tightly to prevent urine leakage. After complete emptying, the bladder was filled with methylene blue-dyed saline via a suprapubic catheter. The saline was infused at either a low (0.5 mL/kg per min) or high (2 mL/kg per min) rate. VUR was identified by direct visualization of methylene blue-dyed saline ascending to the right ureter and pelvis. The other suprapubic catheter was used to monitor bladder pressure and the nephrostomy catheter was used for renal pelvic pressure measurement. Simultaneous and continuous renal pelvic and bladder pressures were recorded and analyzed over bladder filling cycles at low and high infusion rates. Pressure monitoring was achieved using a pressure transducer (TA600 instrument; Gould Instrument Systems; Valley View, CA, USA) and printouts were analyzed with PowerLab version 5.2 for Windows. Cystometric bladder capacity was defined as the infused saline volume before urination, and bladder compliance was calculated as the change in volume divided by the change in pressure. Maximum voiding pressure and post-void residual urine volume were also measured. The individual data were collected into intervals of 10% bladder fullness and plotted against renal pelvic or bladder pressure for comparisons.

Tissue preparation

The animals were sacrificed and the urinary tracts were exposed and assessed. Kidneys were retrieved, trimmed of all fat, and cut into small pieces. Formalin-fixed specimens were embedded in paraffin and sectioned at 6 μm. Standard H&E stains and Masson-Trichrome stains were used for histological evaluation and morphometric analysis.

Histological assessment

Each slide was evaluated in random order by standard light microscopy by two pathologists blind to rabbit grouping. Histological examination was performed to document inflammation, fibrosis, and tubular damage in the kidneys. The mean diameters of distal tubules were measured to evaluate tubular dilation, by examining five fields of cortex on each slide. Internal luminal diameters were measured on circular portions of the distal tubules. Extracellular matrix volume fractions were calculated for evaluation of interstitial fibrosis in the renal cortex. The extracellular matrix was identified as the area that reacted with Masson-Trichrome stain, but excluding glomeruli, tubules, and blood vessels. The results were expressed as volume fractions and measured using the image analyzing software SigmaScan Pro 5.0.

Data analysis

Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Co., Chicago, IL, USA). The results are presented as means± standard errors of the means. Statistical significance was analyzed by the Mann-Whitney test, with $P<0.05$ considered to be

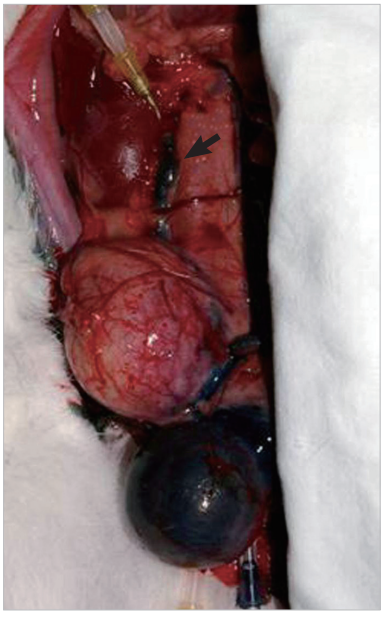

Fig. 1. Detection of right VUR. VUR was detected by direct visualization of methylene blue-dyed saline ascending to the right ureter and pelvis (arrow). The refluxing ureter is dilated and tortuous.

statistically significant.

RESULTS

Of the 40 rabbits, 20 were excluded because of technical complications related to catheter placement that prevented adequate data collection; thus, 20 rabbits (8 VUR, 7 sham-operated, and 5 control) remained for analysis. The culture study performed at the end of the experimental period were negative in all the animals' urine specimens, which showed the experiment was done under sterile condition.

Gross findings and identification of VUR

Grossly, a refluxing kidney was larger than the contralateral normal kidney and the refluxing ureter was dilated and tortuous in VUR rabbits. Right VUR was observed in all eight animals in which ureteral unroofing was performed (Fig. 1). No animal in either the sham-operated or control groups had reflux into the ureter.

Table 1. Cystometric findings of VUR, sham operation, and control group at the low infusion rate

	Mean±SD				
	Sham (n=7)	*P* value	VUR (n=8)	*P* value	Control (n=5)
Capacity (mL)	25.7±5.3	0.26	22.9±4.1	0.001	34.4±5.3
Compliance (mL/cmH_2O)	3.9±2.1	0.68	3.4±2.8	0.02	16.0±12.2
Maximum voiding pressure (cmH_2O)	38.4±11.6	0.28	44.3±8.8	0.11	33.6±13.9
Post-void residual urine (mL)	8.3±3.3	0.20	11.1±4.4	0.01	5.0±1.4

Significance was tested using Mann-Whitney test.
VUR, vesicoureteral reflux.

Urodynamic changes in the bladder

Urodynamic changes in the bladder were evaluated when saline was slowly infused (Table 1). The mean cystometric bladder capacity in the VUR group was significantly less than that of the control group. The cystometric bladder capacity of the sham-operated group was significantly decreased compared with that of the control group (P=0.02). Bladder compliance in the VUR group was less than that of the control group. In the all of the animals of the VUR group, intermittent bladder contraction, suggesting detrusor overactivity, was observed during the filling phase (Fig. 2). Maximum voiding pressure and post-void residual urine were increased in the VUR group compared to the control group.

Renal pelvic pressure at different levels of bladder fullness

At the low infusion rate, renal pelvic pressures in the sham-operated and control groups were stable, but the renal pelvic pressure in the VUR group was stable only until late filling phase and then increased slightly. Renal pelvic pressure increased, along with bladder contraction during the voiding phase, only in the VUR group. At the high infusion rate, renal pelvic pressures in the sham-operated and control groups were stable until late filling phase and then increased slightly, whereas renal pelvic pressure in the VUR group steadily increased from mid filling phase. The renal pelvic pressure increased, along with bladder contraction during the voiding phase, only in the VUR group (Figs. 2, 3). The renal pelvic pressure in the VUR group was significantly greater at the high infusion rate than at the low infusion rate, from the time the bladder capacity approached 80% fullness. The renal pelvic pressure in the sham-operated and control groups did not differ with saline infusion

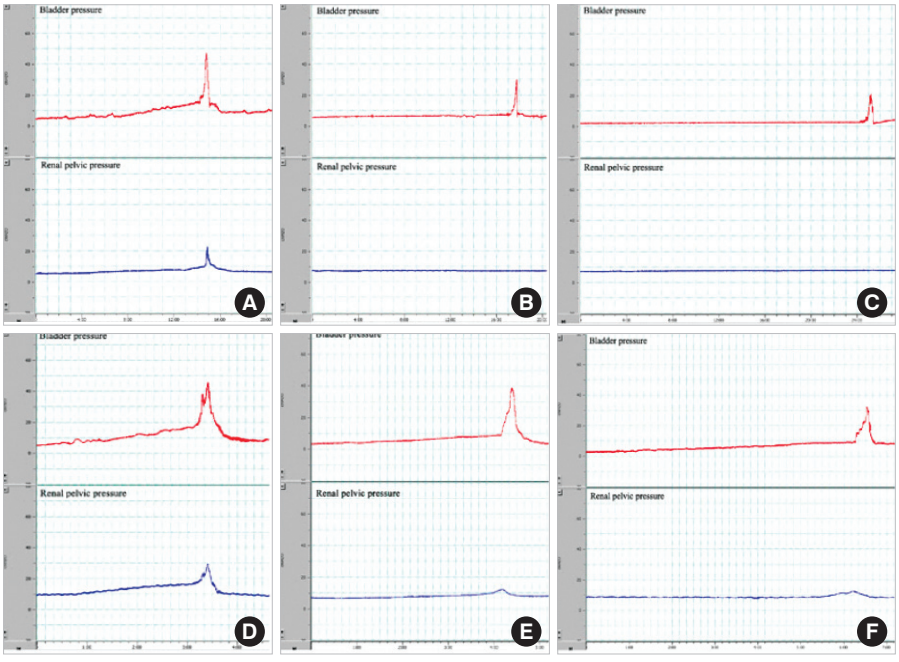

Fig. 2. Bladder and renal pelvic pressures at the low (**A**-**C**) and high (**D**-**F**) infusion rate. At the low infusion rate, the renal pelvic pressure is stable until the late filling phase and then increased slightly, peaking with bladder contraction during the voiding phase in the VUR group (**A**). The renal pelvic pressures are constant in the sham-operated (**B**) and control groups (**C**). At the high infusion rate, intermittent bladder contraction, suggesting detrusor overactivity, is observed during the filling phase and the renal pelvic pressure increase as bladder pressure rise in the VUR group (**D**). The renal pelvic pressures increase slightly during the late filling phase in the sham-operated (**E**) and control groups (**F**).

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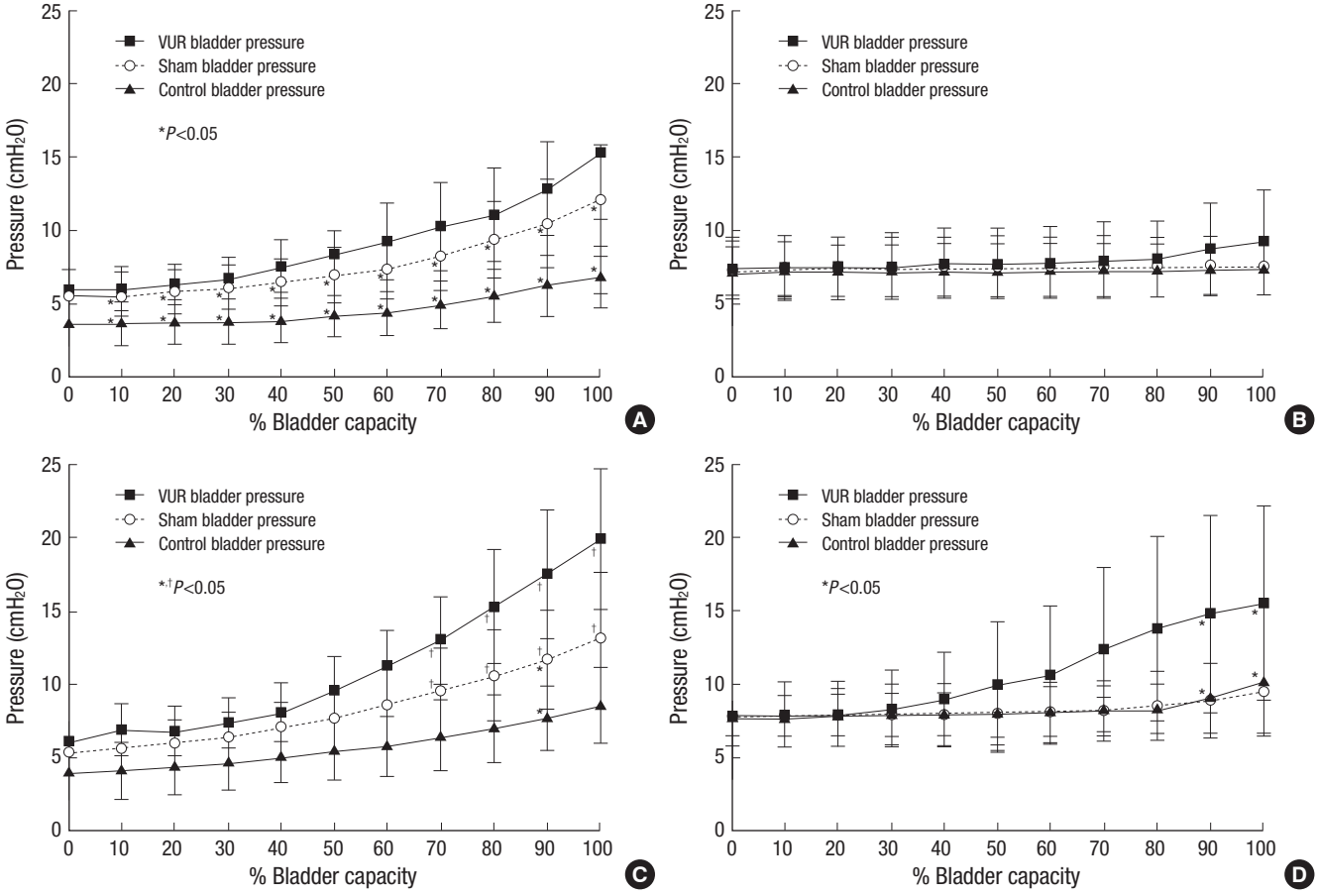

Fig. 3. Bladder and renal pelvic pressures to degree of bladder fullness at the low (**A**, **B**) and high (**C**, **D**) infusion rate. At the low infusion rate, the renal pelvic pressures in the sham-operated and control groups are stable, but the renal pelvic pressure in the VUR group is stable only until late filling phase, and then increase slightly. At the high infusion rate, the renal pelvic pressures of the sham-operated and control groups are stable until late filling phase and then increase slightly, whereas the renal pelvic pressure in the VUR group steadily increase from mid filling phase.

Table 2. Effects of infusion rate on renal pelvic pressure to degree of bladder fullness

% Bladder fullness	Mean renal pelvic pressure (cmH_2O)								
	Sham (n=7)			VUR* (n=8)			Control (n=5)		
	Low	*P* value	High	Low	*P* value	High	Low	*P* value	High
0	7.57	0.76	7.93	7.46	0.86	7.61	7.18	0.49	7.94
10	7.57	0.74	7.96	7.51	0.91	7.61	7.26	0.55	7.94
20	7.59	0.72	8.00	7.56	0.81	7.80	7.22	0.51	7.94
30	7.54	0.69	7.99	7.55	0.51	8.38	7.22	0.49	7.98
40	7.57	0.71	7.99	7.81	0.41	9.00	7.26	0.52	7.98
50	7.60	0.72	8.00	7.81	0.25	9.93	7.26	0.46	8.14
60	7.59	0.64	8.11	7.88	0.15	10.69	7.28	0.44	8.18
70	7.60	0.58	8.21	8.00	0.06	12.40	7.32	0.42	8.26
80	7.59	0.41	8.59	8.08	0.03	13.85	7.34	0.38	8.36
90	7.61	0.28	5.99	8.78	0.04	14.84	7.40	0.23	9.10
100	7.60	0.16	9.59	9.26	0.03	15.55	7.40	0.16	10.18

Significance was tested using Mann-Whitney test.
VUR, vesicoureteral reflux.

rate (Table 2).

Histological assessment

Histological examination of the renal pelvis demonstrated no abnormalities of the collecting system in the sham-operated or control groups. In contrast, histological changes such as dilation of the renal pelvis with flattening of the transitional cell epithelial layer were observed in the VUR group (Fig. 4). No histologi-

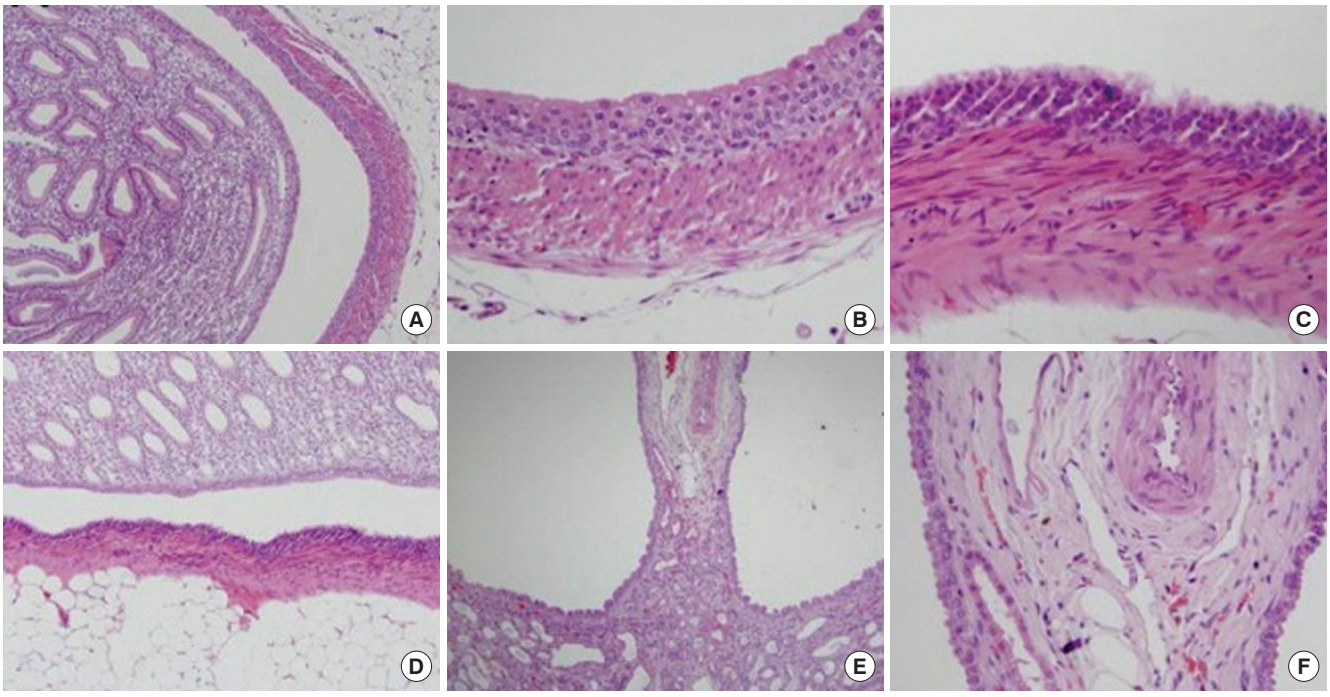

Fig. 4. Histological findings of the renal pelvis (H&E staining). Renal pelvis of control (**A**, **B**) and sham-operated animals (**C**, **D**) show no dilation of the collecting system with a well preserved transitional cell epithelial layer. In the VUR group (**E**, **F**), however, renal pelvis dilation with thinning of the transitional cell epithelial layer is observed. (H&E staining; **A**, **C**, **E**: ×100; **B**, **D**, **F**: ×400).

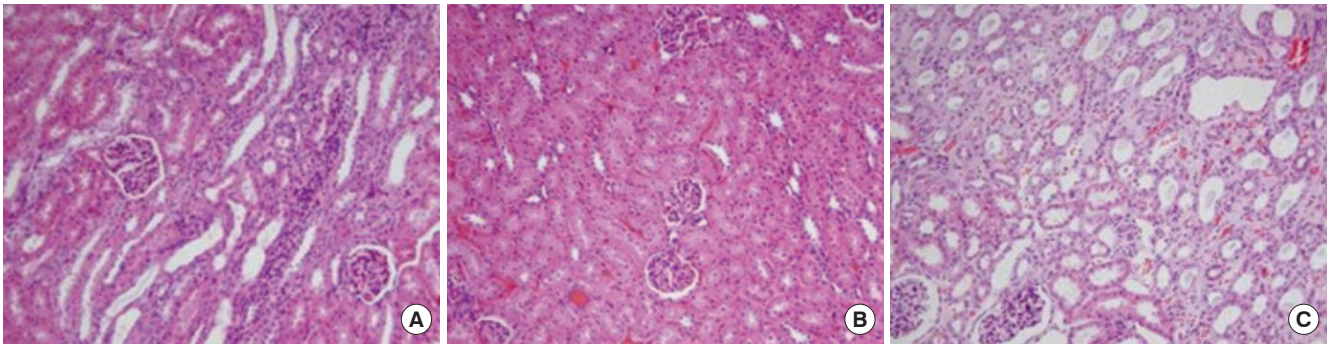

Fig. 5. Histological findings of the renal cortex (H&E staining). Renal cortex of control (**A**) and sham-operated rabbits (**B**) show no histological changes. Focal thinning of the tubular epithelium and interstitial widening are observed, however, in some cortical areas of VUR animals (**C**). There is no inflammatory cell infiltration (H&E staining; ×200).

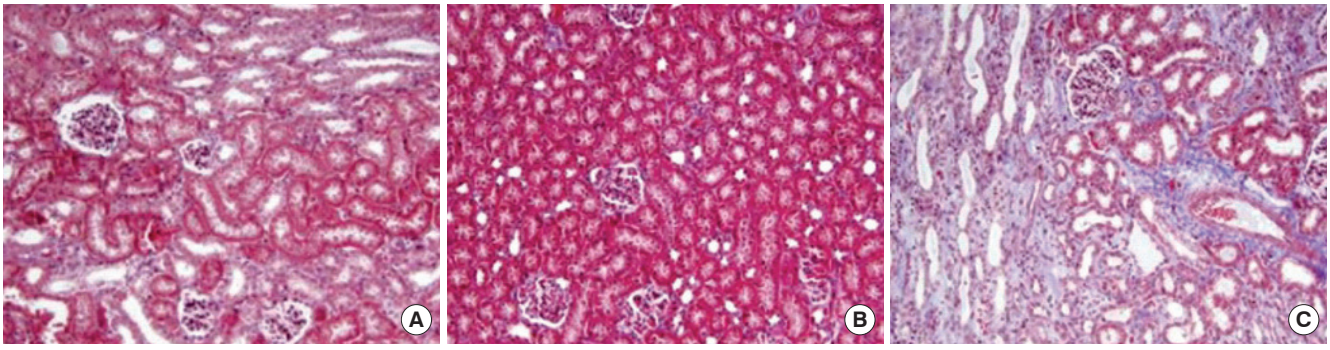

Fig. 6. Histological findings of the renal cortex (Masson-Trichrome staining). Renal cortex in a control (**A**) and a sham-operated rabbit (**B**) demonstrate no histological changes. Interstitial fibrosis is observed, however, in some cortical areas of a VUR animal (**C**) (Masson-Trichrome staining; ×200).

cal changes in the renal cortex were observed in the sham-operated or control groups. Focal thinning of the tubular epithelium and interstitial widening were observed, however, in some cortical areas of 63% (5 of 8) refluxing kidneys. There was no in-

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Table 3. The mean diameters of distal tubules

No. animal	Diameters of distal tubules (μm, mean±SD)		
	Sham (n=7)	VUR (n=8)	Control (n=5)
1	22.4±4.6	22.0±2.3	20.0±2.9
2	20.3±2.3	25.0±3.0	26.7±3.7
3	20.2±2.8	19.8±2.9	17.4±0.9
4	21.9±3.2	27.1±5.1	21.6±3.0
5	19.5±1.4	18.6±1.3	23.1±3.9
6	22.3±3.0	24.0±4.6	
7	23.3±2.6	20.4±2.6	
8		27.9±4.3	
Total	21.4±1.4	23.1±3.5	21.8±3.5
P value	0.25	0.51	

Each value is the mean diameter of distal tubules by counting a total of 5 subsequent fields of cortex per slide (mean±SD).
Significance was tested using Mann-Whitney test.
VUR, vesicoureteral reflux.

Table 4. The extracellular matrix volume fractions

No. animal	Extracellular matrix volume fractions (%, mean±SD)		
	Sham (n=7)	VUR (n=8)	Control (n=5)
1	4.5±1.8	6.5±2.7	3.3±3.3
2	2.8±1.2	4.5±2.4	6.4±3.0
3	2.3±2.0	3.8±2.8	1.6±1.7
4	3.6±2.7	15.6±6.6	4.3±2.5
5	5.3±3.8	2.1±1.2	3.5±2.5
6	4.2±2.6	1.8±2.2	
7	6.9±3.7	3.5±2.5	
8		12.3±6.1	
Total	4.2±2.8	6.3±5.9	3.8±2.9
P value	0.07	0.06	

Each value is the percentage of extracellular matrix volume by counting a total of 5 subsequent fields of cortex per slide (mean±SD). Extracellular matrix was identified as the positive area with Masson-Trichrome stain excluding glomeruli, tubules and blood vessels.
Significance was tested using Mann-Whitney test.
VUR, vesicoureteral reflux.

flammatory cell infiltration (Figs. 5, 6).

Morphometric assessment showed no differences in the mean diameter of distal tubules in the three groups (Table 3). The extracellular matrix volume fraction was also unchanged in refluxing kidneys compared to sham-operated and control kidneys (Table 4). However, subanalysis by renal pelvic pressure disclosed obvious changes in the renal cortex in two highly refluxing kidneys. Compared to other animals, the mean diameters of distal tubules (27.1±5.1 μm and 27.9±4.3 μm) were increased and the extracellular matrix volumes (15.6±6.6% and 12.3±6.1%) were markedly increased in the two animals in which renal pelvic pressures were higher than in others.

DISCUSSION

To date, animals such as puppies, dogs, piglets and sheep have mainly been used as VUR models (1, 2, 7, 8), and rats, 25% of which are well-known to have congenital VUR, have also been employed in several animal VUR studies (4, 9). We used rabbits as an animal VUR model for several reasons. Although rats are more economical than larger animals, the latter are much easier to handle. Moreover, the surgical procedure of open bladder incision of the intravesical ureteral tunnel is well established in the rabbit (5, 10).

In our experiment, urodynamic changes of the bladder were obvious in the VUR groups; these changes included decreases in cystometric bladder capacity and compliance, increases in post-void residual urine levels and maximum voiding pressures, and intermittent bladder contraction during filling phases. Our results are similar to those of a previous study of Gobet and coworkers (8), who reported that bladder dynamics were altered after surgically induced VUR in the sheep fetus, suggesting that cystometric changes can develop after simple operational manipulation of the bladder. Therefore, alteration of bladder dynamics should be considered in any experiment using an animal model of bladder incision.

The renal pelvic pressures in the sham-operated and control groups were stable, whereas the renal pelvic pressure in the VUR group increased steadily during the bladder filling phase. This result differs from the findings obtained in a rat VUR model using a study design similar to ours (4). In the earlier work, renal pelvic pressure increased markedly with increasing bladder fullness, irrespective of the presence of VUR. It may be that the anatomical difference in the ureterovesical junctions of rabbit and rat accounts for this discrepancy in study results. As mentioned above, about 25% of rats have congenital VUR and, even in animals without VUR, the anti-reflux mechanism is very weak and the renal pelvic pressure can increase with artificial bladder filling. By contrast, the inverted "J" course of the intravesical ureter of the rabbit provides a strong anti-reflux mechanism (11, 12), and mitigates against elevation of renal pelvic pressure upon bladder filling.

Our data showing that renal pelvic pressures in the sham-operated and control groups increased slightly in late filling phases, at the high infusion rate, are similar to previous reports that renal pelvic pressure may be significantly higher with a full bladder (13, 14). Tanagho and colleagues have explained this effect caused by the bladder wall stress and obstruction at the ureterovesical junction (15), and we have confirmed that renal pelvic pressure increases as the bladder fills.

The aim of varying the saline infusion rate was to evaluate the effect of bladder instability on renal pelvic pressure. Bladder instability may be caused by fast saline infusion into the bladder (16). We found that bladder pressure was highest in the VUR group, which suggests trigonal irritation occurring after manipulation of the intravesical ureter. In addition, the renal pelvic pressure in the VUR group was significantly increased at the high infusion rate, suggesting that VUR depends not only on anatomical factors but also on functional factors such as bladder irrita-

bility or compliance. In clinical practice, the reflux grade sometimes changes in an individual patient during repeated voiding cystourethrography, which may be explained by the fact that VUR is affected by functional bladder changes resulting from external circumstances such as the rate of infusion of a contrast medium.

The histological changes shown in some cortical areas of refluxing kidneys, such as focal thinning of the tubular epithelium and interstitial fibrosis without inflammatory cell infiltration, suggest that renal damage may be caused by chronic mechanical irritation. Such damage is accompanied by elevated renal pelvic pressure, although there were no between-group statistical differences in mean tubular diameters of distal tubules, or extracellular matrix volume fractions. Moreover, the finding that dilation of the distal tubules and increments in the extracellular matrix volume fractions occurred in animals with higher renal pelvic pressures imply that intrarenal reflux may occur in those animals. Although some evidence suggests that sterile reflux does not cause nephropathy even at high pressures (17, 18), we show in this study that high pressure reflux with bladder instability may result in renal cortical changes.

In conclusion, the renal pelvic pressure in the VUR animal increase with bladder pressure elevation and the changing pattern of renal pelvic pressure is affected by bladder infusion rate, suggesting that VUR does not exclusively depend on anatomical factors. High pressure reflux with bladder instability may result in renal cortical changes, as we present evidence that renal cortical changes occurred in animals with higher renal pelvic pressures.

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