Does Intervention *in Utero* Preserve the Obstructed Kidneys of Fetal Lambs? A Histological, Cytological, and Molecular Study

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ABSTRACT: Ureteropelvic junction obstruction is a common cause of end-stage nephropathy in children. Our aim was to investigate whether relief of obstruction *in utero* can alleviate the development of nephropathy. A silastic tube was tied around the left superior segment ureter to induce unilateral partial ureteral obstruction in 22 fetal sheep at 75–85 d of gestation. Three weeks later, the tubes were removed to relieve the obstruction in 10 of the 22 lambs. A sham operation was performed on four fetuses (the control). At birth, the lambs were killed, and their kidneys were removed to study the changes in histology, podocytes, and expression of paired-box 2 (PAX2) and VEGF. In the obstructed kidneys, we observed cysts of various sizes in the cortex, fibrosis in the interstitial tissue, much decreased number of glomeruli, severe podocyte foot process fusion, and markedly increased PAX2 and decreased VEGF expressions. However, relief of obstruction preserved the number of glomeruli, significantly increased VEGF expression, reduced fusion of the podocyte foot processes, and restored expression of PAX2 to some extent. Thus, relief of obstruction *in utero* may prevent or attenuate the development of nephropathy in lambs. (*Pediatr Res* 66: 145–148, 2009)

Ureteropelvic junction obstruction (UPJO) is a common cause of fetal hydronephrosis [incidence, approximately 1 in 2000 fetuses (1)]. At present, infants suffering from UPJO are either simply followed up or given surgical treatment based on hydronephrosis grade or renal function condition. However, Murer *et al* (2) found that the recovery from renal function injury even after the relief of UPJO was not assured in some children. Intervention *in utero* may help these patients. Chevalier *et al* (3) found that the relief of obstruction markedly reduced tubular atrophy and interstitial fibrosis in developing kidneys. However, Josephson *et al* (4) found that renal damage persisted despite very early efforts to remove obstruction. Because there is no consistent evidence linking recovery from renal damage to relief of UPJO *in utero* in humans, we decided to study this issue in fetal lambs.

Podocytes are needed to maintain normal renal function; yet, they are easily injured by various abnormal situations. To our knowledge, changes in podocytes in obstructed kidneys have rarely been studied.

Paired-box 2 (PAX2) is an important regulatory factor that functions early in the development of the kidney, and is restricted to the developing kidney. In damaged kidneys, PAX2 expression seems to be related to the proliferation of cystic renal epithelia, which is common in obstructed kidneys. VEGF is needed to maintain the renal blood vessel system. Decrease in VEGF will lead to fibrosis, another very common condition in obstructed kidneys.

In this study, whether the relief of UPJO *in utero* in lambs would attenuate the development of nephropathy was investigated by observing changes in histology, podocytes, and expression of PAX2 and VEGF in kidneys after ureteral obstruction relief *in utero*.

MATERIALS AND METHODS

Severe unilateral partial ureteral obstruction was produced in 22 sheep fetuses at 75–85 d of gestation. In brief, open hysterotomy was performed on pregnant ewes (full-term, 150 d) *via* a flank laparotomy, and the fetal lamb hindquarters were then exteriorized. After exposing the upper part of the left ureter of the fetuses, an F6 split silastic tube was wrapped around it to induce obstruction. Methylthioninium chloride (2 mL) was injected into the renal pelvis to see whether it could pass through the obstructed part. Lastly, we repositioned the fetuses and closed the uterine cavity as well as abdominal wall.

Three weeks later, another operation was performed to remove the obstruction (the silastic tubes) in 10 of the 22 fetuses. The lambs born alive were used in the study, and the aborted lambs were excluded. All protocols were approved by Medical Ethics Committee of the first hospital affiliated to Sun Yat-Sen University.

Histologic study. All formalin-fixed and paraffin-embedded kidney tissues (including the contralateral kidneys) were sliced into 5 μm sections and were stained with hematoxylin and eosin. Sections were then observed under light microscope (Olympus CX4, Japan).

Electron microscope study of podocytes. The lambs were killed, and the experimentally obstructed kidneys (not the contralateral kidneys) were removed. Pieces of the superficial cortex (1 mm³) were immediately fixed in 3% glutaraldehyde, dehydrated, embedded, cut into 60-nm sections, stained, and examined for podocytes under a transmission electron microscope (Philips, CM10; Eindhoven, The Netherlands).

Immunohistochemical staining for PAX2 and VEGF. All kidneys (including the contralateral kidneys) were processed for immunohistochemical staining (the SP method). PAX2 and VEGF were detected using rabbit polyclonal anti-PAX2 antibody (1:50; Zymed, San Francisco, CA) and rabbit polyclonal anti-VEGF antibody (1:200; Biosynthese, Biotechnology Co., Ltd., Beijing, China), respectively.

The extent rather than the intensity of PAX2 and VEGF staining was graded semiquantitatively and reflected the percentage of positive renal tubules. There were four scores: 0, absent; 1, less than 25% staining; 2, 25–50% positive staining; and 3, more than 50% positive staining. For each sample, 10 fields (five cortical fields and five medullary fields) were counted under high magnification ($\times 100$), and the average score was calculated. Each slide was scored by an observer who was unaware of the experimental details.

Statistical analysis. Results are expressed as means ± SD. Software SPSS 13.0 (SPSS Inc, Chicago, IL) was used, and one-way ANOVA was adopted

Abbreviations: ECM, extracellular matrix; **PAX2,** paired-box 2; **UPJO,** ureteropelvic junction obstruction; **UTO,** urinary tract obstruction

to analyze the data among the groups. Probabilities of <5% were considered as significant.

RESULTS

Overall, 80.8% (21 of 26) survived: nine lambs survived in the obstructed group, eight in the decompressed group, and four in the control group. After the ureter was ligated with a F6 split silastic tube, 2 mL of methylthioninium chloride was injected into the renal pelvis, and it was then seen to collect in the inferior part of the ureter. Three weeks later, obvious pyeloureterectasis (the diameter of the ureter above the obstruction was more than 7 mm, whereas below the obstruction, it was less than 2 mm) was observed, before the silastic tube was removed from the ureter. When the tube was removed, the inferior segment ureter filled immediately. Furthermore, if the obstruction was not removed, the kidneys became sac as seen in the lambs when they were killed.

Histologic study. Microscopically, there were no dysplastic changes and the basic elements were intact in all kidneys. However, histologic changes were very obvious in the obstructed kidneys. Cysts of various sizes in the renal parenchyma and fibrosis in the interstitial tissue were observed. Furthermore, glomerular capillary loops had collapsed in some glomeruli. Importantly, the number of glomeruli was much decreased.

Removal of the obstruction reversed some of the histologic changes. Collapse of glomerular capillary loops and dilatation of renal tubules and Bowman's capsule were greatly ameliorated, but the number of glomeruli was unaffected and remained unchanged, and the decompressed kidneys exhibited deposits of extracellular matrix (ECM; Fig. 1).

The damage of the podocytes. Podocyte foot process fusion was observed in the obstructed, decompressed, sometimes control kidneys. Possible among-group difference in the severity of foot process fusion was investigated. In each sample, 10 random capillary loops were analyzed under high magnification (×10,000), and the average percentage of loops with foot process fusion was calculated for each sample.

The percentage of foot process fusion was 4.20 ± 1.08% in the control kidneys, 86.79 ± 1.66% in the obstructed kidneys, and 41.18 ± 3.13% in the decompressed kidneys. Multiple comparisons showed significant among-group differences in the severity of foot process fusion ($p < 0.05$). Foot process fusion was more extensive in the decompressed kidneys than the control kidneys, but much less extensive than in the obstructed kidneys (Fig. 2).
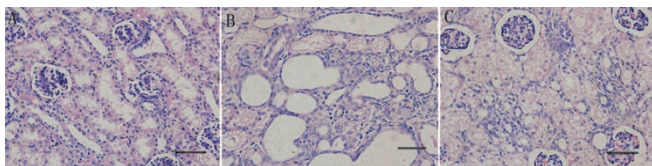

Figure 1. Histologic changes observed in various kidneys. In contrast to the control kidneys (*A*), obstructed kidneys (*B*) had cysts of various sizes, collapsed glomerular capillary loops, fibrosis, and decreased number of glomeruli. Compared with obstructed kidneys, decompressed kidneys (*C*) had a normal number of glomeruli and much less saccular ectasia, but still had ECM deposits (magnification: ×400; bar = 100 μm).
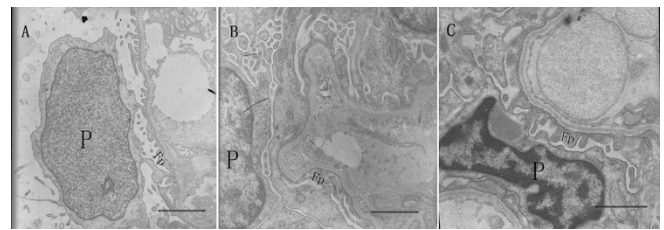

Figure 2. Podocyte foot process fusion in the kidneys of three groups. The podocyte foot process is normal in the control kidney (*A*), nearly completely fused in the obstructed kidney (*B*), and partially fused in the decompressed kidney (*C*) (magnification: ×10,000; bar = 5 μm). *P*: podocyte; *Fp*: foot process.
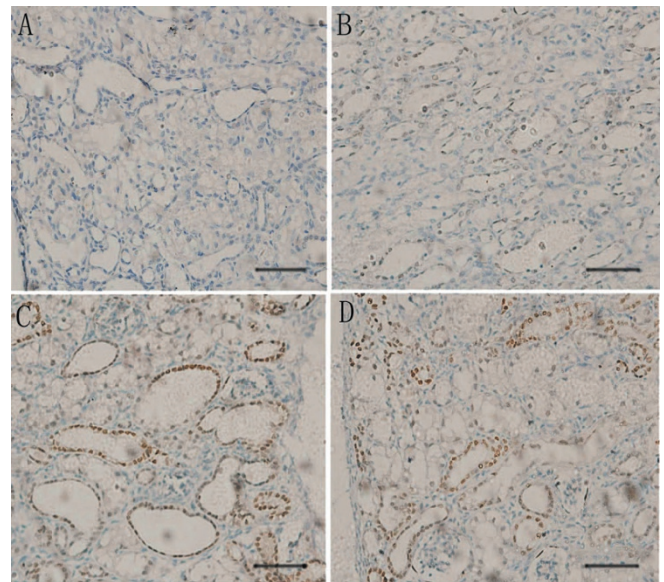

Figure 3. Expression of PAX2 in the kidneys. PAX2 in the control kidneys is absent in the renal cortex (*A*) and confined to the collecting duct and medullary loop (*B*). However, in the obstructed (*C*) or decompressed (*D*) kidneys, PAX2 continues to be highly expressed in the cortex and medulla (magnification: ×400; bar = 100 μm).

Expression of PAX2. As expected for transcription factors, staining for PAX2 was predominantly nuclear and confined to the medullary collecting ducts and medullary loops of Henle in control kidneys and to the kidneys contralateral to the obstructed or decompressed ones. However, PAX2 continued to be highly expressed in the cortex and medulla of obstructed and decompressed kidneys (Fig. 3).

The PAX2 scores are listed in Table 1. Expression of PAX2 was similar in control kidneys and contralateral kidneys (1.43 ± 0.09 *vs* 1.44 ± 0.10 *vs* 1.48 ± 0.10, $p > 0.05$), but increased in obstructed kidneys (2.44 ± 0.09 *vs* 1.43 ± 0.09, $p < 0.05$). Relief of obstruction was able to restore PAX2 expression to some extent (2.05 ± 0.14, $p < 0.05$).

Expression of VEGF. In control kidneys and kidneys contralateral to the decompressed ones, VEGF staining was at a low level and predominantly in the cell membrane and cytoplasm and confined to some cortical renal tubules. In the obstructed kidneys, it was lower and even undetectable. However, in the decompressed kidneys and kidneys contralateral to the obstruction, it was increased in the cortical renal tubules (Fig. 4).

Table 1. *Expression of PAX2 and VEGF (mean staining score ± SD)*

	PAX2	VEGF
CK	1.43 ± 0.09	0.80 ± 0.15
OK	2.44 ± 0.09	0.33 ± 0.14
DK	2.05 ± 0.14	2.08 ± 0.21
KCO	1.44 ± 0.10	2.13 ± 0.26
KCD	1.48 ± 0.10	0.90 ± 0.24

Expression of PAX2 remained at a normal level and similar among the CK, KCO, and KCD groups ($p > 0.05$). However, PAX2 expression was elevated in the OK group ($p < 0.05$) and decreased to some extent after obstruction was relieved ($p < 0.05$). Expression of VEGF remained at low level and was not different between the CK and KCD groups ($p > 0.05$). Compared with VEGF expression in normal kidneys, it was decreased in the OK ($p < 0.05$) and increased in the DK and KCO ($p < 0.05$).

KC, control kidneys; OK, obstructed kidneys; DK, decompressed kidneys; KCO, kidneys contralateral to the obstruction; KCD, kidneys contralateral to the decompressed kidneys.
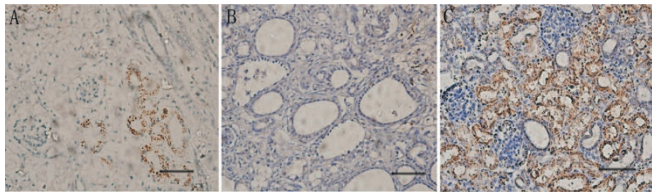

Figure 4. Expression of VEGF in the kidneys. Expression of VEGF in the control kidneys (*A*) is confined to some cortical renal tubules. It is decreased and even lost in the obstructed kidneys (*B*). However, in the decompressed kidneys (*C*), it is increased in the cortical renal tubules (magnification: ×400; bar = 100 μm).

The VEGF scores are listed in Table 1. Expression of VEGF was similar in the control kidneys and the kidneys contralateral to the decompressed ones (0.80 ± 0.15 *vs* 0.90 ± 0.24, $p > 0.05$), it was decreased in the obstructed kidneys (0.80 ± 0.15 *vs* 0.33 ± 0.14, $p < 0.05$) and increased in the decompressed kidneys and the kidneys contralateral to the obstruction (0.80 ± 0.15 *vs* 2.08 ± 0.21 *vs* 2.13 ± 0.26, $p < 0.05$).

DISCUSSION

Congenital urinary tract obstruction (UTO) is an important cause of end-stage nephropathy in children (5). Fetal surgery to relieve obstruction *in utero* has been available for more than 20 years and has prevented the suffering of many patients. Although the results of *in utero* surgical treatment for fetal UTO have been encouraging, the focus of most researchers is on developing treatments for urethral obstruction, which is life threatening, and few are studying treatments for UPJO (another kind of UTO). The rapid development of anesthetic, surgical, and tocolytic techniques for fetal surgery have given new therapeutic options to patients with severe UPJO who would not recover renal function after a postnatal intervention. However, does fetal surgery reverse the renal injury caused by UPJO as effectively as it does in urethral obstruction? We are studying this issue.

The completeness of the obstruction and its timing with respect to the stage of glomerulogenesis at the time of the obstruction determine which pathologic changes occur (6,7).

In this study, severe partial UTO was induced in sheep fetuses at 75–85 d gestation. All obstructed kidneys showed hydronephrosis or cystic change and no dysplastic changes. Removal of the obstruction preserved the number of glomeruli and improved other histologic changes indicating injury such as collapse of glomerular capillary loops, dilatation of renal tubules and Bowman's capsule, and so on. However, ECM deposition was still obvious in the decompressed kidneys. According to Amemiya *et al* (8), VEGF can activate MAP kinase and enhance collagen synthesis. ECM deposition may be attributed to the increased expression of VEGF.

Podocytes cover the external surface of the glomerular basement membrane (GBM) and contribute to various kidney functions. Severe morphologic changes in podocytes including foot process fusion were observed, which may contribute to the collapse of glomerular capillary loops in obstructed kidneys. The morphologic changes in podocytes may occur in sequence, beginning with foot process fusion, cell body contraction, and pseudocyst formation and ending with podocyte detachment from the GBM (9), loss of podocytes, and exposure of the GBM leading to glomerular sclerosis (10). In our study, relief of obstruction *in utero* improved foot process fusion, and thus *in utero* surgery may help to prevent glomerulosclerosis in newborns.

PAX2 is an important regulatory factor of kidney development. During kidney development, it is expressed in the nephric duct, metanephric mesenchyme, ureteric bud, and S-shaped body (11). When the kidney matures, PAX2 is confined to the medullary collecting duct (12). However, if PAX2 continues to be highly expressed in the renal tubule, it will cause the formation of cystic malformations, as observed in the obstructed kidneys of our animal model. Relief of the obstruction could partially reverse PAX2 expression. Consistent with our finding, Edouga *et al* found that the shunting procedure could reverse the expression of PAX2 completely in the obstructed bladder outlet of kidneys from sheep fetuses (13). Nevertheless, there were differences between our results and theirs. The effect of bladder outlet obstruction would be expected to differ from that of ureteral obstruction. Kidney damage, stemming from ureteral obstruction, would occur sooner and be more severe, and so recovery from kidney damage, including normalization of PAX2 expression after removing the obstruction, would take longer.

VEGF is a pluripotent cytokine, which supports functions of mature blood vessels in the kidney (14). In this study, as in other studies (15,16), the expression of VEGF was markedly reduced in obstructed kidneys. Decrease in VEGF will cause loss of peritubular capillaries and fibrosis, which is destined to obstructed kidney. However, in this study, relief of obstruction increased VEGF expression to levels even higher than in control kidneys. Thus, it may prevent kidneys from becoming fibrotic.

Of course, this study has several limitations. First, renal function is not assessed. Second, the results may not apply to humans. The situation in humans is surely much more complex. Harrison and coworkers found improved renal histology and function after relief of urethral obstruction in animals but not in humans (17,18). The main reason, in many cases, is that

UTO is only a part of a more complex defect affecting the nephrourinary system at different levels. Consequently, caution should be exercised before clinically applying fetal surgery.

In conclusion, it seems that relief of obstruction *in utero* can alleviate the development of nephropathy by a histologic, cytological, and molecular study. However, extrapolation of these findings to clinical practice should be done with caution until further evidence is available.

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