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SHORT-TERM FETAL BLADDER OUTFLOW OBSTRUCTION IN THE OVINE MODEL: BLADDER MORPHOLOGY, PHYSIOLOGY AND IN-UTERO URODYNAMICS

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ABSTRACT

Aim: Previous work carried out at the Institute of Child Health revealed that in the fetal sheep, combined urethral and urachal occlusion initiated at 75 days gestation (full term = 145 days) and maintained for 30 days resulted in dilated, hypocontractile and hypercompliant bladders, associated with uniformly disrupted kidney development. The aim of this project was to create a less severe model of fetal bladder outlet obstruction, and to define the role of the urachus. This model would then be utilised to investigate the prenatal onset of obstructive bladder dysfunction by means of in-utero radiotelemetered urodynamics. We hypothesised that short-term obstruction would result in a thick-walled bladder with preserved contractility and compliance, and that urachal ligation alone would result in similar features.

Methods: Male fetal lambs were assigned to urachal ligation and partial urethral occlusion, urachal ligation only, or sham, groups. Histological analyses, filling cystometry and contractility studies were performed following nine days of obstruction. Natural-fill radiotelemetered urodynamics were performed on the urachal and urethral occlusion group,

Results: Nine days urachal and urethral occlusion from mid-gestation caused hydronephrosis and increased bladder weight, protein and DNA content. Detrusor smooth muscle architecture was maintained but urothelia were thickened and showed basal apoptosis. Bladder compliance, wall stress and contractility were not significantly deranged. The thickest, most

compliant bladders were found to be associated with kidneys exhibiting glomerular cysts. Urachal obstruction alone also resulted in similar changes, suggesting that the male fetal lamb urethra is a high-resistance conduit at this gestation. Radiotelemetered urodynamics were only feasible in the obstructed group from 94 days gestation, and revealed the presence of early-onset hypercontractility. Fetal voids became increasingly frequent and prolonged, occurring at higher voiding pressures; baseline filling pressures did not vary significantly over the nine-day period of observation.

Conclusion: Short-term fetal bladder outflow obstruction from mid-gestation generated thick-walled bladders without evidence of contractile failure; these were associated with cystic kidneys. Detrusor hypercontractility and raised voiding pressures were observed within hours of obstruction, although filling pressures remained stable. Future work will investigate the effect of vesico-amniotic shunting on bladder function.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES

DECLARATION

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ABBREVIATIONS

Experimental groups:

U	Urachal ligation
UU	Urachal ligation and urethral occlusion
S	Sham
O	Obstructed

Other:

αSMA	α smooth muscle actin
ABMA	αβ Methylene-adenosine-triphosphate
ATP	Adenosine triphosphate
AUM	Asymmetric unit membrane
BFR	Bladder-fetal weight ratio
BOO	Bladder outlet obstruction
CAKUT	Congenital anomalies of the kidney and urinary tract
COPUM	Congenital obstructing posterior urethral membrane
DSM	Detrusor smooth muscle
EFS	Electrical field stimulation
ESRF	End-stage renal failure
iNOS	Inducible nitric oxide synthase
KCl	Potassium chloride
MCUG	Micturating cystourethrogram
NANC	Non-adrenergic non-cholinergic nervious system
PBS	Prune-belly syndrome

PGP 9.5	Protein-G product 9.5
PKD	Polycystic kidney disease
PLUTO	Percutaenous shunting of lower urinary tract obstruction
PUJO	Pelvi-ureteric junction obstruction
PUV	Posterior urethral valves
TEM	Transmission electron microscopy
TTX	Tetrodotoxin
UP	Uroplakin
UTI	Urinary tract infection
VUJO	Vesico-ureteric junction obstruction
VUR	Vesico-ureteric reflux

1. INTRODUCTION

1.1 Overview

Congenital obstructive uropathy is a leading cause of renal failure in childhood, and currently accounts for 15% of children in end-stage renal failure (ESRF) in the United Kingdom (Lewis *et al.* 2007). The incidence of congenital BOO in the northern region of England is 2.2 per 10000 births, and it is most commonly caused by posterior urethral valves (PUV), with an incidence of 1.4 per 10000 births (Fig.1) (Anumba *et al.* 2005). Other causes of BOO include urethral atresia (incidence 0.7/10000 births) and the Prune Belly Syndrome (PBS). Congenital BOO is still a major cause of fetal and neonatal mortality: recent figures reveal an antenetal termination rate of 60%, resulting in an overall mortality of 75% in antenatally-detected cases. There is also a significant mortality of up to 20% in postnatally diagnosed cases (Anumba *et al.* 2005). Death in the neonatal period is most commonly due to associated pulmonary hypoplasia (Anumba *et al.* 2005).

Congenital obstructive uropathy is also associated with life-long morbidity, which includes renal, bladder and sexual dysfunction. Valve ablation in the neonatal period does not affect prognosis, with the rate of onset of renal failure progressing from 6.5% at five years, to 25% at ten years and 50% at 15 years of age (Woodhouse 2001). So far, prenatal treatment involving bladder decompression via vesicoamniotic shunting has also been disappointing. Holmes et al. (2001) reported that 63% of children who underwent in-utero bladder decompression progressed to renal failure.

These disturbing figures suggest that the pathophysiology, and hence the appropriate management, of these conditions has not been entirely elucidated, and the scope of this thesis is to explore this pathophysiology in more detail.

This introductory chapter begins with an overview of the normal and pathological development of the human urinary tract, PUV in particular. This is followed by an overview of the urinary tract embryology of the sheep, with reference to how this animal can be used to model human nephrogenesis. The clinical aspects of congenital obstructive uropathy are then elucidated. This is followed by a section summarising the genetic and surgical models of fetal BOO. The chapter concludes with my aims and hypotheses.

1 .2 Normal and pathological development of the human urinary tract

1.2.1 Human urinary tract embryology

The human kidneys develop from the intermediate mesoderm, which is situated on either side of the dorsal body wall of the human embryo (Larsen 2001). Three different, slightly overlapping, kidney systems derive from the intermediate mesoderm: the pronephros, mesonephros, and the metanephros, the latter appearing during the fifth week of gestation. The pronephros is transitory and non-functional, and presumably represents a vestige of the primitive kidneys which develop in some lower vertebrates (Fig.2A). The pronephros is succeeded by the mesonephros (Fig.2B), which develops in the thoracic and lumbar regions during the fourth week of gestation (Larsen 2001). The mesonephros is drained by a pair of

mesonephric (Wolffian) ducts, which grow caudally to open into the posterior wall of the primitive urogenital sinus. By the fifth week, a pair of ureteric buds sprout from the distal mesonephric ducts and induce the overlying sacral intermediate mesoderm to develop into the metanephros (Fig. 3A and B), or definitive kidney. Fusion of the ureteric buds and metanephros at about 32 days gestation initiates the process of nephrogenesis via a process known as 'reciprocal induction' (Fig. 4). This process depends on glial-derived neurotropic factor (*Gdnf*) released from mesenchymal cells, which binds to *Ret* and *Gfra1* receptors in the mesonephric duct (Schedl *et al.* 2007). Several hours of direct contact with a ureteric bud ampulla are required to induce nephron differentiation in blastema tissue. If the ureteric bud is abnormal or missing, the kidney does not develop. Conversely, reciprocal inductive signals from the metanephric blastema regulate the orderly branching and growth of the bifurcating tips of the ureteric buds (Larsen 2001).

Induction signals from the ampulla cause the metanephric blastema to condense and form a closed tube of epithelial cells, the nephrogenic vesicle. As this vesicle elongates into a tubule, a capillary glomerulus forms near one end of it. The tubule epithelium near the differentiating glomerulus thins and then invaginates to form a Bowman's capsule that surrounds the glomerulus. The lengthening tubule then goes on to form the remaining elements of the nephron: the proximal convoluted tubule, the descending and ascending limbs of the loop of Henle, and the distal convoluted tubule. The definitive nephron with its renal corpuscle is also called the metanephric excretory

unit. During the tenth week, the tips of the distal convoluted tubules connect to the collecting ducts, and the metanephroi become functional. Further branching and nephron formation continue around the outer rim of the kidney, the nephrogenic cortex, until 34 weeks (Larsen 2001). About two-thirds of nephrons are generated in the last third of human gestation, so that the range of nephrons found in healthy human kidneys is around 0.7 to 1.4 million (Keller *et al.* 2003).

Meanwhile, the cloaca (the distal expansion of the hind-gut) is partitioned into a posterior rectum and an anterior urogenital sinus, the latter continuous superiorly with the allantois via the urachus. It is unknown whether this tubular structure derives from the allantois or urogenital sinus (Zieger *et al.* 1998). During the fourth and fifth months of gestation, the urachus narrows to a small-calibre epithelial tube, and gradually loses its patency as the bladder descends into the pelvis. The apical portion narrows progressively into a fibromuscular strand and often loses its attachment to the umbilicus. The urachus lies between the peritoneum and the transversialis fascia and is composed of three tissue layers: a central lumen lined with transitional epithelium, a submucosal connective tissue layer and an outer layer of smooth muscle (Zieger *et al.* 1998).

In a study of the role of the *Sonic Hedgehog* (*Shh*) gene in urinary tract development, Jenkins et al. (2007) have shown that the human embryo urogenital sinus expresses *Shh* at four weeks of gestation, preceding its separation from the hind-gut and the interposition of the mesonephric ducts

at seven weeks. The appearance of *Shh* precedes the uptake of uroplakin and α-smooth muscle actin associated with the development of the bladder, suggesting that *Shh* initiates bladder smooth muscle development (Jenkins et al. 2007). The expanded superior portion of the primitive urogenital sinus becomes the bladder, whereas its inferior portion gives rise (in males) to the pelvic urethra and to the penile urethra, or (in females) to the pelvic urethra and vestibule of the vagina. During this period, it was thought that the openings of the mesonephric ducts are translocated down onto the pelvic urethra by a process that also emplaces the openings of the ureters on the bladder wall (Larsen 2001). This concept was recently challeneged by Batourina et al (2005), who developed a mutant mouse model to show that the common nephritic duct does not differentiate into the trigone, but instead undergoes apoptosis, a crucial step for ureter transposition, controlled by Vitamin A signals from the primitive bladder. Furthermore, Vitamin A and the *Ret* proto-oncogene are required for the formation of proper connections between the bladder and ureter (Batourina *et al.* 2005). Oswald et al (2006) supported this finding when they investigated the ureterotrigonal units of 38 human fetal specimens at nine to 40 weeks gestation. Bladder specimens were stained with α-smooth muscle actin in order to visualize detrusor smooth muscle. The authors found that as early as week 12, the intravesical ureter exhibited only longitudinal muscle fibres arranged in a single muscular layer. A thin layer of connective tissue separated the detrusor muscle from the muscle layer of the intramural ureter. No longitudinal continuation of the ureteral muscle sheath to the bladder neck was present at any gestational stage. The single muscle layer of the ureters crossed the midline to form the

interureteral fold. The trigone is now thought to develop from the urogenital sinus, as a continuous single circular muscular layer corresponding to the posterior part of the vesical sphincter muscle (Oswald *et al.* 2006).

The adult human urinary bladder is composed of two main components: the bladder body, which is located above the ureteric orifices, and the base, consisting of the trigone, urethrovesical junction, deep detrusor, and anterior bladder wall. It is a hollow smooth muscle organ lined by urothelium, which is composed of a transitional type epithelium and underlying lamina propria. Its muscular wall is composed of detrusor smooth muscle cells orientated in three layers (Andersson & Arner 2004). The cells of the outer and inner layers tend to be orientated longitudinally, and those of the middle layer circularly. Detrusor cells are surrounded by connective tissue rich in collagen. (Andersson & Arner 2004). In adults, the trigone is a triangular-shaped region located at the base of the bladder composed of muscle that helps to prevent backflow of urine to the ureters. The trigonal base, which is in the bladder neck, is bounded by the muscular sheath surrounding the intravesicular ureter; the trigonal apex, which is in the urethra, is the site where the sex ducts join the urogenital sinus (Tanagho 1981).

The pelvic part of the urogenital sinus is a narrow canal which extends to the surface of the genital tubercle, and will give rise to the urethra (Krishnan *et al.* 2006). This endodermal derived groove becomes a solid plate of cells, the urethral plate, which eventually tubularises in a proximal to distal fashion to form the phallic urethra. The appearance is identical in male and female

fetuses until week nine, and by week 14 the male urethra is complete in its development. The urethra is then divided into four distinct regions: the prostatic and membranous parts are derived from the urogenital sinus and are lined by transitional epithelium (like the bladder); the bulbar and pendulous parts are derived from the urethral plate, and are lined by transitional epithelium proximally and squamous epithelium distally – the latter parts are androgen dependent and are only present in the male (Krishnan *et al.* 2006). Haraguchi et al (2007) showed that the coordination of urogenital organ formation is orchestrated by *Shh* signals. They showed that *Shh* mutant mouse embryos display hypoplasia of external genitalia, internal (pelvic) urethra and bladder, and concluded that mesenchymal precursors for multiple urogenital structures derive from peri-cloacal mesenchyme (Haraguchi *et al.* 2007).

Even though the fetal kidneys produce urine from the tenth week of gestation onwards, their main function is not to clear waste products from the blood, a task handled by the placenta. Instead, fetal urine supplements the production of amniotic fluid. Lee et al. (2007) used three-dimensional ultrasound and VOCAL technology to estimate bladder volume and hence fetal urine output. They showed that the mean fetal bladder cycle lasts 25 minutes, and that fetal urine output increases from a mean of 7.3 ml/hr at 24 weeks gestation, to 71.4 ml/hr at 38 weeks.

1.2.2 Pathogenesis of congenital anomalies of the kidney and urinary tract (CAKUT)

Urinary tract maldevelopment may result in a spectrum of abnormalities known as 'congenital anomalies of the kidney and urinary tract' or CAKUT (Woolf et al 2004). The spectrum of diseases encompassed by the term CAKUT is wide, including kidney anomalies such as agenesis (absent kidney), dysplasia (incomplete and metaplastic development), renal hypoplasia (reduced number of nephrons), duplex kidneys (containing two renal pelves each with its own ureter); ureteric anomalies such as megaureter (abnormally dilated and tortuous ureter), pelviureteric junction obstruction (PUJO) or vesicoureteric junction obstruction (VUJO); primary vesicoureteric reflux (VUR) and anomalies of the bladder and urethra (Woolf *et al* 2004). CAKUT is the underlying diagnosis in 40% of children in ESRF; approximately half of these children have an obstructive nephropathy, mainly PUV and other causes of congenital BOO (Lewis & Shaw 2002). It is thought that the primary "hit" in CAKUT is a developmental one: the trigger event could be genetic (i.e. mutation of a gene expressed by the developing kidney); teratogenic (e.g. ACE inhibitor ingestion in pregnancy or fetal alcohol syndrome); 'disruption' (i.e. dysplasia secondary to urinary flow impairment); or a combination of all three events. The renal damage may then be exacerbated by external factors, such as bladder dysfunction, reflux and infection in the case of PUV (Woolf *et al* 2004).

Potter (1972) classified cystic renal malformation into four categories on the basis of microdissection studies. Types I (autosomal recessive polycystic

kidney disease (PKD)) and III (autosomal dominant PKD) do not feature renal dysplasia. Type II kidneys include subtypes A (very small 'aplastic' kidneys) and B (multicystic dysplastic kidneys). Potter type IV malformations were invariably associated with urinary tract obstruction, usually BOO. Kidneys contained subcapsular cysts, each comprising a dilated Bowman's capsule and a primitive proximal tubule; hence cysts derived from forming nephrons ("S-shaped bodies"). Potter postulated that ureteric bud branching is initially normal in type IV malformations. Only nascent nephrons become cystic because they were nearest to ampullae and experienced "pressure (that) extends in a retrograde manner" from the obstructed lower tract; earlier nephrons located at the other end of arcades faced less "backpressure" and remained intact. Potter reasoned that a sudden, severe, obstructive event would rapidly ablate the renal mesenchyme, resulting in one layer of cysts, whereas mild obstruction might allow the renal mesenchyme to generate several generations of cysts (Potter 1972).

It is important to note that type IV kidneys seem to develop following antenatal, rather than postnatal, BOO, correlating with the greater length and limited compliance of a mature versus an immature nephron tubule. Models of fetal urinary tract obstruction in sheep and monkeys emphasise that formation of subcapsular cysts, some of which contain tufts of podocytes, are an early event after obstruction (Attar *et al.* 1998,Tarantal *et al.* 2001,Nyirady *et al.* 2002). These animal studies confirm that urinary flow impairment can generate type IV kidneys and also leave open the possibility

that more profound grades of malformation (e.g. Potter IIA and B) might result from early BOO.

Although most cases of CAKUT are sporadic, kindreds have been described with more than one affected family member. Sometimes, these families have multi-organ syndromes, whereas others have CAKUT anomalies alone. Examples of such syndromes are:

- Fraser syndrome (*FRAS1* mutation, putative cell adhesion molecule expressed by the ureteric bud): renal agenesis and dysplasia, digit and ocular malformations (McGregor *et al.* 2003,Jadeja *et al.* 2005)
- Townes-Brockes Syndrome (*SALL1* mutation, transcription factor): renal dysplasia and lower urinary tract malformations (Woolf *et al.* 2003)
- Orofacial (Ochoa) syndrome (locus on 10q, gene undefined): congenital obstructive bladder and kidney malformation with abnormal facial expression (Ochoa & Gorlin 1987)

Hydronephrosis per se may also be due to a primary genetic event, such as mutations in *Uroplakin IIIa* (*UPIIIa*) and *Sonic hedgehog* (*Shh)* (Yu *et al.* 2002, Jenkins *et al.* 2005). *UPIIIa* was found to be expressed in nascent urothelia in the ureter and renal pelvis of human embryos, suggesting that perturbed urothelial differentiation may generate human kidney malformations, perhaps by altering differentiation of adjacent smooth muscle cells such that the metanephros is exposed to a functional obstruction of urine flow (Jenkins *et al.* 2005). *Shh*, a *Drosophila Hedgehog* (*Hh*) homolog,

is also expressed in the urothelium; its deletion in a genetic mouse model has been associated with kidney defects such as hypoplasia, hydroureter and hydronephrosis (Yu *et al.* 2002). The role of genetic factors in the aetiology of PUV is yet unclear, although a familial or genetic basis has been suggested on the basis of an association of PUV with specific syndromes (trisomy 21, deletion of chromosome 10q) and reports of rare familial or twin cases (Hanlon-Lundberg *et al.* 1994,Maruotti *et al.* 2006). Weber et al reported a consanguineous family with four male descendants affected by PUV and a healthy girl, suggestive of autosomal recessive inheritance (Weber *et al.* 2005).

1.2.3 Posterior urethral valves: developmental anatomy

Morgagni, and Langenbeck, are reported to have described the existence of posterior urethral valves (PUV) as far back as 1717 and 1805, respectively (reviewed by Krishnan *et al.* 2006). However, it was Young et al, in 1919, who first classified "valves" into the three classical types, after reviewing 36 cases of PUV. Type I valves, the most common, were composed of a ridge coursing anterior from the distal verumontanum (the mucosal elevation at the site of insertion of the ejaculatory ducts) and attaching to the anterior urethra. Although type I valves are usually represented in line sketches as two distinct folds, they actually are a single membranous structure with the opening in the membrane positioned posteriorly near the verumontanum. During voiding, the fused anterior portion of the membrane bulges into the membranous urethra, leaving only a narrow opening along the posterior wall of the urethra. Type II valves, the rarest, extended from the proximal

verumontanum toward the internal sphincter and bladder neck. Type III valves consisted of a discoid valve attached to the entire circumference of the urethra with a central opening. They are thought to represent incomplete dissolution of the urogenital membrane (Krishnan *et al.* 2006). Overall, type I valves make up 95% of lesions and type III make up the remainder; it is doubtful whether type II valves actually exist as a clinical entity (Dewan & Goh 1995).

Lowsley (1914) was the first to offer a theory of PUV development based on microscopic examination of an autopsy specimen. In his study of normal prostate development, he described how the ejaculatory ducts continued distally after opening up into the prostatic lumen, their fibres dissipating laterally. In babies with PUV, however, these fibres attached to the entire circumference of the urethra, creating an almost total obstruction of the urethra. Lowsley also noted that the membrane had squamous epithelium, differentiating it from the normally transitional epithelium of that region, leading him to suggest PUV was due to an anomaly of Mullerian duct origin (reviewed by Krishnan *et al.* 2006). In a study of 22 urinary tracts from 12 children who died following obstruction due to PUV, Henneberry and Stephens (1980) showed that the degree of renal dysplasia associated with PUV was dependent on the position of the ureteric orifice in the bladder. Kidneys with a normal ureteric orifice had the potential for excellent renal function; kidneys with ureteral orifices located in diverticula were severely dysplastic; whereas those with their ureteral orifices located laterally in the bladder had a thin cortex, with low glomerular counts. The authors attributed

these differences to ectopic origins of the ureteral buds from the most caudal part of the mesonephric duct. This could lead to induction of defective or sparse mesenchyme of the tail end of the nephrogenic cord with resultant dysplasia and hypoplasia, respectively (Henneberry & Stephens 1980).

The nomenclature of PUV remained wholly unchallenged until the series of Dewan et al. (1994). They thought that the term 'valves' was incorrect because the condition reflected obstruction by a single membrane. They coined the term COPUM (congenital obstructing posterior urethral membrane) to replace PUV (Dewan *et al.* 1994). They also claimed that types I and III valves were the same, with either being mistaken for the other during endoscopy based on the angle of visualisation and prior urethral instrumentation. The only other lesion they recognised which could obstruct the posterior urethra was Cobb's Collar or congenital urethral stricture (Dewan *et al.* 1994). Other mechanical causes of fetal BOO, such as urethral hypoplasia or aplasia, did not fall under the same classification.

1.2.4 Other causes of congenital BOO

Although the focus of this introduction is PUV, it is important to review the features and outcome of the less common causes of fetal BOO. Urethral atresia is a congenital, complete obstruction of the urethra caused by a membrane that is usually located at the distal end of the prostatic urethra. The urethra distal to this point is usually hypoplastic, presumably from lack of fetal voiding (Gonzalez *et al.* 2001). This anomaly is incompatible with life unless an alternative communication develops between the bladder and

amniotic cavity in the form of a patent urachus, a vesico-amniotic fistula or a fistula to the rectum (Gonzalez *et al.* 2001). The possibility of survival with prenatal decompression by vesico-amniotic shunting was reported by Steindhardt et al. in 1990. The fetus was shunted at 30 weeks gestation and although pulmonary function was acceptable, peritoneal dialysis was required in the neonatal period and he eventually received a renal transplant (Steinhardt *et al.* 1990). Freedman et al. included a case of urethral atresia among a series of long-term survivors of prenatal vesico-amniotic shunting (Freedman *et al.* 1999); Gonzalez et al reported the long-term survival of six cases of urethral atresia (all males): three survived following vesico-amniotic shunting in-utero, whereas the remaining three had developed a vesico-cutaneous fistula or patent urachus. Interestingly, five of the patients had a prune-belly phenotype. Ultimately four patients required transplantation and the remaining two are in renal failure (Gonzalez *et al.* 2001).

The Prune Belly Syndrome (PBS) is characterized by a triad of abnormalities including a loose, wrinkled abdominal wall with hypoplastic musculature, urinary tract abnormalities and undescended testicles (Fig. 5) (Reinberg *et al.* 1991). As a consequence, the full syndrome is limited to males; girls presenting with abdominal wall and urinary tract anomalies are rare (Reinberg *et al.* 1991). The condition has an incidence of 1:30,000, and although reports of multiple cases of PBS in the same family have suggested a genetic contribution, no clear mode of inheritance has emerged (Weber *et al.* 2005). The aetiology of abdominal musculature maldevelopment is unknown. It was suggested that the abdominal wall was

deformed by pressure form a distended bladder due to BOO in-utero. However the condition could also arise from a primary defect in mesodermal development (Weber *et al.* 2005).

1.3 Congenital bladder outlet obstruction (BOO): clinical aspects

1.3.1 Congenital BOO: antenatal diagnosis and causes

Antenatal detection of congenital BOO is on the rise, and is reported to have improved from 33% between 1984 and 1990, to 62% between 1991 and 1997, in the north of England (Anumba *et al.* 2005). In fact, a significant prenatal uropathy is found in 1:600 pregnancies (Hutton 2004). Ultrasound findings at the 18 to 20 week anomaly scan may include unilateral or bilateral hydroureteronephrosis and a thick-walled, poorly emptying or persistently full bladder, which may be associated with sonographically echogenic kidneys. The appearance of a full bladder together with a dilated posterior urethra in PUV is reminiscent of a keyhole and is termed the 'keyhole sign' (Fig. 6A). The commonest cause of fetal BOO is PUV in 64% of cases, followed by urethral atresia (39%) and Prune Belly Syndrome associated with obstruction (4%) (Anumba *et al.* 2005). Montemarano et al. (1998) evaluated 21 cases by antenatal ultrasound for the presence of oligohydramnios, posterior urethral dilation, bladder wall thickening, urachal patency, cortical thinning, cortical cysts, and increased renal echogenicity. The postnatal diagnosis turned out to be PUV in only ten cases, the remainder being prune belly syndrome (four cases), primary VUR (four cases), left VUJO (one case), and non-refluxing, non-obstructive megacystis-megaureter (two cases). Oligohydramnios was present in eight

of ten cases of PUV and in one of four cases of prune belly syndrome (Montemarano *et al.* 1998).

There is evidence to suggest that detection of PUV before birth predicts a worse prognosis: Anumba et al. showed that compared to undetected cases, those detected prenatally had higher mortality and a higher rate of chronic renal failure (CRF) at 24 months (17% vs 57%, $p < 0.01$) (Anumba *et al.* 2005). However, more recently other groups have disputed this finding. Thomas (2007) followed up a cohort of PUV patients for a mean of ten years and found no difference in the onset of renal failure in the first ten years of life. However, he suggested that the incidence of late-onset renal deterioration post-puberty may be prevented by antenatal diagnosis and hence close fllow-up and preventive treatment in childhood (Thomas 2007). The likelihood of early-onset renal failure can be largely predicted from the ultrasound appearances of the urinary tract in the second trimester. A study by Hutton et al. (1994) revealed that of 17 infants whose urinary tracts had demonstrated the characteristic ultrasound features of severe urethral obstruction before 24 weeks gestation, 24% subsequently died in infancy from pulmonary and/or renal failure and 35% went on to develop late-onset renal failure. By contrast, all infants with a prenatal diagnosis after 24 weeks survived, and 93% maintained normal renal function in early childhood (Hutton *et al.* 1994).

Cases of PUV not detected antenatally may present in infancy or childhood. Presenting features in the neonatal period are variable. The most severe

cases may present with respiratory difficulties as a consequence of pulmonary hypoplasia, which is thought to be consequent on diaphragmatic compression by the grossly dilated urinary tract, combined with oligohydramnios due to reduced fetal urine output (Nakayama *et al.* 1986). Other modes of presentation include urinary tract infection (UTI) (74%), septicaemia (11%), incontinence (8%), failure to thrive (4%) and abdominal pain and haematuria (3%) (Parkhouse & Woodhouse 1990).

1.3.2 Congenital BOO: in-utero intervention

Management of the distended bladder diagnosed antenatally depends on the underlying aetiology and associated features. In 1994, an algorithm was introduced for the prenatal evaluation and selection of fetal candidates for prenatal intervention and has greatly improved the ability to predict which fetuses might benefit from intervention and those in whom intervention would not improve the clinical outcome (Johnson *et al.* 1994). Three major steps are advised:

1. a fetal karyotype (determination of fetal sex is important as with a female fetus the issue being dealt with may be more complex as in the case of cloacal abnormalities)
2. a detailed sonographic evaluation to rule out other structural anomalies that might impact on the prognosis for the fetus
3. serial urine evaluations to determine the extent of the underlying renal damage present.

A candidate for prenatal intervention would have a normal male karyotype in the absence of other fetal anomalies that would adversely affect prognosis, and maternal oligo/anhydramnios or decreasing amniotic fluid volumes (Johnson *et al.* 1994). The sonographic appearance of the fetal kidneys must also be taken into account, since if these are 'bright' and small then dysplasia is likely and the long-term prognosis is poor. In a study of BOO presenting in the first and second trimester, hyperechogenic kidneys were predictive of renal dysplasia in 95% of cases (Robyr *et al.* 2005). The association of a sagittal diameter of the bladder of at least 40 mm with hydronephrosis before 28 weeks was predictive of PUV with a positive and negative predictive value of 44.4% and 66.6%, respectively (Robyr *et al.* 2005). Three serial vesicocenteses taken at 48-72 hour intervals and showing a pattern of decreasing hypertonicity indicate potential salvage and identify fetuses that hold the greatest potential benefit from in-utero surgical intervention (Johnson *et al.* 1994). Nicolini et al (1992) showed that the highest sensitivity for the detection of renal dysplasia was shown by urinary calcium (100%), whereas urinary sodium showed the best specificity (80%) (Nicolini *et al.* 1992). Muller et al. (2004) proposed that fetal blood levels of β2-microglobulin and cystatin C are significantly higher in cases of bilateral kidney hypoplasia and cystic dysplasia when compared to normal and hyperechogenic, but not dysplastic, fetal kidneys (Muller *et al.* 2004).

Clark et al. (2003) carried out a meta-analysis of studies analysing the effect of prenatal bladder drainage on perinatal survival in fetuses with BOO. Sixteen observational studies that included 9 case series (147 fetuses) and

seven controlled series (195 fetuses) were identified. Among controlled studies, bladder drainage appeared to improve perinatal survival relative to no drainage (Clark *et al.* 2003). However, sub-group analysis indicated that improved survival was predominantly in fetuses with a defined 'poor prognosis' where there appeared to be marked improvement. The authors were unable to comment upon indication or timing of vesico-amniotic shunting due to heterogeneity and shortage of reporting. Also, no outcome data was available regarding the rate of progression to ESRF. Freedman et al (1999) carried out a retrospective study of 34 fetuses with BOO who underwent vesico-amniotic shunt placement. Of the 21 fetuses (62%) that survived the procedure, 57% progressed to CRF or ESRF at a mean follow-up of 54 months (Freedman *et al.* 1999). In a previous controlled study, the same group reported the perinatal survival of prenatally versus postnatally treated infants with PUV to be 60% versus 93% respectively (Freedman *et al.* 1996). However, again the critical factor was the lack of any alteration in the ultimate progression to ESRF in the prenatally treated group in comparison to the postnatally treated infants (31 versus 33%). More recently, Holmes et al (2001) reported outcomes of intervention on 36 fetuses with bilateral hydronephrosis and oligohydramnios at a mean gestational age of 22 weeks. The mean age of the children at follow-up was 54.3 months. Of 14 fetuses who turned out to have PUV, 8 boys survived (43% mortality), of whom five (63%) were in ESRF before puberty (Holmes *et al.* 2001). The rate of progression to ESRF is therefore not different from that quoted in historical studies following postnatal management (Roth *et al.* 2001).

The long-term benefit of antenatal decompression is therefore still questioned– hence the PLUTO (Percutaneous Shunting for Lower Urinary Tract Obstruction) trial (http://www.pluto.bham.ac.uk/) (Kilby *et al.* 2006). This randomised controlled trial is currently recruiting and is being coordinated by Professors Kilby and Khan at the Birmingham Women's Hospital. The plan is to randomise 200 singleton pregnancies with ultrasound evidence of fetal BOO up to 28 weeks gestation (Morris *et al.* 2005). Eligibility for the trial will primarily be based on the "Uncertainty Principle" whereby the fetal medicine specialist is uncertain as to whether decompression would be appropriate. If the fetus is randomized to decompression, a V-A shunt is inserted and the fetus monitored with regular documentation of shunt location, liquor volume, bladder wall thickness and renal pelvic dilatation. The primary outcome measures will be perinatal mortality and serum creatinine at 6 weeks of age, followed by a paediatric nephrology work-up at one year of age (Morris *et al.* 2005). Long-term follow-up, though not mentioned in the study protocol, will be imperative in order to assess outcome in terms of renal and bladder function. Critics of the "uncertainty principle" have argued that because poor prognosis fetuses are the ones likely to benefit from decompression, it is the good prognosis group that need randomization.

Although vesico-amniotic shunting, whereby a catheter drains the fetal bladder into the maternal amniotic cavity, is the most commonly used technique for prenatal bladder decompression (Agarwal & Fisk 2001), other

techniques have been described. Quintero et al. (1995) reported the use of in-utero percutaneous cystoscopy. The advantage of this technique is that it allows distinction between the different causes of fetal BOO. Welsh et al. (2003) published their experience with fetal cystoscopy. The procedure was carried out under local anaesthesia at 14-28 weeks' gestation. A semi-rigid fetoscope was inserted into the fetal bladder via the maternal abdominal wall, and the bladder neck visualised. An attempt was then made to enter the posterior urethra under combined ultrasound guidance and direct vision (Welsh *et al.* 2003). Therapy for valvular obstruction was carried out in 10 of 13 cases by means of hydro-ablation (utilising normal saline flushed at supra-physiological pressure) or by passage of a blunt-ended guide-wire in order to disrupt the valve leaflets. There were no immediate or long-term maternal complications. There were two fetal losses within 2 weeks of the procedure; a third intra-uterine death was related to maternal diabetes. Five cases were complicated by urinary ascites. One infant with urethral atresia died of pulmonary hypoplasia following delivery (Welsh *et al.* 2003). Of the remaining six fetuses, four turned out to have PUV, of whom only two had normal renal function at 16-34 months follow-up. Cystoscopy may therefore have a role in the diagnostic evaluation of fetal obstructive uropathy in-utero, but requires further evaluation, in particular, with equipment modifications to facilitate entry into the posterior urethra. Overcoming current technical limitations seems a prerequisite to definite evaluation of cystoscopic therapy (Welsh *et al.* 2003).

1.4 Posterior urethral valves (PUV)

1.4.1 Postnatal management of PUV

An early treatment priority is to achieve adequate urine drainage by inserting either a fine urethral or suprapubic catheter (Dinneen & Duffy 1996). Attention is paid to the fluid, electrolyte and acid-base balance, and intravenous broad spectrum antibiotics are administered to treat or prevent sepsis. Hyperkalaemic acidosis is a common serious disturbance. In newborns, the initial serum creatinine is related to maternal renal function (because fetal blood is dialysed against the maternal circulation) and will rise over the next 48 hours to reflect the baby's native renal function. Once the baby is in a stable condition, an ultrasound scan is performed followed by a micturating cystourethrogram (MCUG), which may be performed via a urethral or suprapubic catheter (Fig. 6B). The MCUG confirms the diagnosis and permits assessment of bladder size, shape and outline, the presence of unilateral or bilateral vesicoureteric reflux (VUR). Radioisotope renography with MAG3 (mercaptoacetylglycine labelled with ^{99m}Technetium) can be performed after one month of age to provide information about renal function and drainage (Dinneen & Duffy 1996). ^{99m} Tc-dimercaptosuccinic acid (DMSA) renography and glomerular filtration rate (GFR) estimation are useful in assessment of renal scarring and function. Surgical management is normally by endoscopic valve ablation. However, in the presence of a rising creatinine, recurrent urinary infection or doubts over the efficacy of bladder drainage, diversion, usually vesicostomy, may be a safe alternative (Dinneen & Duffy 1996).

1.4.2 PUV and renal impairment

The most common renal diagnosis in children with ESRF is bilateral renal dysplasia, a term that describes organs containing undifferentiated and metaplastic tissues, such as smooth muscle and cartilage (Ikha-Dahmane *et al.* 1997). Just under half the cases of all childhood ESRF in the United Kingdom caused by renal dysplasia are associated with urinary tract obstruction, PUV being overwhelmingly the most common specific diagnosis, accounting for one half of all boys with ESRF (Lewis 1999). Renal impairment in boys with congenital BOO could theoretically be explained by:

1. Primary renal dysplasia/ hypoplasia and/ or
2. Renal dysplasia secondary to:

   antenatal BOO (which is hypothesised to be associated with bladder dysfunction and high-pressure VUR) and

   postnatal bladder dysfunction, high-pressure VUR, hyperfiltration injury and UTI's

Primary renal dysplasia may be consequent on ectopic ureteric budding and abnormal implantation of the ureteric bud into the metanephric blastema, as proposed by Hennebury and Stephens (1980), on the basis of post-mortem findings of a more lateral ureteric orifice position in 22 renal units from boys with PUV (Henneberry & Stephens 1980). Various studies in animal models have documented a deregulation of cell turnover in dysplastic kidneys, with both enhanced proliferation in cystic structures as well as apoptosis in both epithelia and stroma; the latter might represent a loss of precursor cells that

might otherwise form nephrons (Attar *et al.* 1998). The renal expression of diverse cytokines, growth/ survival factors and transcription factors is also deregulated in these organs, including B-cell lymphoma/ leukaemia (BCL2), hepatocyte growth factor, insulin-like growth factor-1 (IGF-1), paired-box (PAX2), tumour necrosis factor α (TNF-α) and transforming growth factor-β1 (TGF-β1) (Winyard *et al.* 1996,Yang *et al.* 2000).

The mechanism of renal injury due to antenatal BOO as yet not known. Cussen (1971) carried out post-mortem examination of 30 children with congenital urethral obstruction (27 PUV and three urethral atresias). Dysplasia was confirmed histologically in 37%, and it was always associated with ipsilateral reflux, suggesting that antenatal high-pressure VUR may be a contributing factor (Cussen 1971). In addition, VUR is found in 37-67% of boys at the time of diagnosis of PUV (Churchill *et al.* 1983). Prenatal bladder dysfunction is difficult to document, but its presence is suggested by the finding of urodynamic abnormalities in infants born with PUV (Holmdahl *et al.* 1995). Holmdahl et al (1995) were able to document bladder dysfunction in neonates and infants up to five months of age, suggesting that the onset of this dysfunction must occur prenatally.

Postnatal progression of renal impairment after valve ablation may be due to a combination of factors including persistent dilatation, VUR, break-through infection, bladder dysfunction and hyperfiltration injury (Cuckow 2006). Holmdahl's (1995) study showed that early renal dysfunction occurs in a significant number of cases: out of 16 infants born with PUV, 11 had a

glomerular filtration rate (GFR) of 80% or less of expected value for age; in seven infants, the GFR was below 60% of expected value. Five patients had elevated creatinine levels between 60 and 90µmol/l (normal less than 60µmol/l). In fact, infants may initially require acute renal replacement therapy until adequate renal function returns. Some patients will experience enough recovery of renal function and compensatory function of the remaining nephrons to come off dialysis once stabilized (Becker & Baum 2006). However, patients who do not require renal replacement therapy, or who come off dialysis, are still at risk of developing renal insufficiency in later life. Scott et al (1985) showed that after their initial management, 60% of patients recovered normal biochemical renal function on short term follow-up. Woodhouse (2001) reported that the proportion of children with PUV and renal dysfunction increased from 6.5% at 5 years of age to 25% at 10 years to 50% at 15 years.

Long-term renal outcome is variable and depends on a number of factors. Roth et al (2001) reported the outcome 10 consecutive, antenatally-diagnosed patients treated for PUV in one centre over a period of 21 years. Seven patients (70%) progressed to ESRF over a period of 9 to 13 years, all received maintenance dialysis and four subsequently received kidney transplantation (Roth *et al.* 2001). Older studies reported less dismal figures; however, patient follow-up was over a shorter period of time, and a greater proportion of patients were diagnosed postnatally. Smith et al (1996) reported a 13% progression to ESRF over 11 years (100 patients, of whom 56% were diagnosed before one year of age), which is the same figure

quoted by Merguerian et al (1992) at ten-year follow-up of 102 patients, of whom only 10% were diagnosed before one year of age (Merguerian *et al.* 1992, Smith *et al.* 1996). It is uncertain at what level of GFR in childhood, renal failure becomes inevitable in adult life. As a clinical guide, Woodhouse (2003) reported that adolescents with a GFR of 20ml/min/1.73m^2 are unlikely to avoid dialysis and transplantation in the long-term; those with a GFR of 20-40 are a serious concern (Woodhouse 2003).

Factors thought to influence this rate of progression of renal dysfunction include bladder dysfunction and persistent high-pressure VUR hyperfiltration injury. Parkhouse and Woodhouse (1990) showed that 58% of boys with bilateral reflux developed ESRF, compared with 22% and 20% for unilateral and no reflux respectively. Dinneen et al (1995) studied urinary concentrating ability, urine production and glomerular filtration rates in 51 boys born with PUV between 5 and 10 years of age. Urine concentrating ability was impaired in 75%, the urine being <800mosm/kg in 59% and <300 mosm/kg in 16%. There were significant correlations between concentrating ability and GFR and concentrating capacity and 24-hour urine volumes or overnight urine production. With an increased daily volume of urine there are more cycles of bladder filling and emptying, and so it is hypothesised that more time is spent with the bladder at a high storage pressure, leading to progressive renal injury (Dinneen *et al.* 1995). Neild et al (2004), however, challenged this concept by comparing the renal outcome of 44 patients with primary VUR and renal dysplasia with 24 patients with congenital BOO and bladder dysfunction and a GFR <60ml/min/1.73m^2. They found no significant

difference in renal outcome or median survival, and proposed that the main cause of progression to ESRF is nephrological rather than urological (Neild *et al.* 2004).

1.4.3 PUV and bladder dysfunction

A normal paediatric bladder would be expected to hold a volume of 30+(agex30) ml, up to 12 years of age, after which estimated bladder capacity is 390ml. It would have a wall thickness of less than 3mm on ultrasound scanning, and would be expected to empty at last 90% of its volume on voiding (Neveus *et al.* 2006). Bladder function may be assessed using urodynamic (cystometric) techniques. Cystometry is used to study both the storage and voiding phase of micturition and is the method by which the pressure-volume relationship of the bladder is measured (Neveus *et al.* 2006). This study involves the insertion of a double-lumen catheter into the bladder (either urethrally or suprapubically) in order to simultaneously permit filling and intravesical pressure recording. A rectal balloon catheter allows the measurement of intra-abdominal pressure. Electronic subtraction of these two pressures gives true detrusor pressure. In normal bladder function there should be little change in intravesical pressure from empty to full. Bladder compliance describes the relationship between bladder volume and bladder pressure ($\Delta V/\Delta P$). In the normal adult bladder with a capacity of 400ml, the change in pressure from empty to full should be less than 10cmH_2O (Abrams 2002). In theory, bladder compliance has a passive and active component. The passive component is dependent on bladder wall composition, and if there are significant quantities of fibrous tissue within the

bladder wall, then compliance may be reduced. Detrusor overactivity exists when, during the filling phase, there are involuntary detrusor contractions that the patient cannot suppress, characterised by phasic contractions in which the detrusor pressure rises and then falls (Abrams 2002).

Ghanem et al. (2004) evaluated the urodynamics of 116 patients born with PUV at a mean follow-up of 10 years post-valve ablation. They found evidence of bladder dysfunction in 80% of cases, namely detrusor overactivity in 38%, poor compliance in 26%, and a combination of the two in 15% (Ghanem *et al.* 2004). Bladder dysfunction may become manifest in the form of incontinence or UTI's; however, as many as 75% of asymptomatic patients can have urodynamic abnormalities, usually hypocontactility (De Gennaro *et al.* 2000). Careful urodynamic evaluation showed that incontinence in boys with PUV was not due to sphincter damage following valve resection, but due to abnormal bladder contractility (Parkhouse *et al.* 1988, Pfister *et al.* 1996, Holmdahl 1997).

The evolution in urodynamic abnormality with age was shown by Holmdahl et al (1996) who compared findings in pre- and postpubertal boys. The detrusor overactivity and reduced compliance noted in infancy improved with age as did the strength of voiding contractions while bladder capacity and residual urine increased. Unstable, poorly compliant, overdistended bladders changed towards decompensation with time, and increased capacity after puberty was attributed to increased prostatic/urethral resistance (Holmdahl *et al.* 1996). De Gennaro et al. (1996) showed that the prevalence of

hypocontractility in boys aged 4 to 7 years was 37%, whereas it was more than 60% in boys older than 12 years. Furthermore, in a study of 30 boys born with PUV and followed up from infancy, the authors showed that bladder dysfunction, mainly hypercontractility, was detected in 21 boys (70%) at the first evaluation and in 18 (60%) at the last (De Gennaro *et al.* 2000). Fifteen boys went on to develop impairment of contractility while voiding at final follow-up. Results suggested that the urodynamic pattern of hypercontractility generally found soon after valve ablation gradually changes to hypocontractility and in many boys this pattern predominates after puberty (De Gennaro *et al.* 2000).

1.4.4 Bladder dysfunction and end-stage renal failure (ESRF)

Parkhouse and Woodhouse (1990) reported an association between continence, which is a marker of bladder dysfunction, and renal outcome. In their series, 45% of 5-year-old boys were incontinent by day, and 46% of these had a bad long-term outcome (death in renal failure, end-stage or chronic renal failure), compared with continent patients of whom only 4% had bad outcomes (follow-up between 11 and 22 years). Ghanem's (2004) study of 116 patients treated for PUV showed that although a combination of loss of compliance and detrusor overactivity was present in only 15% of cases, 53% of this group went on to develop ESRF, suggesting that this combination may be detrimental to the kidneys. Reduced compliance alone also correlated unfavourably with renal function: 40% of patients with loss of compliance developed ESRF (Ghanem *et al.* 2004). A combination of loss of compliance and detrusor overactivity may result in raised intravesical

pressures, which are found in up to 60% of adolescent boys born with PUV (Peters *et al.* 1990). The critical value appears to be a leak-point pressure of 40 cmH_2O, measured during urodynamic evaluation (Woodhouse 2003). For patients with salvageable renal function, management with anticholinergics, or clean intermittent self-catheterisation (CISC) is essential (Woodhouse 2003).

1.4.5 Summary

- In spite of early surgical management of PUV, the secondary effects on the bladder and kidneys are permanent and may be progressive.
- Antenatal diagnosis is associated with a worse prognosis in terms of fetal and perinatal mortality, as well as long-term renal outcome.
- In-utero bladder decompression is feasible and may improve perinatal survival in neonates with an otherwise poor prognosis, but long-term renal outcome is not significantly affected.
- Post-mortem studies suggest that renal dysplasia in PUV may be a primary event, but it is not known whether in-utero bladder dysfunction compounds this damage.

No studies of human fetal bladder function and dysfunction have been reported, yet this seems to be a key piece in this as yet confounding puzzle. Our understanding of the pathophysiology of fetal urinary tract to obstruction has been acquired mainly from the research literature, which will be summarised in the following section.

1.5 Congenital bladder outflow obstruction: research background

1.5.1 Animal models: overview

The use of animal models is expensive, complex and fraught with potential misinterpretation, and raises ethical issues: so why do we continue to use them? The principle goal of all animal research is to improve our understanding of a disease process so that we may treat humans more efficiently. In the field of BOO, it is important to understand the principles of the pathophysiology of the obstructed developing kidney and urinary tract, identify markers of the progression of that natural history, and ultimately to develop more specific treatments for this condition. Treatment should be based more on the impact of obstruction on renal and bladder development rather than the simple 'plumbing' problem producing the obstruction. There are two types of experimental model system in use to examine fetal renal diseases:

1. genetic models, based on spontaneous mutations in isolated animals (in which a specific trait is then propagated) or induced through gene knockout technology; and
2. surgical models of induced disease.

1.5.2 Genetic models of fetal urinary tract obstruction

Spontaneous BOO in animal species is unusual, although recently Singh et al (2007) reported a unique mutant mouse model that develps in-utero megabladder, and variable hydroureteronephrosis and renal insufficiency, secondary to obstructive uropathy. Bladder smooth muscle defects were

evident as early as embryonic day 15 in both genders of affected mice, with male mice exhibiting a more severe phenotype as development proceeds (Singh *et al.* 2007).

Gene knockout technology has presented a new opportunity to study urinary tract obstruction in the developing fetus. Versus the kidney itself, rather less is known about the genes which control the development of the ureter, urinary bladder and urethra. Recent studies have shown that, as the urothelium differentiates, it expresses specialized proteins such as the Uroplakins, which coat the apical surface of cells: part of their function is to make the urinary tract 'water-tight' but they also may be important in directing lower tract growth, possibly even transducing yet-to-be-defined differentiation signals triggered by urinary constituents (Jenkins *et al.* 2005, Jenkins & Woolf 2006). Mice which lack specific Uroplakins have either severe VUR (due to a 'golf-ball' vesicoureteric junction) or anatomical obstruction of the ureter (due to overgrowth of the urothelium). Moreover, rare individuals with vesicoureteric reflux and kidney dysplasia have been shown to have mutations of Uroplakin genes (UPIIIA) (Jenkins & Woolf 2006).

The embryonic urothelium is also remarkable in acting as a 'signalling-centre' because it secretes sonic hedgehog (*Shh*), a growth factor which diffuses into the surrounding mesenchyme and triggers production of detrusor and ureteric smooth muscle (Yu *et al.* 2002). The signal is mediated by a series of other proteins, such as the patched and smoothened

receptors and the GI family of transcription factors. Mutations of these genes in humans cause holoprosencephaly as well as the Pallister-Hall syndrome, and these individuals can sometimes have urogenital sinus defects, absent or small kidneys and vesicoureteric reflux. Mice which are engineered to lack *Shh* in the developing renal tract have small kidneys, probably resulting from poor muscularisation of the ureter and subsequent 'functional' urinary obstruction (Haraguchi *et al.* 2007).

It was recently shown that a transcription factor gene called Teashirt-3 is expressed in the smooth muscle of the ureter: when mice are engineered to have no teashirt gene activity, animals are born with severe hydronephrosis and mimic human PUJO (Gannon *et al.* 2006). Again, there is no overt anatomical blockage of urine flow but the mutant embryonic ureters can be shown to have a very irregular type of spontaneous peristalsis. This type of gene is therefore a candidate for human PUJO, and this disorder may in fact be inherited. Finally, a normal variant (polymorphism) of the Angiotensin II type 2 receptor (*AT2)* gene appears to predispose individuals to a range of lower urinary tract anomalies: this gene may control programmed cell death and hence modulate the final shape of the lower urinary tract (Nishimura *et al.* 1999). Unfortunately to date there are no genetic mouse models which have PUV, although the megabladder model described above my yet prove informative for understanding the human disease (Singh *et al.* 2007).

1.5.3 Surgical models of fetal urinary tract obstruction

Fetal models of urinary tract obstruction have the advantage of reliably reproducing the pathologic condition of choice at a specified time and to a specified degree. This has been successfully accomplished in the mouse, rat, guinea-pig, rabbit, opossum, dog, pig, monkey, and most commonly, the fetal sheep. The larger mammalian models, including the sheep and monkey, exhibit nephrogenesis exclusively during intra-uterine life and have the advantage of longer gestational periods similar to the human condition. In other animals such as rodents, rabbits and pigs, nephrogenesis continues for a variable period postnatally, and therefore neonatal animals may also be used to study the developing kidneys (Matsell & Tarantal 2002). A comparison of periods of gestation and nephrogenesis in these models is presented in Figure 7. Table 1.0 displays periods of nephrogenesis as a percentage of gestation, and hence shows the similarity in the timing of mesonephric and metanephric development in the sheep and human. This implies that, for example, the stage of nephrogenesis at mid-gestation in the ovine model is equivalent to the stage of nephrogenesis at mid-gestation in the human, which is not the case with other animal models (Moritz and Wintour 1999). Tables 1.1 to 1.5 summarise the major fetal/ neonatal BOO studies published in the five most prevalent animal models, namely the lamb, rabbit, rodent, monkey and pig.

1.5.3.1 The fetal lamb BOO model

Prior to reviewing the studies performed on the fetal lamb model, it is useful to review the basics of ovine urinary tract development. Ovine gestation

lasts for 145 days. The urogenital sinus in the fetal sheep is formed by the division of the cloaca and later forms the primitive bladder and urethra (Ward *et al.* 2006). The bladder extends to the umbilicus and opens directly into the allantois. At mid-gestation, the fetal urethra is well-formed but is probably not used as an outflow tract from the bladder as the lumen contains desquamated cells and debris (Ward *et al.* 2006). At this time-point, the urine from the fetal kidneys passes from the bladder in to the allantoic cavity via the urachus until approximately 90 days gestation, when the bladder begins to recede caudally, pulling on the allantoic stalk. Urine progressively passes directly into the amniotic cavity via the urethra until the urachus fully obliterates (the exact time this occurs is unknown but it may remain patent up to term). The delayed closure of thr urachus in the fetal lamb is a key difference from the situation in the human, where the urachus is reported to close between the fourth and fifth months of gestation (Zeiger *et al.* 1998). Ovine fetal allantoic and amniotic sacs remain separate cavities with the former eventually rupturing into the amniotic cavity to form one sac (Ward *et al.* 2006).

During kidney development, the ovine fetus does not develop a pronephros, but forms a "giant glomerulus", consisting of approximately 15 fused glomeruli, at the cranial end of the future mesonephros (Moritz & Wintour 1999). The ovine mesonephros forms around day 17, reaching a peak nephron number by day 30, and then regresses from day 40 to 57. The fetal lamb mesonephros is able to produce hypotonic urine from day 18 of gestation. This urine passes into the allantoic cavity, which increases in

volume from 3ml at day 20 to 20 ml by day 24. Ovine metanephric development begins at day 27. By mid-gestation (75 days), urine production is an impressive 5-6ml pr hour which equates to 0.5 litres of urine/kg body weight per day (Moritz & Wintour 1999). Normal ovine fetal urine is hypotonic, its osmolality being 179 mOsm/ kg (Nijland & Ross 2000). Metanephric formation of new nephrons is complete by day 135 of gestation and therefore complete before birth (Wintour *et al.* 1996).

The first description of ureteric ligation on the fetal sheep was reported by Beck in 1971. He showed that whereas ureteric ligation before mid-gestation results in dysplastic kidney changes, obstruction after mid-gestation results in hydronephrosis but not dysplasia (Beck 1971). Harrison et a.l (1983) later performed urethral ligation on the fetal lamb, and showed that ligation of the urachus is essential in addition to the obstructing the urethra, because the urachus in the fetal lamb, unlike in humans, remains patent throughout gestation (Harrison *et al.* 1983). A comparison of the key studies performed on the fetal lamb BOO model is presented in figures 8 (which summarises the effects of BOO on the kidney) and 9 (effects of BOO on the bladder). Details of these papers may be found in Table 1.1 at the end of the chapter.

Peters et al (1992a) highlighted the profound histopathological effects that BOO in early gestation (before 75 days) had on nephrogenesis, including cystic transformation of the elements of the nephron, abnormal glomerular development, and medullary hypoplasia. They showed that the features of obstructive nephropathy depend on the timing of obstruction. Most notably,

the cystic dysplastic changes and architectural disorganization evident following early obstruction were not noticeable following BOO after 90 days, which was usually associated with cortical thinning but not cystic dysplasia (Peters *et al.* 1992a). Kitagawa et al. (1999) confirmed these findings by performing urachal and urethral ligation on fetal lambs at 60 and 90 days gestation. Most, but not all, obstructed systems were found to have dilated bladders and ureters, as well as vesico-ureteric reflux, on post-mortem cystograms. Obstruction at 90 days was not associated with cystic dysplastic changes, and kidneys were found to have a normal glomerular number (Kitagawa *et al.* 1999). In contrast, obstruction at 60 days was associated with the formation of multiple small cysts and a reduction in glomerular number. The subcapsular cysts seen were thought to reflect damage to the nephrogenic zone, and suggested that in the 60 day model, damage to the developing nephrons occurred while the latter were still in the S-stage of development (Kitagawa *et al.* 1999). The reduction in glomerular number was proposed to be due to a disruption of ureteric duct branching in the developing kidney associated altered glomerular vascularisation and podocyte apoptosis: features also noted in human histological studies (Poucell-Hatton *et al.* 2000).

Further studies on the fetal lamb model have focussed on upper tract obstruction. Attar et al. (1998) and Yang et al. (2001) performed complete unilateral ureteric obstruction in the fetal lamb at 90 days, for a duration of ten days, which resulted in cystic kidneys with a disorganized nephrogenic cortex, with cells separated by oedema and vascular spaces. Cortical

histology was dominated by cysts associated with malformed glomerular tufts. This process was associated with a deregulation of cell turnover, due to a disruption in the balance of genes that promote and inhibit apoptosis. Apoptosis is regulated by an extrinsic pathway and an intrinsic pathway, both of which result in the activation of caspases, which induce apoptosis (Green & Reed 1998). The intrinsic pathway is in turn regulated by BAX, a pro-apoptotic gene, and BCL-2, and anti-apoptotic gene: the relative ratios of pro- and anti-apoptotic proteins determines the sensitivity of the cell to apoptotic stimuli (Reed 2000). The dysregulated tubular epithelial cell cycle observed in the ureteric obstruction model was associated with the sustained expression of PAX2, a cell-prolferation gene, and the over-expression of BCL2, the anti-apoptotic gene (Attar *et al.* 1998). Hence the initiating obstructive event altered the expression of genes necessary for normal tubular epithelial cell differentiation, proliferation, and survival, and was thought to trigger the proliferation of cystic structures, as well as heighten apoptosis in both epithelia and stroma (Attar *et al.* 1998, Yang *et al.* 2001)

Samnakay et al. (2006) published similar findings in a fetal lamb BOO model at 70 days gestation in which the renal status was examined between two and thirty days post-obstruction. Changes in renal morphology were present as early as two days post-obstruction, and progressed over 20-30 days to cystic renal dysplasia. BOO increased the renal cortical expression of BAX relative to BCL-X, a member of the BCL-2 family (Samnakay *et al.* 2006). Gobet et al. (1999) showed that partial BOO at 95 days for two to five

weeks in the fetal lamb resulted in heavier kidneys with increased interstitial fibrosis, as measured by increased extracellular matrix volume fraction. They suggested that a possible mechanism for this process is a shift in the proteolytic activity to reduce matrix degradation associated with increased apoptosis in obstructed kidney (Gobet *et al.* 1999a). Mure et al. (2006) later supported this finding in a fetal lamb study of unilateral ureteral obstruction at 90 days gestation (Mure *et al.* 2006a). They also found increased renal interstitial fibrosis associated with increased type IV collagen deposition (Mure *et al.* 2006b). Peters et al. (1997) showed that increased collagen deposition was also a feature of obstructed fetal bladders (Peters 1997). Since a reduction in collagen degradation could account for the increased content, the authors demonstrated that matrix metalloproteinase-2 (MMP-2) activity, important in collagen degradation, was present in normal, but not obstructed, bladders. This was associated with increased tissue inhibitors of metalloproteinases (TIMP) (Peters 1997).

1.5.3.2 Rabbit BOO models

Details of the studies performed on the rabbit BOO model are presented in Table 1.2. Rabbit nephrogenesis continues during the neonatal period, therefore the neonatal rabbit model may also be employed in order to study the effects of obstruction on the developing kidney. Levin et al. (2002) investigated the effect of BOO on the mature rabbit bladder. Their findings are relevant as they define the terms 'compensation' and 'decompensation', phases of BOO which have not been defined or studied in developmental BOO models. Levin et al. (2002) showed similarities between partial BOO in

mature rabbits and obstructive dysfunction in man, including increased bladder mass, increased fibrosis, reduced compliance, increased incidence of detrusor instability and decreased contractile ability. Gosling et al. (2002) showed that obstructed bladder function in the adult rabbit can remain relatively normal for prolonged periods of time, even though bladder mass is increased, and this defined the *compensated stage* of BOO (Gosling *et al.* 2000). Bladder mass increased rapidly in the first seven days post-obstruction, was constant for the next seven days, then continued to increase gradually. This growth was attributed to bladder wall remodelling with marked urothelial and fibroblast hyperplasia (1 day) and smooth muscle hypertrophy (3-5 days) resulting in a 4-5 fold increase in bladder mass within seven days (Gosling *et al.* 2000). This weight increase could also have been caused by a four-fold increase in blood flow to the mucosa for the first three days post-obstruction, as shown by Lieb et al. (2000).

Levin et al. (1997) defined the *decompensated* stage of BOO in the mature rabbit model, when with prolonged obstruction, bladder function destabilizes and is characterized by progressive deterioration in contractility and function (i.e. ability to empty), a slower increase in mass, and a progressive decrease in the volume fraction of smooth muscle elements in the bladder wall. The end result was either an organ with a thick fibrous wall, low capacity, poor compliance and little or no contractile function; or a dilated bladder with a thin fibrous wall, high capacity, and little or no contractile function (Levin *et al.* 1997). It was hypothesised that the shift from the compensated to the decompensated stage is related to cyclical periods of ischemia followed by

reperfusion (Levin *et al.* 2002). Relative blood flow to the rabbit bladder is increased at one day post-obstruction, due to vasodilatation induced by increased nitric oxide (NO) synthesis and release, then returns to control levels, i.e. blood flow increases with the increase in bladder mass. Using antibodies to the vascular endothelium marker CD31, Levin showed that partial BOO induces a rapid increase and redistribution to small or mid-size blood vessels in the bladder wall. Most notable was the marked neovascularisation of the obstruction-induced proliferated serosal compartment. The end of the compensation stage, Levin argues, is marked by areas of hypoxia revealed using hypoxyprobe-1 (pimidazole), a small molecular weight compound that freely diffuses through tissues and forms protein adducts in hypoxic cells only. The hypoxyprobe-1 adducts can be detected in tissue sections immuno-histochemically (Levin *et al.* 2002). This initiates degenerative membrane effects, which supports the process of bladder decompensation. It seems that relief of obstruction during the compensated phase, at least in animals, can induce a rapid and full restoration of the bladder function. However, if the obstruction is relieved during the decompensated stage, bladder function only partially recovers (Levin *et al.* 2002).

Rohrmann et al. (1997) developed the fetal rabbit BOO model, and evaluated the storage and emptying functions of the fetal bladder obstructed for the last eight days of gestation. Histologically, there was smooth muscle cell hypertrophy and increased connective tissue in obstructed bladders. In whole bladder preparations, the obstructed bladder had increased

compliance. Removing calcium form the organ bath, thus eliminating the active component of compliance, did not affect compliance. This finding suggested that increased compliance was due to the passive properties of the bladder wall rather than smooth muscle tone (Rohrmann *et al.* 1997).

Contractility in the unobstructed bladder was examined by subjecting newborn rabbit bladder strips to various neurotransmitter agonists and antagonists (Levin *et al.* 1981). There was a poor contractile response to methoxamine, an alpha-adrenergic agonist, and isoproterenol, a beta-adrenergic agonist. In contrast, these bladder strips had a normal contractile response to bethanechol, a muscarinic cholinergic agonist, and ATP, a purinergic agonist (Levin *et al.* 1981). As with compliance, contractile function increased as development progressed (Levin *et al.* 1981). Following obstruction, however, Rohrmann et al. (1997) found a *greater* contractile response to bethanecol, in spite of an apparent denervation. These findings led the authors to suggest that this may represent a *denervation supersensitivity* as a result of obstruction. It appeared likely that with prolonged obstruction, the fetal bladder would begin to decompensate, losing both its compliance and contractility (Rohrmann *et al.* 1997).

1.5.3.3 Other developmental BOO models

Table 1.3 summarises BOO studies in the rodent model. Mouse and rat kidneys also continue to develop postnatally and therefore neonatal animals may be used in developmental studies. Lemack et al. (1999) hypothesised that inceased iNOS expression may improve oxygenation during

obstruction-induced ischaemia: they reported that iNOS was non-significantly increased following BOO (Lemack *et al.* 1999). Felsen et al. (2003) went on to show that iNOS inhibition reduced the obstructive effect on bladder growth and spontaneous contractions.

Studies of unilateral ureteric obstruction (UUO) in the neonatal rat have provided many insights in the renal response to obstruction. Thornhill et al. (2005) reported a model of precisely controlled variable partial UOO in the neonatal rat, in which renal growth was impaired in a non-linear fashion, and found that tubular atrophy and interstitial fibrosis develop before significant renal pelvic dilatation. In addition, following a critical partial obstruction of the ureter (70% reduction in ureteral diameter), renal growth was reduced by 60% and the number of glomeruli reduced by 50% (Thornhill *et al.* 2005).

Primate studies are presented in Table 1.4. No primate work has been published in the field of developmental BOO, but considerable work investigating UUO has been performed in this model. Matsell et al. (2002) mimicked pelvi-ureteric junction obstruction (PUJO) by injecting alginate beds into the pelvi-ureteric junction; the beads expand to produce the required partial obstruction. They reported that, as in the sheep model, early kidney obstruction results in disrupted glomerular development, reduced glomerular number, cystic dysplasia, deficient cortical ureteric duct development and branching, and altered glomerular basement membrane formation (Matsell *et al.* 2002a).

The pig model (Table 1.5) was successfully used to test the feasibility of studying pressure-flow changes in unobstructed bladders using dual pressure radiotelemtry devices. Traces were also validated by conventional cystometry (Olsen *et al.* 2001). Olsen et al. (2001) were able to perform conventional urodynamics on unobstructed fetuses in the second and third trimester, accessing the fetal bladder via the urachus. They noted that in early gestation the fetus dribbled continuously but was able to attain significant voiding pressures, by the third trimester, when it voided 2-6 times per minute (Olsen *et al.* 2001). No urodynamic studies on obstructed fetuses have however been documented in the literature.

Bladder compliance has also been studied in the unobstructed fetal calf (Coplen *et al.* 1994, Dean *et al.* 1997). In an isolated whole fetal bovine bladder preparation, total bladder compliance was shown to increase with fetal development (Coplen *et al.* 1994). Compliance with active smooth muscle tone ("active compliance"), however, decreases with fetal age, whereas surgical removal of the detrusor smooth muscle ("passive compliance"), results in an increase in compliance with age (Dean *et al.* 1997). Overall, smooth muscle tension is greater in the youngest fetuses, which therefore have the stiffest bladders. Active smooth muscle tension is a major factor in the compliance of the youngest bladder and decreases as gestation progresses (Dean *et al.* 1997). Connective tissue plays a variable role in compliance during development, and this may be indicative of ongoing remodelling of the extracellular matrix during bladder development (Coplen *et al.* 1994).

Liapis et al (2001) performed UUO in the North American opossum, which resulted in medullary and tubular disruption and a decrease in glomerular number associated with an aberrant increase in fibronectin and collagen I and IV, with a further increase in synthesis with the duration of obstruction.

1.5.4 Models of in-utero bladder decompression

The next step in the investigation of the effect of obstruction on the developing urinary tract is to elucidate to what extent, if any, these changes are reversible following decompression. Kitagawa et al. (2006) created BOO in the fetal lamb at 60 days gestation, and a vesicostomy (female) or urethrostomy (male) fashioned 21 days later. Fetuses were delivered at term, and bladder compliance measured before the lambs were killed. Half the shunted lambs were found to have cystic kidneys, which were not different from the non-shunted ones. Shunted bladder walls were thickened and fibrous, with a thickened muscular layer compared to the obstructed group, and were found to have a poor compliance. The authors concluded that shunting at this gestation was partially effective and would need to be performed earlier to be more effective (Kitagawa *et al.* 2006).

In order to establish the effect that a V-A shint had on the fetal bladder, Kitagawa et al. (2007) studied the effect of shunting on the normal (unobstructed) fetal lamb bladder, carried out from mid-gestation to term. Shunted bladders were noted to have a poor compliance and increased fibrosis, with a mean volume of 4 ml compared to 60 ml in non-shunted

unobstructed bladders. The authors suggested that bladder collapse and lack of cycling due to the shunt could result in damage and consequent fibrotic changes. This led them to experiment with the use of a pressure-limited vesicoamniotic shunt, which, they hypothesised, may preserve the filling and emptying cycle of the fetal bladder (Nagae *et al.* 2006). Both standard and pressure-limiting shunts were found to result in bladder fibrosis; however the latter resulted in a lesser degree of damage.

Edouga et al. (2001) utilised a fetal sheep model to study the effect of de-obstruction on renal development. BOO was performed at 60 days; relief by vesicoamniotic shunting was performed at 90 days gestation. Animals were evaluated at 120 days gestation. Fetal BOO resulted in hydronephrosis (64%) or dysplasia (36%); dysplasia was associated with a reduction in the number of glomeruli. In shunted fetuses, the number of glomeruli increased at 120 days compared with non-diverted fetuses, and the temporal pattern of PAX2, disrupted after obstruction, restored. The authors concluded that in-utero diversion performed before the end of nephrogenesis may allow reversal of the glomerulogenesis arrest observed (Edouga *et al.* 2001).

Duncomb et al. (2002) utilised a similar ovine model of fetal BOO whereby obstruction was performed at 75 days and released at 94 days; animals were studied at 110 days gesatation. BOO resulted in increasing cortical thickness and decreasing glomerular numbers with cystic dysmorphic change. De-obstruction partially reversed these changes although final glomerular counts were in-between but not significantly different from counts

in the obstructed-only or control groups. BOO resulted in both thick and thin-walled bladders but there was no comment about how de-obstruction affected this observation. The authors concluded that there may be at least some renal recovery when diagnosis and intervention occurs in mid-pregnancy (Duncomb *et al.* 2002). Decompression in mid-gestation therefore appears to result in partial reversal of renal, but not bladder, changes.

Lindner et al. (1988) performed short (ten days) and long (six weeks) term BOO in the adult rat. Both models resulted in a rapid increase in bladder weight and DNA and RNA content; after removal of the obstructing ligature, detrusor weight, DNA and RNA content decreased significantly and were almost back at control levels in both groups, within four weeks (Lindner *et al.* 1988).

1.5.5 Summary

- Numerous large and small animal models investigating the effect of BOO on various aspects of kidney and bladder morphology and physiology exist, but results are difficult to assimilate as studies have been performed on different models at different time-points and variable durations.
- Similarly, the effect of obstruction on the bladder and kidney were often performed on separate models, therefore the relationship between findings cannot be commented on.

- The concept of bladder compensation and decompensation, which may be of utmost importance when selecting a time-frame for bladder decompensation, has been defined and studied in the mature, but not fetal, animal model.
- Bladder urodynamics, which are key to precise management of the human PUV bladder, have never been studied on a model of the obstructed fetal bladder.
- Therefore a unifying model which encompasses the above points is essential.

Before moving on to designing this model, a review of relevant bladder physiology, in particular the active role of the urothelium, and the key players in detrusor contractility, is presented.

1.6 Current advances in bladder physiology

1.6.1 The urothelium: "not just a passive barrier"

The urothelium is not merely a passive permeability barrier but an active player in bladder dilatation, responsible for communication of intravesical pressure changes to the underlying nervous system (Apodaca 2004). The epithelium is composed of three cell layers: a single basal cell layer, an intermediate cell layer which may be between one and several cell layers thick, and an umbrella cell layer. The apical surface of umbrella cells contains unique structural and biochemical features (Apodaca 2004). When examined by scanning electron microscopy, the surface of this layer appears pleated and the cells are separated by a tight junction. Higher magnification views show that the surface is covered by raised ridges, also called hinges, or microplicae, and intervening areas called plaques. The membrane associated with the hinge and plaque region is known as the asymmetric unit membrane (AUM) due to the fact that the outer leaflet appears to be twice as thick as the inner leaflet (Apodaca 2004). The AUM is composed of a family of at least five proteins called uroplakins (UPs): UPIa, UPIb, UPII, UPIIIa and UPIIIb. UPIa, UPII and UPIIIa and b are only expressed in the urothelium and are concentrated in the umbrella cell layer . UPIb is also expressed in the cornea and conjunctiva. It is thought that UPs help maintain the permeability barrier associated with the apical membrane of the umbrella cell layer (Apodaca 2004). Lack of UPIII expression in a knockout model resulted in increased permeability to methelene blue, and has been associated with increased cell turnover of the urothelium, vesicoureteric reflux and hydroureter (Hu *et al.* 2001).

Another function ascribed to urothelial plaques is that they may modulate the apical surface area of the umbrella cell during bladder filling and emptying (Truschel *et al.* 2002). UPs are found within fusiform vesicles which occupy the cytoplasm of umbrella cells. These vesicles respond to cyclical changes in hydrostatic pressure as the bladder fills and empties. In the classical model of vesicle dynamics, as the bladder fills, it initially distends by unfolding of its "wrinkled" mucosa, then by a "flattening" of the initially cuboidal umbrella cells. It is hypothesized that this change of shape is associated with exocytosis of the fusiform vesicles, thus increasing the apical surface area of the umbrella cell (Truschel *et al.* 2002). Upon voiding, it is hypothesized that apical membrane added during filling is rapidly internalised, replenishing the pool of discoidal vesicles (Truschel *et al.* 2002). Studies have shown that the volume fraction and numerical density of fusiform vesicles are significantly decreased in filled versus voided bladders and that they acquire a position just underneath the apical plasma membrane in response to increased intravesical pressures (Minsky & Chlapowski 1978). Increased amounts of UPIII are found at the cell surface of umbrella cells exposed to hydrostatic pressure (Truschel *et al.* 2002). A revised model suggests that bladder filling stimulates both endocytosis and exocytosis; endocytosed membrane is delivered to lysosomes whereupon contents are degraded (Truschel *et al.* 2002).

The uroepithelium may communicate bladder fullness to the underlying nervous system through a paracrine system involving ATP release (Knight *et*

al. 2002). Bladder filling increases hydrostatic pressure, stimulating ATP release through an unknown mechanism (Knight *et al.* 2002). The released ATP binds to purinergic $P2X_3$ receptors on afferent nerve processes, increasing nerve firing and relaying bladder filling to the central nervous system (Burnstock 2001). ATP may also bind to purinergic receptors present on the basal/intermediate cells, which stimulate release of 'secretagogues' that act upon umbrella cells to stimulate vesicle exocytosis (Burnstock 2001).

Very little is known about how BOO affects AUM structure, vesicle dynamics and pressure signalling. Studies of obstructed adult human bladders have shown an inverse correlation between the expression of UPs and inducible nitric oxide synthase (iNOS) (Romih *et al.* 2003). A study of bladder neck specimens form adults with benign prostatic hyperplasia has shown that iNOS uptake was localized to areas devoid of an intact AUM. iNOS was not expressed in unobstructed bladder urothelium. As nitric oxide (NO), the product of iNOS, is a vasodilator, NO may regulate bladder mucosal and muscle perfusion, which appears to be inversely related to intravesical pressure. An increase in iNOS may be an initial compensatory response to overcome the effects of ischaemia generated by BOO (Romih *et al.* 2003). In the long term, however, NO could also result in ischaemic damage caused by the formation of peroxynitrite and oxygen free radicals (Romih *et al.* 2003).

Chertin et al. (2004) noted the over-experssion of all forms of nitric oxide synthase (iNOS, eNOS and nNOS) in the early phase of bladder obstruction (2 weeks in an adult guinea pig model), but whereas iNOS was not associated with increased apoptosis, the other two seemed to appear later in obstruction (8 weeks) and where more likely to be associated with areas of apoptosis (Chertin *et al.* 2004).

1.6.2 Bladder contractility and the role of the purinergic nervous system

The fact that autonomic nonadrenergic, noncholinergic (NANC) nerves may play a role in bladder contractility has been known for several years (Ambache & Zar 1970, Taira 1972). A NANC component was recognised in the autonomic nervous system of smooth muscles from a wide range of vertebrate species, and preliminary studies identified purine compounds as transmitter candidates (Burnstock 1977). This led to the view that adenosine and ATP may be released from NANC nerves, and hence the concept of purinergic neurotransmission was proposed (Burnstock 2002). Figure 10 shows how cholinergic and purinergic nerve stimulation results in detrusor contractility. It is widely accepted that there are two major types of purinergic receptor: the P1 receptor, which is more responsive to adenosine, and the P2 receptor, which is more responsive to ATP. Subsequently, the development and use of specific purine nucleotide and nucleoside agonists and antagonists helped to characterise two families of P2 purinoreceptors, namely P2X ionotropic ligand-gated ion-channel receptors, and P2Y metabotropic G-protein-couples receptors (Burnstock 2002). The P2X

receptor is prominent in contractile smooth muscle cells but is not detectable in proliferating smooth muscle cells, in which P2Y receptor expression is substantially increased. Stimulation of purinergic nerves with single pulses produces hyperpolarisations of up to 25 mV (inhibitory junction potentials) in smooth muscle cells. These potentials are unaffected by atropine, adrenergic neuron blocking agents or sympathetic denervation, but are abolished by tetrodotoxin (Burnstock 2002).

In the normal human bladder, atropine will block at least 95% of parasympathetic nerve-mediated contraction, indicating that its innervation is predominantly cholinergic: purinergic signalling is responsible for the atropine-resistant component of contraction (Burnstock 2002). There are a number of examples of the purinergic component of co-transmission increasing in pathological conditions. One is that purinergic nerve-mediated contraction of the human bladder is increased to 40% in the pathophysiological conditions such as interstitial cystitis, BOO, idiopathic instability and possibly neurogenic bladder (Burnstock 2002). Purinergic signalling also appears to play a role in afferent sensation from the bladder. ATP is released from urothelial cells when the bladder is distended. Sensory-nerve recording has indicated that P2X receptors are involved in mediating the nerve responses to bladder distension, providing mechanosensory feedback involving both the micturition reflex and pain. It is not yet known whether these findings are also present in the fetal bladder (Burnstock 2002).

1.7 The unifying model of fetal BOO

1.7.2 The long-term (30 days) fetal BOO model

Nyirady et al. (2002) investigated the effect of urethral and urachal obstruction initiated at mid-gestation (75 days) and lasting 30 days. This long-term fetal BOO resulted in marked hydroureteronephrosis and a floppy, hypocontractile bladder (Fig. 11). There was marked growth of the bladder (both wet and dry weight were significantly increased) compared to unobstructed controls, and a significant increase in both protein and DNA content. Obstructed bladders were characterized by a flattened, single cell-layer urothelium, an attenuated lamina propria, and a significant predominance of connective tissue. Obstructed kidneys showed a morphological disruption of the nephrogenic cortex and subcortical cysts (Nyirady *et al.* 2002). An in-situ end-labelling technique (TUNEL) was used to assess apoptosis, which was noted to be up-regulated in the obstructed detrusor and lamina propria. This was accompanied by a down-regulation of the anti-death protein BCL2 and an up-regulation of the pro-death protein BAX (Thiruchelvam *et al.* 2003a). Moreover, activated caspase-3, an effector of apoptotic death, was increased in obstructed bladders (Nyirady *et al.* 2002).

Ex-vivo cystometry revealed increased compliance and reduced elasticity in the obstructed bladders. Tension-frequency contractility studies showed that obstructed bladder strips were significantly hypocontractile in response to electrical field stimulation (EFS) and to the carbachol, an acetylcholine agonist, α-β methylene-adenosine triphosphate (ABMA), a purinergic

receptor antagonist and KCl, suggesting that both nerve-induced contractility and direct muscle stimulation were reduced. Hypocontractility was more pronounced on nerve-stimulation than on direct muscle stimulation, suggesting that denervation had a greater role to play than detrusor failure per se. This was confirmed on S100 and protein gene product 9.5 (PGP9.5) staining. A greater atropine resistance was noted, suggesting that a purinergic component may be more prominent as cholinergic nerves are failing (Thiruchelvam *et al.* 2003b).

In summary, 30 days of in-utero BOO resulted in an over-compliant, hypocontractile, denervated bladder undergoing active apoptosis of its detrusor and lamina propria layers. Kidneys were cystic and nephrogenesis disrupted. This picture is very similar to the concept bladder decompensation described by Levin et al. (2002) in the mature rabbit model, i.e. "progressive deterioration in contractility and function (i.e. ability to empty), a rapid increase in mass, and a progressive decrease in the volume fraction of smooth muscle elements in the bladder wall" giving an end result of a "dilated bladder with a thin fibrous wall, high capacity, and little or no contractile function" (Levin *et al.* 2002). This type of bladder is more often found in some boys born with PUV who develop a hypocontractile organ around puberty (Holmdahl *et al.* 1996, De Gennaro *et al.* 2000). The next step was to create a model of *compensating* fetal BOO, i.e. the type of bladder found in younger boys born with PUV, then establish a way of recognizing the transition between the two states.

1.7.3 In-utero urodynamics

In-utero urodynamics could potentially be a way of identifying the transition from a compensating to a decompensating bladder. De Tayrac et al. (2003) carried out "traditional" urodynamics using filling cystometrography, on fetal lambs between 87 and 133 days gestation. However, the high fetal death rate (87%) and rate of catheter dislodgement and chorioamnionitis suggested that this model was less than ideal. Some traces were obtainable and revealed a mean resting fetal bladder pressure of 17cm H_2O in both BOO and control fetuses. Mean voiding pressures were 21cm H_2O in control and 20cm H_2O with BOO. Voids occurred every 16 minutes and lasted for 4 minutes in control bladders, and every 25 minutes lasting 5 minutes following obstruction. There was no significant difference between these urodynamic parameters, although instability at end-fill only occurred following BOO (de Tayrac *et al.* 2003). It must be stressed, however, that the "control" fetuses in this study were catheterised via their urachus, which Gobet et al. (1998) have suggested causes BOO at this gestation.

Ghoniem et al. (1997) published the use of telemetry to monitor urodynamics in awake rhesus monkeys, by adapting a system which was originally described for monitoring left ventricular diameter and pressure in dogs (Patrick *et al.* 1974). The advantage of radiotelemetered urodynamics over 'traditional' urodynamics is that it allows bladder pressures to be monitored without the use of external catheters so that dislodgement and infection, and hence fetal death, may be reduced. Transducers were implanted into the primate bladder and abdominal cavity, with all lines

terminating in a subcutaneous transmitter. Conventional urodynamics were performed for comparison and revealed excellent reproducibility (Ghoniem *et al.* 1997). Mills et al. (2000) published the use of radiotelemetered cystometry in adult pigs. Radiotelemetered pressure data were validated by comparison with filling pressures during bladder distension and simultaneous conventional cystometry, and showed a good correlation (Mills *et al.* 2000).

Thiruchelvam et al. (2004) were the first to successfully perform natural-fill radiotelemetered cystometry in a fetal model. The radiotelemetry set-up involved the use of gel-tipped intravesical and intra-abdominal catheters attached to a subcutaneous transmitter. Pressure changes were transmitted to a receiver in the sheep pen in the form of radiowaves; the receiver digitalises the data, which is then stored in a data acquisition system. Four fetal lambs (three at 105 days gestation and one at 75 days gestation) were monitored for four weeks, and the findings validated by ex-vivo cystometry. Insertion of the intra-abdominal and intravesical catheters was performed via an abdominal incision: the tip of the intra-abdominal catheter was intraperitoneal in all fetuses. In two of the 105 day fetuses (one male and one female), the intravesical catheter was inserted through an incision in the bladder wall, whereas in the other two fetuses (both male) the intravesical catheter was inserted into the bladder via the urachus. Traces were obtained from three animals (one trans-urachal catheter displaced before term). Detrusor pressures were derived by calculating the intra-abdominal pressure/ intravesical pressure difference. A normal fetal void was

characterised by a sustained increase in pressure to 10mmHg with superimposed high-frequency, low amplitude activity occurring up to three times per minute at a maximum voiding pressure of 40mmHg, and lasting between 60 and 180 seconds. Discreet detrusor contractions up to 5mmHg, labelled "staccato" activity, were also noted in the unobstructed bladder (Thiruchelvam *et al.* 2004). This study confirmed the resilience of the fetal ovine model, and the feasibility of this set-up for monitoring in-utero cystometry in the ovine model.

1.8 Hypotheses and aims

My hypotheses were that:

1. Short-term (nine days) partial urethral obstruction and urachal ligation in the fetal lamb, initiated at mid-gestation (75 days), would result in a thick-walled bladder with preserved compliance and contractility. In order to clarify the role of the urachus at this gestation, I also hypothesised that urachal ligation alone would result in features of BOO. This hypothesis was tested in Protocol 1.

2. In-vivo radiotelemetered cystometry could be performed in the fetal ovine model of BOO initiated at 94 days gestation for nine days and would identify early-onset bladder dysfunction in the form of high-pressure voiding and detrusor overactivity. This hypothesis was tested in Protocol 2.

Thus the aim of this project was to:

1. develop the ovine model of short-term BOO (as opposed to the long-term BOO model investigated by Thiruchelvam et al. (2003)) at an early stage of the obstructive pathophysiology, such that it could be suitable for subsequent experiments testing a hypothesis of reversibility
2. adapt this model to characterise in-utero bladder dysfunction by means of in-vivo radiotelemetered cystometry

Table 1.0. Mesonephric and metanephric development as a percentage of gestation.

(Adapted from Moritz and Wintour 1999).

Figure 1. Post-mortem appearance of urinary tract associated with posterior urethral valves.

Note bilateral hydroureteronephrosis and thick-walled bladder associated with dilated posterior urethra.

(http://medic.med.uth.tmc.edu)

Figure 2 A. Schematic transverse section through the 50 hour chick embryo showing the pronephric tubule.

Note that the coelom opens into the pronephric tubule via a nephrostome (literally "kidney mouth").

Developmental Biology Online (www.uoguelph.ca)

Figure 2 B. Schematic representation of the mesonephric duct in the chick embryo.

The medial end of the mesonephric duct has expanded and invaginated into itself to form Bowman's capsule.

Developmental Biology Online (www.uoguelph.ca)

Figure 3 A. Schematic representation showing the location of mesonephros and metanephros. B. Metanephric diverticulum (ureteric bud), coming off the mesonephric duct, and the developing metanephros.

Developmental Biology Online (www.uoguelph.ca)

Figure 4. Development of the metanephros into the adult kidney.

(Top right) The dotted outline represents the embryo's body. The ureter elongates and becomes separate from the mesonephric tubule. (Anti-clockwise) Note the increasing branching and complexity of the ureteric bud as it forms the collecting ducts within the medulla of the kidney.

Developmental Biology Online (www.uoguelph.ca)

Figure 5. Infant with prune-belly syndrome: note lax abdominal wall and undescended testicles.

(http://www.emedicine.com/)

Figure 6A. Ultrasound appearance of dilated bladder and posterior urethra (arrow), giving a "key-hole" appearance (www.thefetus.net/)

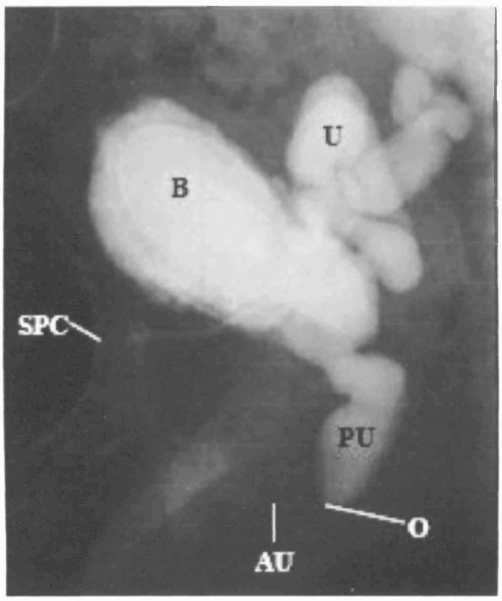


Figure 6B. Micturating cysrourethrogram performed via a supra-pubic catheter (SPC).

Note dilated bladder (B) and ureter (U), and posterior urethra (PU) proximal to the site of obstruction (O). Distal to the valvular obstruction, the anterior urethra (AU) is of normal calibre. (Image courtesy of Mr P Cuckow).

Figure 7. Comparison of periods of gestation and nephrogenesis in various animal models and the human.

(Adapted from Matsell & Tarantal 2002)

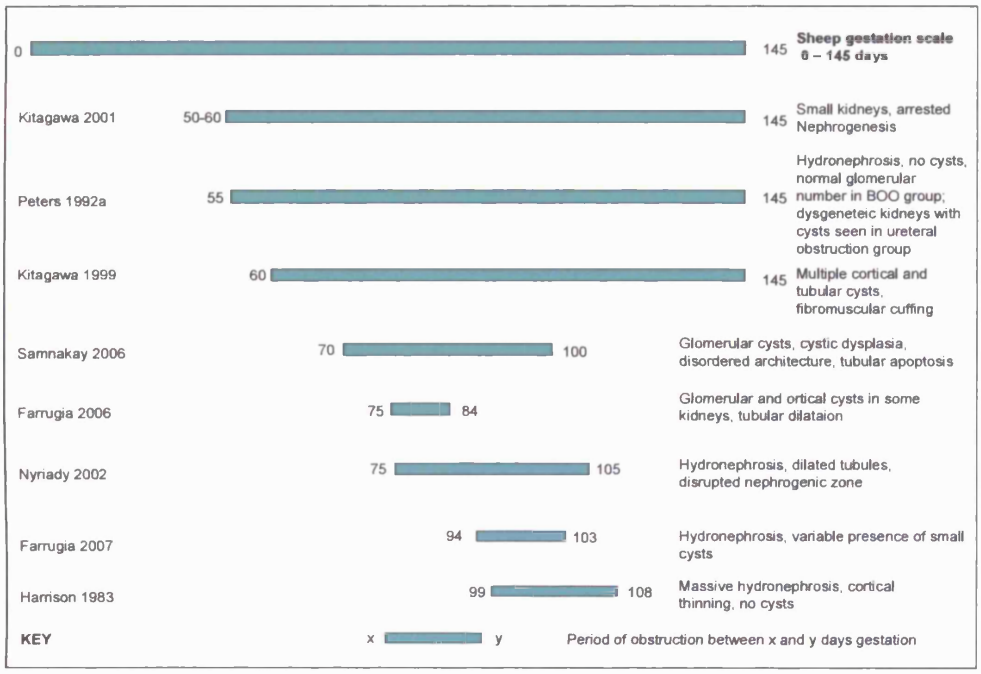


Figure 8. Fetal lamb studies of the effects of BOO on the kidney.

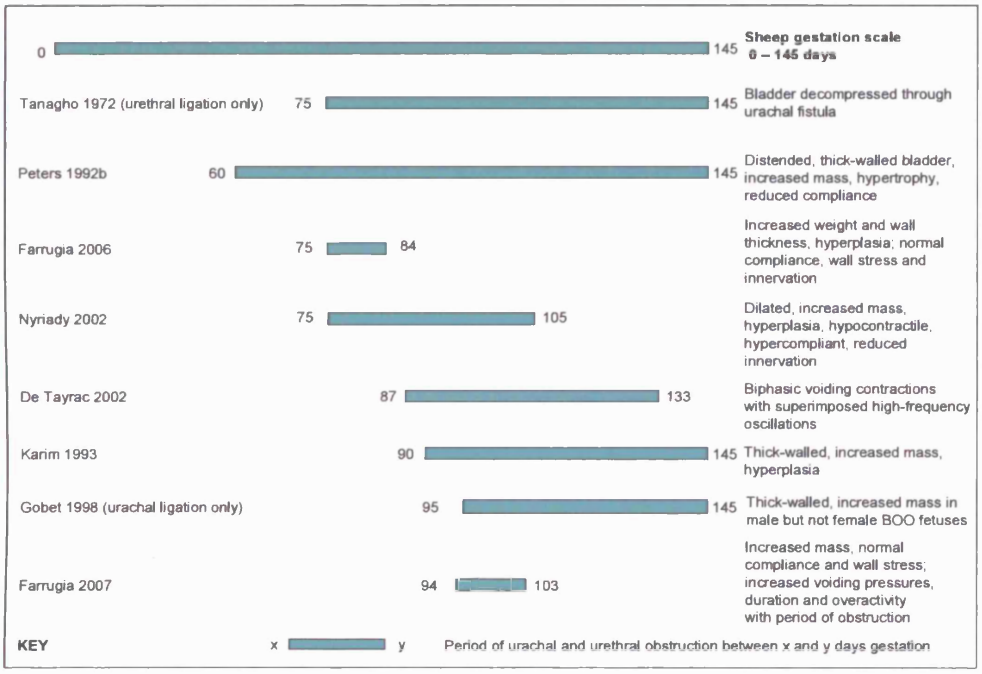


Figure 9. Fetal lamb studies of the effects of BOO on the bladder.

Figure 10. Signal pathways involved in activation of detrusor contraction via the cholinergic (left pathway) and purinergic (right pathway) nervous systems. Acetylcholine (Ach) released at the neuromuscular junction activates the muscarinic M3 receptor wich is coupled to a G-protein; in turn the activated phospholipase C (PLC) triggers inositol triphsphate (IP3) to release calcium from the sarcoplasmic reticulum and hence precipitate contractile coupling. PLC may also activate diacylglycerol (DAG) which results in the opening of non-specific ion channels, allowing influx of calcium into the cell. Alternatively, adenosine triphosphate (ATP) may activate the purinergic P2x receptor, a ligand-gated cation channel that promotes influx of extracellular calcium and hence calmodulin activation as before. Adapted from Fry and Wu 1998.

Figure 11. Comparison of sham and obstructed fetal lamb urinary tracts after 30 days urethral and urachal occlusion initiated at 75 days gestation.

Inset shows a cross-section through the hydronephrotic kidneys. Key: K, kidney; Ur, ureter; Ura, urachus; B, bladder; U, urethra.

(Thiruchelvam *et al.* 2003)

TABLE 1.1 FETAL SHEEP BOO MODEL (145 days gestation)	Publication year	Location	Number studied	Gestational age at BOO	Method of BOO	Gestational age at analysis	Bladder findings	Kidney/Lung findings
Tanagho (Tanagho 1972a, Tanagho 1972b)	1972	San Francisco, USA	N/A	70-75 days	Silastic tubing occluding urethra (partial)	Term	Urethral obstruction became complete as the fetus grew but bladder decompressed via a urachal fistula	-
Harrison, Ross, Noall, de Lorimier (Harrison *et al.* 1983)	1983	San Francisco, USA	5 complete BOO, 8 partial BOO	99-108 days	Urethra was either ligated, or partially occluded with 3mm ameroid constrictor or intermittently occluded with balloon-cuff occluder. Urachus ligated.	Term	-	Complete BOO: massive hydroureteronephrosis and pulmonary hypoplasia, all died Kidneys showed thinning of cortex and medulla with fibrosis; no cyst formation or parenchymal disorganization Partial BOO: hydroureteronephrosis and pulmonary hypoplasia, 4 survived Intermittent BOO: model failed due to inconsistent degree of obstruction
Harrison, Nakayama, Noall, de Lorimier (Harrison *et al.* 1982)	1982	San Francisco, USA	17 BOO of which 9 were decompressed	93-107 days	Urethra occluded with ameroid constrictor (partial) and urachus ligated; decompression via suprapubic cutaneous	Term	-	4 BOO survived to term with respiratory insufficiency and severe hydroureteronephrosis but no cystic dysplastic changes

					cystostomy			7 diverted lambs survived to term with less respiratory insufficiency; hydronephrosis had resolved significantly
Adzick, Harrison, Glick, Flake (Adzick *et al.* 1985)	1985	San Francisco, USA	8 BOO, 9 BOO + cystostomy and 8 controls	95-105 days, suprapubic cystostomy 3 weeks later	Silastic tubing occluding urethra (partial) and urachus ligated	Term	-	4 BOO stillborn, other 4 had respiratory insufficiency due to hypoplastic lungs, only 1 survived 7 decompressed lambs survived, lung weight significantly better Bilateral hydroureteronephrosis but no cystic dysplastic changes in BOO lambs; hydronephrosis had resolved in decompressed lambs
Peters, Carr, Lais, Retik, Mandell (Peters *et al.* 1992a)	1992	Boston, USA	11 BOO, 4 bilateral and 8 unilateral ureteral obstruction	55-60 days	Ureter or urethra and urachus occluded with surgical clip	Term	-	All kidneys were hydronephrotic; most kidneys did not show gross cystic changes but diffuse thinning with normal glomeruli and tubular dilatation on section. Inc urine sodium and normal osmolality in BOO group, no change in ureteral obstruction group 2 BOO, 2 bilateral

								ureteral and 1 unilateral ureteral obstruction kidneys showed cystic changes with distortion of renal parenchyma Dysgenetic kidneys with structural disorganization was only seen in ureteral obstruction groups; urine chemistry significantly altered
Peters, Vasavada, Dator, Carr, Shapiro, Lepor, McDonnell, Retik, Mandell (Peters *et al.* 1992b)	1992	Boston, USA	Unclear as not all animals were analysed	60 days	Surgical clip occluding urethra (complete), urachus ligated	Term	Distended thick-walled bladders, intact muscle bundles, Bladder weight increased x4.6 and muscle mass x5.8 due to hypertrophy (inc protein:DNA ratio) Muscurinic receptor increase x3.2 Reduced compliance	Hydroureteronephrosis
Karim, Cendron, Mostwin, Gearhart (Karim *et al.* 1993)	1993	Baltimore, USA	9 BOO and 6 controls	90-100 days	6mm silver ring (partial), urachus ligated	Term	Thickened bladder wall Significant increase in bladder weigh (x2.7), DNA content (x2.0), and cholinergic nerve density; no change in protein:DNA ratio	No hydronephrosis
Bogaert, Gluckman, Mevorach, Kogan (Bogaert *et al.*	1995	San Francisco, USA	10 BOO and 8 controls	BOO at 90 days, vascular catheters and left renal	Urethra ligated and urachus catheterised, allowing urine drainage into amniotic cavity	120-125 days		Bilateral hydroureteronephrosis in all lambs Higher renal blood flow, increase GFR and urine

1995)				artery flow transducer at 108 days				volume after BOO, no change in sodium excretion Normal architecture but thinned cortex
Gobet, Bleakley, Peters (Gobet *et al.* 1998)	1998	Boston, USA	8 male and 4 female BOO	95 days	Urachus only clipped	109 days (3 male), 116 days (1 male) and term (4 male, 4 female)	7/8 males but no females developed hydroureteronephrosis Significant increase in bladder weight in males only	Cortical thinning and loss of medullary tissue in males, overall renal architecture preserved
Gobet, Park, Nguyen, Chang, Cisek, Peters (Gobet *et al.* 1999)	1999	Boston, USA	12 BOO, 19 controls	95 days	Urethra occluded by surgical clip (complete) and urachus ligated	109 days (n=6) and term (n=6)	-	Increased rennin mRNA (x2.5), angiotensin-2 mRNA (x4.0) and tumour necrosis factor-β1 expression at 109 days
Gobet, Bleakley Cisek, Kaefer, Moses, Fernandez, Peters (Gobet *et al.* 1999)	1999	Boston, USA	33 BOO, 24 controls	95 days	2mm gold ring (partial) and urachus ligated	109 days, 116 days and term	-	Hydronephrosis, distorted nephrogenic blastema Interstitial fibrosis noted 2 weeks post BOO: increased extracellular matrix volume fraction, increased total collagen content and collagen:DNA content Altered proteolytic balance (increased metalloproteinase-1 activity which degrades

								tissue) Increased urine sodium but no change in urine osmolality
Kitagawa, Pringle, Stone, Nakada, Kawaguchi, Nakada, Wakisaka, Furuta, Koike, Seki (Kitagawa *et al.* 1999)	1999	Kawasaki, Japan and Wellington, New Zealand	10 female BOO	60 (n=3) and 90 (n=7) days	Silastic tubing tied around urethra and urachus ligated	Term	-	7/10 hydronephrosis 90 day model: renal architecture maintained, thinned cortex, no cysts, no change in glomerular count 60 day model: multiple small cortical cysts, tubular cysts with fibromuscular cuffing Inc urinary sodium and chloride at 90 days
Kitagawa, Pringle, Zuccollo, Koike, Nakada (Kitagawa *et al.* 2001)	2001	Kawasaki, Japan and Wellington, New Zealand	2 BOO and 1 VUJO at 50 days; and 9 BOO and 9 VUJO at 60 days	50-60 days	Urethra and urachus or ureter ligated with silastic tubing (complete)	Term	-	Three types of renal dysplasia identified: Type A following any obstruction at 50 days: small kidneys, no cysts, paucity of nephrons (arrested nephrogenesis) Type B following BOO at 60 days: large kidneys with large cysts, fibromuscular cuffing – positive keratin staining suggesting distal tubular/ collecting duct origin Type C following VUJO at 60 days: multiple

								small cysts, keratin negative, proximal tubular origin
Levin, Macarak, Howard, Horan, Kogan (Levin *et al.* 2001)	2001	New York and Philadelphia, USA	Not mentioned	90 days	3mm ring around urethra (partial) and 8Fr feeding tube via urachus	93-95 days	Bladder strips subjected to EFS showed biphasic contraction with reduced response at 5 days; no significant difference in response to carbachol, ATP and KCL	-
Edouga, Hugueny, Gasser, Brussieres, Laborde (Edouga *et al.* 2001)	2001	Paris, France	11 BOO only, 5 BOO and V-A shunting, 37 controls	BOO at 60 days, V-A shunting at 90 days	Urethra and urachus occluded by surgical clip (complete)	120 days	-	Control fetal histology at 50 days showed cortical comma- and S-shaped bodies surrounded by undifferentiated mesenchymal tissue; renal cortex became organized in three layers from 60 days. Following BOO, tubular and glomerular microcysts seen in 7 cases, renal dysplasia in 4 cases, subcapsular cysts with distortion in 3 cases. Significant reduction in glomerular number (20% less at 90 days and 25% at 120 days) No change in plasma sodium, potassium,

								creatinine, urea; creatinine clearance significantly lower and sodium excretion higher following BOO Renal morphology in shunted fetuses nearly normal, only residual hydronephrosis but no cysts seen Glomerular count higher than non-shunted group (5% less at 120 days) No significant difference in plasma values or creatinine clearance and sodium excretion
De Tayrac, Cuckow, Devlieger, Deprest, Bogaert, Ville (de Tayrac *et al.* 2003)	2002	London, UK; Leuven, Belgium and Poissy, France	9 urachal occlusion and 2 BOO	87 days	11 with 5 Fr urachal catheter only; 3 also had urethral ring (partial)	133 days	Fetal urodynamic studies via natural fill cystometry were performed weekly under ultrasound monitoring Resting bladder pressure was 14.5cmH20 Mean voiding pressure was 23.0cmH20 Voiding occurred every 19mins and lasted 4.2mins Voiding pattern was biphasic and superimposed by regular low-level	Hydroureteronephrosis and post-void residuals noted in all fetuses (mild in 7 "unobstructed")

							contractions There was no difference between groups	
Nyirady, Thiruchelvam, Fry, Godley, Winyard, Peebles, Woolf, Cuckow (Nyirady *et al.* 2002)	2002	London, UK	8 BOO and 8 controls	75 days	3mm silver ring occluding urethra (partial) and urachus ligated	105 days	Dilated, thin-walled bladder; increased bladder weight (x4.0) Hypocontractile bladder strips in response to EFS, carbachol, KCL and αβMADP Reduced innervation on PGP 9.5 and S100 staining Increased compliance and capacity on ex-vivo cystometry	Progressive hydroureteronephrosis on ultrasonography Disruption of nephrogenic zone, cystic, dilated tubules and loss of normal glomerular formation
Thiruchelvam, Wu, David, 2003Woolf, Cuckow, Fry (Thiruchelvam *et al.* 2003c)	2003	London, UK	5 BOO and 5 controls	75 days	3mm silver ring occluding urethra (partial) and urachus ligated	105 days	Increased bladder compliance and reduced wall stress Muscarinic, purinergic and nitrergic mechanisms present Greater hypocontractility with EFS versus carbachol, suggesting denervation Greater atropine resistance suggesting purinergic component	-
Thiruchelvam, Nyirady, Peebles, Fry, Cuckow, Woolf (Thiruchelvam	2003	London, UK	11 BOO and 11 controls	75 days	3mm silver ring occluding urethra (partial) and urachus ligated	105 days	Increased bladder weight, protein and DNA content Upregulation of apoptosis in detrusor	Progressive hydroureteronephrosis on ultrasonography

***et al.* 2003a)**							and lamina propria; downregulation of anti-death protein Bcl-2 and upregulation of pro-death protein Bax Inc in caspase-3, effector of apoptosis No difference in proliferative index between groups on PCNA staining	
Kitagawa, Pringle, Koike, Zuccollo, Seki, Fugiwaki, Sato, Nagae, Nakada	2003	Kawasaki, Japan and Wellington New Zealand	31 BOO and 8 controls	60 days	Urethra and urachus ligated with silastic tubing (complete)	80-90 days	-	Kidneys had large cysts in 8 lambs, small cysts in 17 and no cysts in 6; 25 kidneys showed early dysplastic changes
Thiruchelvam, Godley, Farrugia, Cuckow (Thiruchelvam *et al.* 2004)	2004	London, UK	2 urachal catheterization, 2 trans-vesical catheterization	105 days (n=3) and 75 days (n=1)	Urodynamic catheter through urachus or bladder wall	Term	Voiding pattern recognized as sustained increase in intravesical pressure with superimpose high frequency low amplitude activity; "staccato" activity: short phasic increase in pressure before and after voiding Urachal catheter at 75 days: voids per hour decreased from 4.0 to 2.0 and duration increased from 25 to 135s Transvesical group:	-

							Voids per hour decreased from 3.0 to 1.5, duration of voids increased from 60 to 100s	
Sato, Kitagawa, Pringle, Koike, Zuccollo, Robinson, Wakisaka, Seki, Nakada (Sato *et al.* 2004)	2004	Kawasaki, Japan and Wellington New Zealand	15 BOO of which 13 shunted but only 3 successful	BOO at 60 days, vesicostomy at 81 days	Urethra and urachus ligated with silastic tubing (complete)	Term	Control BOO bladders had dense subepithelial fibrosis and muscular thickening which were not improved by shunting	-
Kitagawa, Pringle, Koike, Zuccollo, Sato, Sato, Fujiwaki, Odanaka, Nakada (Kitagawa *et al.* 2004)	2004	Kawasaki, Japan and Wellington New Zealand	15 BOO	60 days	Urethra and urachus ligated with silastic tubing (complete)	62/63/65 / 67 days	-	Hydronephrosis noted from 2 days post-BOO Kidneys showed extensive acute tubular necrosis (ATN)-like tubular damage; glomerular cysts were noted in 13 kidneys. Dysplastic changes not seen. No glomerular cysts noted at 2 days, cystic changes in glomeruli and tubules noted at 5 days and glomerular dilatation evident at 7 days
Kitagawa, Pringle, Koike, Zuccollo, Seki, Wakisaka,	2006	Kawasaki, Japan and Wellington New	11 BOO and shunt, 5 BOO controls	BOO at 60 days; Vesicostomy or	Urethra and urachus ligated with silastic tubing (complete)	Term	Shunted bladders had reduced compliance compared to BOO alone; shunted bladder	2 shunted kidneys were small with reduced glomeruli and normal tubules; 3 had cystic

Sato, Sato, Nagae, Nakada (Kitagawa *et al.* 2006)		Zealand		urethrostomy at 81days			walls were thickened with dense fibrosis extending from the submucosal to muscular layers, a feature not present in BOO bladders	dysplastic kidneys; 6 had normal fetal renal histology with macroscopic pelvic dilatation. BOO kidneys were cystic dysplastic.
Samnakay, Orford, Barker, Charles, terry, Newham, Moss (Samnakay N *et al.* 2006)	2006	Subiaco, Australia	33 BOO, 29 controls	70 days	Urethra and urachus ligated with prolene tie (complete)	Fetal kidneys sampled at 72, 75, 80, 90 and 100 days	-	Hydronephrosis in all BOO animals Glomerular cysts were evident from day 2 post-BOO and progressed to cystic renal dysplasia with disordered architecture by day 20 Upregulation of pro-death Bax relative to anti-death Bcl-2 Tubular apoptosis peaked at 2 days; glomerular apoptosis was rare
Farrugia, Long, Godley, Peebles, Fry, Cuckow, Woolf (Farrugia *et al.* 2006a)	2006	London, UK	4 urachal ligation only, 6 BOO and 4 controls	75 days	3mm silver ring occluding urethra (partial) and urachus ligated	84 days	Increase in bladder weight (x5 in urachal group and x3 in BOO group) and bladder wall thickness, no change in protein:DNA ratio Bladder wall architecture preserved Increased urothelial apoptosis in urachal ligation and BOO groups	Hydroureteronephrosis in both urachal alone and complete bladder obstruction. No change in urine sodium and osmolality 2 kidneys in urachal ligation group and 2 in BOO group showed cortical cysts and medullary tubular dilatation

Farrugia, Godley, Woolf, Peebles, Cuckow, Fry (Farrugia *et al.* 2006b)	2006	London, UK	4 urachal ligation only, 6 BOO and 4 controls	75 days	3mm silver ring occluding urethra (partial) and urachus ligated	84 days	Increase in bladder weight, wall thickness and capacity in both obstructed groups No significant difference in compliance and wall stress Contractions blocked by TTX hence nerve-mediated Bladder overgrowth did not result in contractile failure No difference in innervation	Hydroureteronephrosis in both obstructed groups
Wu, Thiruchelvam, Sui, Woolf, Cuckow, Fry (Wu *et al.* 2007)	2007	London, UK	7 BOO and 11 controls	75 days	3mm silver ring occluding urethra (partial) and urachus ligated	105 days	Looked into intracellular calcium regulation by examining the Ca2+ response to agonists in detrusor No difference between resting intracellular calcium but role in down-regulation of muscarinic and purinergic pathways following BOO	-
Farrugia, Woolf, Fry, Peebles, Cuckow, Godley (Farrugia *et al.* 2007)	2007	London, UK	4 BOO and 5 controls	94 days	3mm silver ring occluding urethra (partial) and urachus occluded by urodynamics catheter	103 days	In-vivo fetal urodynamics showed resting bladder pressure of 5-10mmHg with initial voiding pressure of 10mmHg rising to 25mmHg by 3	Hydroureteronephrosis in BOO group ¾ obstructed pairs of kidneys showed cortical cysts but renal architecture well preserved

							days post BOO Voids characterised by sustained rise in pressure with superimposed high frequency low amplitude contractions, and became more frequent and prolonged with time Detrusor overactivity became more prevalent with length of obstruction No significant difference in compliance	

TABLE 1.2 RABBIT BOO MODEL (31 days gestation)	Publication year	Location	Number studied	Age at BOO	Method of BOO	Age at analysis	Bladder findings	Kidney/Lung findings
Rohrmann, Levin, Duckett, Zderic (Rohrmann *et al.* 1996)	1996	Philadelphia, USA	4 animals per group and 4 controls	Mature	Urethra catheterized with 8Fr catheter and tied with silk ligature	1, 7, 14 and 28 days post-obstruction	Increased bladder weight, reduced maximal cholinergic response Contractility at 28 days significantly reduced, suggesting decompensation	-
Rohrmann, Zderic, Duckett, Levin, Damaser (Rohrmann *et al.* 1997)	1997	Philadelphia, USA	8 BOO and 7 controls	23 days gestation	Urethra ligated with silk ligature	30 days gestation	Increased bladder weight and capacity after BOO, no difference in stretch-strain patterns, increased compliance in BOO bladders Spontaneous slow, large amplitude contractions on filling in both groups Removal of calcium from organ bath abolished spontaneous contractions during filling but did not change baseline	-

							pressures or force values	
Geloso, Levin (Geloso & Levin 1998)	1998	Albany, USA	12 BOO and 12 controls	Mature	Urethra catheterized then ligated with silk	14 days post-BOO	Significantly reduced response to EFS after BOO, but increased response in the presence of TTX, unaffected by addition of atropine or ATP suggesting that BOO decreased neurogenic response but increased myogenic response	-
Gotoh, Masuzaki, Taguri, Yoshimura, Ishimaru (Gotoh *et al.* 1998)	1998	Nagasaki, Japan	36 BOO and 10 controls; 3 fetuses decompressed on days 1 and 3	25 days gestation	Urethra ligated with nylon	Fetuses studied on days 1,2,3,4 and 5 post-BOO or 5 days post-decompression	-	Bilateral hydroureteronephrosis from day 1 Hypoplastic cortex and medulla but no dysplasia Renal nephrogenic and glomerular counts lower after 2 days BOO Significant increase in urine microalbumin on day 1 vs day 3 post-BOO; no change in urine β2 microglobulin,

								Na, Cl and osmolality Mild tubular dilatation but less cortical and medullary hypoplasia and better glomerular counts seen in decompressed kidneys
Gosling, Kung, Dixon, Horan, Whitbeck, Levin (Gosling *et al.* 2000)	2000	Albany, USA	28 BOO and 4 controls	Mature	Urethra catheterized then ligated with silk	7,14,28 and 70 days post-BOO	Bladder mass increased rapidly during first 7 days, was constant for next 7 days then gradually increased Decrease in contractile response to EFS greater than decreased response to carbachol Smooth muscle cell hypertrophy and axonal degeneration at 7 days, becoming more extensive with BOO	-
Lieb, Chichester, Kogan, Das, Leggett, Schroder, Levin (Lieb *et al.* 2000)	2000	Albany, USA	20 BOO and 10 controls	Mature	Silk ligature tied loosely around vesical outlet and catheter removed	Left femoral artery and right carotid artery cannulated and rabbits	Rapid increase in bladder weight and capacity post-BOO Mucosal blood flow 4x greater than that of detrusor, more so following BOO with a peak 1 day	No difference in renal blood flow

						studied at 4 hours, 1, 3 and 7 days post-BOO	post-BOO Blood flow in BOO bladders returned to control values at 3 days remaining constant for 7 days	
Schroder, Uvelius, Capello, Longhurst (Schroder *et al.* 2002)	2002	Albany, USA	10 BOO and 4 controls	Mature	Urethra ligated with silk	Cystometry performed pre- and post-obstruction for 2 weeks	Increased bladder weight and post-void residual, no change in compliance After BOO, bladder elongated in long axis Reduced contractile respose to EFS, ATP, carbachol and KCl	-

TABLE 1.3 RODENT BOO MODEL (Mouse 18 day gestation, rat 22 day gestation)	Publication year	Location	Number studied	Age at BOO	Method of BOO	Age at analysis	Bladder findings	Kidney/Lung findings
Uvelius, Persson, Mattiasson (Uvelius *et al.* 1984)	1984	Lund, Sweden	Not mentioned	Mature Sprague-Dawley rats	Urethra ligated	6 weeks and 4 months post-BOO	Bladder muscle mass increased up to 6 weeks post-BOO but stabilizes thereafter, due to both hypertrophy and hyperplasia Increased total collagen	-
Lindner, Mattiasson, Persson, Uvelius (Lindner *et al.* 1988)	1988	Lund, Sweden	Not mentioned	Mature Sprague-Dawley rats	Urethra ligated	10 days and 6 weeks post-BOO, or 2,4 or 6 weeks post-decompression	Increase in detrusor weight after 10 days and 6 weeks BOO Decompression caused reduction in weight up to 4 weeks post-decompression but not thereafter, weight did not return to control values DNA and RNA concentration increased in both BOO groups and decreased post-decompression up to 2 weeks Muscle cell hypertrophy reversed post-decompression	-
Sutherland, Baskin, Kogan, Cunha (Sutherland *et al.* 1998)	1998	San Francisco, USA	48 BOO and 18 controls	Mature Fischer rats	Urethra occluded by ligating over 20G angiocatheter	1,2 and 4 weeks post-BOO	6-7x increase in bladder volume and weight, smooth muscle hypertrophy Increased bladder capacity and voiding pressures post-BOO Diminished AChE-positive fibres within muscle bundles and adrenergic staining in the bladder neck after BOO	-

							PGP nerve staining inc minimally after BOO NADPH staining was similar in both groups	
Lemack, Burkhard, Zimmern, McConnell, Lin (Lemack *et al.* 1999)	1999	Dallas, USA		Mature wild type mice	Urethra occluded by ligating over 22G angiocatheter	1,3 and 5 weeks post-BOO	Increased bladder weight and capacity, decreased cholinergic response at 5 weeks iNOS present in 70% of bladders obstructed for 1 and 3 weeks but in 50% of controls Enhanced iNOS expression may improve oxygenation during obstruction-induced ischaemia	
Kim, Seo, Park, Hwang (Chul *et al.* 2001)	2001	Seoul, Korea	15 mild BOO, 15 severe BOO and 10 controls	Mature Sprague-Dawley rats	Severe: ligature tied loosely around catheter in urethra; Mild: ligature surrounding two catheters and one removed	2 weeks post-BOO	Bladder weight increased in both BOO groups Contractility to EFS inc after mild BOO and decreased after severe BOO Inc in TGF-β1 expression after severe BOO only	-
Felsen, Dardashti, Ostad, Lemer, Gross, Chen, Vaughan, Poppas (Felsen *et al.* 2003)	2003	New York, USA	21 BOO and 22 controls	Mature Sprague-Dawley rats	2mm jeweller jump ring around urethra	2 weeks post-BOO	BOO induced iNOS mRNA expression iNOS-/- mice showed reduced volume at 3weeks iNOS inhibition attenuated increase in bladder size, spontaneous contractions and bladder fibrosis after BOO	-

TABLE 1.4 PRIMATE BOO MODEL (165 days gestation)	Publication year	Location	Number studied	Gestation at BOO	Method of BOO	Gestation at analysis	Bladder findings	Kidney/Lung findings
Tarantal, Han, Cochrum, Mok, DaSilva, Matsell (Tarantal *et al.* 2001)	2001	California, USA and Ontario, Canada	13 unilateral PUJO (contralateral kidney control)	Early: 70, 80 or 90 (second trimester) and late: 100 or 120 (third trimester) days gestation	Ultrasound-guided injection of alginate spheres	90, 120, 140 or 150 days gestation	-	Obstructed kidneys smaller than contralateral side Cortical cysts derived from glomeruli and collecting ducts with disruption of normal glomerular development worse with early than late PUJO but no dysplasia after 120 days obstruction Cortical and medullary interstitial expansion with pericystic and peritubular fibromuscular cuff formation Apoptosis in obstructed kidneys present in cystic glomeruli; tubular apoptosis more pronounced after PUJO
Matsell, Mok, Tarantal (Matsell *et al.* 2002)	2002	California, USA and Ontario, Canada	13 unilateral PUJO (contralateral kidney control)	75 days	Ultrasound-guided injection of alginate spheres	90, 120, 140 or 150 days gestation	-	Ureteric duct branching evaluated with CK-IR and DB lectin staining – reduced expression in outer cortex following PUJO Significant reduction in glomerular number at 150 days; contralateral kidneys displayed fewer glomeruli and ureteric duct area than controls

TABLE 1.5 PIG BOO MODEL (113 days gestation)	Publication year	Location	Number studied	Age at BOO	Method of BOO	Age at analysis	Bladder findings	Kidney/ Lung findings
Speakman, Brading, Gilpin, Dixon, Gilpin, Gosling (Speakman *et al.* 1987)	1987	Oxford, UK	22 BOO, 8 controls	"Recently weaned"	6mm Ω-shaped silver ring occluding urethra	Urodynamic studies ad bladder biopsy performed at 6 weeks, 3,6 and 12 months	Increased voiding pressures and bladder instability after BOO, controls did not have instability Increased contractile responsiveness to carbachol and acetylcholine after BOO, suggesting supersensitvity	-
Nielsen, Andersen, Petersen, Oxlund, Nordling (Nielsen *et al.* 1995)	1995	Aarhus, Denmark	9 BOO, 2 controls	156 days	6mm Ω-shaped teflon ring occluding urethra after bladder wall biopsy	Ring removed 60 days post-BOO	6x increase in bladder weight, 3x increase in smooth muscle number, 2.5x increase in smooth muscle cell size, no difference in muscle and connective tissue proportions, 8x increaes in collage content, increase in collagen I:III ratio All changes incompletely reversed after recovery, except smooth muscle cell number and collagen I:III ratio	-
Kok, Wolffenbuttel, Minekus, Mastrigt, Nijman (Kok *et al.* 2000)	2000	Rotterdam, the Netherlands	34 BOO and 6 controls	3-4 weeks	6mm Ω-shaped silver ring occluding urethra	Pressure flow studies performed before BOO and weekly; animals studied at weeks 1,2,3,4,5,6,8,10 post-BOO	Bladder contractility increased strongly during first 3 weeks, reached plateau weeks 4-7, then decreased Compliance decreased progressively from week 1	-
Mills, Noble, Brading (Mills *et al.* 2000)	2000	Oxford, UK	22 unobstructed	Mature	-	Dual pressure radiotelemetry device implanted for 3 months	Pressure data validated by comparison with conventional cystometry	-

Olsen, Dalmose, Swindle, Jorgensen, Djurhuus (Olsen *et al.* 2001)	2001	Aarhus, Denmark	11 second trimester, 11 third trimester	61 days and 80 days	Urachus catheterized with double-lumen urodynamics catheter, urethra unoccluded	Conventional urodynamics performed for 241 minutes	At mid-term, fetuses dribbled continuously with 1-5 detrusor contractions per minute and maximum flow 1.2ml/min, fill pressure 2-6cmH2O Third trimester pressure increased to mean 5.5cmH2O up to 21cmH2O "staccato" flows 0.2ml/min 2-6 voids per minute	

2. MATERIALS AND METHODS

The aim of Protocol 1 was to develop the ovine model of short-term BOO at an early stage of the obstructive pathophysiology, such that it could be suitable for subsequent experiments testing a hypothesis of reversibility. My hypothesis was that short-term (nine days) partial urethral obstruction and urachal ligation in the fetal lamb, initiated at mid-gestation (75 days), would result in a thick-walled bladder with preserved compliance and contractility, suggesting a stage of bladder compensation. In order to clarify the role of the urachus at this gestation, I also hypothesised that urachal ligation alone would result in features of BOO.

The aim of Protocol 2 was to adapt this model in order to study in-utero radiotelemetered urodynamics on the obstructed fetal bladder. I hypothesised that in-vivo radiotelemetered cystometry could be performed in the fetal ovine model of BOO initiated at 94 days gestation for nine days and would identify early-onset bladder dysfunction in the form of high-pressure voiding and detrusor overactivity.

2.1 Study design

Experiments were conducted in accordance with the Home Office Animals Act (Scientific Procedures) 1986. Protocols 1 and 2 are summarised in Figures 12 A and B. In the section that follows, autopsy, ex-vivo cystometry, in-vitro contractility studies and histology were performed in both protocols and hence only described once. Protein and DNA estimation was only

carried out in Protocol 1, whereas in-vivo radiotelemetered cystometry was only performed in Protocol 2.

2.2 Protocol 1: Fetal BOO from 75 days gestation

2.2.1 Animals

Nineteen pregnant Romney-Marsh ewes (Royal Veterinary College, Potters Bar, UK) were purchased. Ewes were scanned prior to surgery to confirm a viable pregnancy. Establishment of fetal sex was very difficult even in the hands of an experienced obstetrician, therefore all ewes were operated but only male fetuses were included in the study (PUV being a male-limited human disease). When female fetuses were found at laparotomy, these were utilized by our fetal medicine colleagues for a separate gene therapy study.

Animal surgery was performed at the Biological Services Unit, Royal Veterinary College, Camden. The ewes operated in Protocol 1 were the following (numbers are the ewe ID):

Control animals: 2895 (twin 2), 4002, 4007 (twin 1), 4039

Urachal ligation only: 2881 (twin 1), 2891, 4007 (twin 1), 4030

Urachal and urethral ligation: 2881 (twin 2), 2889 (twin 2), 4011, 4084, 4085 (twin 1), 4085 (twin 2)

Female fetuses (excluded): 2886, 2895 (twin 1), 4007 (twin 2), 2889 (twin 1), 4023, 4033

Intra-uterine deaths: 2887 (twins), 4021 (twins), 4023 (twins)

2.2.2 Fetal surgery

Pregnant ewes underwent surgery between 75 and 82 days gestation. They were premedicated with 1 mg/kg sodium thiopentone (Rhone Merieux, Dublin, Ireland) and intubated with a 14Fr endotracheal tube. Anaesthesia was maintained with 2-3% halothane (Fluothane, Mallinckrodt Veterinans, Phillipsburg, New Jersey) in a N_2O/O_2 mixture. After induction, 1 g streptomycin/penicillin (Streptopen, Schering-Plough Animal Health, Omaha, Nebraska) was administered intramuscularly. Laparotomy was performed via a paramedian incision and the peritoneum entered. The pregnant uterus was delivered and single or twin pregnancy confirmed. The uterus was opened using cutting diathermy, carefully preserving all amniotic fluid. Fetal hind-quarters were delivered, and male fetuses were allocated to: a sham control group (n = 4) or one of two groups in which either both urachus and urethra (n = 6) or the urachus-only (n = 4) were obstructed. The urachus, at its confluence with the apex of the bladder, was approached via a para-umbilical incision (Fig.13), and was ligated with a 3/0 silk ligature (Fig.14). In the urethral and urachal ligation group, the urethra was identified as it ran subcutaneously over the abdominal wall in the suprapubic region. A segment was isolated and partially occluded using a standardized 3 mm Ω-shaped silver ring (Fig.15). While the urachal ligation was complete, the urethral ring was expected to constrict but not, at least initially, completely occlude the urethral lumen to produce an incomplete urinary flow impairment. The controls underwent urethral and urachal exposure only. The fetal abdomen was closed using 6/0 prolene interrupted sutures and the fetus returned to the amniotic cavity. Benzylpenicillin sodium (600 mg,

Brittania Pharmaceuticals, Redhill, Surrey, UK) and gentamicin (80 mg, Cidomycin Adult Injectable, Hoechst Marrion Roussel, Strasbourg, Germany) were instilled into the amniotic cavity prior to closure. The uterus was closed with a continuous 2-layer 3/0 silk stitch, and the maternal abdomen was closed in layers, using umbilical tape to secure the fascia, and 0 prolene to skin. Intramuscular buprenorphine (Vetergesic; Alstoe Animal Health, Melton Mowbray, UK) was used for postoperative analgesia. Ultrasonography with a Vingmed CFM 800 device (Vingmed Sound, Horten, Norway) was performed on post-operative days one and seven to confirm fetal viability and monitor urinary tract dilatatation.

2.3 Protocol 2: Fetal BOO model and radiotelemetered urodynamics from 94 days gestation

2.3.1 Animals

Thirteen pregnant Romney-Marsh ewes were operated in the second of our study, with the following ID numbers:

Control animals: 4127, 4132, 4121 (no implants); 4089, 4093 (with implants);

Obstructed animals: 4088, 4091, 4137, 4141;

Animals excluded from study (female fetuses): 4095, 4098, 4105, 4136.

2.3.2 Fetal surgery and radiotelemetry implant insertion

The radiotelemetry set-up used was that employed in our pilot study (Thiruchelvam *et al.* 2004) (Fig.16 and 17). Anaesthesia and surgical approach were carried out as described in section 2.2.2. Pregnant ewes underwent laparotomy and hysterotomy at 94 days gestation. Fetal hind-quarters were delivered, with careful preservation of amniotic fluid, and male fetuses allocated to obstructed (n=4) or sham (n=5) groups. In fetuses assigned to the obstructed group, the intravesical catheter was passed into the bladder via the urachus and secured with a 3/0 silk ligature, while the intra-abdominal catheter was tunnelled beneath the fetal abdominal wall and into the abdominal cavity (Fig.18). The urethra was partially occluded with a standardized 3 mm Ω-shaped silver ring, also seen in Figure 18. The catheters were brought out of the uterus and the implant body was secured on its exterior surface by means of two 2/0 prolene sutures. The uterus was approximated to the inside of the maternal abdominal wall to facilitate switching the implant on and off, performed with a 25T magnet swiped over

the maternal abdomen. In two sham fetuses, the urachus was left patent and the intravesical catheter was inserted via a stab incision on the anterior surface of the bladder, and kept in place by a 6/0 prolene purse-string suture.The intra-abdominal catheter was inserted as described above. The remaining three fetuses underwent exposure only without insertion of a radiotelemetry device. Pregnancy was allowed to proceed for nine days.

2.3.3 In-vivo cystometry

Cystometry traces were recorded continuously for the first 24 hours post-operatively (Day 0), then for two hours daily up to the eighth post-operative day (Days 1 to 8). Traces were stored using the Dataquest software, then analysed at the end of the experiment. 'Voiding' patterns and 'filling' patterns were identified: voids were defined as sustained elevations in detrusor pressure without a concurrent abdominal pressure rise; the filling patterns were traces recorded between voids. The 'voiding' pattern was recognized due to its similarity to that reported in a previous study where fetal voids were visualised by ultrasonography at the same time that direct cystometry measurements were recorded (de Tayrac *et al.* 2003). The same pattern was recognized during the pilot study, performed on unobstructed fetuses at 105 days gestation (Thiruchelvam *et al.* 2004). More than three consecutive acute rises in pressure lasting less than 0.1 of a minute were defined as 'detrusor overactivity'. Representative episodes of good quality recordings devoid of movement artefacts were then copied onto a Microsoft Excel® database for further analyses. An Excel worksheet was created for each animal. Three baseline intra-abdominal and intra-vesical pressure readings

per two minutes of sampled trace were measured and documented. The median detrusor pressure over a six minute period was then calculated by subtracting the median intra-abdominal pressure from median intravesical pressure for that period. The voiding pressure per void was calculated by subtracting the baseline bladder pressure from the peak intra-vesical pressure. The duration of the void was defined as the time taken for the voiding pressure to return to baseline. The interval between the start of one void and that of the subsequent void was measured in order to derive the frequency of voids. Where clearly distinct voiding patterns were identified but no time at baseline was identifiable between voids, the voids were described as "successive". Pressures and duration of episodes of detrusor overactivity were also measured.

2.4 Autopsy

Ewes in both protocols were sacrificed after nine days by means of 1 ml/kg intravenous pentobarbitone sodium injection (Euthatal; Rhone Merieux, Athens, Georgia). Ewes were sectioned and the uterus incised, taking note of, but not formally measuring, amniotic fluid volume. Fetuses were removed, weighed, and their occipito-snout length (OSL) and crown-rump length (CRL) measured. A mid-line para-umbilical incision was then performed and the bladder identified. Both ureters were then ligated at the vesico-ureteric junction and bladder urine was aspirated and its volume noted, then stored in dry ice (for later sodium content and osmolality estimation). After ex-vivo cystometry (see below), bladders were dry-blotted and weighed. Dome strips were used for in-vitro physiology while samples

from bodies of the same organs (defined as the superior portion of the bladder excluding the trigone) were placed in 10% paraformaldehyde (BDH, Poole, UK) for histology or were weighed (after blotting) and frozen in liquid nitrogen pending biochemical analyses. Kidneys were weighed intact (i.e. containing urine in the pelvis) and then again with urine drained; kidney-related weights in *Results* are totals for the two kidneys of each fetus.

2.5 Ex-vivo cystometry

The fetal bladder was catheterised with a 5-French double-lumen tube, inserted through an incision in the proximal urethra, and connected to a PDCR 75 water-filled transducer (Druck Ltd, Groby, UK) placed level with the bladder (Fig.19). One lumen filled the bladder and intravesical pressure was measured via the second lumen, using a Lectromed chart recorder (Letchworth, Hertfordshire, UK). Pressure was recorded at baseline and during intermittent filling with Ca-free Tyrode's solution at room temperature, during stepwise increments of 0.1-0.5 ml. Filling was stopped when initial bladder volumes were reached or when the bladder became tense on visual observation.

Compliance (Δvolume/Δpressure) curves were constructed using raw pressure-volume data. However these curves were not comparable due to the different empty bladder intravesical volumes and variablility in wall thickness. Therefore, standardisation of the curves was carried out by calculating wall stress (σ; a measure of circumferential tension in the bladder wall). A modified Laplace relation $\sigma = P.r_1/2.d$ was used, where P is luminal

pressure, r_1 the bladder inner radius and d wall thickness; r_1 was calculated from bladder volume (V) using the relationship $V = (4\pi/3).r_1^3$. Tissue volume (W) of the bladder wall (bladder weight/density, ρ; ρ = 1.05 g.ml^{-1}) was used to calculate d, assuming that tissue formed a concentric sphere around the bladder lumen, using the formula $d = \left(\sqrt[3]{\frac{3W}{4\pi} + r_1^3}\right) - r_1$. Plots of wall stress, σ, versus bladder volume were linear and the slope was a measure of the physical properties of the bladder wall, a decreased slope indicated that for a given change of volume the consequent change of wall stress was smaller.

2.6 In-vitro contractility studies

Full-thickness bladder dome strips, less than 1mm in diameter, were mounted in a horizontal trough between a fixed hook and an isometric force transducer (Fig.20), and superfused at 4 ml.min^{-1} with Tyrode's solution at 37°C. Electrical field stimulation (EFS) used 3 second trains (1-60 Hz; 0.1 ms pulses) every 90 seconds. The effect of tetrodotoxin (TTX, 1 μM) and atropine (10 μM) in response to EFS, was recorded. Carbachol (10 μM) and α-β methylene-adenosine triphosphate (ABMA, 10 μM) were added to the Tyrode's in the absence of EFS. A high-K (80 mM) solution was made by adding solid KCl to Tyrode's solution, no osmotic correction was made. At the end of the experiments, the preparation diameter and weight were recorded. Tension values are expressed as mN per unit cross-section area (mm^2) of muscle to enable the normalised level of contractility to be compared between specimens.

2.6.1 Solutions

Tyrode's solution contained (mM): NaCl, 118; KCl, 4.0; $NaH_2PO_4.2H_2O$, 0.4; $NaHCO_3$, 24, $MgCl_2.6H_2O$, 1.0, $CaCl_2$, 1.8; Na pyruvate, 5.0; glucose, 6.1; gassed with 95%O_2-5% CO_2. Ca-free solution contained (mM): NaCl, 105; KCl, 3.6; $NaH_2PO_4.2H_2O$, 0.4; HEPES, 19.5; $NaHCO_3$, 22.3; $MgCl_2.6H_2O$, 0.9; glucose, 5.4; Na pyruvate, 4.5, pH 7.1 adjusted with 1 M NaOH. All chemicals were from Sigma-Aldrich Co Ltd, Poole, Dorset, UK.

2.7 Histology

For light microscopy, fixed tissues were washed in PBS (1x30 minutes, 2x1hour, 2x24 hours), dehydrated in alcohols of increasing concentration (30 minutes in each of 70%, 85%, 95% then 100% for 1 hour followed by 1 hour in xylene at 56˚C), left overnight in wax at 56˚C, then embedded in blocks. Blocks were cooled in ice and sectioned at 5 μm using a microtome. Prior to staining, sections were dewaxed by placing in xylene (2x15 minutes) then rehydrated by placing in alcohols of decreasing concentration as follows: 100% alcohol for 5 minutes, 90% for 2 minutes, 80% for 2 minutes, then 70% for 10 minutes. A selection of slides was taken to the histopathology department (Great Ormond Street Hospital) for staining with Masson's trichrome (which differentiates between collagen (blue), cytoplasm and smooth muscle (red) and nuclei (black)) and Elastic von Geison (stains elastin fibres and nuclei black, collagen red and other tissue elements yellow).

2.7.1 Immunohistochemistry

Further sections were used for immunohistochemistry. Endogenous peroxide was quenched by immersing the slides in 3% H_2O_2 in methanol for 30 minutes. After rinsing in buffer (0.1% triton in PBS) for 30 minutes, 10% fetal calf serum was used to block non-specific binding of immunoglobulin (30 minutes). When final staining was not intense enough, the sensitivity of the tissue to antigen binding was increased by microwaving the sections in 6% citric acid at high power for 6 minutes, then rinsed in buffer solution. After excess buffer was allowed to drip off carefully from the slides, tissue sections were encircled using a wax pen, and each slide was placed on a humidified tray. Two hundred microlitres of antibody solution per specimen were required. Negative controls were also created by omitting primary antibodies or replacing them with pre-immune IgG. As a positive control, slides known to stain positively with antibody (courtesy of Nyriady (2002) and Thiruchelvam (2003)) were used.

Primary antibodies employed were:

- Mouse monoclonal anti-human α-smooth muscle actin (α SMA; 1:200; DAKO, High Wycombe, UK) – labels smooth muscle cells, myofibroblasts and myoepithelial cells
- Rabbit anti-human inducible nitric oxide synthase (iNOS; 1:100; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) – identifies the enzymes responsible for NO synthesis
- Rabbit polyclonal anti-human PGP 9.5 (1:500; DAKO) – expressed in vertebrate neurons and neuroendocrine cells

- Mouse anti-human uroplakin III (UPIII; 1:50; courtesy of TT Sun, NYU medical centre, New York), rabbit anti-human UPIb (1:1000) and rabbit anti-human asymmetric unit membrane (AUM; 1:1000) – taken up by the respective uroplakin component of the AUM or by the entire AUM

Primary antibodies were detected with Envision + System-HRP (DAKO). The system is based on an HRP-labelled polymer which is conjugated with secondary antibodies. Staining was completed by a 5-10 minute incubation with 3,3'-diaminobenzidine (DAB) substrate chromagen which results in a brown-coloured precipitate at the antigen site (DAB is a potential carcinogen and precautions were taken). Once stained, the reaction was stopped by rinsing in water twice for ten minutes. Slides were counterstained in Haematoxylin and rinsed in water for ten minutes. Slides were then dehydrated by placing in alcohols of increasing concentrations (70%-80%-90%-100%) followed by xylene twice for 15 minutes. The slides were transferred to a fume cupboard, and DPX adhesive used to fix the coverslip in place. Slides were allowed to dry overnight prior to microscopy. The intensity of the iNOS signals were graded as 0 (absent), 1 (weak) or 2 (strong) by two 'blinded' observers.

2.7.2 TUNEL

Apoptosis (programmed cell death) was detected using an *in-situ* cell death detection kit based on labelling of DNA strand breaks (TUNEL - Roche Applied Science, Penzberg, Germany). Tissue sections were rehydrated as

described above and rinsed in buffer solution. Sections were then incubated with a solution of trypsin (one tablet per ml of water) (Sigma Aldrich, Dorset) for an hour at 37˚C, then rinsed in PBS. Sections were then incubated with the TUNEL kit solution for one hour at 37˚C and rinsed in PBS. Nuclei were counterstained with 1:10,000 propidium iodide (Invitrogen, Paisely) for 5 minutes, rinsed and mounted using immunofluorescence mountant media (Citifluor, London). Fluorescein labels incorporated in nucleotide polymers were detected and quantified by fluorescence microscopy. In order to generate an 'apoptotic index', apoptotic and total number nuclei were counted in six high power fields per specimen, and the percentage of apoptotic cells (the 'apoptotic index') calculated. Numbers of 'nuclear layers in urothelium' were established by counting propidium iodide-labeled nuclei visualised in the shortest distance between lumen and lamina propria: counts quoted per bladder were averages of six separate measurements.

2.7.3 Transmission electron microscopy

For transmission electron microscopy (TEM), tissues were fixed in 3% glutaraldehyde and sections visualised with a JEOL 1200EX electron microscope.

2.8 Protein extraction and quantification

2.8.1 Protein extraction

Bladder samples were weighed and 100 mg of tissue added to 1 ml of radioimmunoprecipitation buffer (RIPA, 150 mM sodium chloride, 1.0% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulphate

(SDS), 50 mM Tris-HCl pH 8.0 and fresh inhibitors (30 μl/ml of 2.2 mg/ml aprotinin; 10 μl/ml of 10 μg/ml phenylmethyl-sulfonyl fluoride; 10 μl/ml of 100mM sodium orthovandate)). The RIPA buffer enables efficient cell lysis and protein solubilisation while avoiding protein degradation and interference with the proteins' immunoreactivity and biological activity. RIPA buffer also results in low background immunoprecipitation and molecular pull-down assays. The samples were homogenised for two minutes on ice in a falcon tube. After each sample, the homogeniser was wiped with tissue and washed in a series of tubes of distilled water. Samples were left to stand for 5 minutes on ice. The supernatant was transferred to 1.5 ml centrifuge tubes and centrifuged at 13,000 x g for 30 minutes at 4˚C to remove any solid debris. The supernatant was transferred to fresh tubes and a further 10 μl of 10 μg/ml PMSF was added and the supernatant stored at -70˚C.

2.8.2 Protein quantification

The concentration of protein was quantified using a bicinchoninic acid protein (BCA) assay (Pierce, Rockford, IL, USA). BCA is a highly sensitive and selective detection agent for the cuprous cation Cu^{+}. This assay combines the reduction of Cu^{2+} by protein to Cu^{+} in an alkaline medium with the cuprous ion detecting property of BCA. For each sample, 50 μl of protein was incubated with 1 ml of working reagent (50 parts of BCA Reagent A and 1 part of BCA reagent B). The mixture was incubated at 37˚ C for 30 minutes in a water bath and absorbance readings measured at 562 nm. 1 ml of sterile water was used as a negative control. A standard curve was

generated using 2 mg/ml of bovine serum albumin (BSA) in serial dilutions and protein concentrations read from this graph.

2.9 DNA extraction

0.1 mg bladder samples were dissolved in 300 μl of extraction mix (20 mM Tris pH 7.4, 100 mM EDTA pH 8.0 and 0.3 mg/ml proteinase K) at 55 °C overnight. Samples were then vortexed to make sure the tissue had fully dissolved. 20 μl of 3M sodium chloride was added to each sample and then 300 μl of phenol. Samples were vortexed and centrifuged at 13,000 x g for 30 minutes. The top (aqueous) phase containing DNA was carefully removed using a 1000 μl pipettor and transferred to a new tube. DNA was precipitated by adding 1x volume of isopropanol. After vortexing and centrifuging at 13,000 x g for 30 minutes a pellet was formed at the bottom of the tube. The pellet was washed in 1 ml ice-cold 70% ethanol and then air-dried. The pellet was then re-suspended in 50 μl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) and quantified at 260 nm using a spectrophotometer.

2.10 Statistics

Due to the small numbers in both experiments, measurements were expressed non-parametrically as medians (ranges) and groups compared using two-tailed Mann-Whitney U-tests. The null hypothesis was rejected at $p<0.05$. Correlation between different variables was assessed by calculation of a correlation coefficient, r, estimation of a t-value from $t = r\sqrt{(n-2)/(1-r^2)}$, to allow estimation of p.

2.11 Summary

- Short-term (nine days) BOO was carried out in the fetal lamb model at 75 days and at 94 days gestation
- Prenatal ultrasonography was performed on post-operative days one and seven
- Radiotelemetered urodynamics (in Protocol 2 only) were monitored at intervals throughout the experiment
- Following autopsy, gross fetal inspection, in-vivo cystometry, contractility studies and histological analyses were performed
- Results were analysed non-parametrically, and will be discussed in the following chapter.

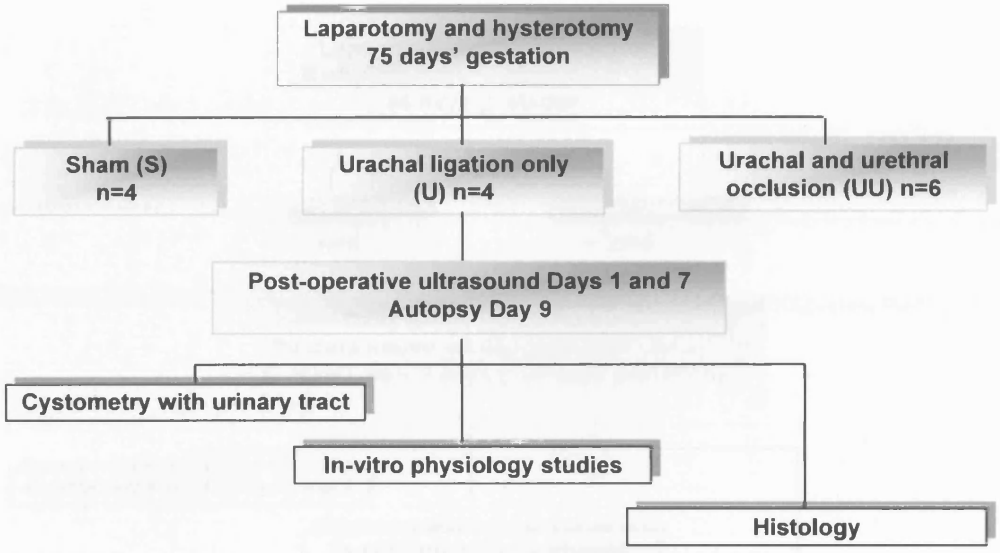


Figure 12 A. Summary of Protocol 1 study design.

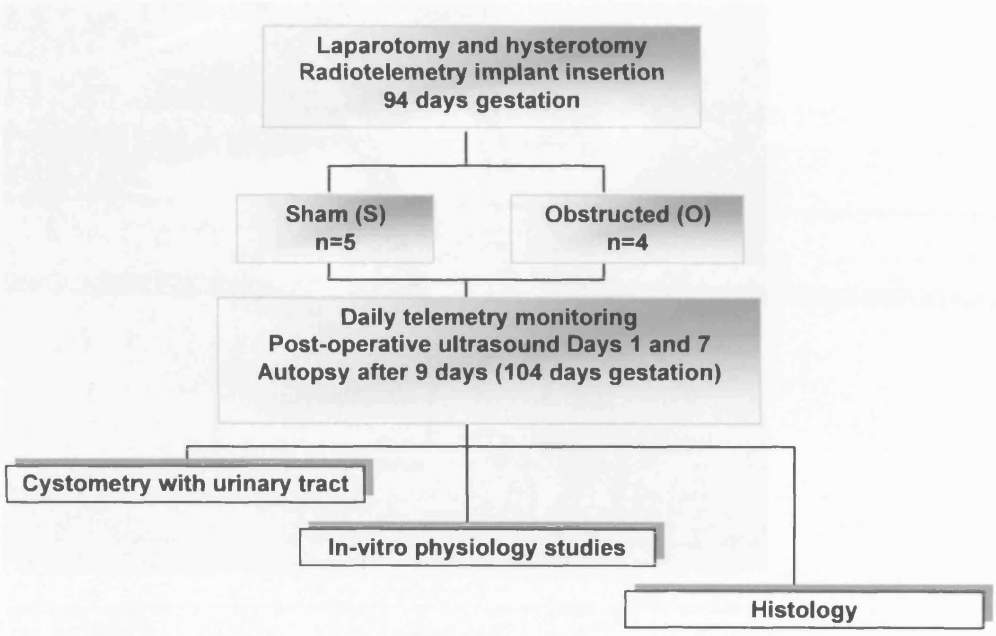


Figure 12 B. Summary of Protocol 2 study design.

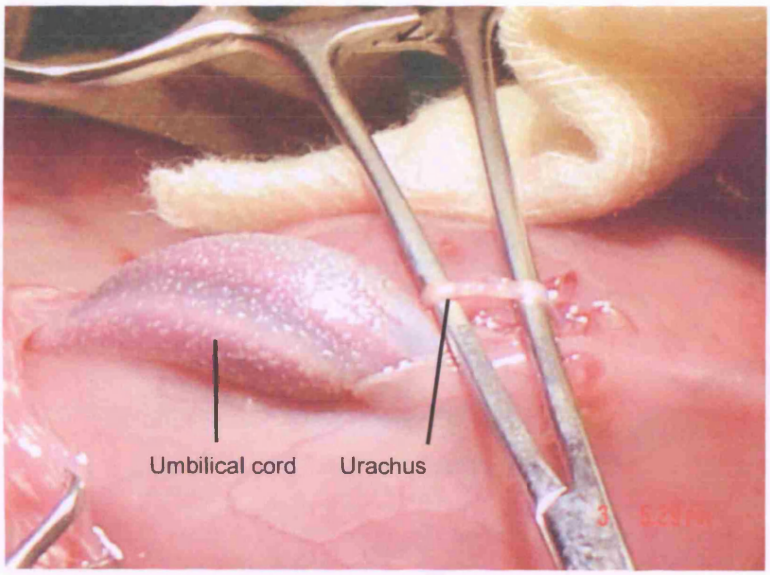


Figure 13. Exposure of urachus lateral to umbilical cord.

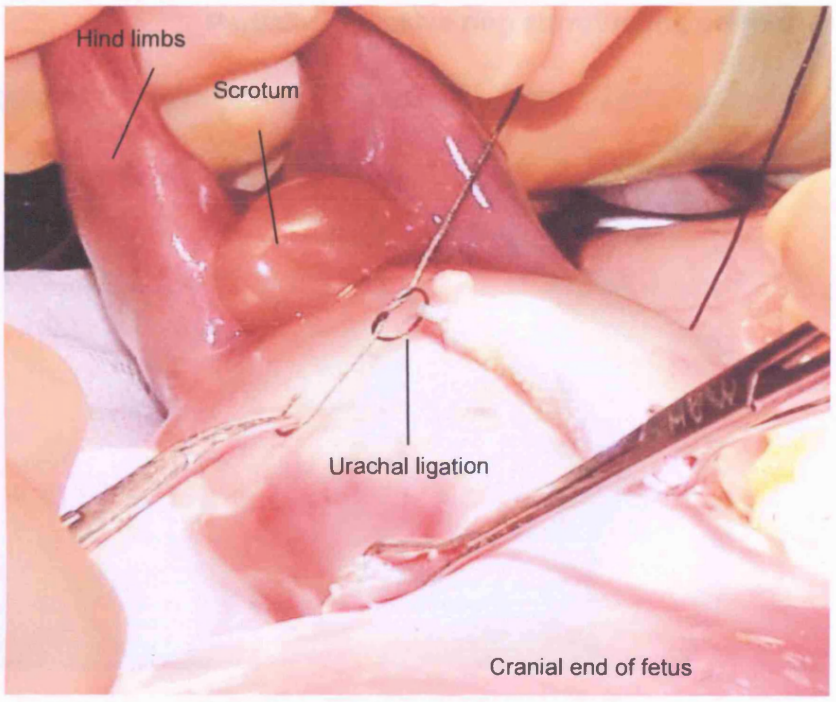


Figure 14. Urachal ligation.

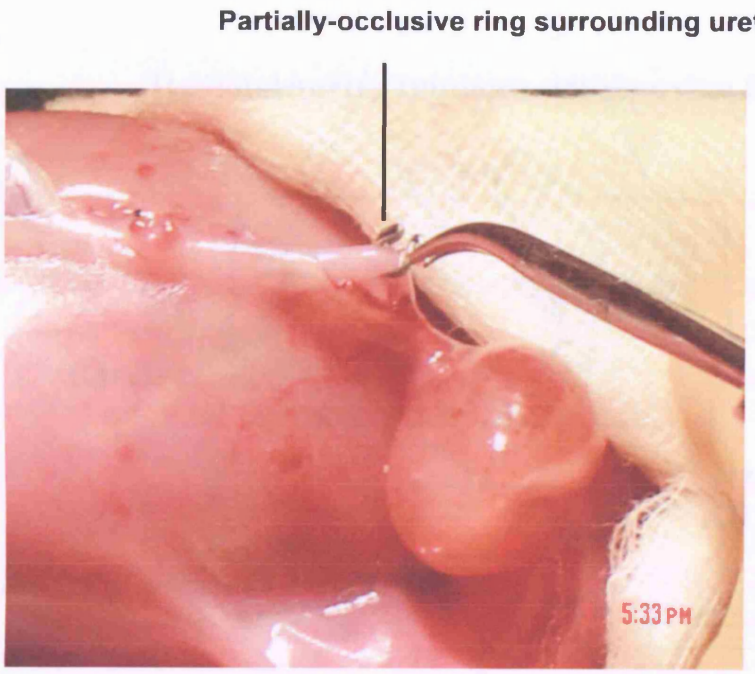


Figure 15. Partial urethral occlusion with Ω-shaped ring.

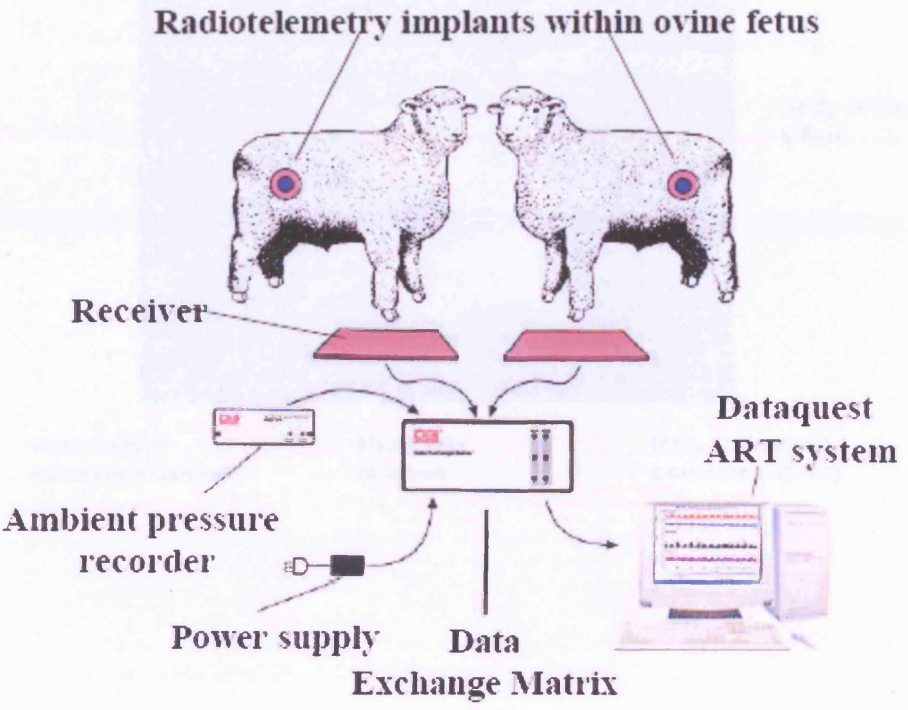


Figure 16. Radiotelemetered urodynamics set-up.

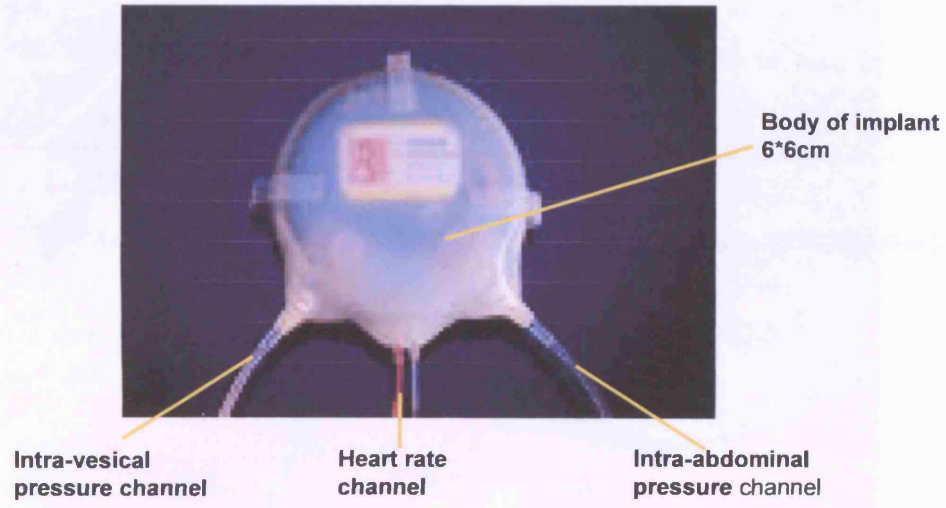


Figure 17. Radiotelemetry implant.

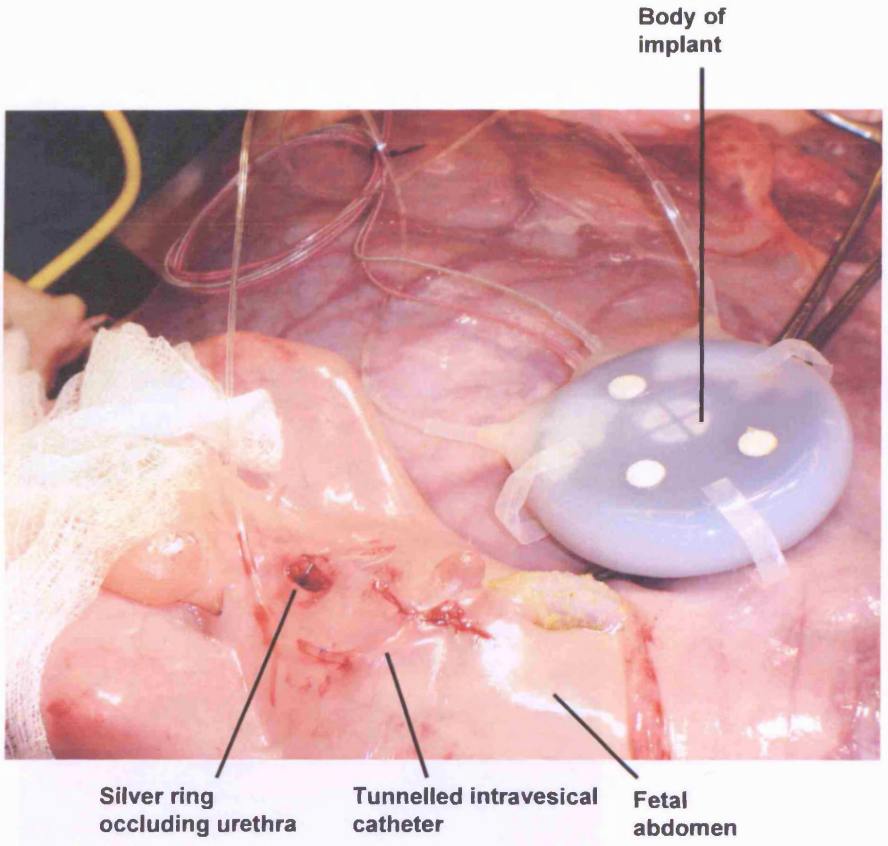


Figure 18. Insertion of intravesical and intra-abdominal catheters.

Implant body seen lying over uterus.

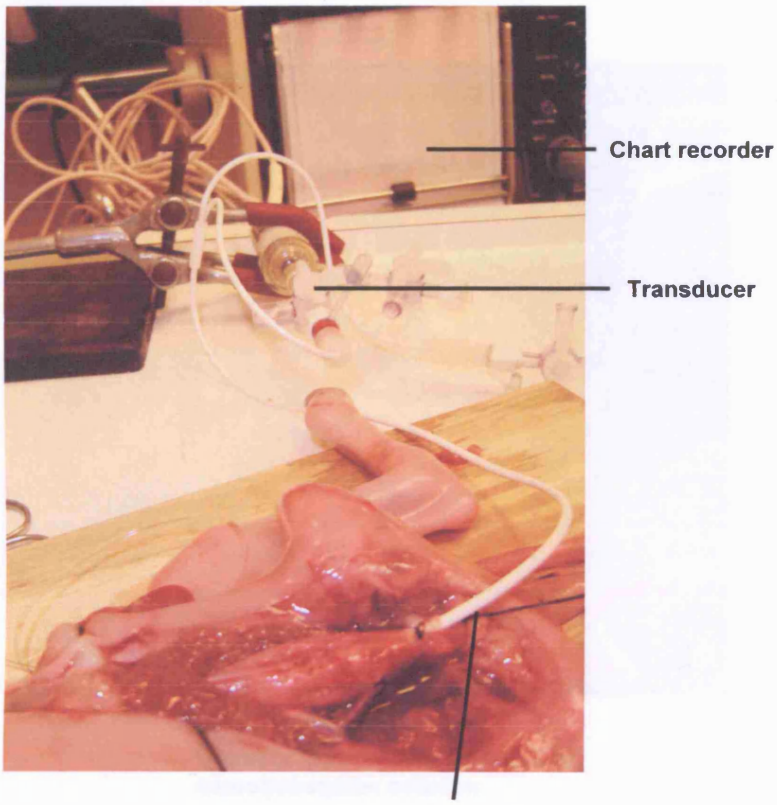


Figure 19. Ex-vivo cystometry set-up.

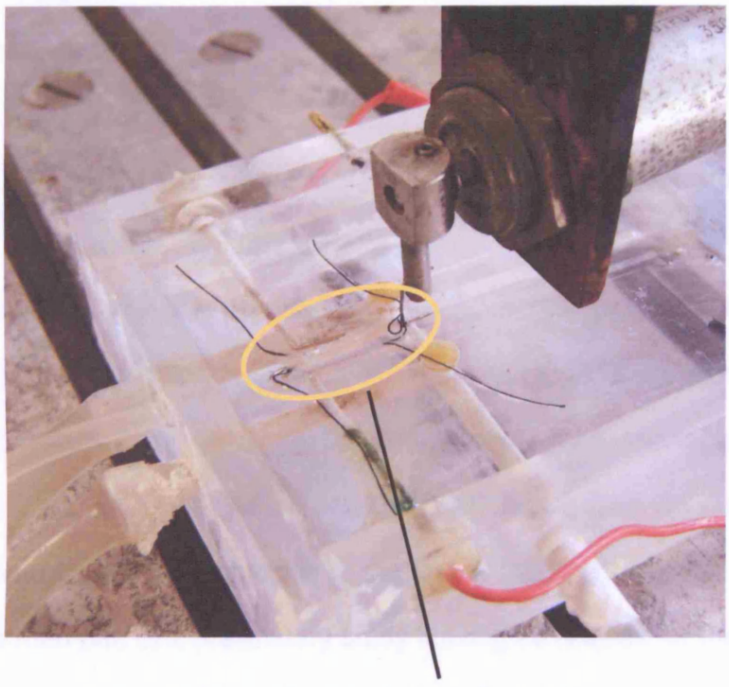


Figure 20. In-vitro contractility set-up.

3. RESULTS

The results presented in this chapter have been published (Farrugia *et al.* 2006 a and b, Farrugia *et al.* 2007) and reprints are enclosed at the end of this thesis. Key results are discussed here and summarised in Tables 2 to 4 and Figures 21 to 24. Raw data may be found in the appendix at the end of the thesis.

3.1 Protocol 1 results: Fetal BOO from 75 days gestation

3.1.1 Surgical and anaesthetic outcome

Nineteen pregnant ewes were operated on in Protocol 1 – two (4021 (twins) and 4022 (twins)) arrested on the table due to difficulty with intubation and maintaining the airway – giving a 10% anaesthetic mortality. Of the 24 fetuses operated, one set of twins (2887 (one male)) was lost following a maternal chorio-amnionitis giving an 8% fetal mortality rate. Four fetuses formed part of a preliminary study leaving 20 fetuses for the actual study, of which 12 were female. Eleven male fetuses were therefore utilised in Protocol 1.

3.1.2 Ultrasonography

Ultrasound examinations were carried out on postoperative days one and seven by Boaz Weiss, research fellow in Fetal Medicine. On the first postoperative day, all fetal bladders were identifiable on ultrasonography (Fig.21); kidneys were also identifiable but not the renal pelves. An attempt was made to measure renal size, renal pelvic diameter and bladder volume,

but measurements could not be made due to technical limitations of the ultrasound machine available at the Biological Services Unit and due to movement of the ewe, which was difficult to restrain. On the seventh post-operative day, bladders in the urachal ligation only and in the urachal and urethral ligation group were noted to be comparatively larger than the sham bladders (Fig.22). Hydronephrosis was visible in both ligation groups (Fig.23) but not in shams. This appearance suggested that both interventions resulted in hydroureteronephrosis, henceforth the urachal ligation only and urachal and urethral ligation groups will be referred to as "BOO" groups.

3.1.3 Gross anatomy

At autopsy, fetal weight and size were not significantly different between the three groups (Table 2). Urine aspirated from all bladders was snap frozen and submitted to the biochemistry laboratory at the Institute of Child Health for analysis. There were no significant differences in urine osmolality and sodium content between the three groups. After removing urine, bladder weights (absolute and normalised for fetal weight) were significantly greater after BOO, with a significant response in the urachal-only group (Table 2).

Kidneys were weighed intact (with ureters), then bivalved in order to drain the urine retained in the renal pelvis, and re-weighed. We hypothesised that the difference in weight would reflect the volume of retained urine, and hence of that of the renal pelves. Amounts of renal pelvic urine were increased in both BOO groups, confirming the ultrasonographic impression

of hydronephrosis. The weights of kidneys (after urine drainage) were increased in both BOO groups but, after normalising for body weight, this remained significant in only the urachal-only ligation group (Table 2).

3.1.4 Protein and DNA content

Absorbance and concentration data for bladder protein and DNA estimation are shown in Appendices 5 and 6 respectively. Total bladder protein was significantly greater in the double-ligation group compared with the urachal ligation only group. The median DNA content was increased in both BOO groups but this only attained significance in the urethral and urachal ligation group: BOO had no affect on the protein/DNA ratio in the BOO groups versus shams (Table 2).

Figure 24 shows scatter plots of each measurement, providing a visual guide to be considered along with Table 2, containing medians/ranges for all parameters. Unless otherwise stated, similar differences were recorded in both BOO groups versus sham controls.

3.1.5 Histology

Bladders from all three groups had distinct urothelial, lamina propria and detrusor smooth muscle (DSM) bundle layers, the latter expressing α-smooth muscle actin (αSMA) (Fig.25 A and B). DSM appeared similar in all specimens, as assessed by light microsopy (Fig.25 C and D). An attempt was made to quantify the amount of DSM in each specimen using Image-J software, but this method was deemed to be inaccurate as the programmed

tended to pick up "background" SMA staining which did not correspond to DSM. On observation, however, DSM remained intact following nine days of BOO. Individual obstructed muscle bundles appeared to be wider and were separated by more connective tissue when visualised by TEM (Fig.25 E and F).

The urothelium, on the other hand, differed markedly between sham and BOO bladders. The *nuclear layers* in urothelium in obstructed groups were increased versus shams (Fig.26 A and B; Table 2). iNOS immunostaining was detectable in both the sham and BOO groups (Fig.26 C and D), with no difference in signal intensity on blind analysis. TUNEL staining revealed a striking difference in the number of apoptotic nuclei present in BOO urothelia, where apoptosis was pronounced particularly in the basal zone, compared to sham ones (Fig.27). Apoptotic cells were noted in the lamina propria and DSM layers of all samples but were too rare to quantify accurately.

A continuous asymmetric unit membrane expressing UPIb (Fig.28 A and B) and UPIII (not shown) was demonstrated in all bladders. Areas of denudation were occasionally present in some bladders (Fig.28 C) but this may have been due to fracturing during processing of the specimens. When viewed in greater detail under TEM, however, the asymmetric unit membrane appeared to be intact in obstructed specimens. Umbrella cells appeared to be larger, and contained a higher concentration of cytoplasmic vesicles, in BOO bladders (Fig.29).

Nerve bundles were present in the detrusor from all three groups, as identified by PGP 9.5 immunostaining. These were situated mainly between the detrusor layer and the serosa, with some neurovascular bundles interspersed between muscle fascicles (Fig.30). Nerve bundle count in the double ligation group was significantly reduced compared with the other two groups.

Sham renal histology revealed a nephrogenic zone, a deeper cortex with formed glomeruli (Fig.31 A) and medullary collecting ducts (Fig.31 B). In six out of ten kidney pairs from the BOO groups, these appearances were maintained (Fig.31 C and D). In two sets of kidneys from the urachal-only ligation group and in two sets from the urachal and urethral ligation group, however, the cortex contained cysts, some with glomerular tufts, and medullary ducts were dilated (Fig.31 E and F). Kidneys with cysts tended occur in fetuses with the highest bladder weights and protein contents.

3.1.6 Compliance and wall stress

Bladder compliance was measured from ex-vivo filling cystometry data. End-fill volumes were determined by measuring the volume of urine aspirated from each bladder at post-mortem. Bladder volume was greater in the obstructed groups compared to control. Figure 32 A shows ex-vivo pressure-volume curves for bladders from the three groups. The curves from sham-operated bladders were similar and are shown as a single curve; pressures did not exceed 7 cm H_2O at maximum volumes of 2 ml. Corresponding

curves from obstructed bladders were variable: two bladders (one from each intervention group) had low compliance, and the remainder had a similar or greater compliance compared to the sham group. Part of this variability may be due not only to differences in the unit physical properties of the bladder wall, but also to its thickening in response to obstruction.

Estimated (empty) bladder wall thickness was significantly increased in both BOO groups compared to control. Wall stress, normalised to unit cross-section area, was calculated as a function of filling volume (see Methods) to compensate for any increase of wall thickness. Figure 32 B shows that the transformation linearised the curves; the shallower the line the smaller was the wall stress generated per unit change of volume, i.e. wall stiffness was reduced. Despite the standardized median wall stress values being smaller in the obstructed groups there was no statistical difference between them. However, the scatter of data may hide any trend that may develop with obstruction.

Morphology results showed a good correlation between bladder: fetal weight ratio (BFR) and bladder protein/DNA content, indicative of bladder overgrowth with obstruction. The question arises if normalised wall stiffness is a function of increasing bladder weight with obstruction. Figure 32 C plots this estimate of wall stiffness as a function of BFR, irrespective of the interventional group. The data show a significant negative correlation between BFR and wall stress, with the exception of a single datum point for one of the obstructed bladders, with a correlation coefficient of -0.76

($p<0.01$). It appears, therefore, that an increase of BFR, as occurred during this short-term BOO, is associated with a more compliant bladder wall.

3.1.7 Contractility

Dimensions and weights of detrusor strips from all groups were similar. Contractions elicited by electrical field stimulation (EFS) were completely blocked by the neurotoxin TTX, indicating they were nerve-mediated. Atropine completely abolished nerve-mediated contractions in detrusor strips from all groups. However, application of 1 μM ABMA generated transient contractures in all preparations, of similar magnitude in control and obstructed groups. The data from the two obstructed groups have been combined because of the relatively small data sets. In the presence of atropine, EFS generated small transient relaxations in nearly all preparations from both sham-operated and obstructed bladders.

Another important hypothesis to be tested was if bladder overgrowth was associated with contractile failure. Plots of BFR as a function of tension generated by various interventions did not show a significant correlation: nerve-mediated stimulation at 40 Hz ($r = 0.02$, $p>0.05$), 10 μM carbachol ($r=0.14$, $p>0.05$) or an increase of extracellular [KCl] ($r=-0.04$, $p>0.05$). Figure 33 A shows the median values (with the 25% and 75% interquartiles) of tension generated by carbachol, and in part B that by nerve-mediated stimulation at 40Hz, T_{40}, and by high-KCl solution. Because of the similarity of data in the two obstructed groups, they have been combined to one experimental data set. With all three interventions the hypothesis was

rejected, as the median values were not significantly different between the sham-operated and obstructed data sets. It should be noted that one preparation in the sham-operated group yielded a much larger value for the KCl contracture than the others and hence has generated a large 75% interquartile range. However, even if this individual value is removed from the statistical evaluation a similar conclusion is drawn, i.e. that the median values in the sham-operated and obstructed groups are statistically similar.

3.1.8 Innervation

In the morphology section, it was shown that nerve bundles were present in the detrusor from all three groups, as identified by PGP 9.5 immunostaining. Nerve bundles in the urachal and urethral occlusion group appeared to be smaller and were significantly reduced in number compared to the other two groups. However, there was no significant relationship between PGP nerve count and either BFR ($r=-0.42$, $p>0.05$) or tension generated by electrical field stimulation at 40 Hz ($r=0.50$, $p>0.05$). These correlations are consistent with the observation that bladder overgrowth at this period of obstruction is not associated with reduced contractility. Wall stress also showed no correlation with PGP counts ($r=0.44$, $p>0.05$). However, it is of interest to note that if the single point associated with the very high wall stress observed in Figure 32 C was omitted a strong positive correlation was observed ($r=0.74$, $p>0.01$).

3.1.9 Protocol 1 results: summary

- Ultrasonography on the pregnant ewe revealed progressive hydronephrosis in both the combined urachal and urethral ligation and the urachal ligation only groups, hence the combined groups are henceforth referred to as "BOO" groups
- BOO bladders were significantly heavier and had a greater DNA content than controls, but there was no significant difference in protein: DNA ratio between groups
- BOO kidneys were hydronephrotic; histologically, some kidneys in both groups exhibited glomerular cystic change and medullary tubular dilatation
- BOO bladder histology revealed an intact detrusor with a reduced nerve bundle count in the double ligation group only; the urothelium appeared to have an increased number of nuclear layers, and exhibited marked apoptotic change
- Compliance was variable in both BOO groups and was standardised by calculating the circumferential bladder wall tension, or wall stress, which was not significantly different
- Detrusor contractility was not significantly affected following BOO

3.2. Protocol 2 results: Fetal BOO and radiotelemetered urodynamics from 94 days gestation

3.2.1 Surgical and anaesthetic outcome

All BOO fetuses survived the full nine days of the experiment, as did the three sham fetuses which did not receive telemetry devices. The two sham fetuses implanted with radiotelemetry devices, however, died within 48 hours. The cause of death was undetermined as autolysis was too advanced for analysis at the time of autopsy. Fetal demise was probably related to the fact that, at 94 days gestation, the fetuses were too small for direct insertion of the telemetry catheter through the delicate bladder wall. In-vivo cystometry was, therefore, only obtained in the BOO group, although three sham bladders were available for all other analyses. Operative mortality in this protocol was 22%.

3.2.2 Ultrasonography

On the first postoperative day, all fetal bladders were identifiable on ultrasonography as previously described; kidneys were also identifiable but not the renal pelves. On the seventh post-operative day, BOO bladders were comparatively larger than the sham bladders. Dilated renal pelves were visible in obstructed, but not in sham, fetuses (not shown).

3.2.3 Gross anatomy

On direct inspection, BOO fetuses were noted to have tortuous ureters and a dilated posterior urethra proximal to the occlusive ring. There was no significant difference in fetal weight and size (Table 3).

3.2.4 Histology

As assessed by Masson's trichome staining and αSMA immunostaining, all sham and BOO bladder cross-sections were clearly demarcated into urothelium, lamina propria and detrusor layers (Fig.34 A-C). Detrusor muscle bundles were well-preserved in three obstructed bladders; one BOO bladder, however, was found to have thinner bundles (Fig.34 B). Microscopically, sham kidneys contained a nephrogenic zone, directly below the kidney capsule, with several layers of glomeruli deeper in the cortex (Fig.34 D-F). One BOO kidney pair had a similar appearance as the shams (Fig.34 E), whereas the three other BOO bladders contained subcapsular cysts, often with glomerular tufts on their walls (Fig.34 F), and medullary tubular dilatation (not shown). The non-cystic set of kidneys (Fig.34 E) was associated with the bladder with the attenuated detrusor (Fig.34 B). There were no consistent differences between sham and BOO groups regarding fetal or bladder weights, bladder end-fill volumes, urothelial layers and incidence of apoptosis, or kidney weights (Table 3).

3.2.5 In-vivo radiotelemetered cystometry

A total of 36 urodynamic trace recordings were performed, of which 19 were amenable to accurate analyses. The remaining traces could not be analysed due to a software malfunction (which could not be repaired by the DSI Technical Support team, support@datasci.com 1-800-262-9687). Analysis of the available traces provided a composite picture of events that occurred

during the period of observation. A summary of medians obtained per obstructed animal are summarized in Table 4.

The first 24 hours post-BOO (Day 0) were characterized by a filling pattern with stable, low baseline detrusor pressures of less than 10 mmHg. Voids occurred at intervals of one to two minutes at median voiding pressures of 12 to 14mmHg (Fig.35 A), and resembled the voids documented in our preliminary study (Fig. 36) (Thiruchelvam *et al.* 2004). The 'filling' pattern between voids was either a flat trace or consisted of a series of low-pressure 'undulations' (Fig.35 B). A gradual increase in voiding pressures occurred over the ensuing days, such that there was a significant difference between voiding pressures on days 0-1 versus days 6-7 (sheep 4137 and 4141 compared), when voiding pressures up to 38 mm Hg were recorded. Meanwhile, baseline detrusor pressure did not differ significantly between days 0-1 and 6-7 (medians of 3 to 8 mmHg) (see Table 4). Voids also became significantly more prolonged with obstruction. By day five, voids typically lasted three minutes (Fig.35 C) and occasionally occurred in successive runs (Fig.35 D). Days 6-8 were characterized by 'haphazard' excursions of elevated detrusor pressure of varying amplitude and duration (Fig.35 E). It was thought this represented repeated efforts to void in view of the fact that "normal" voiding patterns became much less frequent.

Detrusor overactivity was characterised by runs of three or more detrusor pressure elevations lasting less than 0.1 of a minute (Fig.35 F). Detrusor overactivity was identified in all animals starting from day 0, apart from

sheep 4091. There was no significant difference in the pressures reached or duration of instability between days 0 and 7.

Overall, there were marked changes in the patterns of bladder activity throughout the period of study. There was a significant progression in the frequency and duration of 'voiding' activity, such that more time was spent at higher pressures of emptying activity rather than at the baseline with a non-contracting detrusor. The patterns between individual BOO animals were not substantially different, although, notably, the one animal with the non-cystic kidneys (see below) maintained a more moderate frequency and duration of voidings.

3.2.6 Ex-vivo compliance, contractility and innervation

Compliance curves for the obstructed and control bladders are shown in Figure 37 A; there was no significant difference between the two groups. The least compliant bladder was the one which did not develop cystic kidneys. Derived wall stress values were plotted as a function of bladder volume and are shown in Fig.37 B. The median values of the slopes were not different between the two groups. As expected, the least compliant bladder from the animal without cystic kidneys had the greatest wall stress per unit volume. The most severe hydronephrosis was associated with least wall stress per unit volume. Contractions elicited by 1-60Hz EFS were blocked by the neurotoxin TTX, indicating they were nerve-mediated. Atropine, a muscarinic receptor antagonist, also abolished nerve-mediated contractions in all detrusor strips. The contractions elicited by 20Hz EFS and

contractures generated by the acetylcholine analogue are shown in Figure 38 A and B: although BOO bladders appeared to be hypercontractile, the difference did not reach significance. Nerve bundle (PGP 9.5 staining) count (per high power field) was not different in the two groups.

3.2.7 Protocol 2 results: summary

- BOO at 94 days did not result in a significant difference in bladder or kidney growth between obstructed and non-obstructed groups
- Similarly, ex-vivo compliance and wall stress were not significantly different between the two groups
- In-utero fetal cystometry (via urachal catheterisation) was only feasible in the obstructed group
- Urodynamic changes were noted within hours of obstruction: these were characterised by voids at increasing pressures and frequency with a baseline disrupted by detrusor overactivity. The filling/storage pressure did not alter significantly throughout the nine days of obstruction
- Histologically, glomerular cysts were evident in three of four sets of kidneys; bladder wall architecture was well-maintained, although there was evidence of an apoptotic response

Thus Protocol 2 has raised further questions, which I will attempt to explain in the forthcoming discussion:

- Why does obstruction at 94 days result in less growth than obstruction at 75 days?
- Why does ligation of the urachus with a patent urethra result in obstructive changes?
- How does short-term BOO for nine days compare with long-term BOO for 30 days?
- What is the clinical implication of the antenatal cystometry findings?

Table 2. Effects of short-term fetal BOO at 75 days on renal tract morphology and biology. Measurements are median (range) * and ** respectively indicate P<0.05 and P≤0.01 for each obstructed group versus sham controls; + and ++ respectively indicate P<0.05 and P≤0.01 for the urachal and urethral obstruction group versus the urachal obstruction-only group. Gestation at surgery refers to the day when sham or obstruction surgery was performed; all other measurements were taken at sacrifice.

	Sham operation (n=4)	Urachal and urethral occlusion (n = 6)	Urachal ligation only (n = 4)
Fetal weight at sacrifice (g)	450 (350-650)	635 (400-850)	500 (400-700)
Gestational at surgery (days)	75 (75-82)	79.7 (75-82)	78.5 (75-82)
Bladder weight (g)	0.41 (0.34-0.46)	**1.20 (0.85-2.60)	**2.04 (1.37-2.59)
Bladder/fetal weight ($x10^{-3}$)	0.9 (0.7-1.1)	**/++2.10 (1.4-3.1)	**3.8 (3.0-4.7)
Bladder protein (mg)	39.8 (30.3-42.7)	*/+96.4 (33.2-144.3)	*159.5 (136.0-166.4)
Bladder DNA (mg)	0. 40 (0.24-0.41)	*1.0 (0.56-2.24)	*1.87 (0.69-2.56)
Protein/DNA	104 (101-128)	77 (30-239)	73 (65-230)
Urothelial layers	1.4 (1.3-2.6)	*3.6 (2.4-8.3)	**6.4 (4.3-10.1)
Urothelial apoptotic index (%)	0.09 (0.08-0.20)	**1.66 (0.81-4.59)	0.98 (0.09-2.27)
Kidney weight (both) (g)	5.38 (4.29-6.14)	*7.76 (5.10-10.00)	**7.33 (6.90-13.04)
Kidney/fetal weight ($x10^{-2}$)	1.2 (0.9-1.3)	1.2 (1.0-1.9)	**1.6 (1.4-1.7)
Renal pelvic urine weight (g)	0.30 (0.10-0.40)	**1.05 (0.46-2.16)	**0.68 (0.54-4.21)
Urine osmolality (mOsmol/kg water)	150 (131-165)	157 (139-257)	143 (113-294)

Table 3. Effects of short-term fetal BOO at 94 days on renal tract morphology and biology. Values for sham and BOO groups at sacrifice, nine days after the experiments were initiated at 94 days gestation. Values are shown as median (ranges). The 'apoptotic index' is the percent of urothelial nuclei undergoing apoptosis.

	Sham (n=3)	BOO (n = 4)
Fetal weight (g)	1063 (380-1860)	1319 (798-1472)
Bladder weight (g)	0.6 (0.4-1.3)	1.0 (0.8-1.3)
Bladder/fetal weight ($x10^{-3}$)	0.70 (0.56-1.1)	0.79 (0.54-1.5)
Bladder end-fill volume (ml)	2.8 (1.0-11.0)	6.0 (1.1-10.5)
Urothelial layers	3.0 (-)	3.5 (3.0-4.0)
Urothelial apoptotic index (%)	0.79 (0.26-0.93)	0.72 (0.56-2.10)
Two-kidney weight (drained of urine (g)	9.5 (5.2-15.6)	10.7 (8.3-16.6)
Total drained kidney/fetal weight ($x10^{-2}$)	0.89 (0.84-1.37)	0.95 (0.71-1.21)

Table 4. Radiotelemetered cystometry results. * signifies a significant difference between values.

Day	Sheep ID	Baseline Filling / Storage pressures (mmHg)			Detrusor Overactivity (mmHg)		Voiding Phase pressures (mmHg)	
		PAbd	PVes	Pdet	Rise in PDet	Duration (Mins)	Voiding Pressure PDet	Duration of void (mins)
0	4141	22	25	3	8	<0.1	12	1.1
	4137	18	20	4	4	<0.1	12	0.8
	4088	14	22	8	-	-	-	-
1	4141	20	25	5	8	<0.1	17	1.4
	4137	17	20	2	18	<0.1	16	1.8
	4088	10	23	9	17	<0.1	42	2.4
2	4141	16	26	11	10	<0.1	5	5
	4137	15	21	6	18	<0.1	19	5
3	4141	14	24	10	6	<0.1	14	2
	4137	15	21	6	20	<0.1	23	3.6
	4091	10	20	10	-	-	25	2
4	4141	18	29	11	6	<0.1	17	3
	4091	10	23	15	-	-	25	2
5	4141	14	24	8	14	<0.1	18	3
	4088	16	33	15	41	0.2	29	2.7
	4091	15	28	10	-	-	25	2
6	4141	16	21	5	16	<0.1	32	2.7
	4137	18	24	8	20	<0.1	24	2.4
7	4141	18	24	7	18	<0.1	32	3.4
	4137	16	24	8	-	-	26	4
Day 0/1 and 6/7 differences (Sheep 4137 and 4141 data only)				0.05	0.16		0.03*	0.03*

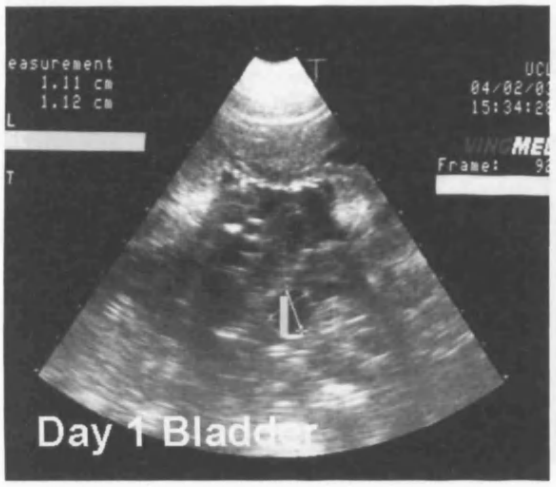


Figure 21. Ultrasound appearance of a 75-day fetal bladder one day post-urachal ligation only.

Bladder diameter marked with yellow line (Scale 1:2).

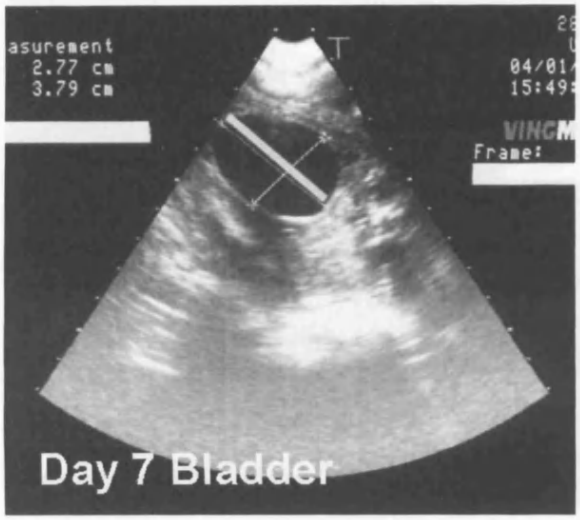


Figure 22. Ultrasound appearance of a 75-day fetal bladder seven days post-urachal ligation only.

Bladder diameter marked with yellow line (Scale 1:2).

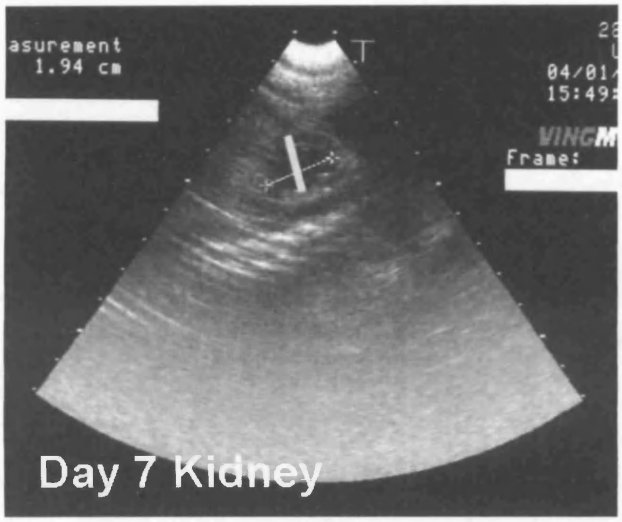
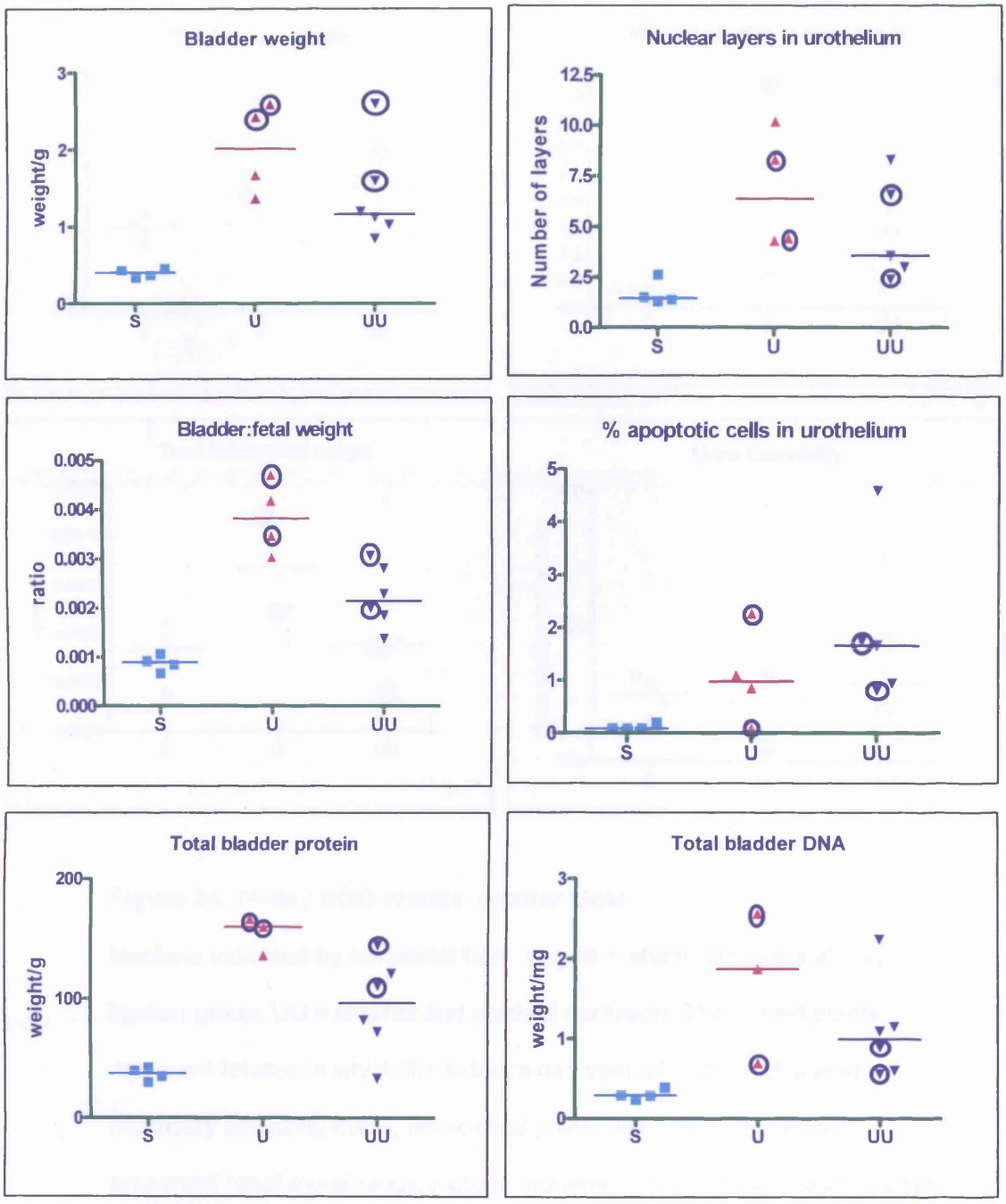


Figure 23. Ultrasound appearance of a 75-day fetal renal pelvis seven days post-urachal ligation only.

Renal pelvic diameter marked with yellow line (Scale 1:2).


Bladder weight
weight/g
3
2
1
0
S
U
UU
Nuclear layers in urothelium
Number of layers
12.5
10.0
7.5
5.0
2.5
0.0
S
U
UU
Bladder:fetal weight
ratio
0.005
0.004
0.003
0.002
0.001
0.000
S
U
UU
% apoptotic cells in urothelium
5
4
3
2
1
0
S
U
UU
Total bladder protein
weight/g
200
100
0
S
U
UU
Total bladder DNA
weight/mg
3
2
1
0
S
U
UU

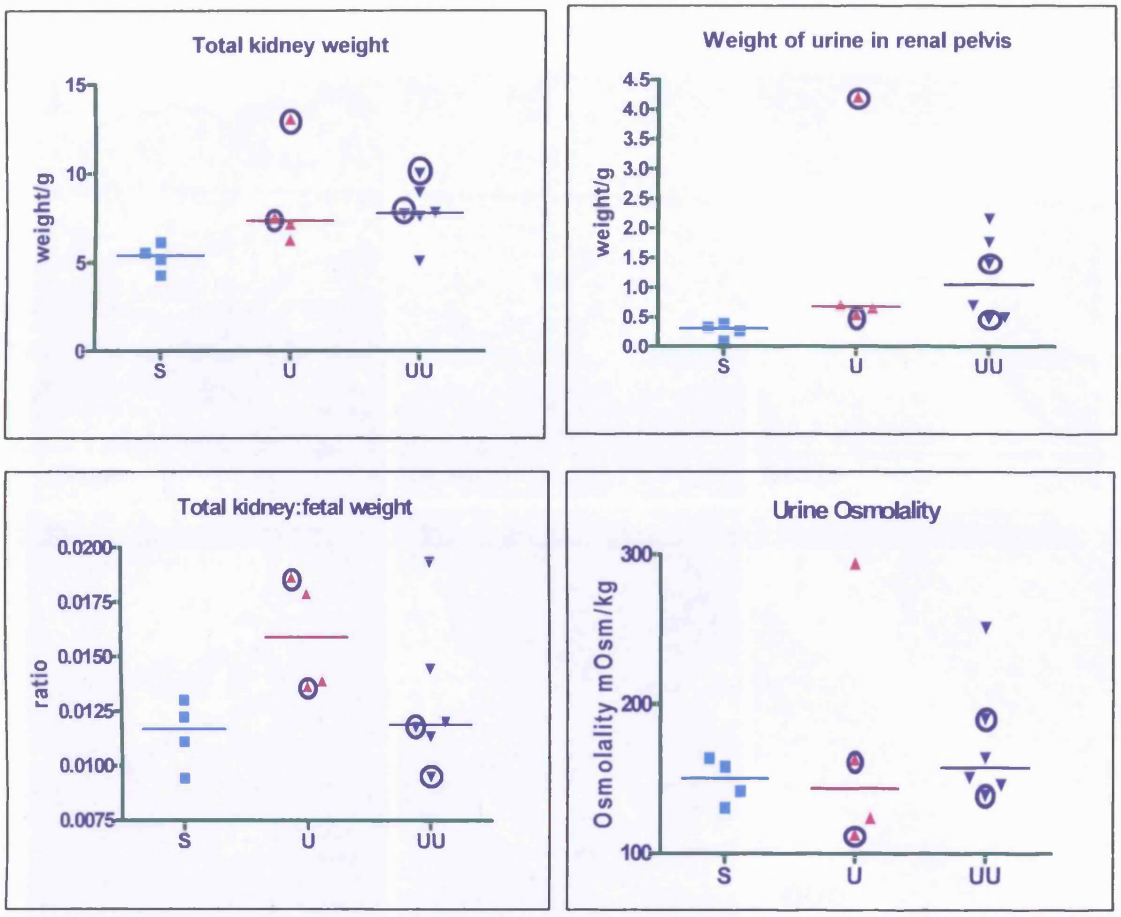


Figure 24. 75-day BOO results: scatter plots.

Medians indicated by horizontal bars. Key: S = sham; U = urachal only ligation group; UU = urachal and urethral occlusion. The circled points represent fetuses in which the kidneys had cortical cysts and dilated medullary collecting ducts; non-circled points were from fetuses with preserved renal morphology. Note on numbers: for technical reasons, only three of the four bladder samples in the U group were available for analysis of protein and DNA. In the UU group, only five of the six bladders were available for analyses of the nuclear layers in urothelium and apoptosis.

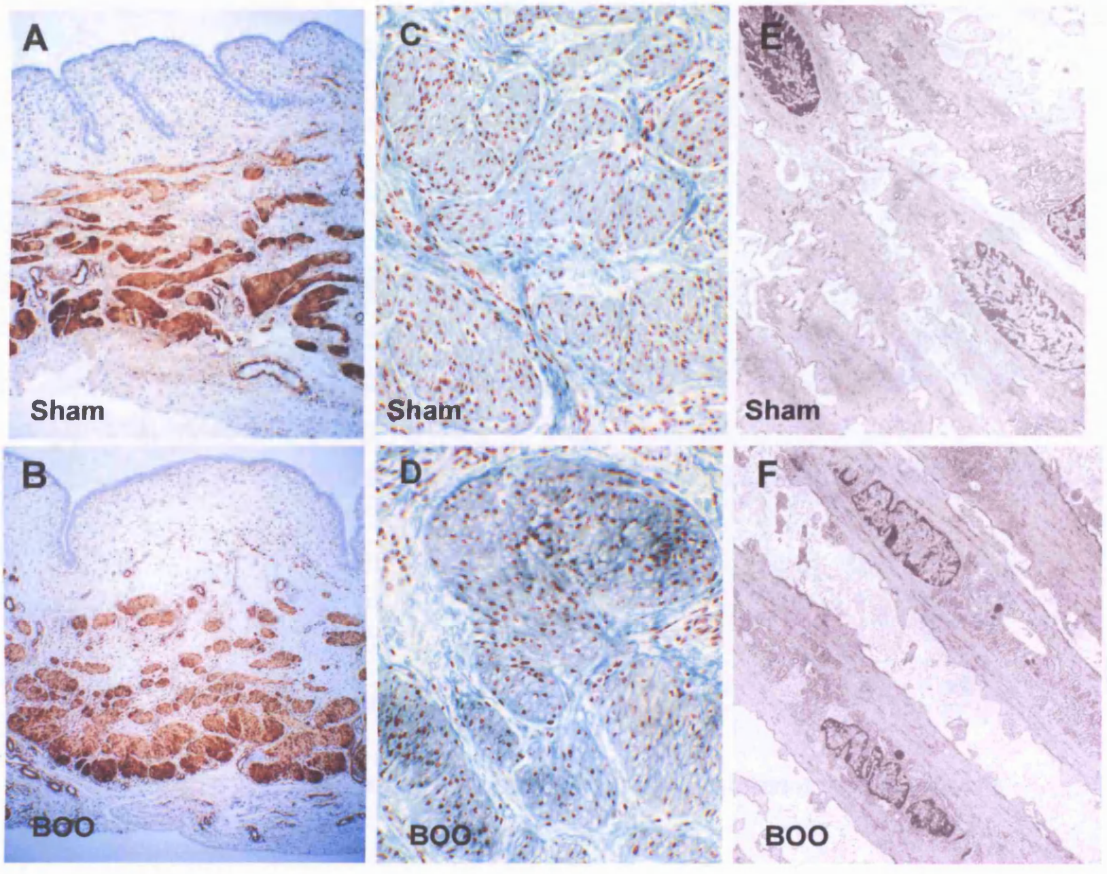


Figure 25 A-F. Detrusor smooth muscle (DSM).

A and B were immunostained for α-smooth muscle actin (brown) and counterstained with hematoxylin (x12.5 original magnification); C and D were stained with Masson's trichrome (original magnification x50); E and F are TEM images (original magnification x3000). A, C and E are from sham bladders and B, D and F are from bladders with urachal ligation and urethral rings. Note the generally-preserved DSM morphology in the BOO versus sham samples.

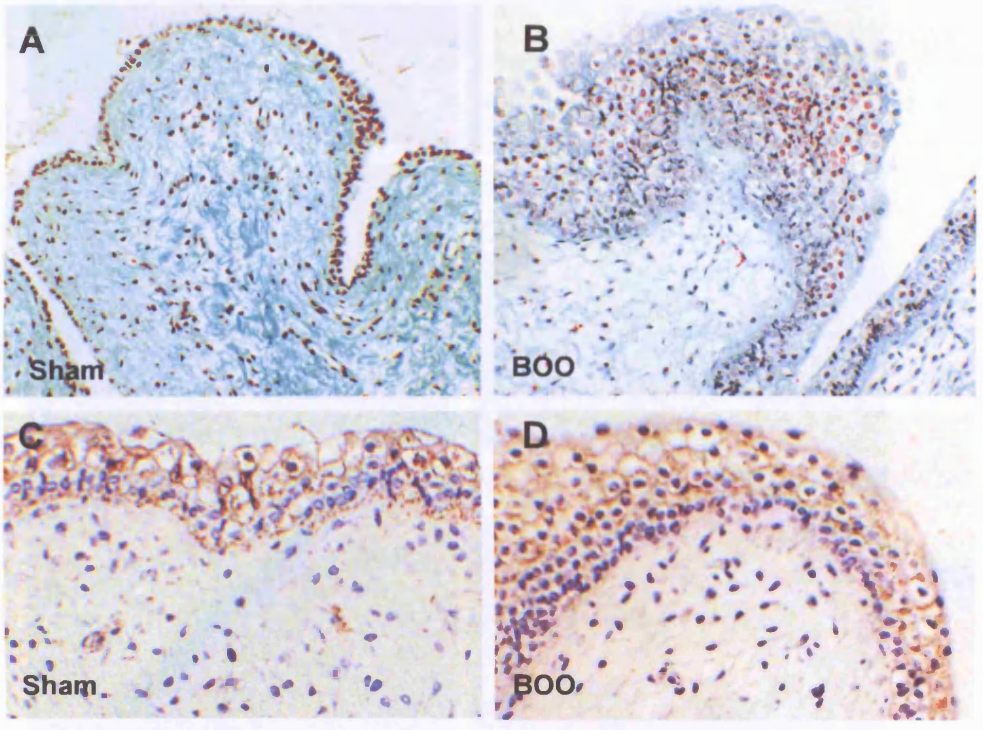


Figure 26 A-D. Urothelium visualized by light microscopy: Masson's trichrome and iNOS immunostaining.

A and B were stained with Masson's trichrome (original magnification x50); C and D were immunostained for iNOS (brown) and counterstained with hematoxlin (original magnification x100). Note that BOO bladders appeared to have more nuclear layers (between lumen and lamina propria) and that iNOS protein could be immunolocalized to both sham and BOO urothelium.

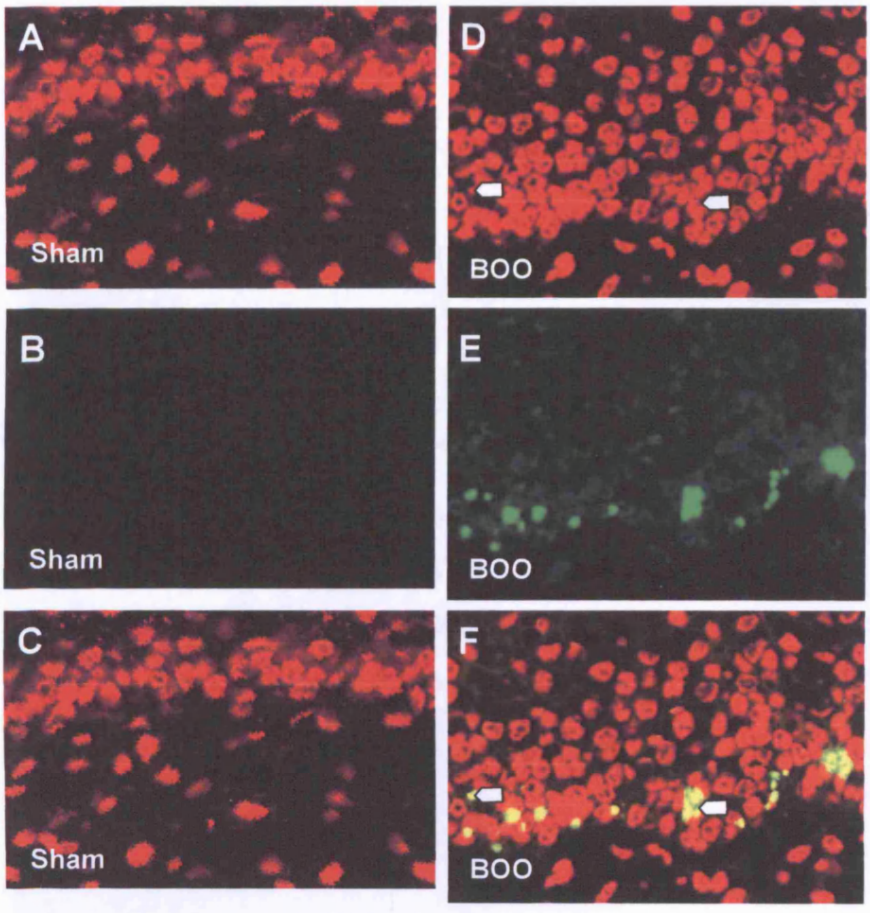


Figure 27 A-F. In-situ end-labelling for apoptosis in the urothelium.

All views are from a confocal microscope, original magnification x260. A-C represent sham bladder wall sections, whilst D-F are from a BOO bladder. The uppermost, dense layer of cells is the urothelium, while the lamina propria, with sparser cells, is below. A and D have a 'wavelength window' set to detect all nulcei (propidium iodide – red), B and E have a window set to detect apoptotic nuclei (green), and C and F are merged images. Note the apparent increase in nuclear layers of BOO urothelium, which has many apoptotic nuclei in the basal zone (two examples are marked with arrows in D and F).

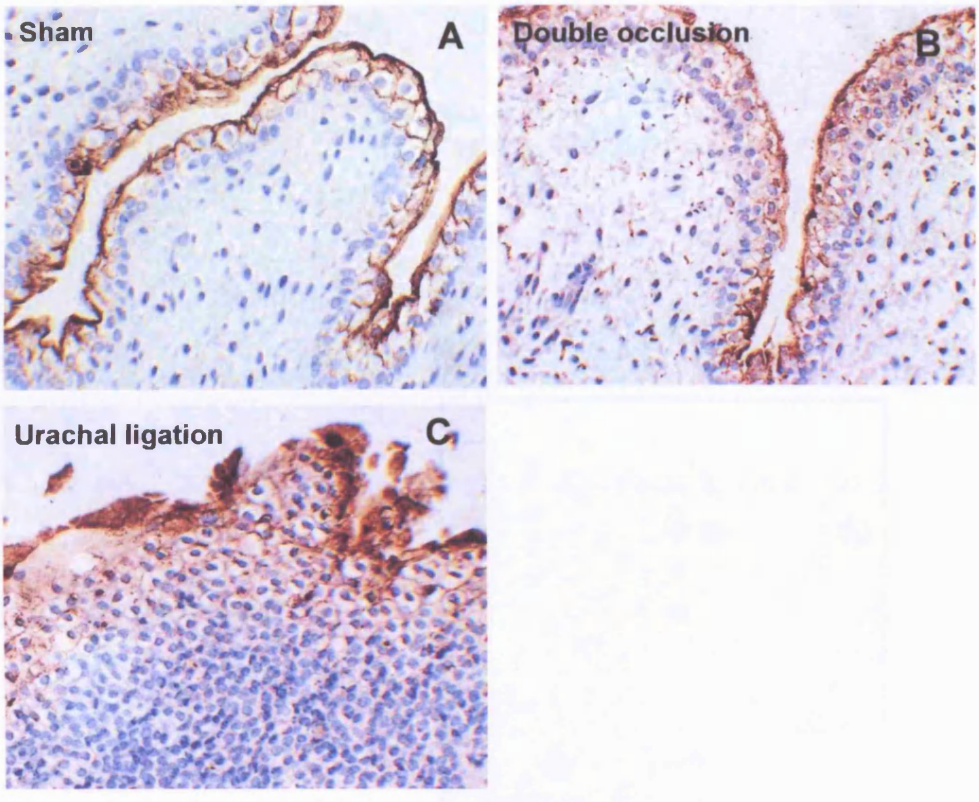


Figure 28 A-C. Uroplakin Ib immunostaining.

A and B show an intact asymmetric unit membrane (AUM) expressing Uroplakin Ib in both control and obstructed bladders (original magnification x200). C shows an obstructed bladder with a potentially disrupted AUM, although this may be due to a processing artefact.

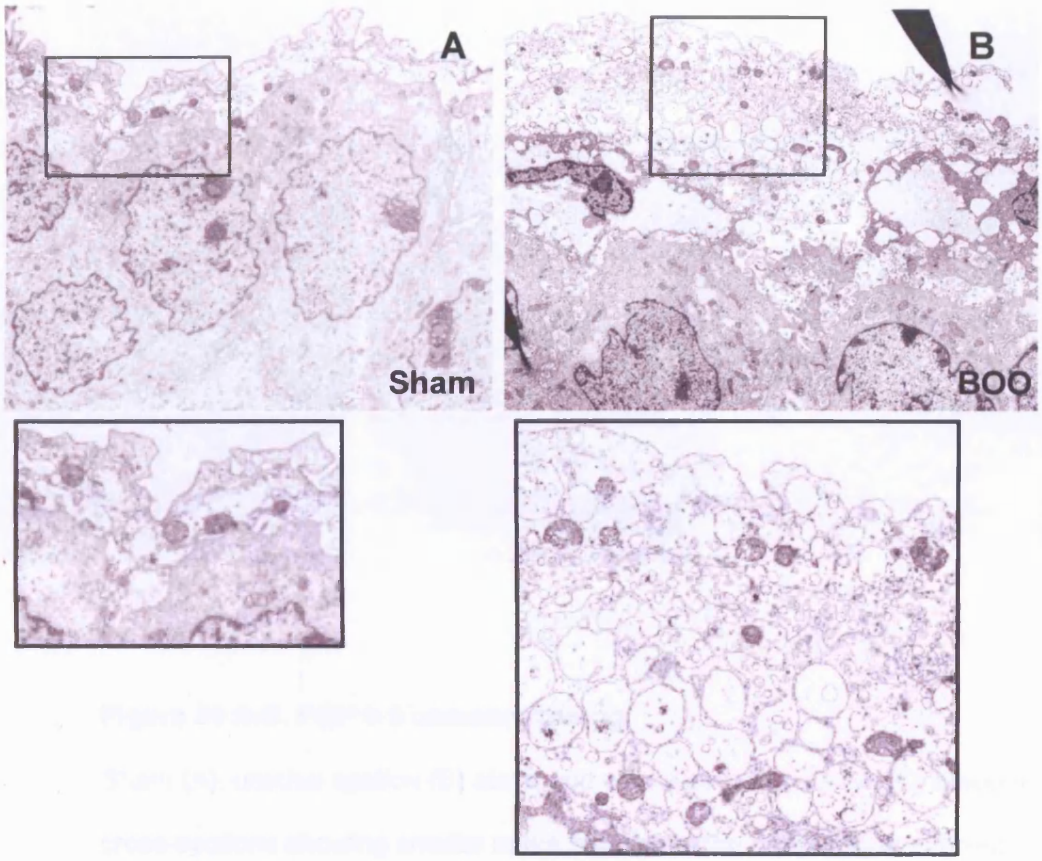


Figure 29 A-B. TEM of urothelium.

TEM of sham (A and enlarged inset x2.7 magnification) and BOO (B and enlarged inset x2.7 - combined urachal and urethral ligation) bladders. Note the flat umbrella cell layer sitting on top of cuboidal-shaped urothelial cells in the sham bladder. In the BOO sample, the umbrella cell layer is very prominent and contains many vesicles.

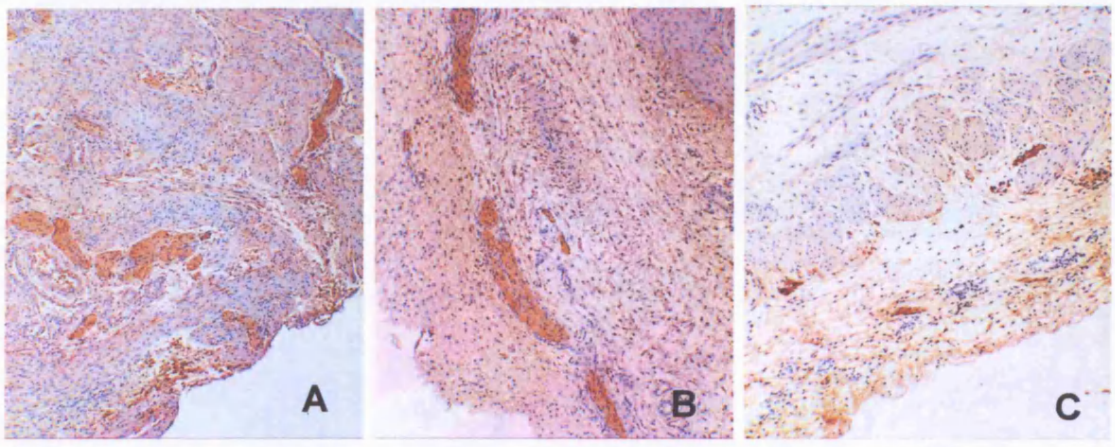


Figure 30 A-C. PGP 9.5 immunostaining.

Sham (A), urachal ligation (B) alone and with urethral occlusion (C) bladder cross-sections showing smaller nerve bundles in the double ligation group.

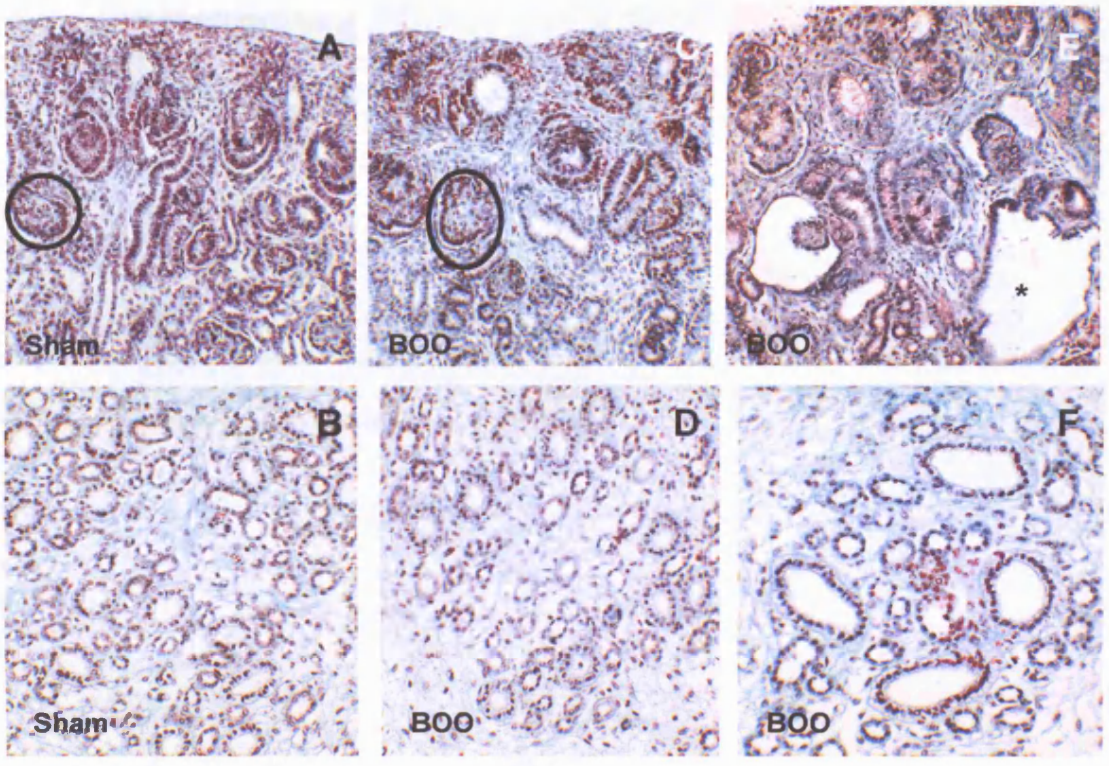
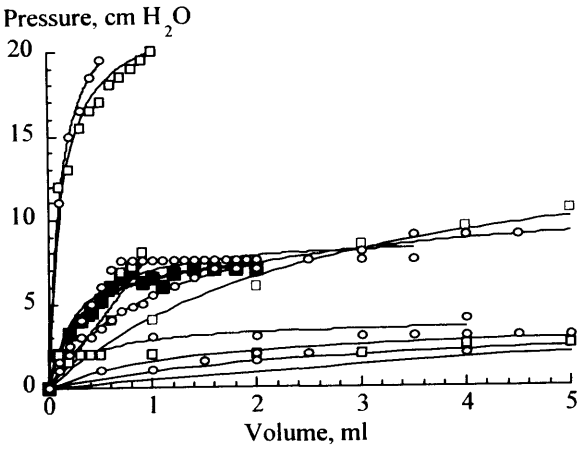


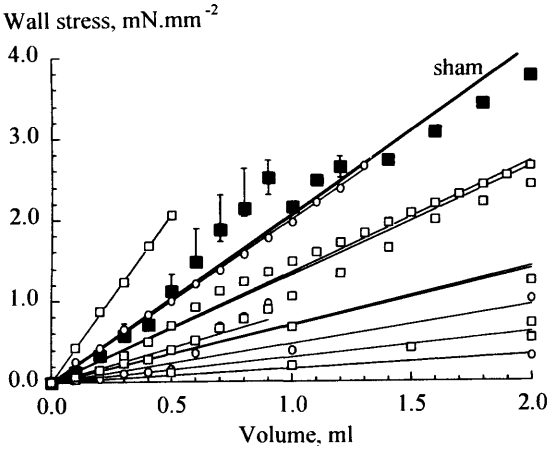
Figure 31 A-F. Kidney histology.

All samples stained with Masson's trichrome (original magnification x50). A and B are from a sham kidney, and C-F are from two fetuses with BOO. In the sham sample, note the nephrogenic zone surrounding the first layers of forming glomeruli (circled in A), with normal calibre ducts in the medulla (B); in six out of the ten fetuses with BOO, similar appearances were noted (C and D). In four of the fetuses with BOO, abnormal renal morphology was noted with a cysts in the outer cortex, many of which contained a glomerular tuft (indicated by * in E); in these kidneys, dilated ducts were always found in the renal medulla (F).

A


Pressure, cm H 2O
20
15
10
5
0
0
1
2
3
4
5
Volume, ml

B


Wall stress, mN.mm -2
4.0
3.0
2.0
1.0
0.0
sham
0.0
0.5
1.0
1.5
2.0
Volume, ml

C

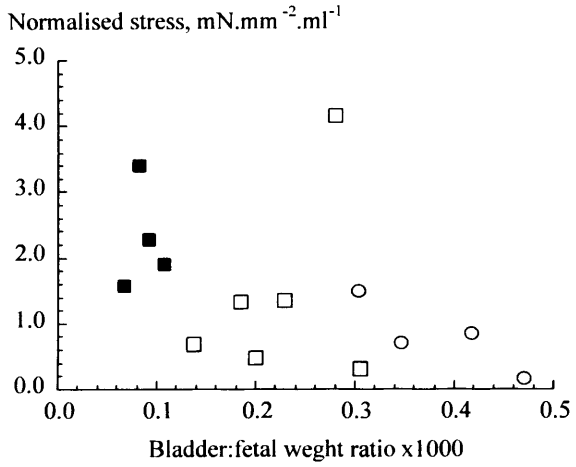
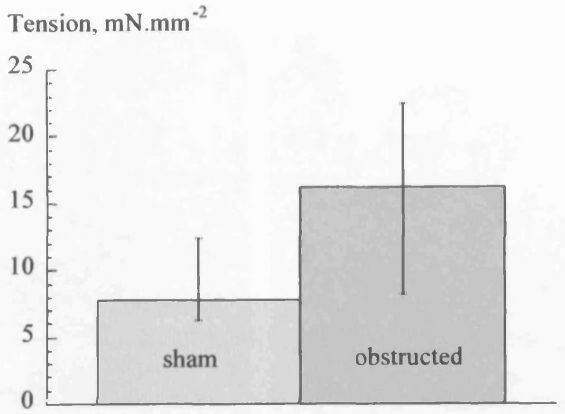


Figure 32 A-C. Pressure-volume relationships.

The median [25, 75% interquartiles] data for the sham-operated animals are shown as closed squares. Data from the bladders undergoing urachal ligation (open circles) and urachal ligation with partial urethral occlusion (open squares) are shown for individual animals. A. Compliance curves constructed from raw data. B. Transformed data from part A showing calculated wall stress as a function of filling volume. C. The relationship between normalised wall stress (slope of lines in part B) and bladder-to-fetal weight ratio: symbols the same as in parts A and B.

A



B

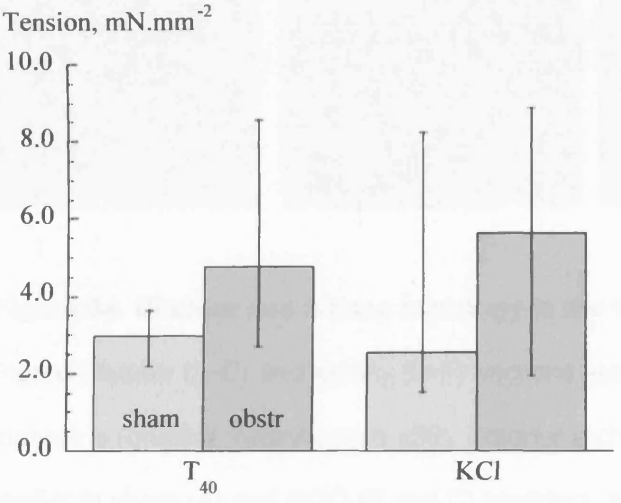


Figure 33 A-B. Contractile function of isolated detrusor.

A. Contractions evoked by 10 μM carbachol in strips from sham-operated and obstructed bladders. Data from the two obstructed groups have been combined – see text. B. Contractions evoked by electrical field-stimulation at 40 Hz or raised superfusate [KCl]. Median values with 25% and 75% interquartiles.

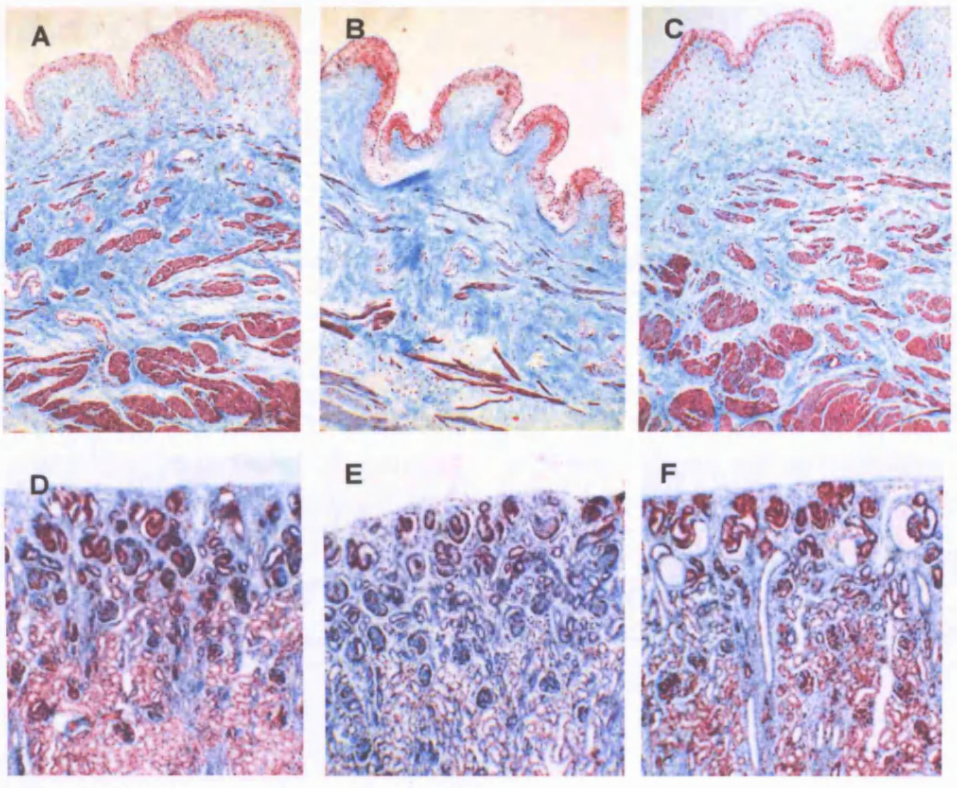
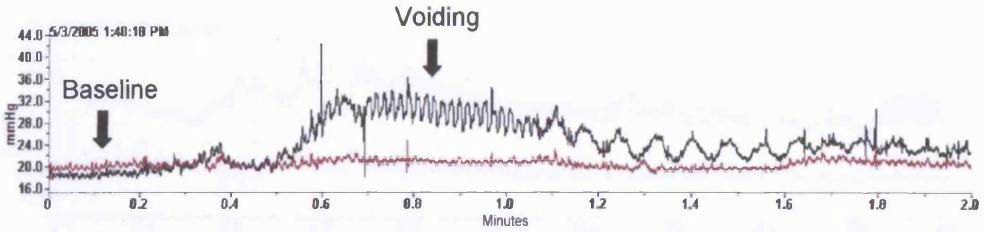


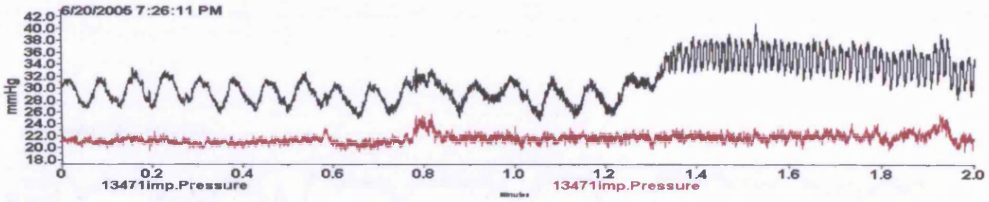
Figure 34. Bladder and kidney histology in the 94-day model.

Paired bladder (A-C) and kidney (D-F) sections stained with Masson's trichrome (original magnification x50). Bladder architecture was generally similar in sham (A) and BOO (B and C) bladders, although apparently thinner detrusor bundles were found in B. Representative sham renal cortex (D). The kidneys (E) attached to one BOO bladder (B) appeared normal, whereas kidneys attached to the three other BOO bladders contained subcapsular cysts (F).

A


Voiding
Baseline
5/3/2005 1:40:18 PM
mmHg
Minutes

B


6/20/2005 7:26:11 PM
mmHg
13471imp.Pressure
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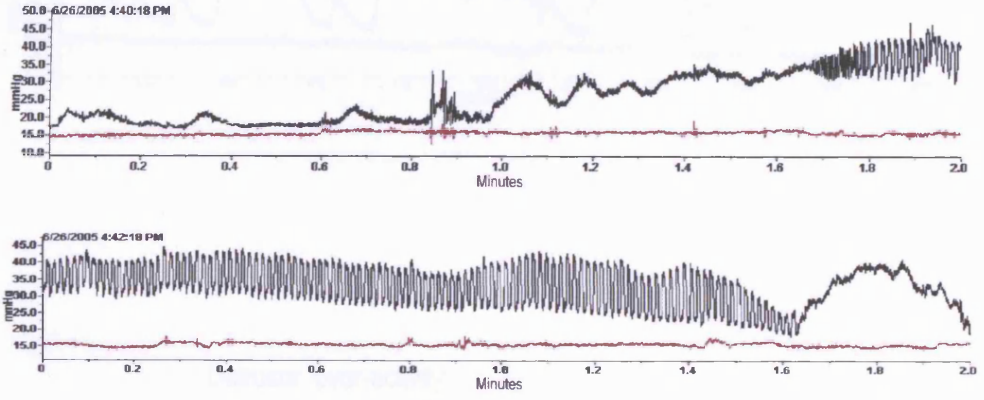
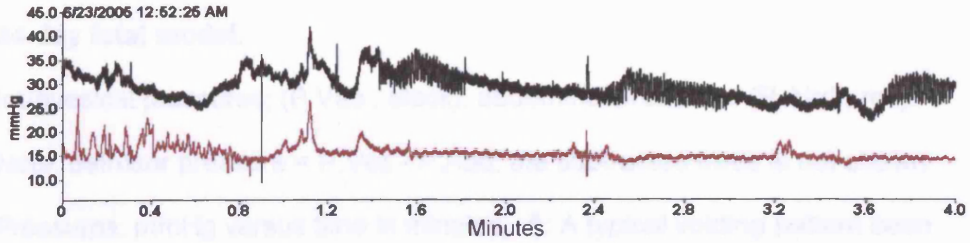
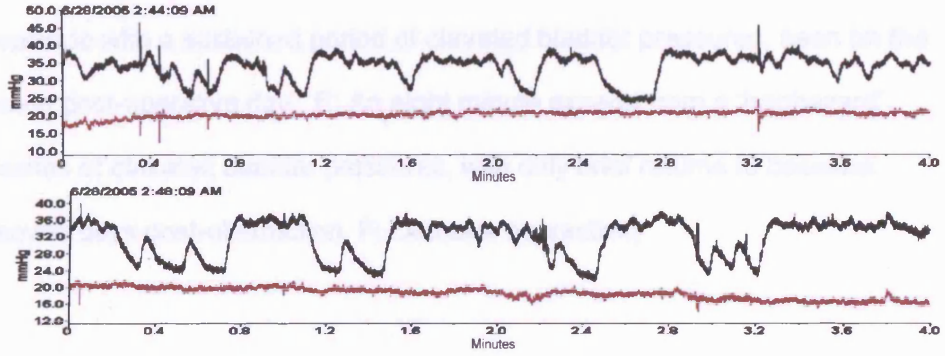
C


6/26/2005 4:40:18 PM
mmHg
Minutes
6/26/2005 4:42:18 PM
mmHg
Minutes

D


6/23/2006 12:52:25 AM
mmHg
Minutes

E


6/28/2006 2:44:09 AM
6/28/2006 2:48:09 AM
mmHg
Minutes

F

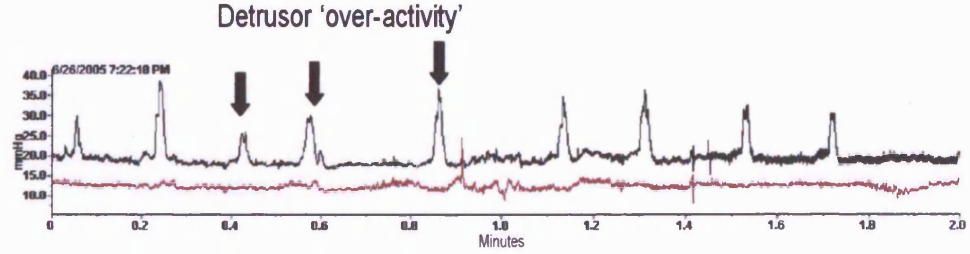

Detrusor 'over-activity'
6/26/2006 7:22:18 PM
mmHg
Minutes

Figure 35 A-F. Radiotelemetered cystometry traces in the obstructed 94-day fetal model.

Intravesical pressures; (P.Ves.; black), abdominal pressures (P.Abd.; red). Note: detrusor pressure = P.Ves - P.Abd; the subtracted trace is not shown. Pressures: mmHg versus time in minutes. A: A typical voiding pattern seen within an hour of surgery and terminating in an 'undulating' baseline; the void was repeated after two minutes. B: The 'undulating' pattern seen in A was often seen preceding the onset of a void. C: Prolonged void; day six post-obstruction. D: A four minute excerpt from a prolonged 'voiding' episode with a sustained period of elevated bladder pressures, seen on the sixth post-operative day. E: An eight minute excerpt from a 'haphazard' series of elevated bladder pressures, with only brief returns to baseline; seven days post-obstruction. F: Detrusor overactivity.

Figure 36. Example of a normal void (105 day fetus).

Pattern is typically that of a sustained rise in intravesical pressure (P_{ves}) with superimposed high-frequency, low-amplitude activity with a gradual return to baseline filling pressure (Thiruchelvam *et al.* 2004)

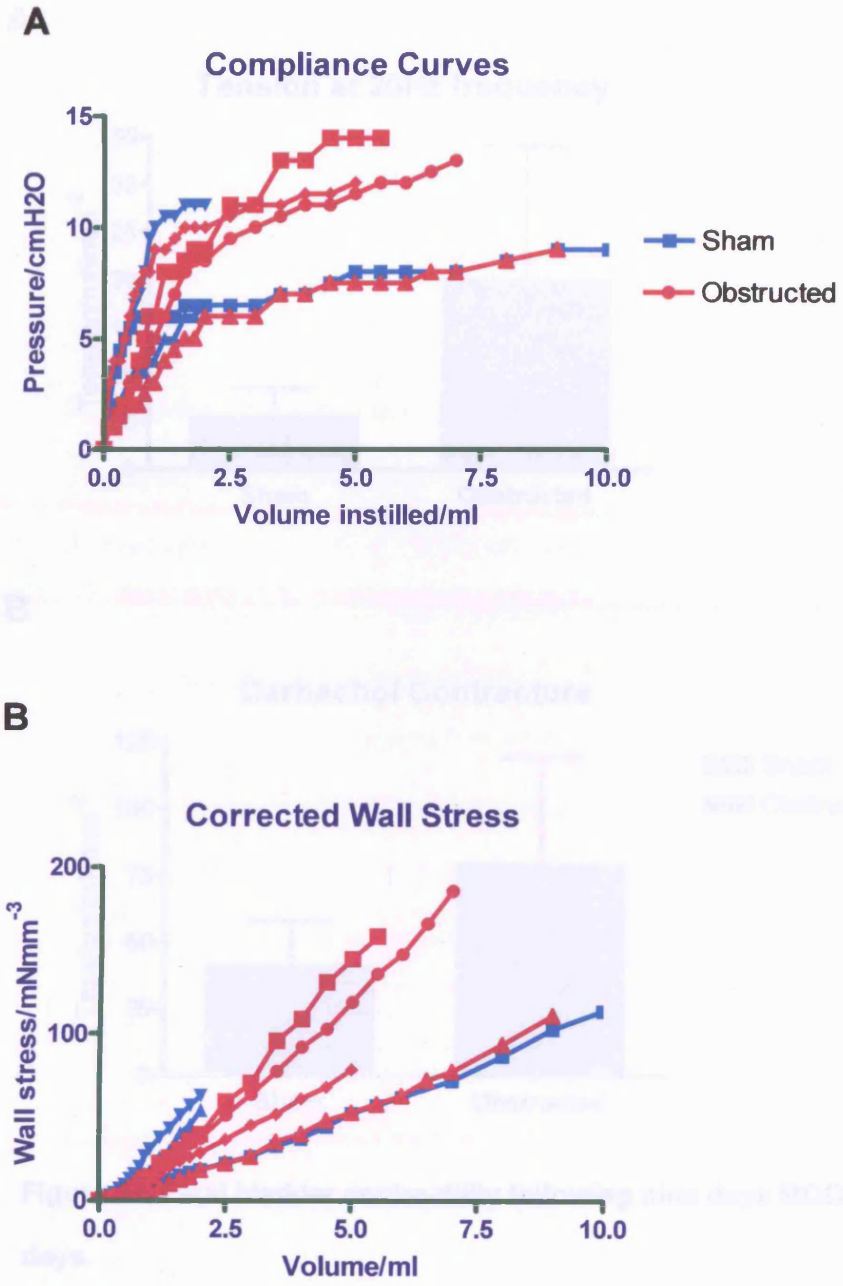
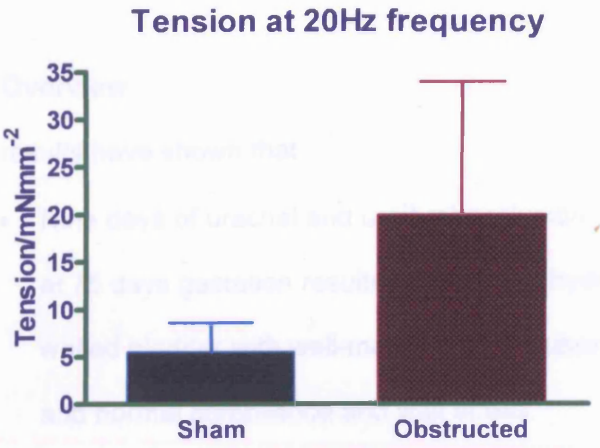


Figure 37. Pressure-volume plots for ex-vivo fetal bladders.

Compliance curves shown in A, and corrected wall stress shown in B.

Shams are shown in blue, and BOO data in red.

A



B

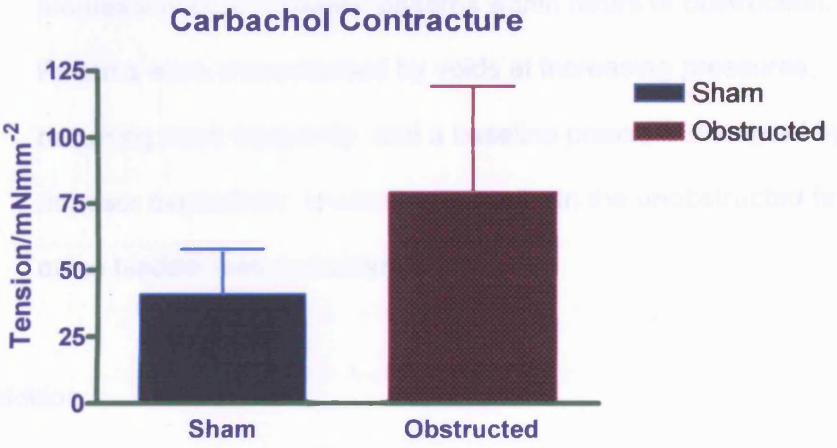


Figure 38. Fetal bladder contractility following nine days BOO at 94-days.

Bar charts illustrating muscle tension achieved by EFS at 20Hz (A) and by direct muscle stimulation with the acetylcholine agonist carbachol (B).

4. DISCUSSION

4.1 Overview

My results have shown that:

- Nine days of urachal and urethral occlusion, in the fetal lamb, initiated at 75 days gestation resulted in bilateral hydronephrosis, a thick-walled bladder with well-maintained detrusor structure and function, and normal compliance and wall stress.
- Radiotelemetered fetal cystometry was feasible in the obstructed fetal ovine bladder from 94 days gestation (for nine days), and revealed a progression of urodynamic patterns within hours of obstruction. Patterns were characterised by voids at increasing pressures, occurring more frequently, and a baseline pressure disrupted by detrusor overactivity. In-utero cystometry in the unobstructed fetal ovine bladder was unsuccessful.

In addition:

- Urachal ligation alone from 75 days gestation resulted in the same urinary tract appearance and bladder physiology as in the double ligation group.
- Nine days of BOO initiated at 75 days caused marked growth of the obstructed fetal urinary tract compared with shams, a feature which was not noted when BOO was initiated at 94 days gestation.

- The appearance and function of the bladder obstructed for nine days from 75 days contrasted markedly with that found after 30 days of BOO

Protocols 1 and 2 confirm the reproducibility and resilience of the fetal ovine BOO model. This is the first time morphology, function and in-vivo cystometry have been studied in the same model. This work, together with that of Nyirady et al. (2002) and Thiruchelvam et al. (2003a and b), constitutes the first time that BOO at 75 days has been studied since Tanagho's pioneering work in 1972 (Tanagho 1972). Samnakay et al (2006) published findings on a 70-day model but only renal, and not bladder, changes were described.

My findings are discussed in the following sections:

- BOO initiated at 75 days (bladder morphology and physiology followed by kidney morphology)
- BOO initiated at 94 days gestation (bladder morphology and physiology and comparison between short- and long-term fetal BOO)
- Interpretation of radiotelemetered urodynamics
- Clinical implications
- Future work

4.2 Protocol 1 results: Fetal BOO from 75 days gestation

4.2.1 Bladder morphology

BOO initiated at mid-gestation, and maintained for just nine days, produced marked accelerated bladder growth, as assessed by weight, protein and DNA content. Bladder weights increased four-fold in spite of the fact that fetal growth was not significantly different between the normal and obstructed groups. Amounts of protein and DNA in the obstructed bladders reached levels expected of normal term organs. Considering the bladder as a whole, the growth response was predominantly hyperplasia, as judged by lack of increase in protein/DNA ratios. Detrusor smooth muscle (DSM) in BOO bladders appeared grossly normal on light microscopy, with no evidence of disruption. Ultrastructurally, individual detrusor muscle bundles, as well as intervening connective tissue, appeared greater than controls, though this difference was not formally quantified. These findings reflect work performed by other groups. Peters et al. (1992) described a significant increase in bladder mass following BOO in the fetal lamb at 60 until term – and showed the ratio of hyperplasia to hypertrophy to be 3.3:1. Buttyan and Levin (1997) used ^{3}H-thymidine staining to show that hypertrophy and hyperplasia occur in different cellular compartments: the former in detrusor smooth muscle, and the latter in fibroblasts and mucosal cells.

When analysing the bladder in greater detail, my results revealed significant changes in the urothelium, whereby the "urothelial cell layers" increased almost three-fold in the obstructed bladders compared with shams. It is possible that the measured increase in urothelial nuclear layers was due to

bladder collapse prior to fixation, rather than growth. However, Gosling et al. (2000) and Lieb et al. (2000) also noted this phenomenon: they described urothelial and fibroblast hyperplasia following BOO in the mature rabbit, which was later followed by detrusor hypertrophy. Monson et al. (1995), who also studied BOO in the rabbit, proved that the increase in urothelial nuclear layers was due to urothelial hyperplasia, by means of a thymidine-labelling technique. My urothelial studies also showed prominent apoptosis in the basal urothelial zone in BOO groups but not in sham urothelia, a feature not previously reported. The apoptotic count in the other layers of the fetal bladder, namely the lamina propria and DSM layers, was sparse. iNOS was immunolocalized in urothelia of sham fetal bladders with no change in signal intensity after BOO: hence, in this context, no correlation was noted between levels of iNOS and apoptotic index, as in the guinea-pig bladder (Chertin *et al.* 2004).

BOO at 75 days also resulted in the undisrupted expression of uroplakin (UPIb and III) proteins in urothelia from both sham and obstructed bladders: ultrastructural studies will be needed to establish whether these proteins are found in vesicles or apical membranes of the umbrella cells. Although Truschel et al. (2002) showed that UPIII expression is increased in apical membranes exposed to increased hydrostatic pressure, my work showed no difference between BOO and control UPIII.

When visualised by transmission electron microscopy, urothelial umbrella cells appeared larger than control cells, and contained a higher

concentration of apical vesicles (Fig. 29 A-B). This finding is very interesting because although bladders would have been drained, and hence "un-stretched" prior to fixation, they may have maintained their "stretched" appearance ultrastructurally. We know from previous work that bladder distension results in the accumulation of vesicles beneath the apical membrane (Minsky & Chlapowski 1978), and that exocytosis of vesicle contents occurs in order to increase the urothelial apical membrane surface area (Truschel *et al.* 2002) – so it is possible that continuous distension results in an increased production of apical vesicles for this purpose.

4.2.2 Bladder physiology

Analysis of the data sets indicated that nine days of BOO from 75 days gestation generated an evolving pattern of changes in bladder physiology. In terms of conrtactility, standardization of bladder data for initial intravesical volume and wall thickness suggested that there was no significant difference between the three groups (sham, urachal ligation only, urachal and urethral occlusion) - a finding which correlated with the well-preserved DSM. Since electrical field stimulation depends on an intact nerve supply, this finding suggests that the degree of BOO was not sufficient to cause a functional denervation. Variability of the raw data was generally greater in the obstructed groups than the control set. For example, the variance ratio of normalised wall stress values in the obstructed and control groups was, as indicated by Fisher's test, highly significant. This indicated that there was an inherent variability in the extent of obstruction between animals and this led to a spectrum of responses. This made comparison of group data sets

difficult, as the large inherent variability may have masked any trends. To minimise variability, different parameters were assessed as a function of the bladder-to-fetal-weight ratio (BFR). Furthermore, as no differences could be identified between the two obstruction groups, (urachal ligation only and urachal and urethral occlusion), the data from the two groups were amalgamated.

The change in the passive properties (compliance) of the bladder wall, as assessed by measurement of normalised wall stress per unit change of volume, precedes functional (contractility) changes: an increase of BFR was associated with a reduced wall stress per unit change of volume, i.e. the bladder wall was easier to extend per unit volume change as BFR increased. The implications are that an increase in bladder wall thickness appears to facilitate filling by reducing wall stress, and hence reduce the risk of a raised intravesical pressure. A reduced circumferential tension implies a reduction in the ability of detrusor tension to be transmitted through the tissue mass, and so again reduces the ability of the bladder to raise pressure during active contraction. This compensatory mechanism may not last throughout a more prolonged obstructive period, and hence intravesical pressures may be expected to rise in spite of a thickened bladder wall. Whether wall stress recovers after a period of de-obstruction remains a further question to address.

The hypothesis that a short period of obstruction would result in compensation, i.e. bladder overgrowth of the bladder without failure of

function (Gosling *et al.* 2000) was confirmed by the lack of association of nerve counts or contractile function with increased BFR. Two modes of contractile activation were tested, i) via the cholinergic motor nerves, ii) by direct activation through depolarisation with elevated [KCl] or with muscarinic or purinergic agonists. Again, data from the two obstructed groups have been combined because of the relatively small data sets. Contractions elicited by EFS and by direct muscle stimulation with charbacol, confirmed that a cholinergic mechanism is active at this gestation. Contractions elicited by EFS were completely blocked by the neurotoxin TTX, indicating they were nerve-mediated. Atropine completely abolished nerve-mediated contractions in detrusor strips from all groups, and actually resulted in mild relaxation. This finding would suggest that no mechanism for detrusor contraction (e.g. purinergic) other than via cholinergic neurotransmission was in place (Burnstock 2002). However, application of ABMA, a purinergic agonist, generated transient contractures in all preparations, which were not significantly different between BOO and control bladders. This suggested that purinergic receptors may in fact be present at this gestation, although ATP-induced contraction is not necessarily active in normal or obstructed fetal bladders at this gestation. Studies in older fetal lamb models (90 days and above), on the other hand, have suggested a possible active purinergic component (Levin *et al.* 2001, Thiruchelvam *et al.* 2003c). Receptors may also develop in response to an increased amount of ATP, which is released by bladder distension (Burnstock 2002). The relaxation component may suggest that a nitrergic mechanism may also exist but was not studied in the current set-up. Plots of

BFR as a function of tension did not show a significant correlation suggesting that bladder overgrowth was not associated with contractile failure.

4.2.3 Kidney morphology

BOO for nine days had variable effects on renal histology, even though hydronephrosis was uniformly documented. Sham renal histology revealed a nephrogenic zone with S-shaped bodies, a deeper cortex with formed glomeruli and medullary collecting ducts. In six out of ten kidney pairs from the BOO groups, these appearances were maintained. In two sets of kidneys from the urachal-only ligation group and in two sets from the urachal and urethral ligation group, however, the cortex contained cysts, some with glomerular tufts, and medullary ducts were dilated. These changes were typical of fetal renal obstruction early on in gestation in ovine and other models (Kitagawa *et al.* 1999, Matsell *et al.* 2002). Kidneys with cysts tended to occur in fetuses with the highest bladder weights and protein contents, although in this study I have not shown whether this relationship was due to a direct transmission of intravesical pressure due to reflux. In human congenital obstructive nephropathy, the observation that urine osmolality concentrations approach plasma values indicate a failure to dilute urine; however, this is a normal function of the sheep kidney in the second half of gestation (Nijland & Ross 2000). In this study, urine osmolality and sodium concentration were not significantly different between BOO and sham fetuses.

4.2.4 Role of the urachus

An unexpected outcome of Protocol 1 was the revelation that urachal ligation with patent urethra produced similar changes in bladder growth, morphology, compliance and contractility as combined urachal and urethral obstruction. Gobet el al. (1998) had also suspected and investigated this finding. They performed urachal-only ligation in the fetal lamb at 95 days gestation: at term, only the male lambs had hydronephrosis and increased bladder weight. Collectively, Gobet's and our current results strongly suggest that the male sheep fetal urethra is a high-resistance conduit in mid-gestation. We found that some paramaters, such as bladder protein and bladder/fetal weight, had less variability in the urachal-only group, and this finding remains unexplained.

4.2.5 Comparison between BOO at 75 days maintained for nine days and BOO at 75 days maintained for 30 days

It is interesting to compare effects of short- versus longer-term fetal BOO induced by combined urachal and urethral obstruction (Table 5). Nine days BOO caused bladders to be 3-4 times heavier than controls, whereas 30 days BOO led to an even greater increase versus time-matched shams (Nyirady *et al.* 2002): hence the accelerated growth response is sustained. Although bladders obstructed for either nine or 30 days were both overgrown, their histology differed markedly. More prolonged BOO led to a flattened urothelium, with DSM bundles separated by prominent matrix and increased apoptotic index in the DSM and lamina propria; short-term BOO generated a urothelium with more cell layers than normal, with a generally

well-maintained detrusor layer and prominent urothelial apoptosis (Nyirady *et al.* 2002). The trajectory of morphological/cell biological changes is paralleled by physiological observations, with well-maintained contractility at nine days but hypocontractility after 30 days. Hence, there is an evolution from an overgrown but contractile ('compensating') bladder to a hypocontractile ('decompensated') organ. These findings are again in parallel with Levin et al.'s (1997) definition of decompensation as the phase characterized by progressive deterioration in contractility and function (i.e. ability to empty), a slower increase in mass, and a progressive decrease in the volume fraction of smooth muscle elements in the bladder wall . Levin et al. (1997), however, describe two possible end-results: either an organ with a thick fibrous wall, low capacity, poor compliance and little or no contractile function; or a dilated bladder with a thin fibrous wall, high capacity, and little or no contractile function: the findings in the study by Nyriady et al. (2002) were consistent with the latter. Importantly for my future work, Levin et al. (2002) showed that relief of obstruction during the compensated phase, at least in animals, induced a rapid and full restoration of the bladder function. However, when the obstruction was relieved during the decompensated stage, bladder function only partially recovers (Levin *et al.* 2002).

4.2.6 Summary

In summary, the results from Protocol 1 proved the hypothesis that nine days urachal and urethral occlusion in the fetal lamb, initiated at mid-gestation, result in a thick-walled, compliant, contractile bladder similar to the compensating obstructed bladders found in infants born with PUV

(Holmdahl *et al.* 1995; see 4.4.2). These findings contrast with the decompensated features noted following BOO for 30 days.

4.3 Protocol 2: Fetal BOO and radiotelemetered urodynamics from 94 days gestation

4.3.1 Morphology and physiology

The most striking difference between the morphology and physiology outcomes of BOO initiated at 75 days and that initiated at 94 days is that although the same BOO methodology was used in both sets of experiments, early-onset BOO resulted in significant increases in bladder weights, urothelial layers and incidence of urothelial apoptosis, as well as profound renal cystic changes, while late-onset BOO, maintained for the same period of time, failed to elicit such pronounced responses. These findings are in keeping with previous experimental BOO studies: several groups have shown that fetal ovine BOO at 75 days gestation or earlier, results in kidneys with microcystic dysplastic changes, structural disorganization, a reduction in glomerular number and medullary tubular dilatation (Kitagawa *et al.* 1999,Kitagawa *et al.* 2001,Edouga *et al.* 2001,Nyirady *et al.* 2002,Kitagawa *et al.* 2004), whereas fetal BOO later in gestation results in hydronephrosis with larger cyst formation and a normal glomerular count, but no parenchymal disorganization, although a thinned cortex was often observed (Harrison *et al.* 1982,Harrison *et al.* 1983,Adzick *et al.* 1985,Gobet *et al.* 1998,Kitagawa *et al.* 1999).

Although bladder morphology appeared rather resilient in the 94 day BOO group, three of the four sets of BOO kidneys had developed cysts in nascent glomeruli in the subcapsular area: the reason why these just-forming nephrons appear to be more easily distorted following fetal BOO versus

more mature nephrons in the deeper cortex is unknown but may theoretically relate to less resilient basement membranes. In fact, the formation of similar cortical cysts has been shown to initiate after just a few days of experimental complete unilateral ureteric obstruction in sheep fetuses aged 90 days gestation (Attar *et al.* 1998), and is also a characteristic of experimental urinary flow impairment in primates (Matsell *et al.* 2002a). Protocol 2 showed that generation of cortical cysts correlated with episodes of raised detrusor pressure; however, it remains to be determined whether the underlying mechanism involves mechanical obstruction of the ureters by dilated bladders, or high pressure VUR secondary to BOO. Surprisingly, the obstructed fetus which did not develop cystic kidneys was found to have the least compliant bladder, which goes against VUR being the mechanism of transmission of pressure to the upper tracts.

4.3.2 Radiotelemetered natural-fill urodynamics

This model represents a fresh insight into the application of the fetal ovine model to antenatal urodynamic studies, and the characterization of fetal cystometry at various stages of obstruction. The major advantage of radiotelemetered natural-fill urodynamics versus traditional urodynamics utilising external catheters (de Tayrac *et al.* 2003) is that in the absence of external lines, the pregnant ewes are free to move around the pen without restraint, and there is also less risk of dislodgement and infection. The disadvantages of the radiotelemetry technique are the relatively large size of the implant body (6 by 6 cm, approximately the size of a 94-day fetal

abdomen) and the diameter of the catheters (1.2 mm); factors which clearly limit the size of fetuses which can be realistically studied. Bladder catheterisation through an incision in the bladder wall was successful in the pilot study performed by Thiruchelvam et al. (2004) in the fetal lamb at 105 days gestation, but failed in our 94 day-old fetuses, suggesting that the younger bladders were too delicate to sustain direct catheterisation. Inserting catheters through the urachus is not an accurate way to study 'normal' unobstructed bladders, as we now know that urachal occlusion per se causes a degree of BOO (Gobet *et al.* 1998,Farrugia *et al.* 2006a). Until radiotelemetry technology is able to provide smaller catheters, a substitute method of measuring intravesical pressure is required that is tolerated by animals of younger gestational age. Difficulties identified during an earlier pilot study were overcome in this study: intra-abdominal catheter dislodgement was prevented by tunnelling the intra-abdominal catheters, and a previous inability to activate the implant's on/off switching mechanism was overcome by attaching the implant body to the internal surface of the maternal abdomen making it accessible to the required magnetic induction.

An inevitable limitation of monitoring in-vivo fetal urodynamics chronically in an ambulatory situation was that voiding could not be directly visualised. Thus 'voids' were recognised as sustained elevations in pressure, also characterised by a high frequency superimposed low-amplitude activity (thought to arise from the activity of a urethral pump mechanism in the long posterior urethra of sheep) (Fig. 35 and 36). The same pattern of voiding has been noted in a previous fetal sheep study with voiding observed during

ultrasonograpy (de Tayrac *et al.* 2003) and in an acute study of normal fetal pigs in which synchronous voiding and urinary flow were observed (Olsen *et al.* 2001). Thiruchelvam at al. (2004) reported the same sustained normal voids in a study of in-vivo urodynamics in two unobstructed animals (one male, one female) using the same radiotelemetry technique in 105 day gestation sheep with bladder catheters inserted directly through the bladder wall. The voids had variable durations between 60 and 180 seconds and occurred about three times per hour. The maximum detrusor voiding pressures at 105 days gestation were 20 to 28 mmHg (27 to 38 cmH_2O) (Thiruchelvam *et al.* 2004).

4.3.3 Interpretation of in-vivo cystometry

Due to the unavailability of control traces in this study, the obstructed traces obtained were compared with the 'normal' traces reported by Thiruchelvam et al. (2004) at 105 days (which is close to the age at which my study was concluded). This comparison was not ideal, as only one of the two fetuses with trans-vesical catheterisation in that study, was male. My results showed that within 24 hours of obstruction, voids occurred at median voiding pressures of 12-14 mmHg (16-19 cmH_2O) and lasted for one minute, with a pattern similar to that seen by Thiruchelvam et al. (2004). Conversely, the low-pressure 'undulations' noted in-between voids had not been previously documented. The gradual increase in voiding pressures that occurred over the ensuing days, reaching a median of 24 to32mmHg at 101 days, was also a new finding, as Thiruchelvam's work showed that the unobstructed male fetus at 105 days maintained stable voiding pressures of 27-28 mmHg

throughout 30 days of observation (Thiruchelvam *et al.* 2004). Of course one cannot conclude, without directly comparable control animals, whether the increasing detrusor pressures seen in this study were actually a result of obstruction or a feature of normal intra-uterine development.

The frequency and duration of voids also differed markedly between the two studies. The frequency of voids in the unobstructed male fetus was observed to decrease from 3.0 to 1.5 voids per hour over a period of 30 days (Thiruchelvam *et al.* 2004); mean duration of each void increased from 1 to 1.5 minutes. Fetuses in Protocol 2 were attempting to void initially every 1 to 2 minutes and even more frequently with prolonged obstruction; the duration of each void increased significantly to a median of 3.4 to 4 minutes after seven days of obstruction. Notably, only one obstructed fetus maintained a more regular voiding frequency (one void every two or three minutes), and this was the only BOO fetus which did not develop glomerular cysts, possibly because the bladder spent less time at raised intravesical pressures. The 'haphazard' contractile activity noted later in the obstructive period, is also a new finding. These excursions may represent frequent voiding attempts by the fetus. Overall, the obstructed bladders spent increasing amounts of time at sustained high pressures usually associated with bladder emptying activity.

Despite the heterogeneity of renal cystic changes and voiding patterns in the BOO group, it should be emphasised that all four BOO fetuses had dilated renal tracts on ultrasonography. Overall, the resting baseline detrusor

pressure remained low throughout the nine day study period: this finding is consistent with the normal compliance found on ex-vivo cystometry and implies that the bladders were able to compensate by accommodating increasing intra-vesical volumes.

4.3.4 Summary

Protocol 2 results have proven the hypothesis that radiotelemetered fetal cystometry is feasible in the obstructed ovine bladder at 94 days gestation and suggest that bladder dysfunction associated with congenital BOO begins in-utero. In the fetal lamb, this occurs within hours of obstruction and is characterized by a pattern of more frequent and prolonged voids. Voiding pressures inceased during the period of obstruction; however, in the absence of any controls, it is unknown whether this was the effect of obstruction or response to fetal and bladder growth. Baseline storage pressure, however, does not change significantly throughout the nine days and is consistent with the normal bladder compliance noted following ex-vivo cystometry. Radiotelemetered cystometry failed in the 94-day gestation unobstructed fetus.

4.4 Clinical implications

4.4.1 Human versus experimental fetal BOO

The morphological and physiological findings following fetal ovine BOO closely reflect the human scenario where, although the typical infant "valve" bladder is thick-walled and trabeculated with a variable compliance, the extent of renal dysplasia differs considerably. Cussen (1971) peformed post-mortem studies of 30 renal units from children with congenital urethral obstruction, and demonstrated cystic renal dysplasia in only 37%. The nature of the dysplasia found experimentally is typical of prenatal obstruction, i.e. Potter type IV malformations (Potter 1972). Potter (1972) described the characteristic findings, which are subcapsular cysts, each derived from S-shaped bodies, and comprised of a dilated Bowman's capsule and a primitive proximal tubule. She postulated that ureteric bud branching is initially normal in type IV malformations and that a sudden, severe, obstructive event would rapidly ablate the renal mesenchyme, resulting in a single layer of cysts, as seen in this study (Potter 1972).

A further result from Protocol 1 which is relevant to clinical management is the fact that urine concentrating ability remained normal in spite of the fact that changes in bladder morphology and compliance, as well as renal cystic change, had already become manifest. However, fetal medicine criteria for antenatal intervention as established by Johnson et al. (1994) state that three serial vesicocenteses taken at 48-72 hour intervals and showing a pattern of decreasing hypertonicity are required to identify fetuses that hold the greatest potential benefit from in-utero surgical intervention. My findings,

however, suggest that decompression may have to be carried out earlier than this in order to better preserve bladder and kidney function.

Overall, it is evident that patients with PUV share only a common label; each obstruction is unique, and the resulting course varies. This is important to remember in the laboratory setting, where each obstruction that is surgically produced will also be quite variable.

4.4.2 Human pre- and post-natal bladder function

Sillen et al. (2000) evaluated the voiding pattern of preterm neonates at a mean of 32 weeks gestation, by means of four-hour voiding observation. The neonates voided once an hour, and voided a mean of 53% of total capacity with a 58% frequency of "interrupted voiding". Interrupted voiding was defined as two or three voids in less than 10 minutes, and was documented in 60% of preterm neonates, and later in 30% of term babies (Sillen *et al.* 2000, Sillen 2001). Natural fill urodynamic studies have shown that interrupted voiding remained prevalent in babies at six months of age; however, the bladder at this age empties to near completion (Yeung *et al.* 1995). Yeung et al. (1995) attributed this voiding pattern to a dyscoordination between the sphincter and the detrusor, and also noted that detrusor instability is rare in the normal infant bladder. Mean voiding pressures at this stage were noted to be higher than those expected in older children with values reaching 117 cm H_2O in males and 75 cm H_2O in females (Yeung *et al.* 1995). Thus the standards for voiding pressures in

healthy infants are imprecise and can be a median of more than 100 cm H_2O in males, and 60-70 cm H_2O in females (Wen & Tong 1998).

Urodynamic studies in babies born with PUV have revealed even higher voiding pressures (162 cm H_2O) than those found in normal bladders described above, with a high incidence of instability, leading the authors to describe these bladders as "hypercontractile" (Holmdahl *et al.* 1996). Instability was present in 88% of patients at presentation, at a mean pressure if 60 cmH_2O. Compliance was calculated from the increase in basic detrusor pressure at expected normal bladder capacity for patient age: a basic pressure increase of >20 cmH_2O was considered to represent a poorly compliant bladder. Non-compliant bladders were noted in 50% of babies (Holmdahl *et al.* 1996).

Wen et al. (2006) compared valve bladder urodynamics in boys aged below and above two years of age. Changes in urodynamic patterns were noted at two years of age, when voiding pressures and bladder compliance were noted to be significantly reduced, whereas post-void residual and bladder capacity were increased. Interestingly, almost half of the older boys showed intermittent detrusor contraction during voiding, which the authors described as a "special voiding pattern": the pattern was remarkably similar to the 'superimposed' contractions typical of the fetal void (Fig. 39) (Wen *et al.* 2006).

It is of interest that Yeung et al. (1998) reported the clinical application of continuous real-time ambulatory urodynamic monitoring using infrared telemetry, in infants and young children. The system works via an ambulatory urodynamic recorder that converts digital pressure signals to modulated infrared waves which are then picked up by a receiver attached to the clinic ceiling. Thus the child is able to perform normal play and activity, while the clinician monitors the natural-fill urodynamics in a separate room (Yeung 1998). Therefore as technology is refined and radiotelemetry is adapted to allow human ambulatory urodynamics, the concept of its use in the prenatal setting may not be such a distant one.

In conclusion, it appears that neonatal bladder dysfunction may in fact be a continuum of the damage initiated in-utero. Although urodynamic findings in the fetal model and those in human neonates are not strictly comparable, it is interesting to note that the absence of bladder instability in normal newborns suggests that the finding of detrusor overactivity in utero may in fact be abnormal. On the other hand, "dyscoordinated" voids occurring two or three times per ten minutes was not unusual in the term baby, hence the significance of the frequent voids in the fetal model becomes questionable in the absence of a suitable control. Also, voiding pressures in human neonates were found to be considerably higher than those measured in utero, even in the presence of BOO. Holmdahl et al. (1996) documented a non-compliant bladder in 50% of infants born with PUV, implying that the other half may have a normal compliance: this variation was also noticed in the experimental setting. Of course one can only speculate on how the

human fetal bladder functions, as antenatal urodynamics in the clinical setting are as yet not feasible.

4.4.3 Long-term outcome

The long-term effect of close follow-up and management of neonatal bladder dysfunction is as yet unknown, as the infants on whom urodynamic studies have been performed (largely in the last 10-15 years) have not as yet reached adulthood. Retrospective reviews have shown that boys with bladder dysfunction in childhood, particularly those with persistent poor compliance and instability, are more likely to develop ESRF (Lopez-Pereira *et al.* 2002). Holmdahl et al. (1996) retrospectively reviewed outcome in boys born with PUV and followed up from 4 years of age: a changing urodynamic pattern was noted, with decreasing instability, increasing bladder capacity, and poor bladder emptying. Postpubertal PUV patients had high capacity bladders with low contractility. Renal concentrating ability was normal in only 17% of patients studied (Holmdahl *et al.* 1996). It is hoped that the greater awareness of antenatal and neonatal bladder dysfunction and its careful management will improve outcome in this groups of patients.

4.5 Future work

My results have shown that antenatal urodynamics are feasible in the fetal lamb BOO model from 94 days gestation. However, my work in this thesis, and that of others, has shown that obstruction at this gestation induces a lesser degree of renal dysplasia than obstruction earlier in gestation. Hence

it would be more useful to study the correlation between onset of bladder dysfunction and renal dysplasia in a younger fetus at or before mid-gestation. This would entail a combination of improvement in technology with smaller implants and thinner lines, and better fetal operative skills to reduce fetal loss. It would also be useful to observe these changes for a period longer than nine days, when the bladder retains its compliance and contractility, but shorter than thirty days, when the bladder becomes hypocontractile and over compliant. My work in Protocol 1 showed that two bladders exhibited a reduced compliance (which is what occurs in a proportion of neonatal PUV bladders) whereas there was a large variability in the rest, possibly implying that the "ideal" period of observation should run a few days longer: I would hypothesise 15 days. In order to study the effect of vesico-amniotic shunting on bladder dynamics, further observation would then be required.

I therefore hypothesise that 15 days of BOO from 70 to 85 days gestation would result in a thick-walled bladder with a variable compliance as seen in newborns with PUV, and would result in associated urodynamic changes. Decompression at 90 days gestation would result in an improvement in intravesical pressures and detrusor overactivity over a further ten day period to 95 days. In addition, following observations by Kitagawa et al. (2007), it would be essential to study the effect of a shunt *per se* on bladder morphology and function. Four experimental groups would be required: a control non-obstructed group (70-95 days gestation); a non-obstructed shunted group in which a shunt is inserted at 85 days and observed for a

further 10 days; and two BOO groups, of which one is shunted at 85 days and observed for 10 days. Radio-telemetered urodynamics would be observed throughout, and ex-vivo cystometry performed following sacrifice.

Figure 39. Intermittent detrusor contractions (arrow) during voiding noted during a urodynamic study on a 6 year old boy with a valve bladder.

(Wen *et al.* 2006)

Table 5. Comparison between nine and thirty days BOO commencing at 75 days gestation.

Bladder feature	9 days BOO (75-84 days)	30 days BOO (75-105 days)
Weight	Increase	Increase
Wall thickness	Increase	Decrease
Urothelium	Increase 4-8 cell layers	Flattened, single cell layer
Urothelial apoptotic index	Increased	No difference
Asymmetric unit membrane	Intact AUM and UP1b	-
Lamina propria	Thickened	Flattened
Lamina propria apoptotic index	No difference	Increase
Detrusor muscle	Intact, no difference	Disrupted, increased area
Nerve bundles	Reduced	Reduced
Total protein/DNA	Increase in total protein and DNA but no difference in protein/ DNA ratio	Increase in total protein and DNA but no difference in protein/DNA ratio
Compliance	Normal	Increased compliance
Contractility	No difference, contractility cholinergic in origin. No purinergic component. Evidence of nitrergic relaxation.	Hypocontractile throughout; evidence of purinergic and nitrergic component

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6. PUBLICATIONS AND PRESENTATIONS

Publications (see insert)

Radiotelemetered urodynamics of obstructed fetal bladders: Correlations with ex-vivo cystometry and renal histopathology. Farrugia MK, Cuckow PM, Woolf AS, Peebles DM, Fry CH, Godley ML. BJU.Int. 99(6):1517-22, March 2007.

Experimental short-term fetal bladder outflow obstruction: I. Morphology and cell biology associated with urinary flow impairment. Farrugia MK, Long DA, Godley ML, Peebles DM, Fry CH, Cuckow PM, Woolf AS. Journal of Pediatric Urology, 2 (4): 243-253, August 2006.

Experimental short-term partial fetal bladder outflow obstruction: II. Compliance and contractility associated with urinary flow impairment. Farrugia MK, Godley ML, Woolf AS, Peebles DM, Cuckow PM, Fry CH. Journal of Pediatric Urology, 2 (4): 254-260, August 2006.

The sensitivity of antenatal ultrasound for predicting renal tract surgery in early childhood. Bhide A, Sairam S, Farrugia MK, Boddy SA, Thilaganathan B. Ultrasound in Obstetrics and Gynecology 25(5):489-92, August 2005.

Natural fill, radiotelemetered fetal sheep cystometry.Thiruchelvam N, Godley M, Farrugia MK, Cuckow P. BJU International 93:382-387, February 2004.

Presentations

In-utero radiotelemetry to monitor urodynamics in a fetal model of posterior urethral valves: results following short-term obstruction. Farrugia MK, Woolf AS, Godley ML, Fry CH, Peebles, D, Cuckow PM. British Association of Peadiatric Surgeons annual meeting, Stockholm, July 2006.

Live In-utero urodynamics in ovine fetal bladder outlet obstruction: a feasibility study. Farrugia MK, Woolf AS, Godley ML, Fry CH, Peebles, D, Cuckow PM. European Society of Pediatric Urology annual meeting, Athens, April 2006.

Posterior urethral valves: clinical and experimental aspects. Farrugia MK. Kings College Hospital neonatology grand round, November 2005.

Modelling posterior urethral valves: features of the obstructed fetal bladder during compensation and at onset of decompensation. Farrugia MK, Woolf AS, Godley MI, Jenkins D, Sun TT, Fry CH, Cuckow PM. British Association of Peadiatric Surgeons annual meeting, July 2005.

The compensating fetal bladder: structure, compliance and contractility following eight days in-utero obstruction. Farrugia MK, Woolf AS, Godley ML, Fry CH, Cuckow PM. European Society of Paediatric Urology annual meeting, Uppsala, June 2005.

Modelling posterior urethral valves: fetal detrusor physiology following short-term bladder outflow obstruction. Urological Research Society Meeting, Royal College of Surgeons of England, January 2005.

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Fetal bladder outlet obstruction – the model. Farrugia MK, Woolf AS, Godley ML, Fry CH, Cuckow PM. Institute of Urology and Nephrology, Middlesex Hospital, August 2004.

Effects of in utero bladder outflow obstruction. Farrugia MK, Woolf AS, Godley ML, Fry CH, Cuckow PM. Institute of Child Health, March 2004.

Natural fill, radiotelemetered fetal sheep cystometry. Thiruchelvam N, Godley M, Farrugia MK, Cuckow P. Urological Research Society annual meeting, Royal College of Surgeons of England, January 2003.

7. APPENDICES

In all tables, * indicates a significant difference ($p<0.05$) and ** indicates a highly-significant ($p\leq0.01$) difference between groups.

Key

SvU = Sham versus urachal ligation only

SvUU = Sham versus urachal and urethral occlusion

UvUU = Urachal ligation only versus urachal and urethral ligation

SvO = Sham versus obstructed (protocol 2 only)

M-W = Mann Whitney analysis

Appendix 1. Protocol 1 fetal data.

	Sham	Urachal ligation	Double ligation	P values (M-W)		
				SvU	SvUU	UvUU
Fetal age (years)	82	82	82			
	75	75	75			
	75	75	75			
	82	75	82			
			75			
			75			
Median	78.5	75	75	0.65	0.74	0.91
Fetal weight (g)	650	700	620			
	400	400	400			
	500	450	450			
	350	550	800			
			650			
			850			
Median	450	500	635	0.56	0.23	0.45
Occipito-snout length (cm)	77	72	72			
	67.5	67.5	68			
	68	69	69			
	63	71	68			
			78			
			80			
Median	67.75	70	70.5	0.56	0.16	0.59
Crown-rump length (cm)	22.5	26.5	27			
	19.8	21.5	20.8			
	23	24	20.6			
	20	22.8	24			
			24			
			25			
Median	21.25	23.4	24	0.20	0.11	0.91

Appendix 2. Protocol 1 urine osmolality and sodium content.

	Sham	Urachal ligation	Double ligation	P-values (M-W)		
				SvU	SvUU	UvUU
Osmolality (mOsm/Kg)	164	113	151			
	148	294	164			
	169	124	146			
	131	163	251			
			139			
			190			
Median	156	143.5	157.5	0.73	0.46	0.61
Sodium (mmol/L)	67	0	56			
	48	117	65			
	54	0	47			
	36	51	116			
			37			
			82			
Median	51	25.5	60.5	0.90	0.25	0.48

Appendix 3. Protocol 1 fetal bladder data.

	Sham	Urachal ligation	Double ligation	P values (M-W)		
				SvU	SvUU	UvUU
Bladder weight (g)	0.43	2.42	0.85			
	0.34	1.67	1.12			
	0.46	1.37	1.03			
	0.37	2.58	1.60			
			1.20			
			2.60			
Median	0.41	2.04	1.16	*0.03	**0.01	0.15
Bladder:total fetal weight ratio	0.67	3.46	1.37			
(*10-3)	0.85	4.17	2.81			
	0.92	3.03	2.29			
	1.07	4.70	2.00			
			1.84			
			3.06			
Median	0.89	3.81	2.14	*0.03	**0.01	**0.01
Volume of urine drained from bladder	1.20	8.00	1.50			
(ml)	1.20	0.40	0.50			
	2.50	1.30	1.20			
	1.60	22.0	3.50			
			3.40			
			40.50			
Median	1.40	4.65	2.45	0.69	0.59	1.00
Bladder wall thickness (empty) (mm)	0.46	0.82	0.58			
	0.42	0.72	0.63			
	0.47	0.68	0.62			
	0.52	0.84	0.71			
			0.65			
			0.84			
Median	0.47	0.77	0.64	*0.03	**0.01	0.17

Appendix 4. Protocol 1 kidney data.

	Sham	Urachal ligation	Double ligation	P values (M-W)		
				SvU	SvUU	UvUU
Right + left whole kidney weights (g)	6.24	17.25	10.70			
	5.48	7.85	9.88			
	5.96	6.90	5.55			
	4.62	8.04	8.10			
			8.20			
			10.50			
Median	5.72	7.95	9.04	*0.02	*0.03	0.60
Right + left kidney weights (g)	6.14	13.04	8.94			
(after urine drained)	5.20	7.15	7.72			
	5.56	6.24	5.10			
	4.29	7.50	7.60			
			7.80			
			10.00			
Median	5.38	7.32	7.76	*0.02	0.06	0.60
Right + left renal pelvis weights (g)	0.10	4.21	1.76			
(i.e. weight of drained urine as	0.27	0.70	2.15			
reflection of degree of hydronephrosis)	0.34	0.65	0.46			
	0.33	0.54	0.50			
			0.70			
			1.40			
Median	0.30	0.68	1.05	*0.02	**0.01	0.91
Total drained renal: fetal weight ratio	9.44	18.62	14.42			
(*10-3)	13.02	17.88	19.30			
	11.12	13.88	11.33			
	12.25	13.64	9.50			
			12.00			
			11.76			
Median	11.68	15.88	11.88	*0.02	0.60	0.25

Appendix 5. Protocol 1 protein estimation data.

Experimental Group	Whole bladder weight (g)	Sample weight (g)	Absorbance 1	Absorbance 2	Concentration 1	Concentration 2	Mean
Sham	0.438	0.012	0.213	0.216	1140	1157	1148
	0.341	0.019	0.346	0.273	1875	1473	1676
	0.462	0.015	0.270	0.220	1457	1179	1318
	0.373	0.013	0.220	0.280	1326	1171	1249
Urachal ligation	2.420	0.014	0.173	0.177	918	940	929
	1.668	0.012	0.190	0.163	1012	862	937
	2.584	0.016	0.202	0.182	1079	968	1023
Double ligation	0.850	0.024	0.173	0.177	918	940	929
	1.125	0.023	0.325	0.303	1762	1640	1701
	1.031	0.014	0.181	0.192	962	1023	993
	1.600	0.014	0.193	0.201	1029	1073	1051
	1.200	0.011	0.190	0.177	1012	940	976
	2.600	0.027	0.261	0.292	1407	1579	1493
Total bladder protein (µg)	Total protein (mg)	Median	P-values				
42645	42.64		SvU				
30250	30.25						
39808	39.80						
35029	35.03	37.41	0.06				
159521	159.52		SvUU				
136018	136.01						
166398	166.39	159.52	0.07				
33194	33.19		UvUU				
82162	82.16						
72121	72.12						
121055	121.05						
110566	110.56						
144337	144.33	96.36	*0.04				

Appendix 6. Protocol 1 DNA estimation data. Note on numbers: for technical reasons, only 3 of the 4 bladder samples were available for DNA analysis in the urachal-only group.

	Whole bladder weight (g)	Sample weight (g)	Absorbance 1	Absorbance 2	Mean	Concentration (ng/ul)	DNA per sample
Sham	0.438	0.029	0.433	0.437	0.435	1087	27187
	0.341	0.026	0.305	0.280	0.293	731	18281
	0.400	0.031	0.304	0.296	0.300	750	18750
	0.462	0.023	0.301	0.333	0.317	792	19812
Urachal ligation	2.420	0.035	0.156	0.160	0.158	395	9875
	1.668	0.029	0.515	0.516	0.516	1288	32218
	2.584	0.040	0.636	0.639	0.638	1593	39843
Double ligation	0.850	0.038	0.398	0.400	0.399	997	24937
	1.125	0.042	0.351	0.365	0.358	895	22375
	1.031	0.027	0.476	0.480	0.478	1195	29875
	1.600	0.044	0.480	0.480	0.480	1200	30000
	1.200	0.028	0.337	0.338	0.338	843	21093
	2.600	0.038	0.518	0.522	0.520	1300	32500
	Total bladder DNA (ng)	**Tot bladder DNA (mg)**	**Median**	**P-values**			
	410625	0.41		SvU			
	237030	0.23					
	242718	0.24					
	396250	0.39	0.32	0.06			
	692681	0.69		SvUU			
	1872504	1.87					
	2561100	2.56	1.87	**0.01			
	562251	0.56		UvUU			
	603642	0.60					
	1153600	1.15					
	1095890	1.09					
	894434	0.89					
	2241379	2.24	0.10	0.38			

Appendix 7. Protocol 1 protein: DNA ratio estimation data.

	Total protein	Total bladder	Protein:DNA ratio	P-values
	(mg)	DNA (mg)		
Sham	42.64	0.41	103.85	SvU
	30.25	0.23	127.62	
	39.80	0.24	164.01	
	35.03	0.39	88.40	0.63
Urachal ligation	159.52	0.69	230.29	SvUU
	136.01	1.87	72.64	
	166.39	2.56	64.97	0.35
Double ligation	33.19	0.56	59.03	UvUU
	82.16	0.60	136.11	
	72.12	1.15	62.51	
	121.05	1.09	110.46	
	110.56	0.89	123.61	
	144.33	2.24	64.39	0.80

Appendix 8. Protocol 1 urothelial apoptotic index. Six propidium iodide-stained and TUNEL-stained counts per field (x200 magnification) per fetus were made. The total of six counts per fetus is shown for each experimental group. The apoptotic index for each experimental group was then derived.

	Sham	Urachal ligation	Double ligation	P-values		
				SvU	SvUU	UvUU
Total number of urothelial	998	3170	1807			
cells counted (total of six	1217	1815	1219			
counts per fetus)	2257	2219	1333			
	1197	1165	1972			
			1272			
			1089			
Total cell count	5669	8369	8692			
per experimental group						
Number of apoptotic nuclei	2	72	30			
(Total of six counts per fetus)	1	20	56			
	2	19	29			
	1	21	16			
			12			
			19			
Total apoptosis count	6	132	162			
per experimental group						
Apoptotic index	0.11	1.58	1.86	*0.03	**0.01	0.67

Appendix 9. Protocol 1 PGP 9.5 nerve bundle count. Six counts per x200 magnified field were performed per fetus. The sum of each count is shown and the median per experimental group derived.

	Sham	Urachal ligation	Double ligation	P-values		
				SvU	SvUU	UvUU
Total nerve bundle	44	9	4			
count per fetus	60	44	13			
(Sum of six counts	39	56	38			
per x200 field)	33	5	22			
			18			
			5			
Median	41.5	26.5	15.5	0.56	*0.02	0.52

Appendix 10. Protocol 1 ex-vivo cystometry data.

Intravesical volume (ml)	Intravesical pressure (cmH2O)													
	Sham				Urachal ligation				Double occlusion					
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.1	1.0	2.5	2.0	2.0		2.0	12.0			11.0	1.0		1.5	
0.2	2.0	4.0	3.5	3.0		2.0	13.0	1.5		15.0	2.0		2.5	
0.3	3.0	5.0	4.0	4.0		2.0	15.5			16.5	3.0		4.0	
0.4	3.5		4.5	5.0		2.0	16.5			18.5	3.0		5.0	
0.5	4.0.	6.0	5.0	5.5		2.0	17.0	2.0		19.5	3.5	1.0	6.0	1
0.6		6.5	5.0	6.0		4.0	18.0				4.0		7.0	
0.7		7.0	6.0	6.5		6.8	18.5				4.5		7.5	
0.8		7.0	6.0	7.0		7.2	19.0				4.8		7.5	
0.9		6.5	6.0			8.0	19.5				5.0		7.5	
1.0			6.0	7.0	4.0		20.0	2.0	3.0		5.5	1.0	7.5	1.0
1.1			6.0				21.0						7.5	
1.2			6.5	7.0			21.0				6.0		7.5	
1.3							22.0						7.5	
1.4				7.0							6.5		7.5	
1.5												1.5	7.5	1.5
1.6				7.0							7.0		7.5	
1.7													7.5	
1.8				7.0							7.0		7.5	
1.9													7.5	
2.0				7.0	6.0			2.0	3.0		7.0	2.0	7.5	1.5
2.5											7.5	2.0	7.5	
3.0					8.5			2.0	3.0		8.0	3.0	7.5	
3.5											9.0	3.0	7.5	
4.0					9.5			2.5	4.0		9.0	3.0		2.0
4.5											9.0	3.0		
5.0					10.5			2.5				3.0		
5.5											9.0			
6.0					11.0			2.5				3.0		2.5
7.0					11.0			2.5				3.5		
8.0					11.0			3.0				3.5		2.5
9.0								3.0				3.5		
10.0								3.0				3.5		2.5
12.0								3.0				3.0		
14.0								4.0				3.0		3.0
16.0								5.0				3.0		
18.0								6.0				3.0		
20.0								6.5				3.0		4.0
22.0								7.0						
24.0														
														5.0
														5.0
														5.5
														6.0

Appendix 11. Protocol 2 fetal data.

All measurements taken at autopsy.

	Sham	Obstructed	P-value (SvO)
Fetal weight (g)	1860	1372	
	380	1472	
	1063	1266	
		798	
Median	1063	1319	0.86
Occipito-snout length (cm)	100	94	
	67	94	
	90	115	
		88	
Median	90	94	0.63
Crown-rump length (cm)	32	35	
	20	29	
	27	30	
		26	
Median	27	29	0.63

Appendix 12. Protocol 2 fetal bladder and kidney data.

	Sham	Obstructed	P-value (SvO)
Whole bladder weight (g)	1.30	1.30	
	0.40	0.80	
	0.60	0.80	
		1.20	
Median	0.60	1.00	0.48
Bladder: fetal weight ratio x10^3	0.70	0.95	
	1.05	0.54	
	0.56	0.63	
		1.50	
Median	0.70	0.79	1.00
Total kidney weight (g)	16.70	18.10	
	5.70	11.30	
	10.30	11.40	
		10.00	
Median	10.30	11.35	0.63
Total bivalved kidney weight (g)	15.60	16.60	
	5.20	10.40	
	9.50	10.90	
		8.30	
Median	9.50	10.65	0.63
Total weight of urine drained from kidneys (g)	1.10	1.50	
	0.50	0.90	
	0.80	0.50	
		1.70	
Median	0.80	1.20	0.48
Bivalved kidney: fetal weight ratio x10^3	8.39	12.10	
	13.68	7.07	
	8.94	8.61	
		10.40	
Median	8.94	9.51	0.86
Volume of urine aspirated (ml)	11.00	5.00	
	1.00	7.00	
	2.80	1.10	
		10.50	
Median	2.80	6.00	0.86

Appendix 13. Protocol 2 urothelial and detrusor apoptotic indices.

	Sham			Total	Obstructed				Total	P-value (SvO)
Urothelial cell count (sum of six counts per x200 field)	1325	1456	1518	4299	1248	1218	1312	1240	5018	
Apoptotic nuclei count (sum of six counts)	4	4	12	20	14	6	6	18	44	0.23
Urothelial apoptotic index				0.47					0.88	
Detrusor muscle count (sum of six counts)	1534	1312	1478	4324	1680	1532	1286	1422	5920	
Detrusor apoptotic nuclei	53	14	20	87	78	33	14	29	154	0.72
Detrusor apoptotic index				2.01					2.60	

Appendix 14. Protocol 2 ex-vivo cystometry data.

Intravesical volume (ml)	Intravesical pressure (cmH2O)						
	S1	S2	S3	O1	O2	O3	O4
0	0	0	0	0	0	0	0
0.1	2.0	1.0	2.0	3.0	1.0	1.0	1.0
0.2	3.5	1.5	3.5	4.0	1.0	1.0	1.0
0.3	4.5	2.0	4.0	4.0	1.5	1.5	1.5
0.4	5.0	2.5	5.0	5.0	2.0	2.0	2.0
0.5	5.5	3.0	5.5	6.0	3.0	2.0	2.0
0.6	6.0	3.5	6.5	7.0	3.0	3.0	2.0
0.7	6.0	3.5	7.0	7.5	4.0	4.0	2.0
0.8	6.0	3.5	8.0	8.0	4.0	5.0	2.5
0.9	6.0	4.0	9.5	8.0	4.5	6.0	3.0
1.0	6.0	4.0	10.0	9.0	5.0	6.0	3.0
1.1							
1.2	6.0	5.0	10.5	9.0	6.0	8.0	4.0
1.3							
1.4	6.0	5.0	10.5	9.5	7.0	8.0	4.5
1.5							
1.6	6.5	6.0	11.0	10.0	8.0	8.5	5.0
1.7							
1.8	6.5	6.0.	11.0	10.0	8.5	9.0	5.0
1.9							
2.0	6.5	6.5	11.0	10.0	8.5	9.0	6.0
2.5	6.5			10.5	9.5	11.0	6.0
3.0	6.5			11.0	10.0	11.0	6.0
3.5	7.0			11.0	10.5	13.0	7.0
4.0	7.0			11.5	11.0	13.0	7.0
4.5	7.5			11.5	11.0	14.0	7.5
5.0	8.0			12.0	11.5	14.0	7.5
5.5	8.0				12.0	14.0	7.5
6.0	8.0				12.0		7.5
6.5					12.5		8.0
7.0	8.0				13.0		8.0
8.0	8.5						8.5
9.0	9.0						9.0
10.0	9.0						

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