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ORIGINAL ARTICLE

Early bladder wall changes after creation of obstructive uropathy in the fetal lamb

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Abstract Vesico-amniotic shunting of obstructive uropathy in fetal lambs produced a thick-walled, poorly compliant bladder. We report the early histological changes in the obstructed bladder wall. We created an obstructive uropathy in fetal lambs at 60 days gestation by ligating the urethra and urachus. Vesicostomy or vesico-amniotic shunt tube insertion and biopsy of the bladder wall were performed 21 days later. The fetuses were delivered at term (145 days) and the kidneys and bladder sampled for histology. Colloidal iron (Col Fe), and alpha-smooth muscle actin (α-SMA) immunohistochemical stains were used for these samples. Seventeen fetuses were shunted with 15 biopsies taken at that time. Six (shunt failure or missed urachal ligation) were excluded. All biopsies taken at shunting had positive Col Fe and α-SMA. Term lambs had mild multicystic dysplastic kidney (MCDK) in five, severe MCDK in two, and hydronephrosis in four. All bladders had small volume and were severely fibrotic. Fetal shunt operations 3 weeks after the creation of obstructive uropathy provided partial preservation of renal histology but did not preserve normal bladder histology. We suggest that the high hyaluronic acid synthesis activity or hyperplasia of the myofibroblasts in the dilated fetal bladder wall at the time of shunting results in irreversible damage to the developing bladder muscle and fibrosis.

Keywords Fetal surgery · Obstructive uropathy · Bladder function · Posterior urethral valve

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Introduction

Obstructive uropathy is the leading cause of end stage renal failure before the age of 4 years and a significant cause of chronic renal failure throughout childhood and adolescence [1]. Fetal bladder drainage may improve perinatal survival in these fetuses and evaluation of fetal renal function is well documented in the literature [2]. Fetal surgery for obstructive uropathy was started at 1980s but these obstructive uropathy patients had bladder dysfunction described in the long-term follow-up results in 1999 by Freedman et al. [3, 4]. In the 1980s, the emphasis was on the survival of the fetus with obstructive uropathy. It is only more recently that consideration has been given to the long-term outcomes for these children [3, 4]. We created a model of obstructive uropathy in the fetal lamb and then placed vesico-amniotic shunts to investigate bladder function after this type of intervention. We have shown that this

approach results in marked hypertrophy of the bladder wall muscle with severe interstitial fibrosis [5–7]. We repeated our obstructive uropathy model in fetal lambs at 60 days gestation and biopsied the bladder wall at the time of shunting to determine the state of the bladder wall at that time and to attempt to define the optimal timing of prenatal treatment for obstructive uropathy in this model.

Materials and methods

After approval was obtained from the Wellington School of Medicine and Health Sciences Animal Ethics Committee (Application 8-03), timed gestation ewes (60 days gestation) were transported from the farm 24–48 h before operation. They were examined by ultrasound to confirm pregnancy and avoid unnecessary operations. These ultrasound examinations were repeated before each intervention. Once the ewes had recovered from anesthetic, they were returned to the farm and then returned to the laboratory 24–48 h prior to the next procedure. The ewes were induced using intravenous thiopentone (20 mg/kg) and intubated. Anesthesia was maintained using nitrous oxide, oxygen and halothane, as previously described [8]. The surgical techniques used to create obstruction have previously been described [5, 6].

Our previous studies suggested that the optimal timing for shunting the bladders in our fetal lamb model of obstructive uropathy would be 3 weeks after the obstruction had been created [9]. At this time, therefore, the uterus was re-opened under general anesthesia using the previous incision and the fetuses were exposed. The fetus's lower extremities and abdomen were delivered through the uterus and a vertical incision was made just below the umbilical cord. The peritoneum was opened and a stitch was inserted into the bladder wall and this was used as a stay-suture around which a small piece of bladder wall was excised ($n = 15$). Biopsies were also taken from normal lambs at the same gestation. In most cases these were unoperated twins of lambs that had previously undergone an obstructive procedure at 60 days gestation. Normal unoperated term lambs were used as controls ($n = 5$). Most of these lambs were also unoperated twins of experimental lambs.

These biopsies were immediately placed in 10% formal saline. A purse string suture was placed in the bladder wall and the peritoneal end of a Pudenz catheter (Integra NeuroSciences^{TM} Pudenz Peritoneal Catheter, REF NL850–1380, low pressure 15–54 mmH_2O) was inserted into the bladder. The fetuses were returned to the uterus and the uterus and ewe's abdominal wall were closed with Biosyn® or Polysorb® (US Surgical, Tyco Healthcare, Japan).

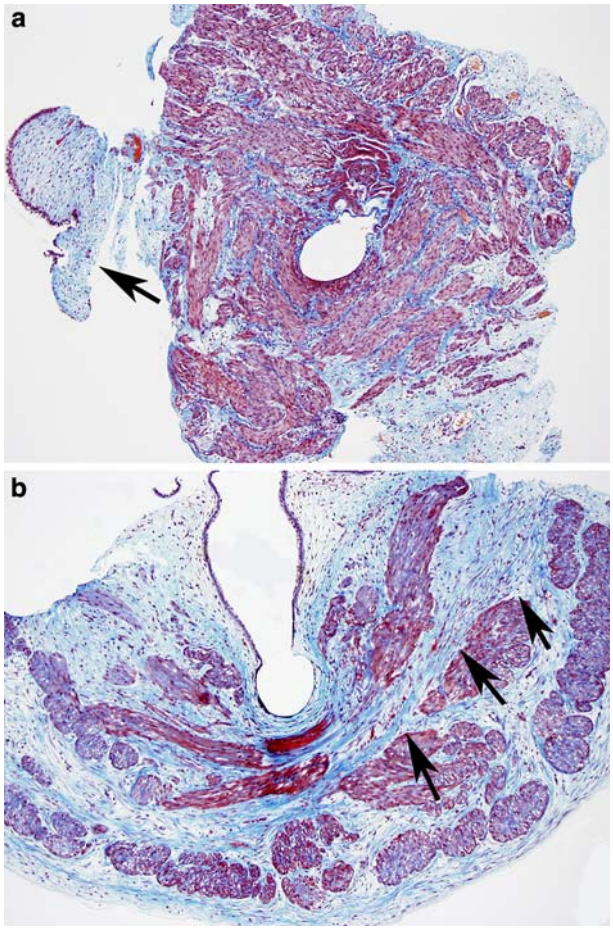

Fig. 1 Histological findings of control and obstructive uropathy model at 81 days (21 days after creating the obstruction) (Masson's trichrome stain: original magnification ×100). **a**, **b** The Masson stain. There are three layers including mucosal epithelium, the submucosal layer and the muscular layer in the normal bladder wall in the control animals (**a**). **b** The myxoid change in the submucosal layer and in the muscle layers, the muscle bands are separated (*arrows*) by the myxoid change

At term (145 days), the ewes were anaesthetized and the fetuses were delivered by caesarean section. The lambs were sacrificed using pentobarbital injected into the umbilical vein. The lamb's entire renal tract was removed en block and initially fixed in 10% formal saline. Samples were taken from the kidneys, ureters and the bladder wall. Histological sections of the bladder wall were stained with H&E (Hematoxylin & Eosin), Masson's Trichrome, α-SMA and Col Fe.

Results

Seventeen fetuses were shunted with 15 biopsies taken at 3 weeks after obstruction (60 + 21 days). Six

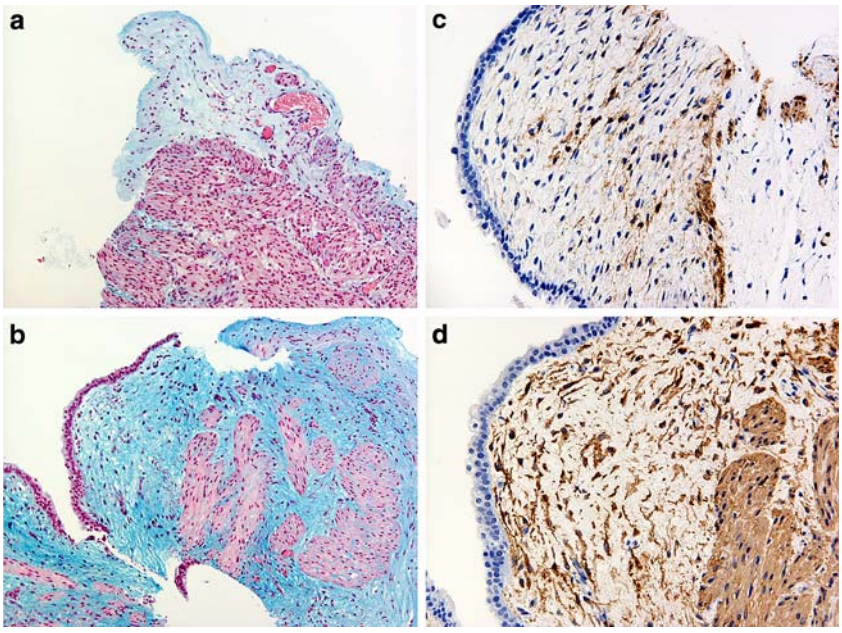

Fig. 2 Accumulation of extracellular matrix in control and obstructive uropathy model (Colloidal Fe: original magnification ×200). In the normal control bladder wall (**a**) there are small amounts of colloidal Fe-positive extracellular matrix accumulation in the sub-mucosal layer. In the obstructive uropathy model, extracellular matrix accumulation is increased in both the sub-mucosal and muscle layers (**b**). **c–d** Demonstration of myofibroblasts using immunohistochemical stains for α-SMA (original magnification ×400). There are very small numbers of myofibroblasts in the submucosal layer in the normal bladder (**c**). In the obstructive uropathy model there is marked proliferation of myofibroblasts in the submucosal and muscle layers (**d**)

were excluded because of shunt failure or missed urachal ligation. Two died after the shunt operation. The histological findings of control and obstructed bladder biopsies at 81 days (Masson stain original magnification 100×) are shown in Fig. 1. In the control bladders, there are three layers including the mucosal epithelium, the submucosal layer and the muscular layer (Fig. 1a). Myxoid transformation is seen in the submucosal layer in the bladder of the obstructive uropathy model. The muscle bundles within the muscular layer are separated (arrows) by myxoid tissue (Fig. 1b). Our interpretation is that this myxoid tissue is the precursor of the dense fibrosis seen in term bladder walls. Col Fe stain demonstrates accumulation of extracellular matrix in both the control and obstructive uropathy bladders shown in Fig. 2 (Col Fe stain original magnification 200×). Small amounts of colloidal iron-positive extracellular matrix accumulation is seen in the submucosal layer in the normal bladder wall of the control lambs (Fig. 2a). In the obstructive uropathy model, extracellular matrix accumulation is increased in the submucosal and muscular layers (Fig. 2b). Immunohistochemical stains for α-SMA allow detection of myofibroblasts. These are shown in Fig. 2c, d (original magnification 400×). There are very small numbers of myofibroblasts in the submucosal layer in control bladders (Fig. 2c), but marked proliferation of myofibroblasts in the submucosal and muscular layers in the obstructive uropathy model (Fig. 2d). This is contrasted with the bladder wall of a normal term lamb of Masson's trichrome stain (Fig. 3a). The appearances of the bladder wall in non-shunted term lambs are shown in Fig. 3b. There is marked thickening of the bladder wall in the obstructed uropathy bladders compared to the control bladder. In the obstructive uropathy model, positive α-SMA staining extends from the submucosal layer into the muscular layers.

In term lambs, five had mild multicystic dysplastic kidney (MCDK), two had severe MCDK, and four lambs simply had hydronephrosis.

Discussion

Antenatal treatment of obstructive uropathy, although widely performed, remains controversial. Recent reports of the short-term and long-term outcomes following fetal intervention for posterior urethral valves highlight that these interventions carry a considerable risk to the fetus, with a fetal mortality rate of 43% [10]. The immediate threat to life resulting

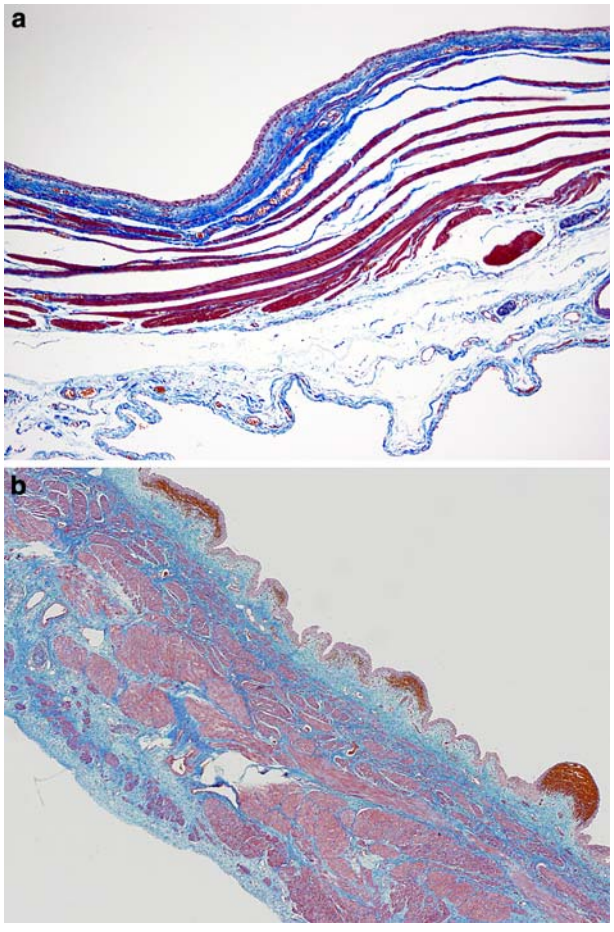

Fig. 3 Term bladder after obstructive uropathy. **a** Samples from the bladder wall of normal term lambs. **b** Samples from the bladder wall of term lambs with bladder outlet obstruction created at 60 days and not shunted. There is marked muscle hypertrophy and interstitial fibrosis in the muscle layer

from obstructive uropathy is pulmonary hypoplasia immediately after birth and renal failure in infancy. However, in the longer term, bladder function, both the ability to void normally and maintain urinary continence, is vital in achieving a good quality of life after fetal intervention. Our previous data demonstrated that shunting the bladder after creating an obstructive uropathy, early in fetal life, resulted in a severely fibrotic and poorly compliant bladder [6, 7, 11].

Bagli et al. [12] showed that the hyaluronic acid receptor is induced by stretch injury of rat bladder in vivo and influences smooth muscle cell contraction in vitro**.** Our models suggest that the congenital bladder failure that occurs in children suffering in utero bladder outlet obstruction results from high-pressure stretch damage to the bladder muscle wall. The molecular mechanisms that incite fibrosis and loss of compliance in the bladder are not yet understood. Bladder dysfunction is reported in 75% of boys with posterior urethral valves on long term follow up [13]. This is established during gestation as a response to urethral obstruction and remains throughout childhood and adolescence even when obstruction is removed in early infancy. These results suggest that the emphasis for fetal intervention for bladder outlet obstruction has moved from salvaging a life-threatening situation toward the preservation of bladder function. Our results clearly show that within 21 days of the onset of a bladder outlet obstruction, there is increased accumulation of extracellular matrix, as demonstrated by our positive Col Fe in both the sub-mucosal and muscle layers of the bladder wall. Earlier experiments have shown that the unshunted bladder develops a thickened, fibrotic bladder wall with smooth muscle hypertrophy and interstitial fibrosis. This effect is even more marked if a vesico-amniotic shunt is created 21 days after the onset of the obstruction [6, 7].

Peters et al. [14] reported that the most evident response of the fetal bladder to congenital bladder obstruction is that of an increase in smooth muscle mass in which there is both an increase in smooth muscle cell volume and an increase in the Myosin content in each muscle cell. Capolicchio et al. [15] reported that collagen gene transcription is rapidly responsive to stretch injury of the developing bladder. Their model is an infant rat bladder and not a fetal bladder but at it does, at least, demonstrate the spectrum of changes and the mechanism by which these changes result in changes in the extracellular matrix collagen gene expression in the obstructed bladder which, we suggest, progresses to fibrotic decompensation. We reported previously that the shunted bladder at term had severe fibrosis extending from the sub-mucosal layer into the intramuscular region and we have expressed our opinion that this result has important implications for the voiding problems reported in human children after fetal intervention for PUV [4]. Our results, from this study, demonstrate that 3 weeks after creating a bladder outlet obstruction, biopsies of the bladder wall demonstrated positive Col Fe stain in the sub-mucosal layer. Our view is that this is related to the stretch injury that increases hyaluronic acid and that this may be facilitating new collagen assembly, thus generating the fibrosis that is such a prominent feature in these bladders at term. Granulation tissue fibroblasts (myofibroblasts) develop several ultrastructural and biochemical features of smooth muscle cells, including the presence of microfilament bundles and the expression of α-SMA [16]. Our bladder biopsies also demonstrated positive α-SMA in the submucosal layer 3 weeks after the onset

of obstruction. Comparison with term (145 days) bladder wall biopsies stained with Masson's trichrome reveal that in the fetal bladder biopsy samples (81 days) there was less fibrosis in the fetal bladder but the positive Col Fe and α-SMA staining demonstrated that the fetal bladder could be expected to have significant bladder dysfunction after birth. This suggests that even earlier shunting may be required to preserve bladder function after placing fetal shunts in bladder outlet obstruction. Our goal is to not just preserve renal function but also preserve bladder function for obstructive uropathy patients.

We conclude that fetal shunt operations after obstructive uropathy 3 weeks after obstruction fail to preserve normal bladder histology. Shunted bladders had increased activated myofibroblasts (α-SMA positive) and these produce high hyaluronic acid synthesis in the extracellular matrix (positive Col Fe) in the submucosal and muscle layers of the bladder.

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