Ph.D. School of Semmelweis University

Experimental fetal urinary bladder outflow obstruction perturbs electromechanical properties of detrusor muscle

Ph.D. thesis

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Contents:

5. Results:

5.1. Intrauterine ultrasonographic results of severe bladder outflow obstruction

5.2. Morphological disruption of the nephrogenic cortex with dilated and cystic tubules

5.3. Histological effects of infravesical obstruction on upper urinary tract

5.4. Increased capacity and compliance in obstructed bladders

5.5. Electrophysiology shows hypocontractility in obstructed bladder detrusor muscle

5.6. Urinary bladder innervation is decreased in response to outlet obstruction

5.7. Female fetuses

6. Discussion

6.1. The normal fetal ovine bladder

6.2. The fetal ovine bladder subjected to severe BOO

6.3. Comparison with human fetal BOO

7. References

8. Acknowledgment

9. Conclusion

10. Summary

Összefoglaló

11. Articles and chapter connected to Ph.D. thesis

1. Introduction

The human urinary tract performs a number of functions essential for normal post-natal life: kidneys regulate secretion of nitrogenous waste products, homeostasis of water, electrolytes and acid base balance and the production of hormones, whilst the lower urinary tract forms a low pressure, continent storage container which empties completely during micturation.

The bladder develops from the urogenital sinus and its surrounding mesenchyme concurrently with the metanephros and, in both kidney and bladder, mutual interactions between the different tissue types are essential for normal development. A number of key regulatory molecules have recently been identified using descriptive studies of normal and abnormal human development and, increasingly, from mice with targeted null mutations which disrupt urinary tract development (2).

Antenatal research and experimental urinary tract obstruction is a growing scientific topic because understanding of developmental and physiological changes might help us to find the right time and the right way to treat antenatally or postnatally children with severe congenital urinary tract obstruction like posterior urethral valve.

To exam morphological and physiological changes of the obstructed fetal bladder one has to be aware about the normal ontogeny. Furthermore morphological and physiological basis of the lower urinary tract is also essential which will allow us to place the growing data of the obstructed fetal urinary tract into context.

1.1 Embryology, morphology and physiology of the urinary tract

1.1.1. Normal bladder development

The first stage of bladder development involves partitioning of the cloaca: at around 28 days post fertilisation, an in growth of mesoderm from the point of confluence of the allantois and the hindgut forms an advancing septum, which progresses towards the cloacal membrane. This septum, aided by the in-growth of lateral Rathke's folds, divides the cloaca into an anterior primitive urogenital sinus that receives the mesonephric ducts, and a posterior rectum. Septation is completed when the advancing edge of the urorectal septum reaches the cloacal membrane during the sixth week,

dividing it into an anterior urogenital and a posterior anal membrane. The urogenital membrane breaks down during the seventh week, establishing continuity between the developing urinary tract and the amniotic cavity. The upper part of the primitive urogenital sinus, located between the allantois and the mesonephric ducts, is called the vesicourethral canal and this will form the definitive bladder. This process involves mutual inductive interactions between endoderm of the urogenital sinus, which will give rise to the bladder urothelium, and the adjacent mesenchyme, which develops into the lamina propria connective tissue and the detrusor smooth muscle layer. Growth of the anterior abdominal wall between the allantois and the urogenital membrane is accompanied by an increase in size and capacity of this bladder precursor. The allantois remains attached to the apex of the fetal bladder and extends into the umbilical root, although it loses its patency and persists as the urachal remnant, the median umbilical ligament (1,2). By 13 weeks, the interlacing circular and longitudinal strands of the smooth muscle of the trigone are discernible. At 16 weeks these are refined into discrete inner and outer longitudinal layers and a middle circular layer and at this time continence may be possible. The definitive urothelium is visible by 21 weeks gestation. It means that halfway through gestation the urinary bladder is already morphologically developed and in the second part of intrauterin life maturation of the created bladder is in result.

During the process of cloacal septation, the mesonephric ducts distal to the ureteric bud origins become progressively incorporated into the posterior aspect of the primitive urogenital sinus. The ureteric orifices are then incorporated into the posterior wall of the developing bladder, and become separated from the mesonephric duct orifices. As further incorporation of more proximal mesonephric ducts occurs the ureteric orifices move superolateraly relative to the mesonephric duct orifices. These stay close to the midline and descend into the developing posterior urethra. Eventually, the mesonephric ducts fuse in the midline and the triangular area between them and the ureteric orifices defines the trigone.

During the ascent of the kidneys from the pelvis the ureters rapidly elongate. Initially their lumen is not apparent but it develops cranially and caudally from the midpoint until early in the eighth week when only a membrane persists between the lower ureter and urogenital canal. By the middle of the eighth week this membrane (also known as

Chwalla's membrane) disappears. The muscularisation of the ureter is probably induced by drainage of the first secreted urine at about the 9th week of development. At 18 weeks of gestation intrinsic narrowing can be discerned at the PUJ and UVJ.
The distal part of the primitive urogenital sinus will form the definitive urogenital sinus. In females this gives rise to the entire urethra and the vestibule of the vagina. In males it gives rise to the posterior urethra, whilst the anterior urethra is formed from the closure of the urethral folds (1,2).

1.1.2. The structure of the bladder

The bladder consists of three layers: an epithelial layer, a muscular layer and an adventitial layer, which on its posterior aspect is covered with peritoneum. The urothelial layer is a multilayered transitional epithelium that is continuous with the ureters above and the prostatic urethra below. Under the light microscope, the urothelium is similar in the bladder and the prostatic urethra, but there are differences on scanning electron microscopy that become more marked the further one proceeds down the urethra. The urothelium has numerous so-called tight junctions, which make it impermeable to fluids and solutes.
The smooth muscle of the bladder, which accounts for most of the thickness of the bladder wall, is called detrusor layer. There have been various attempts to identify specific bundles of muscle within the bladder wall, largely in order to substantiate a hypothetical mechanism to account for the opening of the bladder neck at the initiation of voiding, but there is no good evidence that such layering exists (2,3). The bladder should be considered as a single homogenous layer of relatively large muscle bundles in a relatively small amount of connective tissue. These bundles have no particular orientation. As one approaches the bladder base from above down, there is a flimsy additional superficial layer of smooth muscle quite separate from the underlying smooth muscle, which is typical detrusor. This additional trigonal layer is derived from the ureters and shows several distinct characteristics. The muscle fibres form bundles that much smaller with a higher connective tissue component and a fairly dense adrenergic innervation (4). The trigonal muscle was once thought also to have a role in opening the bladder neck, but this too has not been substantiated. As one approaches closer to the bladder neck, the disposition and orientation of the detrusor change

uniformly around it. The muscle bundles get smaller, there is a relatively greater amount of connective tissue, and the orientation of the muscle bundles becomes more uniform, to loop obliquely around the bladder neck. Below this level in the male there is the pre-prostatic sphincter in which adrenergically innervated smooth muscle bundles form a distinct sphincter around the urethra to prevent retrograde ejaculation (5). It should be emphasised that this adrenergically innervated smooth muscle component – the pre-prostatic sphincter – which is present in males but not in females, is a genital sphincter and not the bladder neck urinary sphincter mechanism in the strict sense, which is presumably the same in both sexes. The pre-prostatic sphincter and the trigone are only areas to show a distinct adrenergic innervation; otherwise the only adrenergic neurons in the bladder are those that supply the blood vessels (6). This means there is no evidence that there is a functionally important innervation to these receptors in normal individual.

1.1.3. The innervation of the bladder

Four separate neuronal pathways to the lower urinary tract have been alluded to. The principal one is derived from cell bodies in the intermediolateral column of the second, third and fourth sacral segments of the spinal cord. These are preganglionic parasympathetic fibres that run out of the anterior primary rami of S2, S3 and S4 and then separate out from the somatic component which runs to the sacral plexus, to run as the nervi erigentes to the pelvic plexus. These preganglionic parasympathetic neurons end by synapsing in ganglia on the cell bodies of the postganglionic parasympathetic nerves, which then run to the bladder and to the urethra. In humans, 50% of the ganglia of the pelvic plexus are in the adventitial tissue around the base and posterolateral aspects of the bladder and 50% are within the bladder wall itself. For this reason it is, strictly speaking, technically impossible to denervate the bladder because the 50% within the bladder wall itself will still remain and there will therefore still be reflex activity, even if this is not physiologically significant (4).
The somatic nerve fibres that arise from Onuf's nucleus and that travel with otherwise autonomic parasympathetic nerve fibres of the nervi erigentes.

The third component is the sympathetic nerve component that arises from the intermediolateral column of the 10^{th}, 11^{th} and 12^{th} thoracic and the 1^{st} and 2^{nd} lumbar segments of the spinal cord.
Finally, there is the pudendal nerve component, also arising from S2, S3 and S4, in this instance from typical anterior horn motor neuron cell bodies, which innervates the urethra and the pelvic floor musculature and provides afferent innervation to the urethra.

1.1.4. Functional aspects of the innervation of the bladder

Cholinergic nerves can not be stained for microscopical study – only indirectly by staining for acetylcholinesterase, the enzyme that breaks down acetylcholine at the neuromuscular junction. Staining for acetylcholinesterase shows a dense "presumptive-cholinergic" innervation of the detrusor smooth muscle with the nerve to muscle cell ratio of about 1:1, and less dense cholinergic innervation of the urethral smooth muscle (4). Under the electron microscope, the terminal branches of the parasympathetic neurons within the bladder wall are seen to contain varicosities. These varicosities are there because of the presence of numerous vesicles within the terminal neuron at that point and these vesicles contain neurotransmitter substances. Alongside the varicosity is the specialised area of the smooth muscle that constitutes the receptor sites, although this is not very specialised by comparison with the receptor site – the neuromuscular junction – in striated muscle. Adjacent smooth muscle cells are connected by so-called "regions of close approach" like gap junction in effect, which could, at least theoretically, allow electrotonic spread of activity from one smooth muscle cell to the next and which thereby confer so-called "cable properties" on the smooth muscle fibres. This would supplement the spreading neuronal stimulus as a sort of "parallel pathway", and help to ensure a uniform simultaneous contraction of the entire bladder smooth muscle cells at the time of voiding.
Vesicles are of two main types (6): *small clear vesicles* and *larger vesicles* with a dense core.
The *small clear vesicles* are thought to contain so-called "fast" neurotransmitter substances, which are released directly into the area between the neuron and the adjacent neuron across a synapse or the adjacent smooth muscle cell across a

neuromuscular junction to open ligand-gated ion channels on the receptor site that will initiate an action potential. Outside the central nervous system, the commonest neurotransmitter to be found in these small clear vesicles is acetylcholine and the commonest receptor is the nicotinic acetylcholine receptor, although acetylcholine does not exclusively act as a fast neurotransmitter to open ligand-gated ion channels in this way.

Large, dense-cored vesicles contain so-called "slow" neurotransmitters, which are called "slow" because they are released non-specifically from around the area of the varicosity rather than specifically into the receptor site; and because they generally act by binding to G-protein-linked receptors and initiating smooth muscle contraction through second messengers systems. This is so called "pharmacomechanical" coupling of the neuronal stimulus to smooth muscle contraction, as distinct from the "electromechanical" coupling initiated by the binding of ligand-gated ion channels by fast neurotransmitters.

A single nerve impulse will empty about half the vesicles in a varicosity, each of which will release about 5000 molecules of acetylcholine as a quantum. These vesicles empty their contents in response to a rise in cytosolic calcium within the varicosity as a result of the opening of membrane calcium channels induced by the neuronal electrical impulse (voltage-gated calcium channels), causing an influx of extra cellular calcium (7).

There are other ways of demonstrating the primacy of parasympathetic cholinergic activity in the excitatory innervation of the bladder other than by showing its density on light microscopy and electron microscopy. The most convincing way is by the physiological study of strips of detrusor smooth muscle in an organ bath (Figure 1).

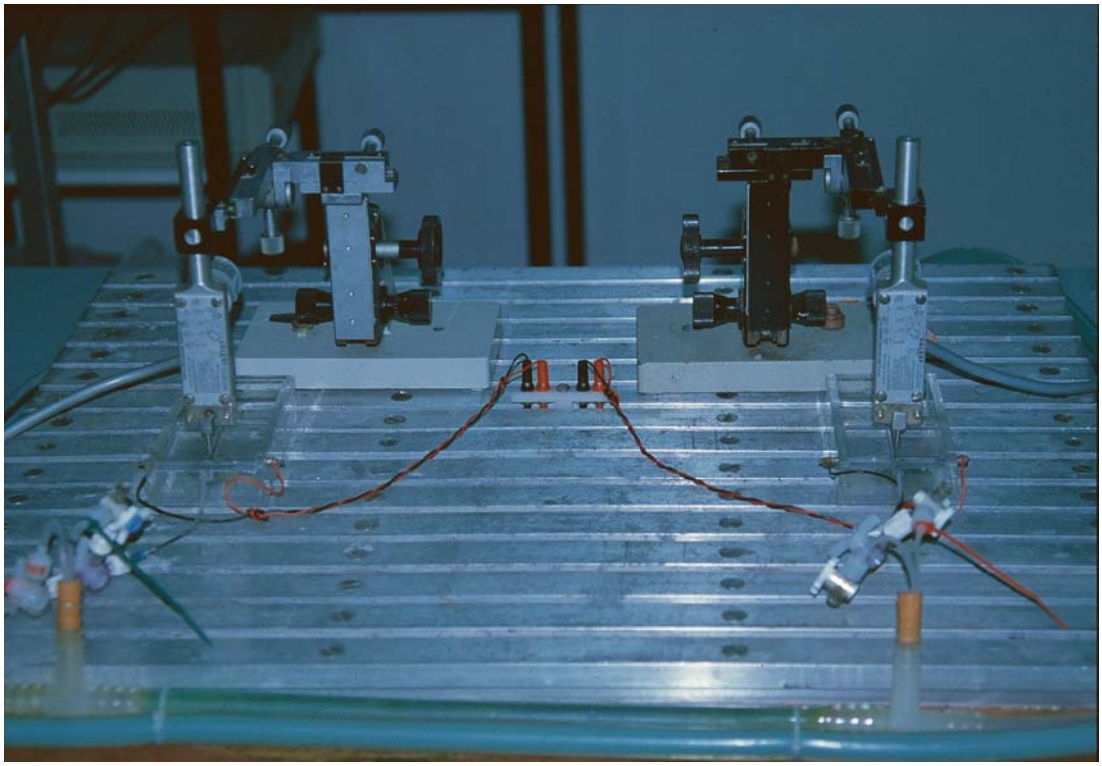
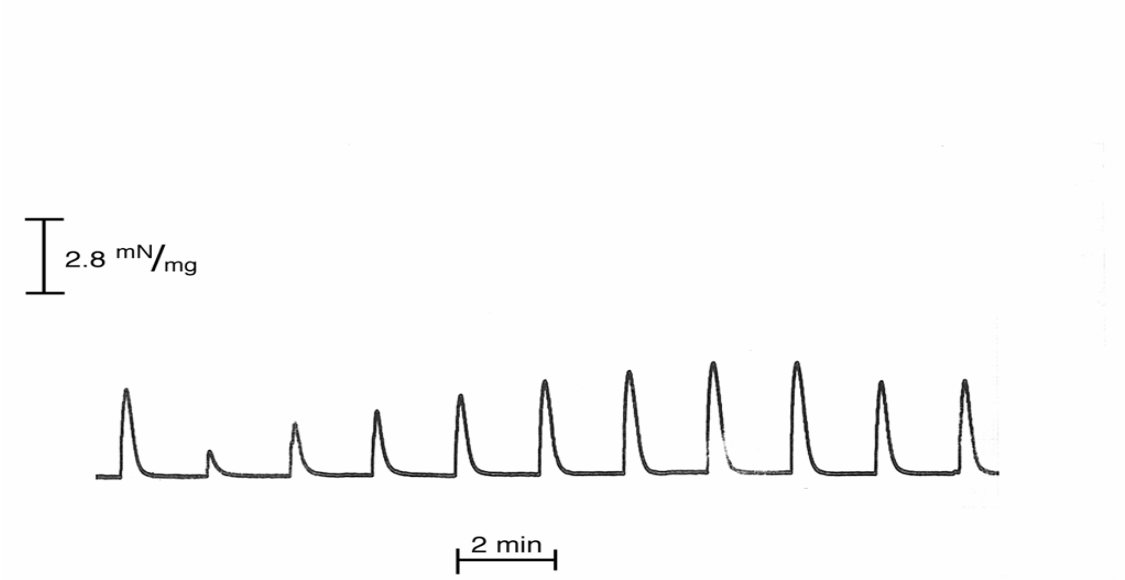


Figure 1. Organ bath for smooth muscle strips

The organ bath keeps the muscle strip at the right temperature, sufficiently oxygenated and in the right fluid medium to keep it viable sufficiently long for adequate study. Using an electrical impulse stimulates the excitatory nerve supply to the muscle fibres within the muscle strip. If the strength and amplitude are optimised and the frequency of the electrical stimulus is then varied, a frequency-response curve is produced between about 0.5Hz and 60Hz (Figure 2), above which the response rate plateaus until the electrical impulse is of sufficiently high frequency to cause damage.



16Hz 1Hz 2Hz 4Hz 8Hz 16Hz 24Hz 40Hz 60Hz 16Hz 16Hz

Figure 2. Force-frequency relation between 1 and 60 Hz of frequency

This response can be abolished by the application of tetrodotoxin, which is sodium channel blocker, which therefore blocks nerve conduction. If frequency-response curve is plotted after the application of tetrodotoxin, there will be very small response at higher frequencies that is not nerve mediated but due to direct stimulation of the smooth muscle cell membrane itself. If the frequency-response curve performed after the application of atropine at a sufficient dose to give complete cholinergic blockade, then the only response left will be the same as that after tetrodotoxin. In other words, in the normal human specimen, there is no excitatory component left after atropine blockade and there is no “atropine-resistant” component to excitatory neurotransmission. Normal excitatory neurotransmission in the human bladder is exclusively muscarinic cholinergic. In other mammals, it is a completely different story because in some small mammals, there may be some atropine resistant frequency-response curve. In most animal species showing this type of excitatory

neurotransmission, is thought to be adenosin triphosphate (ATP). In the normal human bladder it does not appear to be a component of normal human voiding. It may; however be a cause of abnormal detrusor function in detrusor instability and hyperreflexia.

In summary, initiation of detrusor contraction in the human bladder is controlled by the parasympathetic nervous system. Release of acetylcholine from the postganglionic fibres activates muscarinic (M_3) receptors on the smooth muscle surface membrane, which in turn activates phospholipase C thus generating intracellular inositol triphosphate (IP_3) (8). In normal human detrusor muscle, contractile activation is exclusively controlled by this cholinergic elicited by nerve-mediated contractions, i.e. contractions blocked by tetrodotoxin (TTx) and hence mediated by initial activation of excitatory nerves.

1.1.5. Excitation-contraction coupling and contractile apparatus

The two types of excitation-contraction couplings are: electromechanical coupling, in which an electrical impulse causes the release of a neurotransmitter that, binds to a ligand-gated ion channel, which causes membrane depolarisation and contraction and onward spread of the impulse by the generation of an action potential. (Figure 3)

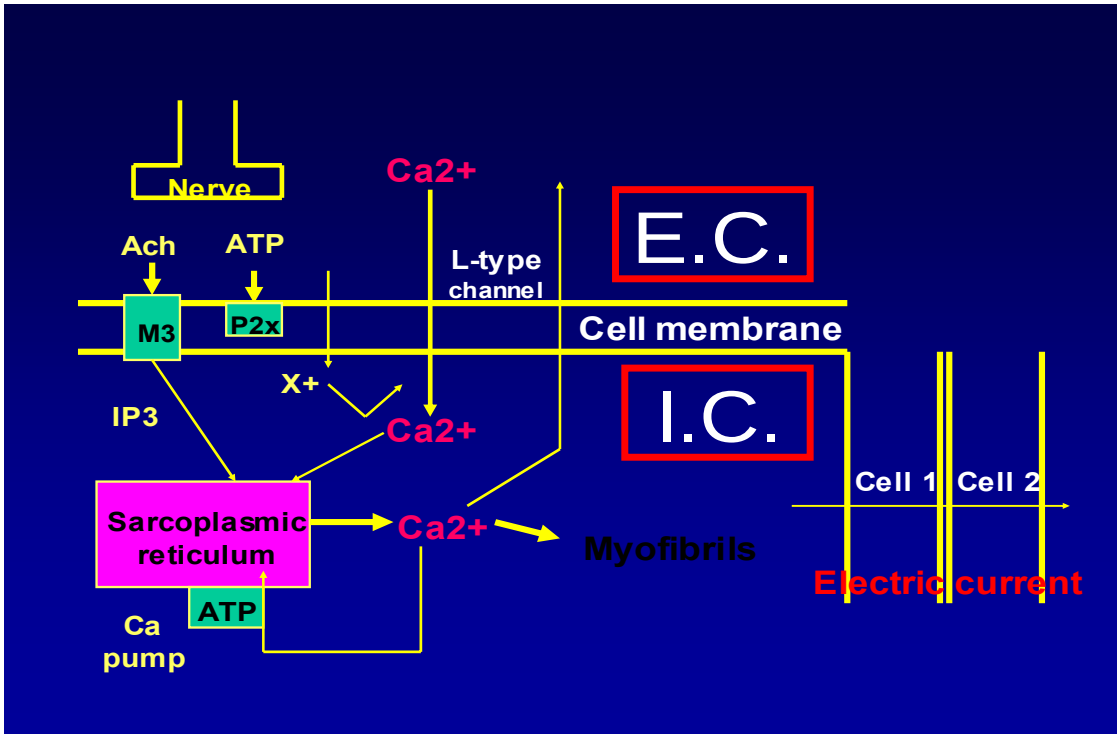


Figure 3. Schematic representation of the factors involved in contractile activation and relaxation of detrusor smooth muscle

Pharmacomechanical coupling is the other one, in which G-protein-linked receptor is bound and there is release of a second messenger – inositol triphosphate (IP_3) – which causes the release of intracellular calcium from calcium stores (9), mainly in the endoplasmatic reticulum, which in turn causes contraction and also opening of ligand-gated ion channels in the cell membrane and generation of action potential. In either case, intracellular calcium rises and is bound by calmodullin, a ubiquitous intracellular calcium-binding protein, which is thereby activated. This in turn activates myosin light chain kinase, which activates myosin, causing it to bind to actin, and this produces the so-called "power-stroke" in which the actin and myosin filaments shorten, leading to contraction (10). The relative importance of electromechanical coupling and pharmacomechanical coupling in the human bladder is not clear, but it is thought that they are probably equally important. Either way, the most important factor is the occurrence of a rise of cytosolic calcium levels. In electromechanical coupling, the calcium comes initially from outside the cell, firstly in response to membrane depolarisation, which opens voltage-sensitive calcium channels, and secondly in

response to the calcium binding of calcium-gated calcium channels. In pharmacomechanical coupling, the calcium initially comes from intracellular calcium stores via a G-protein-linked receptor and a second messenger system using IP_3 as the second messenger. The G-protein-linked receptor that is activated by cholinergic neurons in pharmacomechanical coupling is the muscarinic acetylcholine.

There are three types of muscarinic receptors: M1, M2 and M3. The highest percentages of receptors in the bladder are of the M2 type, accounting for more than 80% (11), but physiological these are the least important and it is the M3 muscarinic receptor that is most important for bladder physiology.

The final pathway of excitatory neurotransmission in the lower urinary tract is therefore as follows. Preganglionic parasympathetic nerve activity causes the release of acetylcholine in the parasympathetic ganglia. Acetylcholine activates the nicotinic receptors of the postganglionic neurons, which are ligand-gated ion channels. The postganglionic neurons also release acetylcholine at their terminals, which in their case are neuromuscular junctions within the detrusor. On the smooth muscle cell membranes, the acetylcholine receptors are muscarinic receptors, of which the most important are M3 receptors, which are G-protein-linked receptors. These causes the generation of inositol triphosphate from membrane phospholipids, which in turn causes the release of calcium from intracellular calcium stores. This has two principal effects: firstly, bound to calmodullin, it causes smooth muscle contraction by pharmacomechanical coupling and, secondly, it opens calcium-gated calcium channels in the plasma membrane. This causes an influx of extra cellular calcium to perpetuate the contraction and to replenish intracellular calcium stores.

The contractile apparatus of striated muscle is clearly defined by the striations visible under the light microscope that have been shown to be interlinking fibres of actin and myosin. Smooth muscle also contracts because of the relationship between actin and myosin, but the disposition of these fibres is not as clearly defined as in striated muscle and is not visible by light microscopy.

Under the electron microscope “dense bodies” are visible that are the closest smooth muscle gets to revealing its contractile apparatus. However, the principle of action is the same as in striated muscle except less well organised and less efficient. The calcium-calmodullin complex in smooth muscle activates the enzyme myosin light

chain kinase, which causes the myosin fibres to straighten out and align themselves with actin fibres and form cross-linkages through the myosin heads.
Phosphorylation causes the myosin heads to flex in relation to their tails and this causes the myosin fibres to move over the actin fibres (or vice versa). The effect of this is for the contractile apparatus to shorten, thereby generating contraction. The flexion of the myosin head is known as the "power stroke". A dephosphorylation switch off this process and relaxation occurs passively as a result.

1.2. Abnormal development of the lower urinary tract

1.2.1. Lower urinary tract malformations

Agenesis: Unilateral agenesis of the bladder and trigone can occur in conjunction with failure of ureteric bud development and renal agenesis.
Hydronephrosis: Dilatation of the renal pelvis is a relatively common and non specific finding, which may be associated with reflux or obstruction of the lower urinary tract. Renal dysplasia and/or hypoplasia may coexist.
Vesicoureteric reflux: Retrograde flow of urine from the bladder into the ureters and kidney is very common (1/50-100), and usually resolves as the patients grow older, although reflux nephropathy causes up to 15% of end-stage renal failure in older children and adults. Autosomal dominant inheritance has been described and several potential genetic loci have recently been identified (12).
Bladder exstrophy: Failure of growth of the lower abdominal wall between the allantois and the urogenital membrane, coupled with breakdown of the urogenital membrane leaves a small, open bladder plate, a low placed umbilical root and diastases of the pubic bones. The genital tubercle is probably placed lower in these patients so the cloacal membrane ruptures above it, leading to a penis with an open dorsal surface that is continuous with the bladder plate.

1.2.2. Posterior urethral valves (PUV)

Outflow obstruction in the posterior urethra causes aberrant bladder development, often with a small capacity and thickened walls, which may be associated with

ureteric/kidney abnormalities and the Potters sequence, when severe. With an incidence of between 1 per 8000 and 1 per 25.000 births, posterior urethral valves (PUVs) represent the commonest congenital obstruction of the lower urinary tract, exclusively in males (13). Obstruction of the posterior urethra, above any other urinary abnormality, has the capacity to affect the development and function of the whole urinary tract (13).

History: Although the anatomic features of posterior urethral valves were mentioned as early as 1717 and 1802 by Morgagni and Langenbeck, no clinical significance was attributed to this anomaly until Bud (1840) published the autopsy report of a young sailor who died from uraemia due to advanced hydronephrosis. The cause of death was directly linked to the presence of congenital valves obstructing the prostatic urethra (14). Tolmatschew (1870), reporting the autopsy findings in a male infant who died from renal failure shortly after births, gave an accurate description of the valves. In November 1913, Young reported the Johns Hopkins Hospital Medical Society the first clinical recognition and operative cure of congenital valves of the prostatic urethra. The patient was a 20-month-old boy suffering from great difficulty and frequency of urination. Young's interest in the subject led to his classical paper of 1919, in which he reviewed his own 12 cases in addition to the 24 case reports that he was able to collect from the literature (14).

Antenatal diagnoses: Hutton et al (15) demonstrated that gestational age at detection is a predictor of outcome; 53% of patients detected at or before 24 weeks died or were in renal failure at a median follow-up of 3.9 years. Of those detected after 24 weeks, only 1/11 patients had a poor outcome.

Our understanding of this problem has evolved over the past decades and has included the identification, classification and treatment of the obstruction; recognition and management of the degrees and types of renal impairment that may be associated with it; the approach to associated vesicoureteric reflux and dilated upper urinary tracts; the function and development of the '"valve bladder"; and finally the impact on our practice of antenatal diagnosis and the opportunities that it offers not only in the identification but also the early treatment of PUVs.

Presentation, imaging and initial management: In the past, approximately one-third of cases have presented during the neonatal period, one-third between 1 month and 1

year, and one-third over one year of age. More recent reviews have demonstrated increased presentation during the neonatal period and relate this to greater awareness of the condition, quite apart from antenatal diagnosis. The most severe cases may present in the puerperium with respiratory difficulties, limb deformities, Potter's facies, and a distended, palpable urinary tract, reflecting the severe form of obstruction and maternal oligohydramnios.

In common with other anomalies causing dilatation of the fetal urinary tract, PUVs are increasingly recognized by routine prenatal ultrasound. The characteristic findings are bilateral hydronephrosis and a distended thickened bladder, although a dilated prostatic urethra can occasionally be seen. Low amniotic fluid volume and bright renal parenchyma (suggestive of dysplasia) provided important clues for severity. Often the diagnosis is only suggested antenatally and relies on postnatal assessment for confirmation.

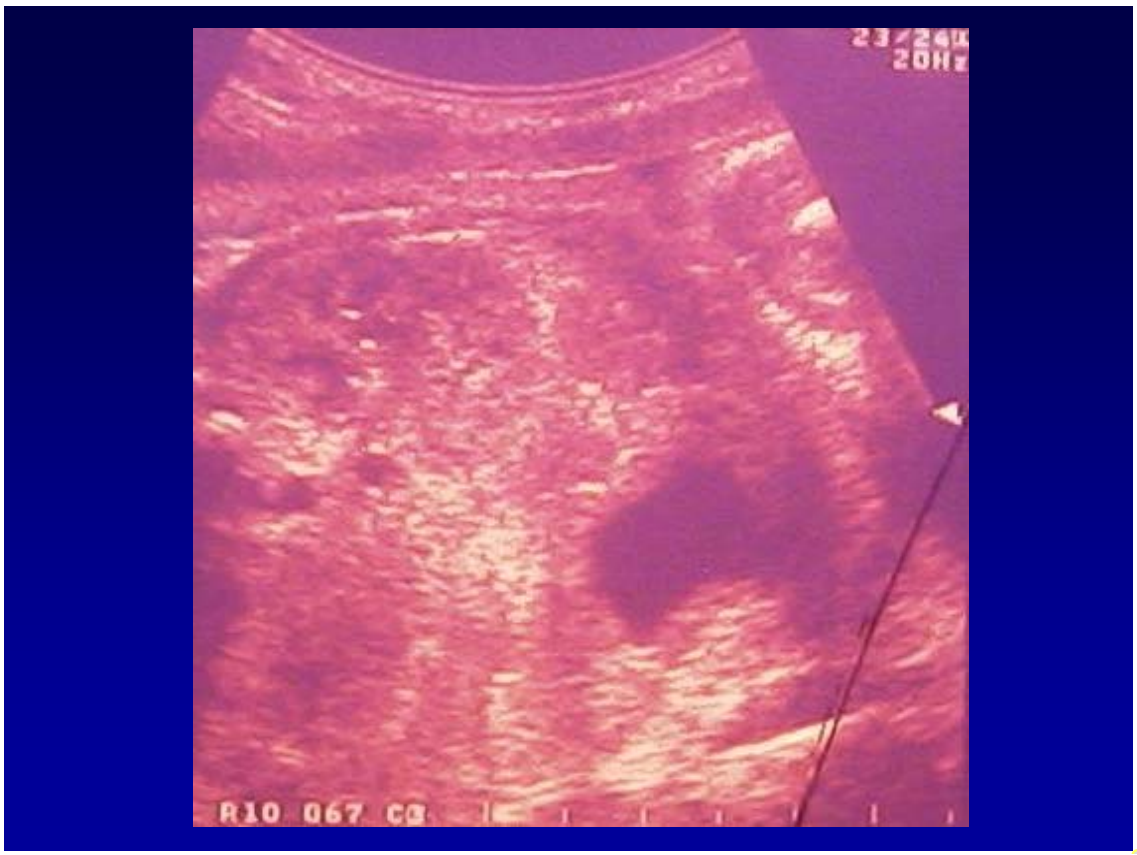


Figure 4. Antenatal US of PUV in human

Ultrasound is the first investigation of antenatally and postnatally presenting PUVs (Figure 4). It demonstrates hydroureteronephrosis, a thick walled bladder, and occasionally a dilated posterior urethra or bright kidneys with loss of corticomedullary differentiation suggestive of dysplasia. The diagnostic "gold standard", however remains the micturating cystourethrogram which may be performed via a urethral or suprapubic catheter.

1.2.3. Bladder dysfunction

Bladder or detrusor instability is a symptomatic condition which is characterized by uncontrollable rises in pressure within the bladder lumen leading to a feeling of urgency of micturation and, if severe enough, urinary incontinence (16).
Bladder dysfunction, manifested as incontinence, is present in 13-38% of patients following resection of posterior urethral valves (17,18). Two cohort studies performed at Great Ormond Street Hospital of 42 boys (aged 1 month to 14 years) (18) and 51 boys (aged 6 to 9 years) (19) have both shown urodynamic abnormalities in 75%. Studies in patients with valves and bilateral reflux demonstrate an inverse relationship between filling detrusor pressure and GFR.

2. Aims

The bladder dysfunction has been implicated in postnatal renal functional deterioration that occurs in some boys with PUV (17). It is unknown however, how prenatal or postnatal bladder dysfunction, or features of maldevelopment, contribute to the ensuing deterioration in renal function.
Intrauterine fetal operation is a very challenging and relatively new, not yet discovered part of surgery which needs very fine manipulation. To perform the less invasive handle for fetus and also for mother in the right time is essential to avoid any harm of pregnancy. Therefore the aim of our work was to study the effects of intrauterine performed bladder outflow obstruction (BOO) in a reproducible experimental model.
In the last decade the best way for monitoring fetal development and maturation is US. However, it is not established when the consequence of infravesical obstruction occurs

and how fast is the process. Therefore our point was to monitor regularly fetuses, underwent sham operation or obstruction, and record morphological changes on US.

Our further intention was to be acknowledged about the relationship between storage volume and intravesical pressure of normally developed and obstructed bladders.

The main goal of our experimental study was to determine the electromechanical changes of detrusor smooth muscle of obstructed, instable bladder. Because first, a through investigation of the cellular basis of contractile activation will permit the development of more specific therapeutic agents to regulate detrusor function; second, if any functional alterations can be documented and the cause identified, a strategy of prevention could be easier formulated.

Induction of BOO in fetal sheep causes changes in structural, cell biological and molecular properties of the bladder: these include increases in bladder weight, detrusor DNA content, detrusor cell volume and protein-to-DNA ratio and smooth muscle cell cholinergic receptor number, as well as an alteration in myosin heavy chain isoform composition and deregulated collagenase activity (24,25,26). Therefore our target was to prove these statements and compare to our electromechanical findings.

In conclusion, using an experimental fetal sheep model, the aim of our work was to examine the physiological properties of the fetal bladder after 30 days of fetal BOO. We characterised the in vitro contractile properties of bladder detrusor smooth muscle by constructing force-frequency relationship, we performed ex vivo filling cystormetry to determine the relationship between storage volume and pressure, and we examined key neuronal proteins by immunohistochemistry and western blotting, as indices of tissue innervation.

3. Hypothesis

A relatively brief period of physical obstruction of fetal bladder outflow will significantly perturb the anatomical structure, electrophysiological properties, ex vivo cystometry characteristics of the urinary bladder in addition to neuronal proteins existence and biomechanical quality of its detrusor muscle.

4. Methods

Chemicals were from Sigma (Poole, Dorset, UK) unless otherwise stated. All work was conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

4.1. Experimental design

Investigators generally used fetal sheep models (24,27). Ovine gestation lasts around 145 days and the normal development of the urinary tract has been well-documented (28,29). At mid-gestation, fetal sheep bladders receive about 5-6 ml of urine each hour from the developing kidneys (28). In contrast to humans, in which the urachus obliterates at four-five months gestation (30), the sheep urachus remains patent until late gestation (31); this structure constitutes another outflow tract from the developing bladder into the uterine cavity and must be ligated in sheep, in addition to obstruction the urethra, to generate severe bladder outflow obstruction (BOO).

Pregnant ewes were purchased from the Royal Veterinary College herd (Potters Bar, Hertfordshire, UK). The main experiment used 16 male Romney Marsh sheep fetuses; eight were sham-operated control fetuses (the *sham group*) and eight had induced partial bladder outflow obstruction (the *obstructed group*). Male fetuses were used as human fetal BOO occurs almost exclusively in the male population. Surgery was performed at mid-gestation (75 days) and animals sacrificed 30 days after surgery (105 days) (Figure 5). Urinary bladder samples from all 16 male fetuses underwent contractility, immunohistochemistry and western blot studies. Of these, four obstructed fetal bladders and six sham bladders were examined by ex vivo filling cystometry, to determine bladder storage characteristics. Finally, fetal bladder strips from six sham female fetuses were examined by contractility studies, in order to investigate possible sex differences in fetal bladder function.

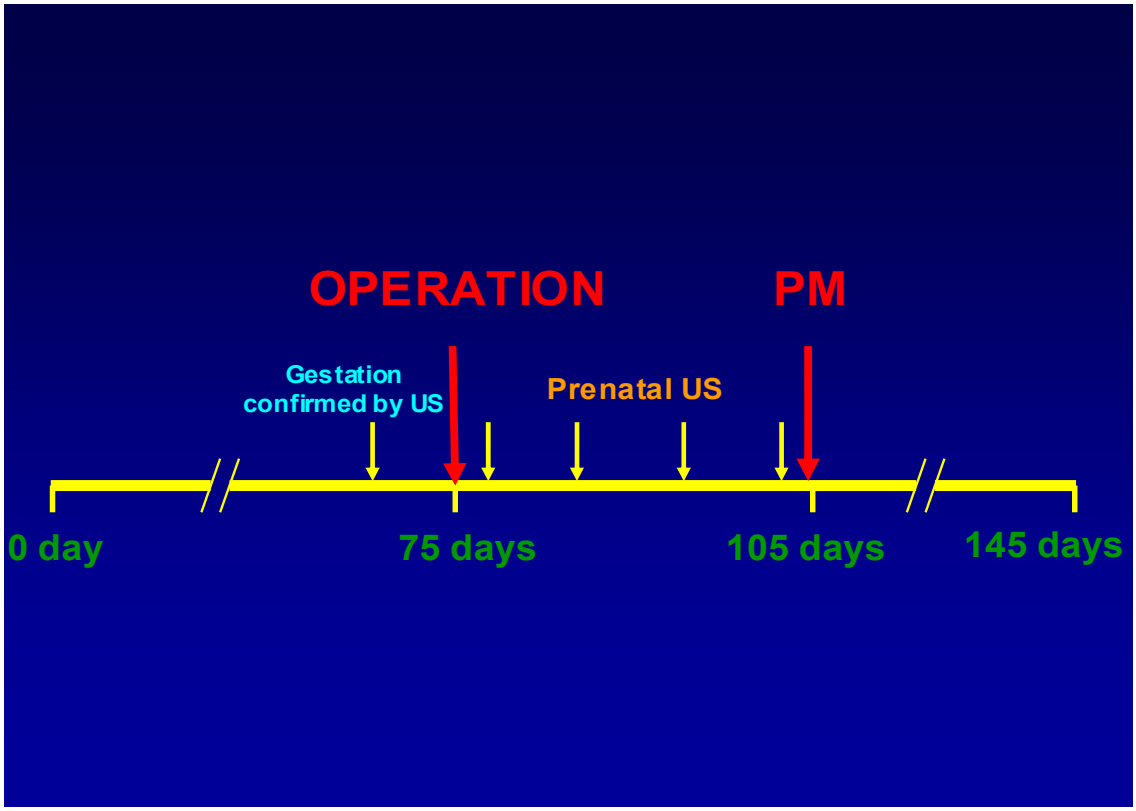


Figure 5. Experimental protocol

4.2. Fetal surgery

Date-mated Romney Marsh ewes bearing fetuses at 75 gestation underwent surgery. Ewes were premedicated with 1 g thiopentone sodium (Intraval Sodium; Rhone Merieux, Dublin, Ireland), intubated and anaesthetised with 2-3% halothane (Fluothane TM ; Mallinckrodt Veterinans, Phillipsburg, USA) in a nitrous oxide and oxygen mixture. Following induction, 1 g of streptomycin/penicillin (Streptopen; Schering-Plough Animal Health, Omaha, USA) was administered intramuscularly. The uterine horn was delivered through a medial laparotomy incision and hysterotomy performed parallel to the uterine vessels over the fetal tail. The fetal hindquarters were then delivered exposing the umbilical root. Abdominal wall muscles in the midline were exposed by blunt dissection and carefully incised to expose the patent urachus, which was ligated with 3/0 silk. The urethra was exposed by a small longitudinal incision made at the perineal end of the abdomen and constricted by a 2 mm omega-shaped, silver ring secured with a 3/0 silk ligature (Figure 6). Both abdominal incisions were

closed with 6/0 Prolene. Before closure, 80 mg gentamicin (Cidomycin ® Adult Injectable; Hoechst Marion Roussel, Strasbourg, Germany) and 600 mg benzylpenicillin sodium (Britannia Pharmaceuticals Limited, Redhill, Surrey, UK) were administered into the amniotic cavity. The uterus was then closed in two layers with 0/0 silk followed by maternal fascia and skin closure with cloth tape and 2/0 silk respectively. Post-operatively, regular analgesia (a paracetamol/opoid combination) was administered and ultrasonography, using a scanner equipped with an annular array multifrequency sector transducer, center frequency 5 MHz (Vingmed CFM 800; Vingmed Sound, Horten, Norway).

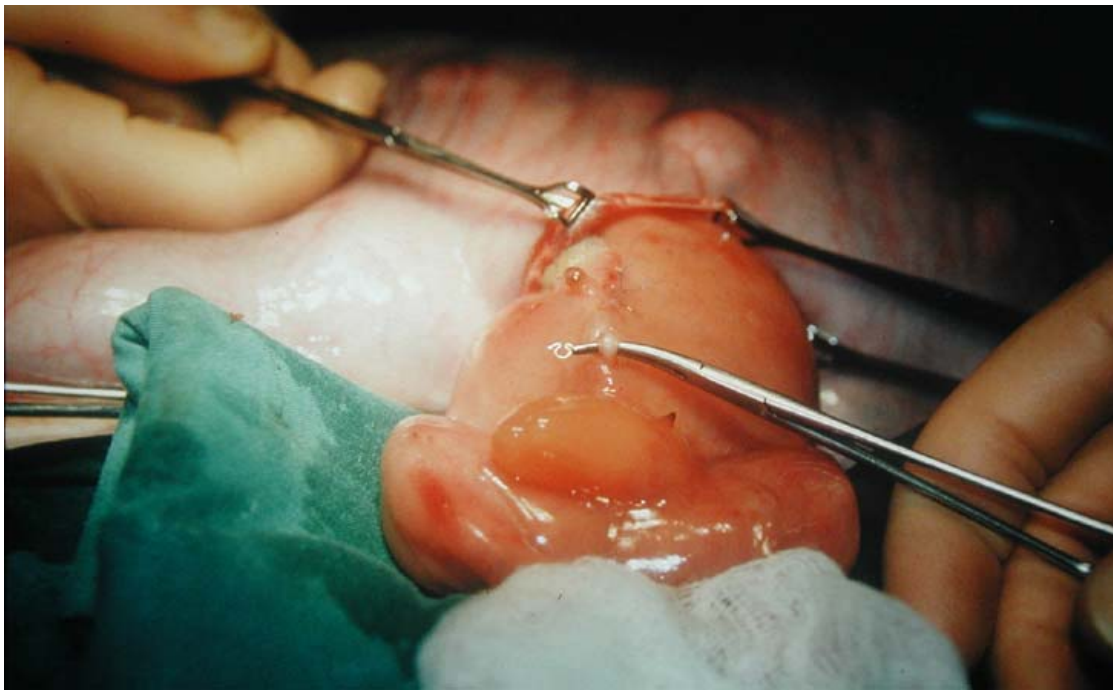


Figure 6. Intraoperative view as putting a 2 mm omega-shaped, silver ring on the urethra. Urachus – below the urethra – is already ligated

4.3. Antenatal ultrasonography

Ultrasound examination was performed first on the 5th postoperative day. Afterwards, to confirm outcome of surgical procedure and fetal viability ultrasonography was done weekly. Mother sheep had to be held on her back and abdomen should be shaved previously (Figure 7). We recorded the status of the kidneys, ureters and urinary bladder. When fetal viability was questionable, flow in the umbilical vessels were examined by colour-Doppler probe (Figure 8). We also paid attention to account the amount of amniotic fluid.

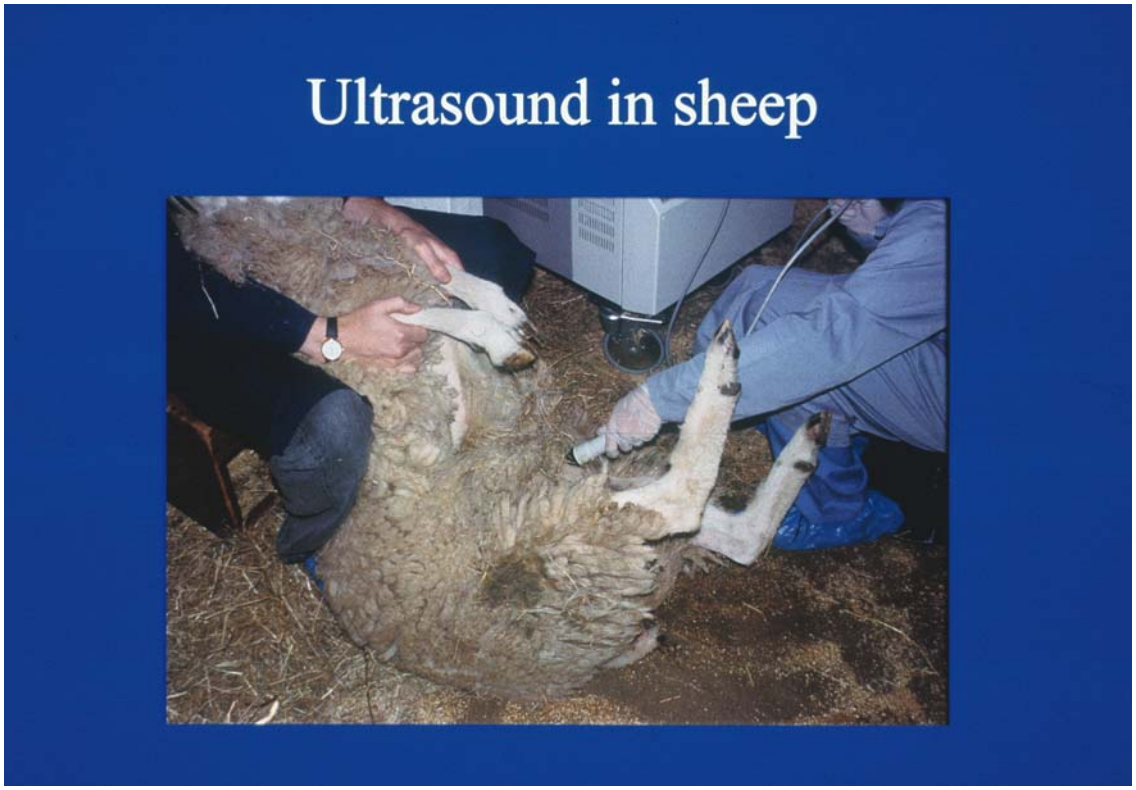


Figure 7.

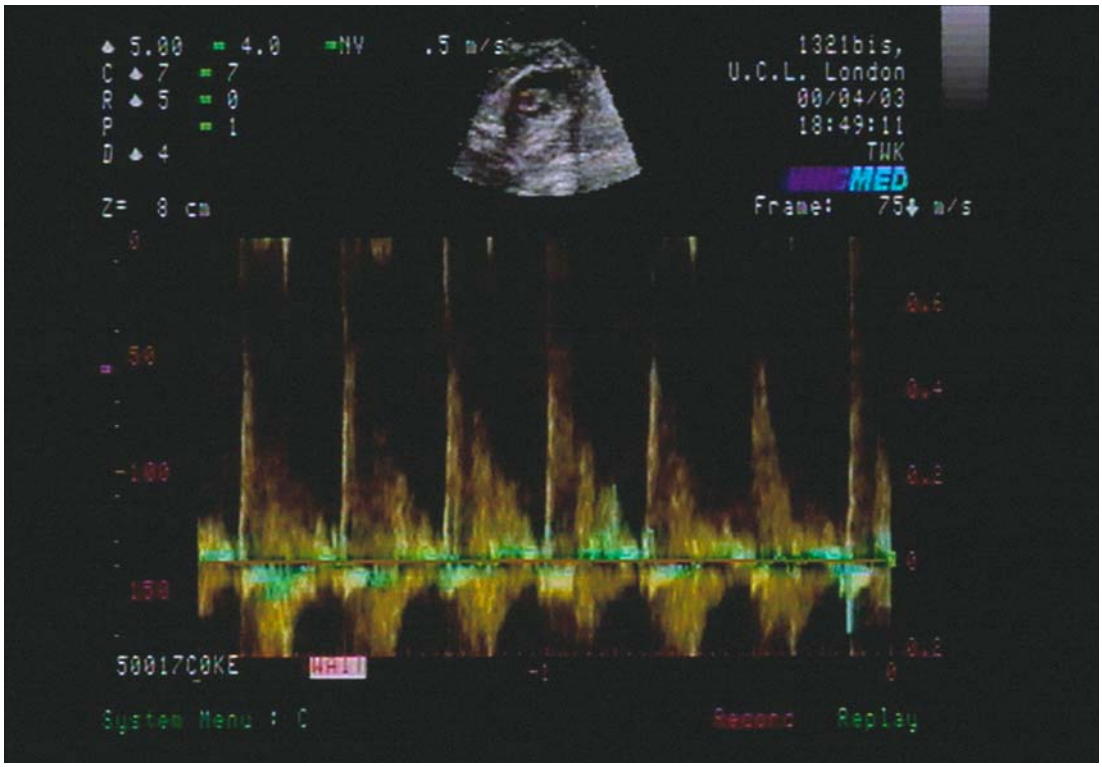


Figure 8. Colour-Doppler US from the umbilical artery to prove fetal viability

4.4. Post-mortem examination and sample collection

In our hands, this surgical technique is associated with a 15% (3 of 19) fetal mortality. Thirty days after surgery, ewes were sacrificed by intravenous injection of pentobarbitone sodium (Euthatal; Rhone Merieux, Athens, USA). Fetuses were weighed and then underwent post-mortem examination. In some, filling cystometry was performed, as described below. All bladders were blotted dry and weighed. Separate samples of bladder tissue from the bladder dome were then either snap-frozen for western blot, fixed in 10% paraformaldehyde (BDH, Poole, UK) for immunohistochemistry or placed in Ca^{2+} free Hepes Tyrode's solution (see below, for composition) for contractility studies. In addition, fetal kidneys were preserved for histology.

4.5. Ex vivo filling cystometry

Ex vivo filling cystometry was used to determine the relationship between storage volume and pressure in six sham and four obstructed male fetuses. With the urinary tract in situ, the urethra and the urachus were ligated to avoid urine loss; the ureters were also ligated close to the vesico-ureteric junctions. Two catheters were placed in the urinary bladder, one for filling, a 5F feeding tube, and the other, an 1.2 mm external diameter vinyl tubing, for pressure recording. Access was by a cut-down technique either through the urachus if the luminal size was adequate or through the urethra, and the tubes held with 2/0 silk ligatures. Intravesical pressures were recorded with reference to air (i.e. atmospheric pressure = zero) by a water-filled transducer (PDCR 75; Druck Ltd, Groby, Leicestershire, UK) placed level with the bladder and connected to a chart recorder (Lectrome**d;** Letchworth, Hertfordshire, UK). The pressure at the outset was recorded, and the bladder then drained noting the volume. The bladder was intermittently hand-filled by syringe with isotonic saline at room temperature using step-wise increments of 0.1 to 1.0 ml for sham bladders and increments of up to 5.0 ml for obstructed bladders. Intravesical pressures were recorded continuously and documented from stable plateaux following stress-relaxation. Filling was stopped when the initial bladder volumes were reached or when the bladder became tense on visual observation. Over-distending the bladder was avoided to prevent tissue damage, as samples were subsequently used for in vitro studies. The complete procedure took

about 10 minutes. There was no significant difference in results obtained from contractility studies between fetal bladders that were or were not exposed to filling cystometry (data not shown).

4.6. Contractility studies

Smooth muscle contractile function was examined using excised strips of detrusor tissue (diameter <1 mm) from the mid-region of bladders, after removal of the urothelium and adventitia by dissection. Strips were superfused with normal Tyrode's solution gassed with 95% O_2 / 5% Co_2 (pH 7.4, 37 °C). Muscles were electrically-stimulated (3 sec tetanic trains; 1-60 Hz; 0.1 ms pulses; every 90 sec) in the presence or absence of tetrodotoxin (1 mM); atropine (1 mM) or a-β methylene-ATP (ABMA) (10 μM) or exposed unstimulated to KCl (120 mM), carbachol (10 μM) or ABMA (10 μM). At the end of the experiment the muscle was weighed and contractile force expressed in units of mN.mg $^{-1}$ wet weight of tissue.

Force frequency relations were fitted to the empirical equation (3):

$$T = \frac{T_{max} \cdot f^n}{f_{1/2}^n + f^n}$$

where T is the tension, T_{max} is the estimated maximum tension at high frequencies, f, of stimulation, $f_{1/2}$ the frequency required to achieve $T_{max}/2$ and n is a constant. The solutions and chemicals used were: Tyrode's solution, mM: NaCl, 118; $NaHCo_3$, 24; KCl, 4.0; $MgCl_2.6H_2O$, 1.0; $NaH_2Po_4.H_2O$, 0.4, $CaCl_2$, 1.8; glucose, 6.1; Na pyruvate, 5.0 and Ca^{2+} -free HEPES Tyrode's, mM: NaCl, 105; HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), 19.5; KCl, 3.6; $MgCl_2.H_2O$, 0.9; $NaH_2Po_4.H_2O$, 3.6; $NaHCo_3$, 21.5; glucose, 5.5; Na pyruvate, 4.5mM; pH 7.1 adjusted with 1M NaOH. Interventions used were: KCl, added as a solid to Tyrode's solution to a final concentration of 120 mM (no osmotic correction was made), stock solutions in water of TTX (1 mM), atropine (1 mM), carbachol (10 mM) and ABMA (10 mM) were stored at 4°C and samples added to Tyrode's solution to achieve the required final concentrations.

4.7. Histological analyses

Fixed bladders and kidneys were dehydrated in alcohol, wax-embedded and sectioned at 4 μm. Bladder sections were dewaxed, rehydrated, and stained with Masson's trichrome; this stains myocytes purple-red, erythrocytes bright red and collagen blue. Nerves were visualised by immunohistochemistry with antibodies to neuronal proteins, S100 and PGP 9.5. S100 is a calcium-binding protein, found in neuroepithelial and neural crest derived cells; it is present in all Schwann cells and in most neurons (36). PGP 9.5 is a cytosolic protein that is highly expressed in neurons (17). Sections were microwaved on high power for 10 minutes in 0.2% citric acid (pH 6.0). Sections were then exposed to 3% hydrogen peroxide in methanol for 30 minutes, to quench endogenous peroxidase activity, then washed in phosphate-buffered saline (PBS) (Oxoid Ltd, Basingstoke, Hampshire, UK) for five minutes and then PBS-0.1% tween20 (BDH, Poole, UK) for 10 minutes. Sections were blocked in 10% fetal calf serum (FCS) (GIBCO BRL, Life Technologies, Paisley, Strathclyde, UK) with 2% bovine serum albumin (BSA) and 1% tween-20 for one hour. Sections were incubated overnight at 4ºC in a humidified chamber with anti-S100 antibody (1:500; DAKO, Ely, UK) or anti-PGP 9.5 antibody (1:500; DAKO). After three further PBS washes, biotinylated goat anti-rabbit antibody (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) was applied at 1:200 dilution for 1 hour. Slides were washed with PBS and reacted with streptavidin-conjugated horseradish peroxidase (HRP) for one hour. Peroxidase activity was developed with diaminobenzidine for five minutes, generating a brown colour. Sections were counterstained in haematoxylin, to detect cell nuclei, dehydrated and mounted in dextropropoxyphene (BDH). Slides were examined and photographed on a Zeiss Axiophot II microscope (Carl Zeiss, Oberkochen, Germany). Finally, sections of fetal kidneys were examined after hematoxylin staining. PGP 9.5 western blot Sections of bladder domes were homogenised at 4ºC in radioimmunoprecipitation assay buffer containing protease inhibitors- 30 ml/ml aprotinin (2.2 mg/ml), 10 ml/ml phenylmethylsulfonyl fluoride (10mg/ml) and 10 ml/ml sodium orthovanadate (100 mM in 95% ethanol). After centrifugation at 13,000 rpm at 4ºC, protein concentration in supernatants was measured using a BCA protein assay (Pierce, Rockford, USA). Samples containing 20 mg of protein were denatured at 95ºC for five minutes and separated on SDS-12% polyacrylamide electrophoresis gels.

Proteins were transferred to nitrocellulose membranes (Hybond-P; Amersham Pharmacia Biotech) by electroblotting (Bio Rad, Hemel Hempstead, Hertfordshire, UK). Consistent protein transfer was shown with Ponceau S staining (data not shown) and rainbow markers (Amersham Pharmacia Biotech) were used to size proteins. After a PBS-0.1% tween-20 wash, membranes were blocked for 1 hour at room temperature with 5% fat-free milk powder in PBS-0.1% tween-20. Membranes were incubated overnight at 4ºC with anti-PGP 9.5 antibody (1:1000; DAKO) or b-actin (1:10000), a 'house-keeping' protein. Membranes were washed with PBS-0.1% tween-20, blocked and incubated for an hour either with HRP-linked goat anti-rabbit antibody (1:1000), for PGP 9.5 detection, or HRP-linked sheep anti-mouse antibody (1:5000), for b-actin detection. Bands were detected by chemiluminescence (Amersham Pharmacia Biotech), quantified using the Phoretix ID program (Phoretix International, Newcastle upon Tyne, UK) and expressed as the ratio of PGP 9.5/b-actin chemiluminescence. S100 protein binds calcium and as such, also reacts with calcium-binding proteins such as calmodullin and myosin light chain (2) found in detrusor muscle; hence, in this study, it was appropriate only for immunohistochemistry.

4.8. Statistical Analysis

Results were expressed as the mean ± SD. Independent samples Students t-test (SPSS 8.0 for Windows) was used to examine differences in values between sham and obstructed groups. The null hypothesis was rejected at $p<0.05$, $p<0.01$ or $p<0.001$ levels respectively.

5. Results

5.1 Intrauterine ultrasonographic results of severe bladder outflow obstruction

At weekly intervals following surgery, ultrasonography showed no hydronephrosis in sham fetal kidneys, and sham fetal bladders were of insufficient size to be definitively demonstrated. In the obstructed group, all fetal kidneys became hydronephrotic and urinary bladders were dilated; this appearance was evident at the first ultrasound examination and the severity progressed during the 30 days of observation. It was

already obvious the difference among urinary tract between obstructed and sham fetuses at the 5th day. In control animals however, normal echogenic kidneys and empty bladder was visible (Figure 9). At this time there was no difference in amniotic fluid between our two groups.

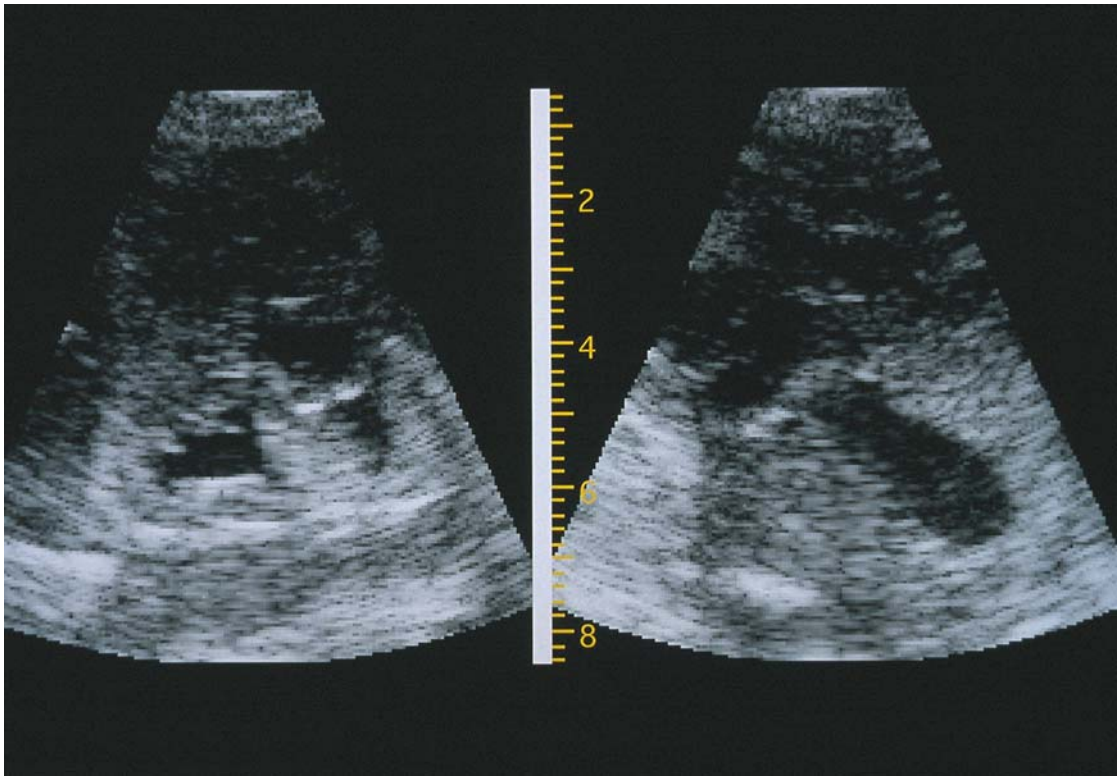


Figure 9. intrauterin US 5 days after bladder outlet obstruction (left kidney, right bladder)

In contrast 1 and 2 weeks later even more severe contrast was studied in not only the dilatation of the kidneys and bladder but also the amount of amniotic fluid as well. However there was no any evidence in abnormality in sham animals (Figure 10). Ultrasound examination, performed before post-mortem examination, showed the most marked inequality between sham and obstructed fetuses (Figure 11,12,13). There was oligo-hydramnion also observed in operated animals.

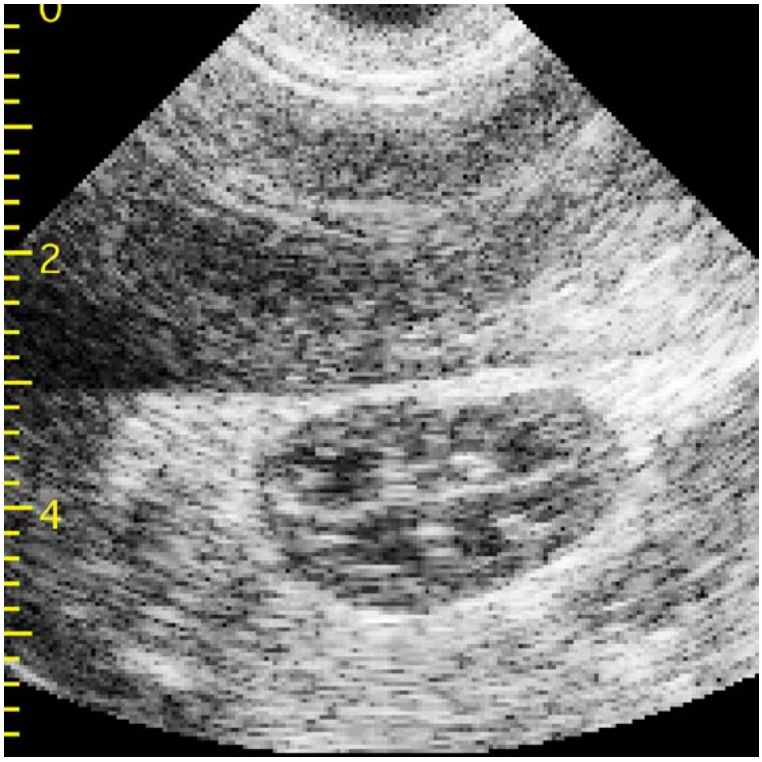


Figure 10. Kidney US scan 1 month after sham operation (longitudinal view)

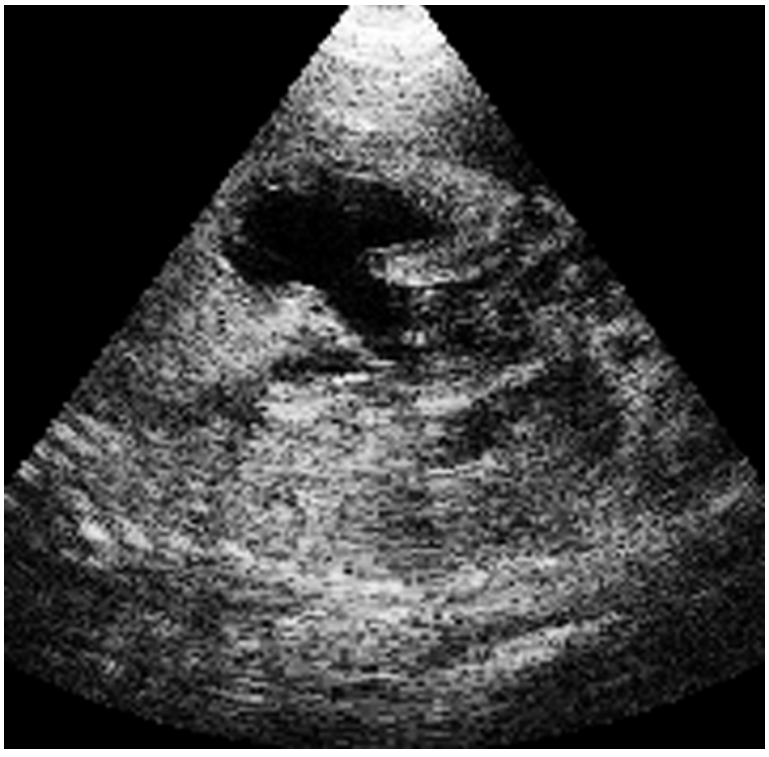


Figure 11. Longitudinal view of the obstructed kidney 1 months after surgery

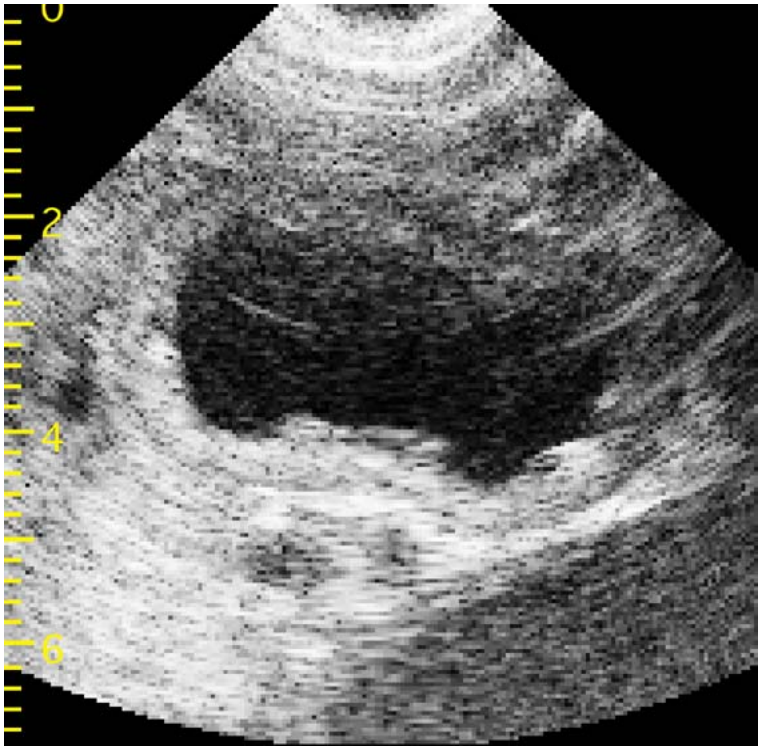


Figure 12. Dilated bladder 1 month after obstruction

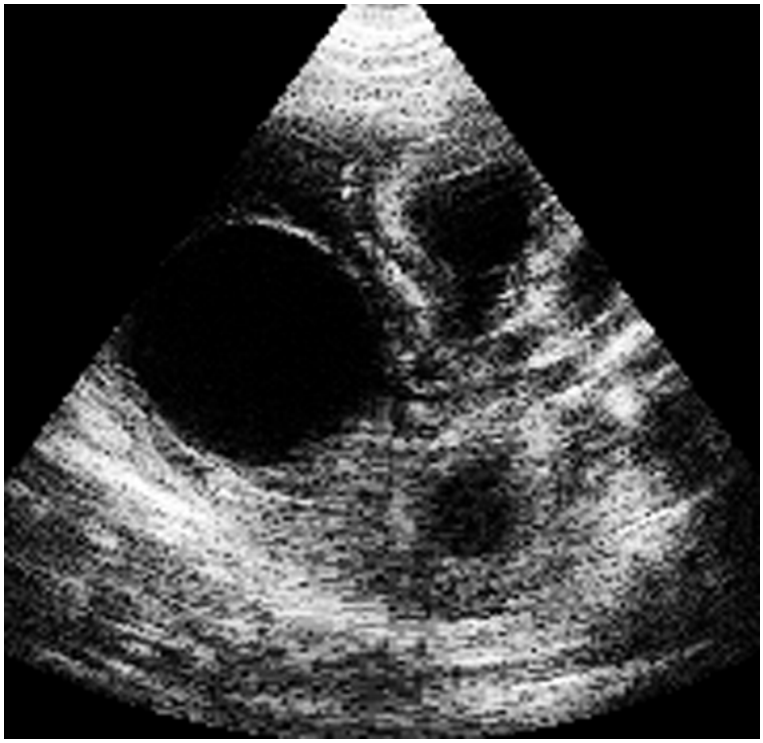


Figure 13. Bilateral hydronephrosis and dilated bladder (typical intrauterin view of a severe posterior urethral valve)

5.2. General morphological effects of bladder outflow obstruction on the urinary tract

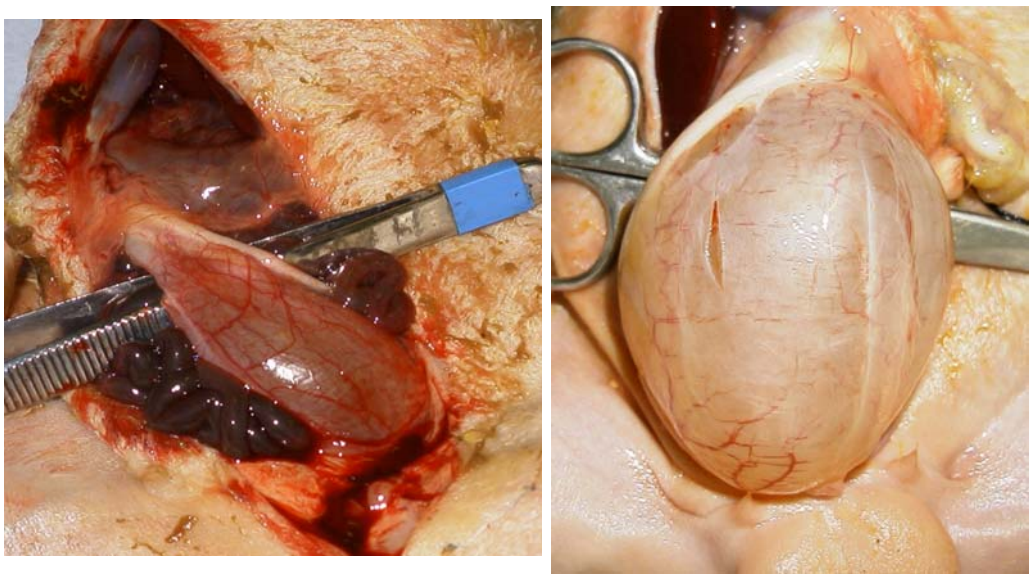


Figure 14. Sham and obstructed bladder after post-mortem

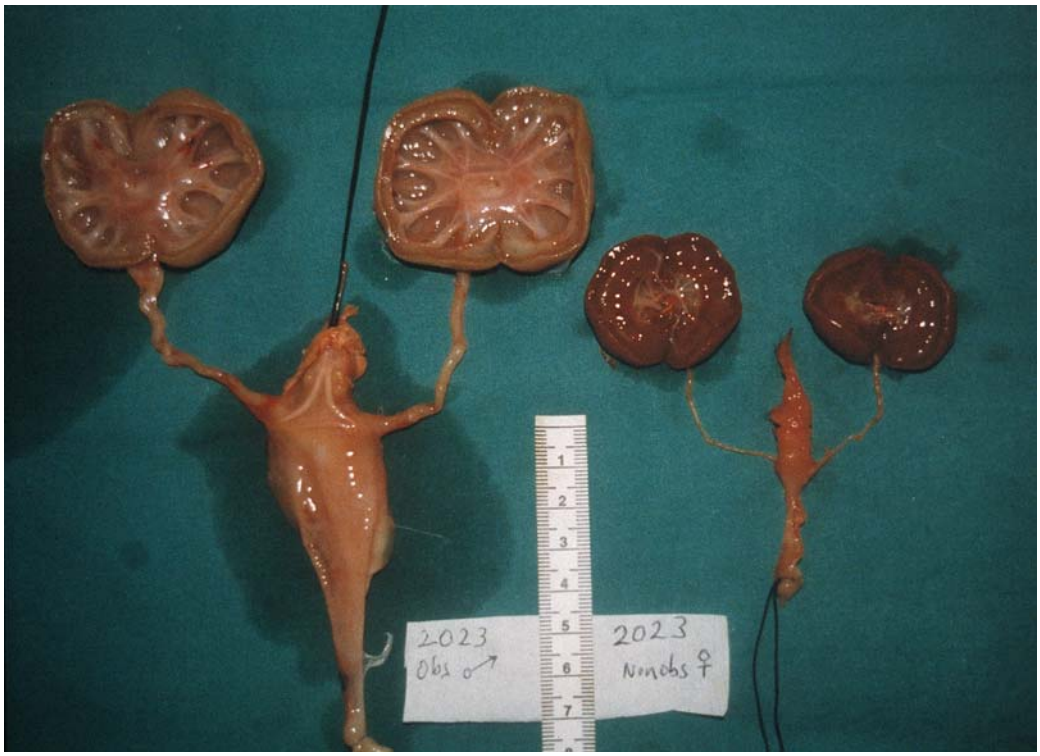


Figure 15. Obstructed and control fetal urinary tracts (bilateral renal dilatation)

5.3. Major morphological disruption of nephrogenic cortex with dilated and cystic tubules

On histological examination, sham fetal kidneys at 105 days gestation (Figure 16A) showed a prominent nephrogenic zone, with normal calibre tubules and developing glomeruli. By contrast, in obstructed kidneys, a major perturbation of nephrogenesis was noted (Figure 16B), with morphological disruption of the nephrogenic cortex with dilated and cystic tubules and a loss of normal glomerular formation.

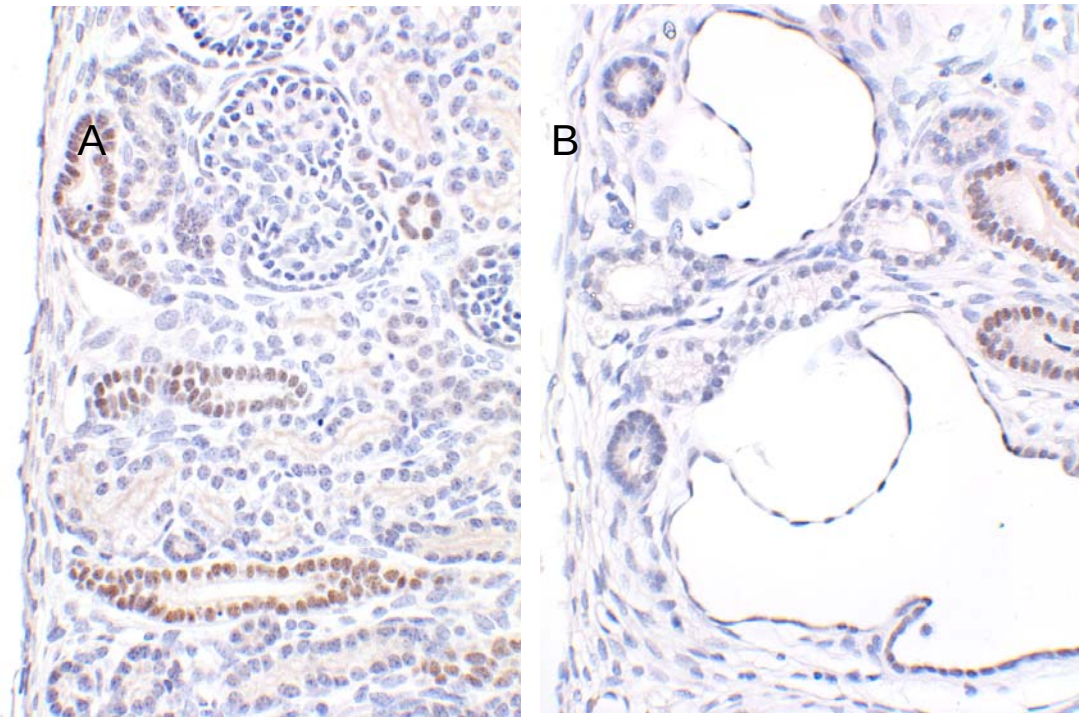


Figure 16. Histology of nephrogenic cortex of fetal kidney

Sections were stained with hematoxylin. **A:** Sham male fetal kidney contains a nephrogenic zone with normal calibre tubules and nascent glomeruli. **B:** Obstructed male fetal kidney contains cystically dilated tubules and there was a lack of glomeruli.

5.4. Increased capacity and compliance in obstructed bladders

Obstructed bladder weights (8.56±1.36 g, n=8) were significantly increased ($p<0.001$) versus sham bladders (1.15±0.29 g, n=8). Fetal weight was greater ($p<0.001$) in the obstructed group as compared to sham fetuses (1.53±0.37, n=8 versus 2.09 kg ±0.30, n=8); bladder weights factored for fetal weights were significantly greater ($p<0.001$) in

the obstructed versus the sham group (respectively, 4.41±0.62, n=8, and 0.77±0.16 g.kg $^{-1}$, n=8).

Intravesical volumes found at post-mortem examination were greater ($p<0.001$) in the obstructed group than in the sham group (63.5±21.9, n=4, versus 5.6±3.0 ml, n=6). With the bladders empty at the outset, the total volumes instilled in the obstructed bladders (61.3±14.4, n=4) were greater than in the sham bladders (10.3±2.3, n=6) for similar increases in pressure (11.5±2.5, n=4, versus 12.1±1.8 cm H_2O, n=6). Both the sham and the obstructed bladders demonstrated stress-relaxation of the pressure change after an injection of fluid, i.e. pressure rose rapidly and partially recovered over several seconds to a stable level (figure 17).

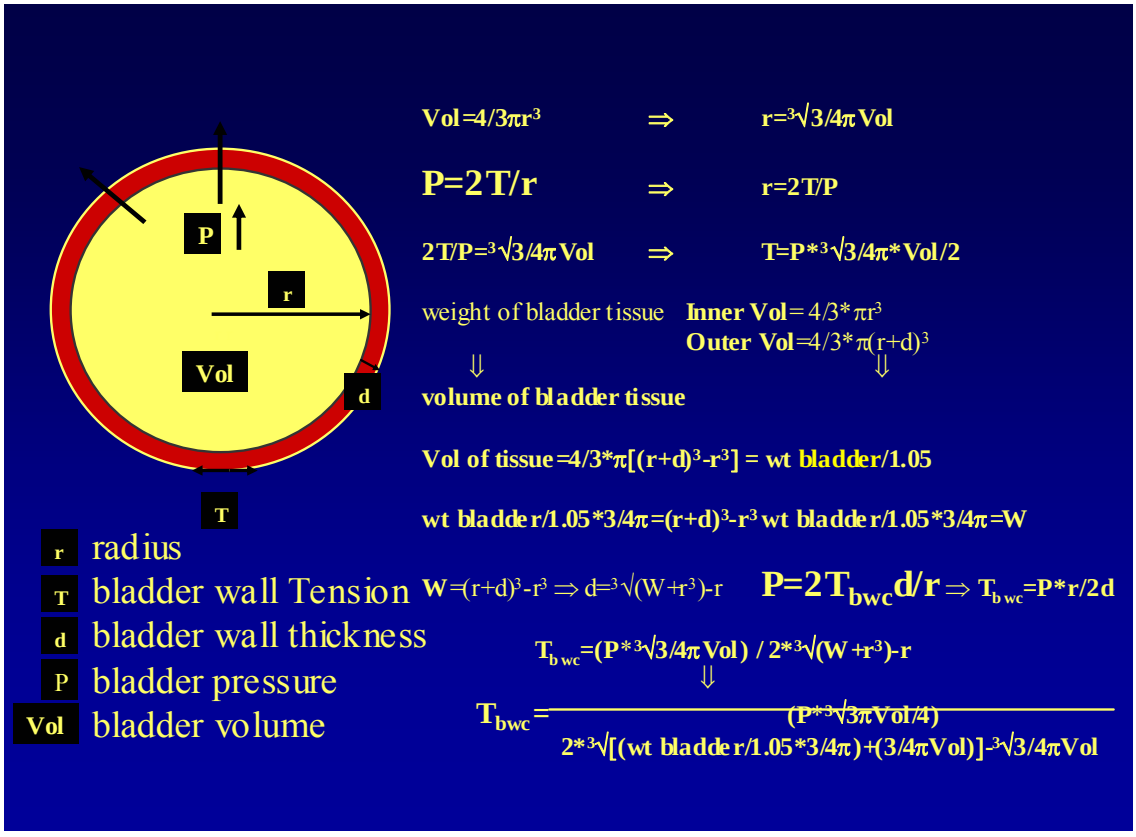


Figure 17. Bladder wall tension calculation

The steady-state pressure values were used to calculate compliance. Figure 18A shows representative examples of pressure-volume curves from a sham-operated and an obstructed bladder. The fitted straight-line is equivalent to 1/C (C = compliance =DV/DP) and the figure shows that compliance was greater ($p < 0.001$) in the

obstructed bladders (mean 6.42±0.91 ml.cm H_20 $^{-1}$, n = 4) compared to those in the sham group (1.03±0.28 ml.cm $_H20$ $^{-1}$, n=6). To examine further the compliance of the bladder wall and take account of disparate bladder volumes between the two groups, wall stress was derived. Wall stress, s, was measured using a modified Laplace relation: $\sigma = P.r1/2.d$, where P is luminal pressure, $r1$ inner radius of the bladder and d the wall thickness; $r1$ was calculated from the volume V in the bladder from the relation $V = (4p/3).r1^3$. The tissue volume, T, of the bladder wall (bladder weight/density; density=1.05 g.ml $^{-1}$) was used to calculate wall thickness assuming the tissue formed a concentric sphere around the bladder lumen and using the relation:

$$d = \sqrt[3]{[(3T/4p)+r1^3]} - \sqrt[3]{[3V/4p]}$$

A plot of wall stress versus bladder volume is shown in figure 18B with examples from a sham-operated and an obstructed bladder. Wall stress was a linear function of volume until a limit was reached when σ rose more steeply in a curvilinear fashion; this limit was approximately 10 kN.m $^{-2}$ in the sham bladder and approximately 6.5 kN.m $^{-2}$ in the obstructed bladder. From the initial linear part of the curve, bladders from the obstructed group were more flaccid than sham bladders (wall stress 0.24±0.04, n=4, versus 1.72±0.30 kN.m^{-2} .ml $^{-1}$, n=6, respectively, $p<0.001$). Figure 18C shows that the initial amounts of instillate had no significant effect in raising wall stress, s. This volume was estimated from the intercept of the straight-line fit with the abscissa and was significantly ($p<0.001$) smaller in the sham bladders (0.41±0.40,n=6, versus 5.37±2.19 ml, n=6, respectively).

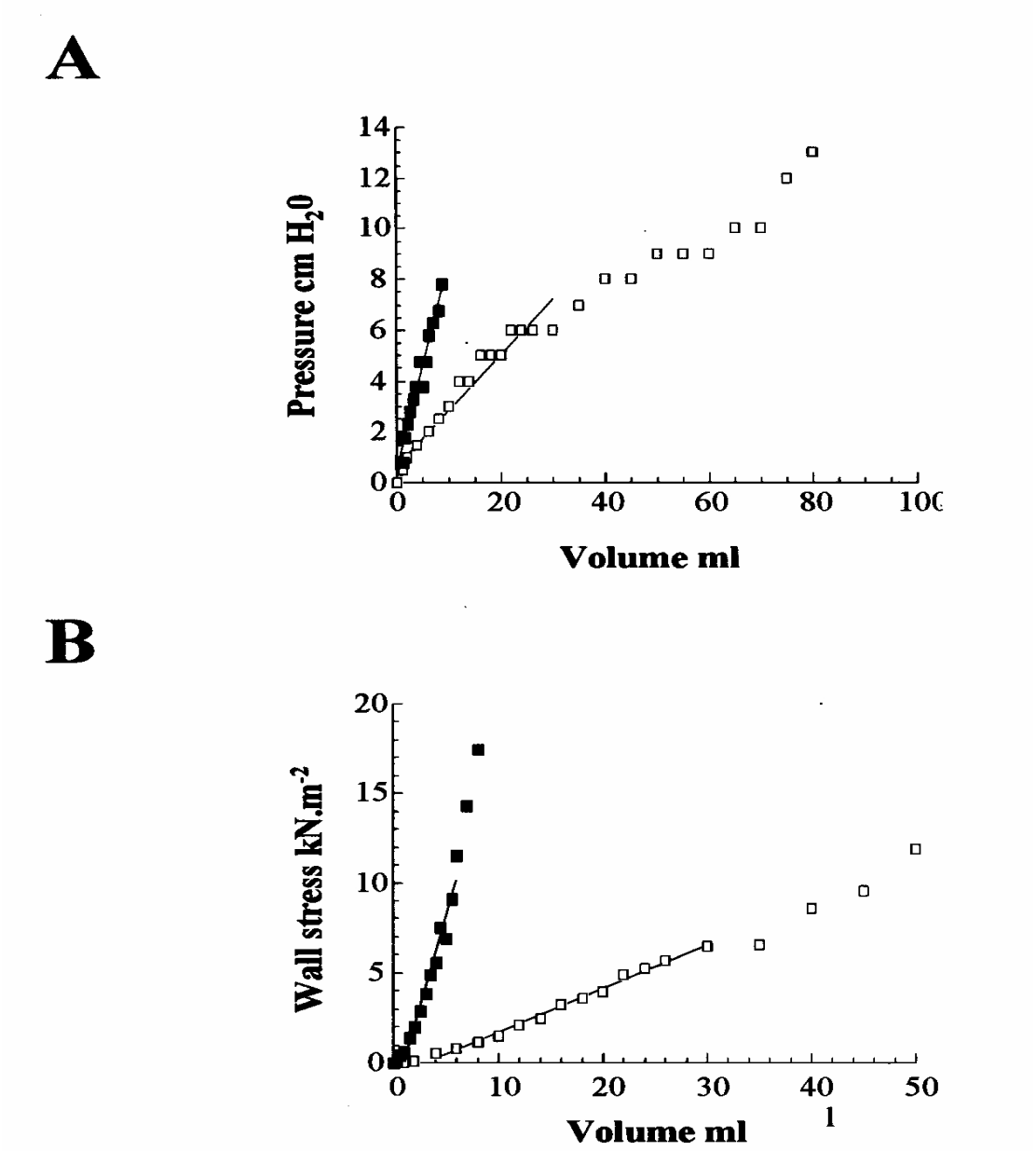


Figure 18. Ex vivo pressure-volume relationships in sham-operated (closed symbols, n=6) and obstructed (open symbols, n=4) fetal bladders. A: shows representative examples of pressure-volume curves from a sham-operated and an obstructed male bladder. The fitted straight-line is equivalent to 1/C (C = compliance =DV/DP), compliance was greater in the obstructed bladder. B: A plot of wall stress, s, versus bladder volume, with examples from a sham-operated and an obstructed bladder. Note wall stress was a linear function of volume until a limit was reached and that sham bladders were stiffer than obstructed bladders.

5.5. Electrophysiology shows hypocontractility in obstructed bladder detrusor muscle

The sizes and weights of the bladder strips used in the contractile studies were the same in obstructed and sham groups (data not shown). Spontaneous contractions were not recorded in any preparation. Steady-state base-line contractions were recorded at 16 Hz stimulation rate. The normalized force generated by muscle strips from obstructed fetal bladders (0.37±0.19 mN.mg tissue wet weight^{-1}, n=8) was significantly less ($p<0.001$) than that from sham fetal bladders (3.23±1.62 mN.mg tissue wet weight^{-1}, n=8) (Figure 19). The difference between obstructed and sham operated fetuses was significant in all frequencies (Figure 20). The contraction profile was also different in the two groups with a greatly prolonged relaxation time course in samples from obstructed bladders (Figure 21). Force-frequency relationships (1-60 Hz) were generated by increasing the tetanic stimulation frequency from 1-60 Hz in normal Tyrode's solution, in the presence of 1 mM atropine and then in the presence of 1 mM tetrodotoxin (TTX). Nerve-mediated tension was taken as the difference between total and TTX-resistant force; atropine-resistant contraction as the difference between nerve-mediated responses and those in the presence of atropine. Atropine-resistant contractions were recorded in only one preparation from one sham bladder (20% of the total nerve-mediated contraction) and in none from the obstructed bladders.

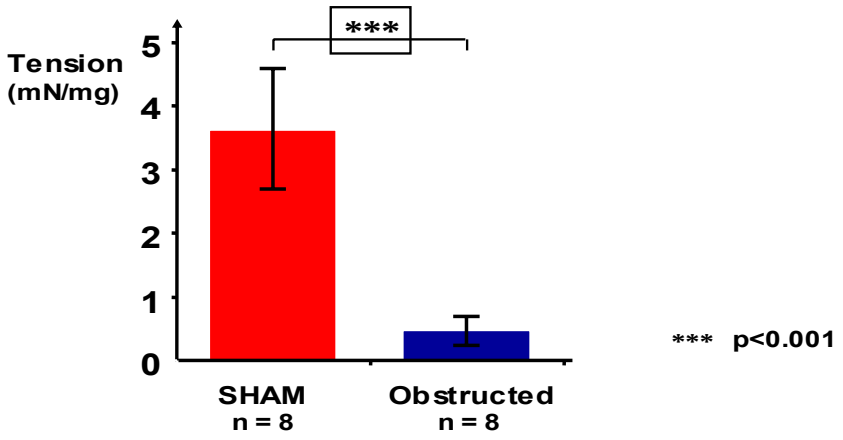


Figure 19. T_{16Hz} response

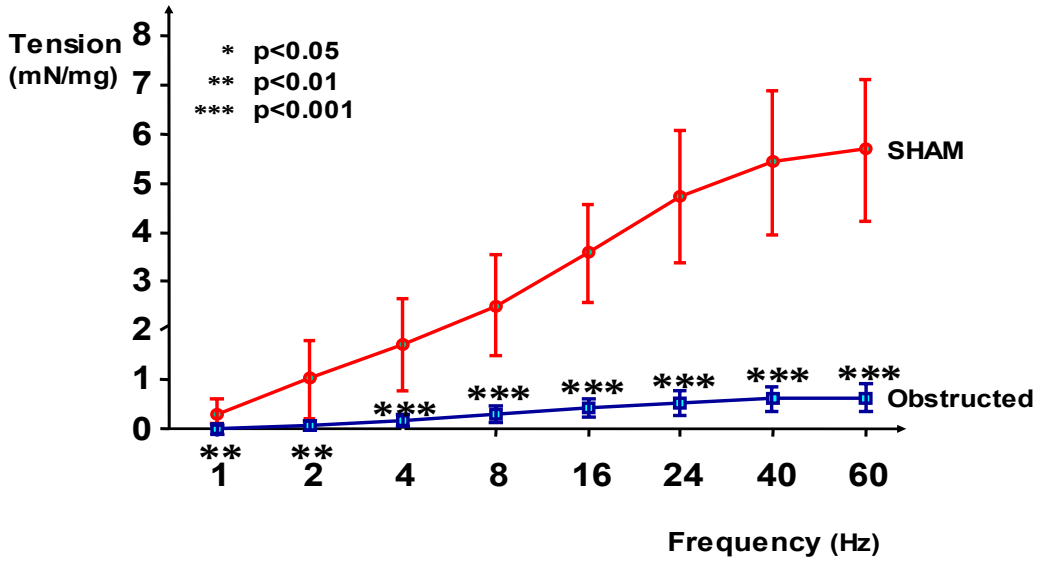


Figure 20. Force-frequency relation

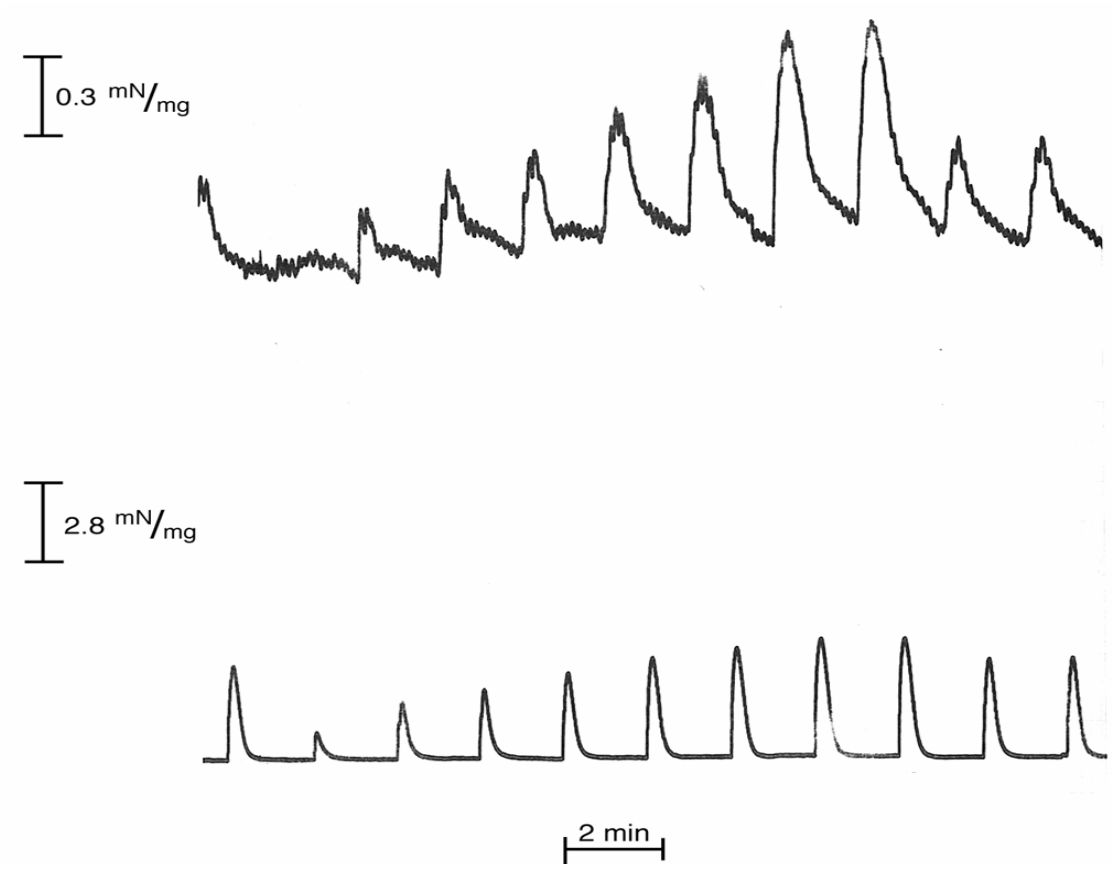


Figure 21. Force-frequency records between 1 and 60Hz of frequency in Tyrode's

Contractures were also elicited in unstimulated preparations by 10 mM carbachol, 10 mM ABMA and 120 mM KCl (Figure 22A and 22B). Figure 22A shows the magnitude of the force developed by these interventions as well as the nerve-mediated contraction at 16 Hz. The absolute forces developed by these interventions were all significantly less ($p<0.001$) in preparations from obstructed bladders compared to sham bladders.

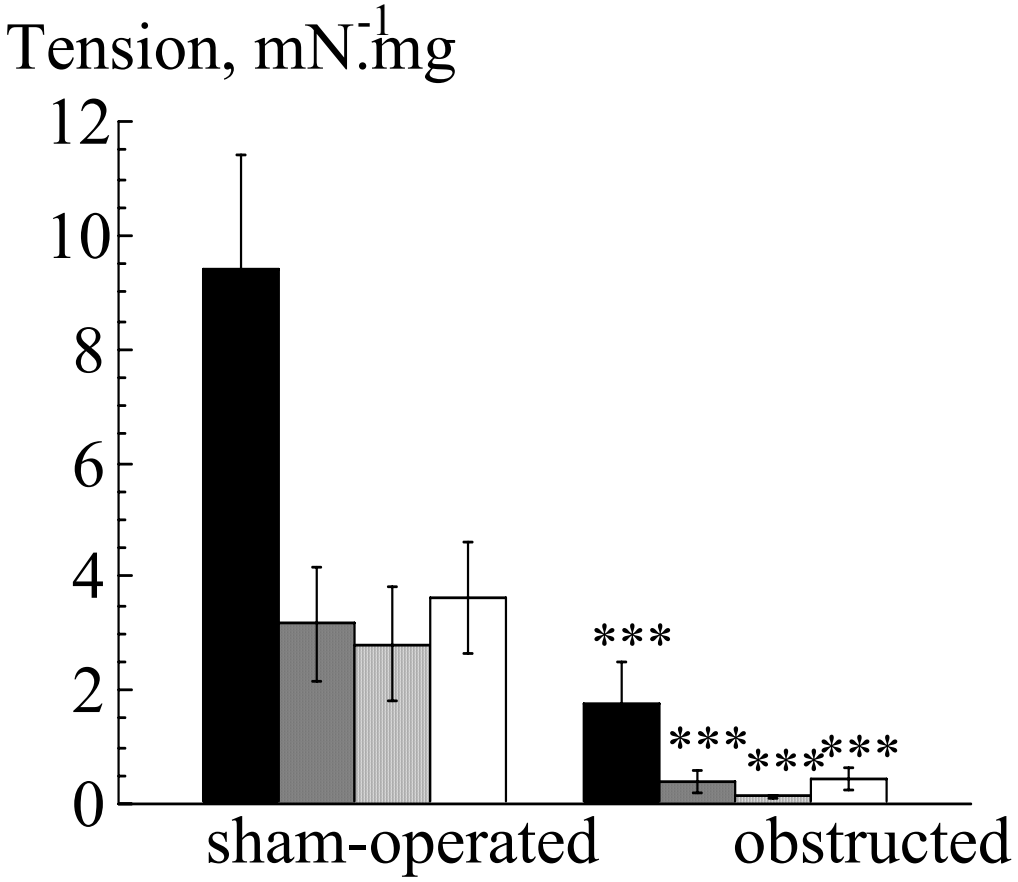


Figure 22A.

Histograms showing absolute tension evoked by10 μM carbachol, electrical field stimulation at 16 Hz (T16), 120 mM KCl and 10 μM ABMA. Closed bars from sham (n=8) and open bars (n=8) from obstructed male fetal bladders. Note the significant differences ($p<0.001$) between sham and obstructed groups.

Figure 22B shows the ratio of force developed by the three agonists compared to the nerve mediated contraction at 16 Hz (i.e. T16). In the sham group, the contractile

response to carbachol was significantly greater than the response to nerve-mediated contraction (i.e. ratio of carbachol: T_{16}>1.0).

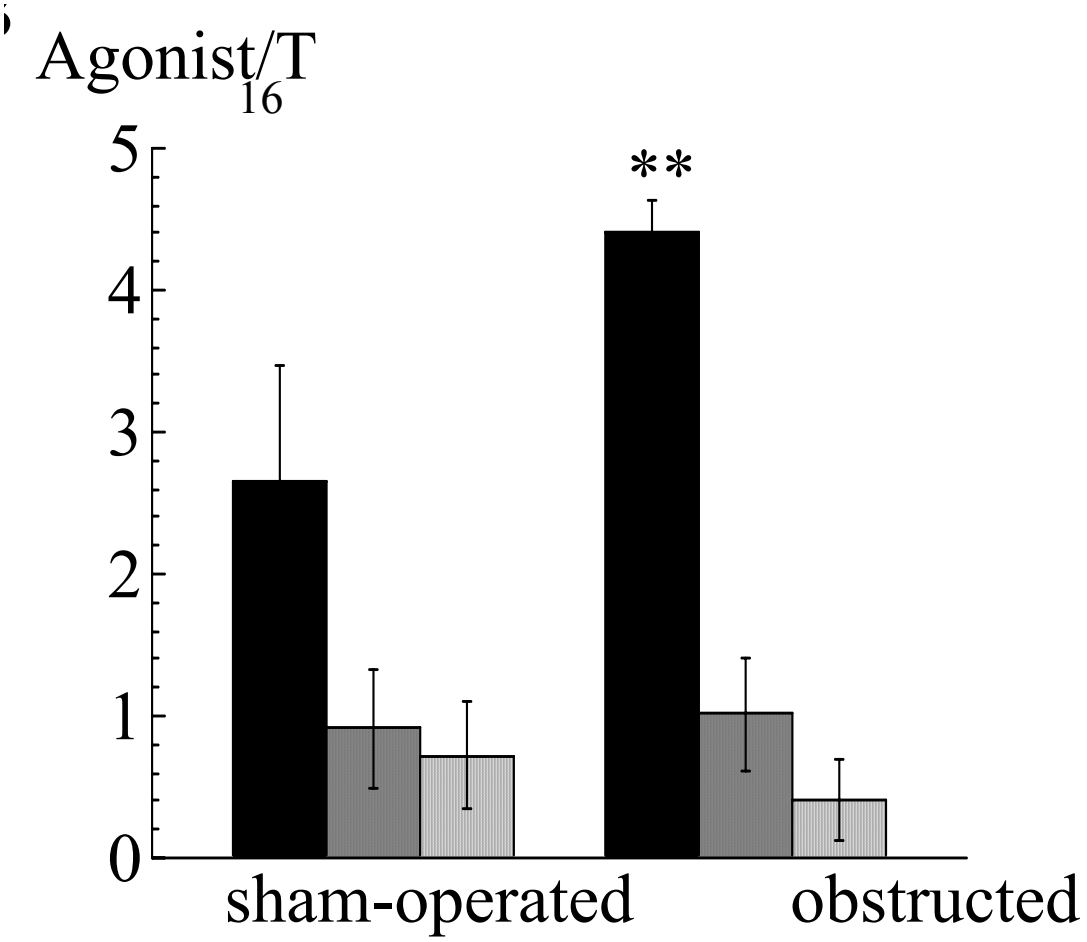


Figure 22B.

Histograms showing the ratio of force evoked by either carbachol (10 μM), KCl (120 mM) or ABMA (10 μM) to that generated by electrical field stimulation at 16 Hz (T16). Closed bars from sham (n=8) and open bars (n=8) from obstructed male fetal bladders. Note the significant difference ($p<0.05$) between the sham and obstructed group for the carbachol/T16 ratio.

The response of sham bladder strips to stimulation to KCl, and to ABMA, was of the same magnitude as that achieved by nerve mediated stimulation (i.e. ratio of agonist: T_{16}~1). The ratio of the responses, carbachol to EFS (at 16 Hz), in the obstructed

bladders was significantly greater ($p<0.05$) than the comparable ratio in the sham group (Figure 23). Therefore the decline of the nerve-mediated contraction was significantly more than the decline of the carbachol contracture in the obstructed group. The ratios of response of KCl and ABMA to EFS at 16 Hz were not significantly different between the sham and obstructed groups.

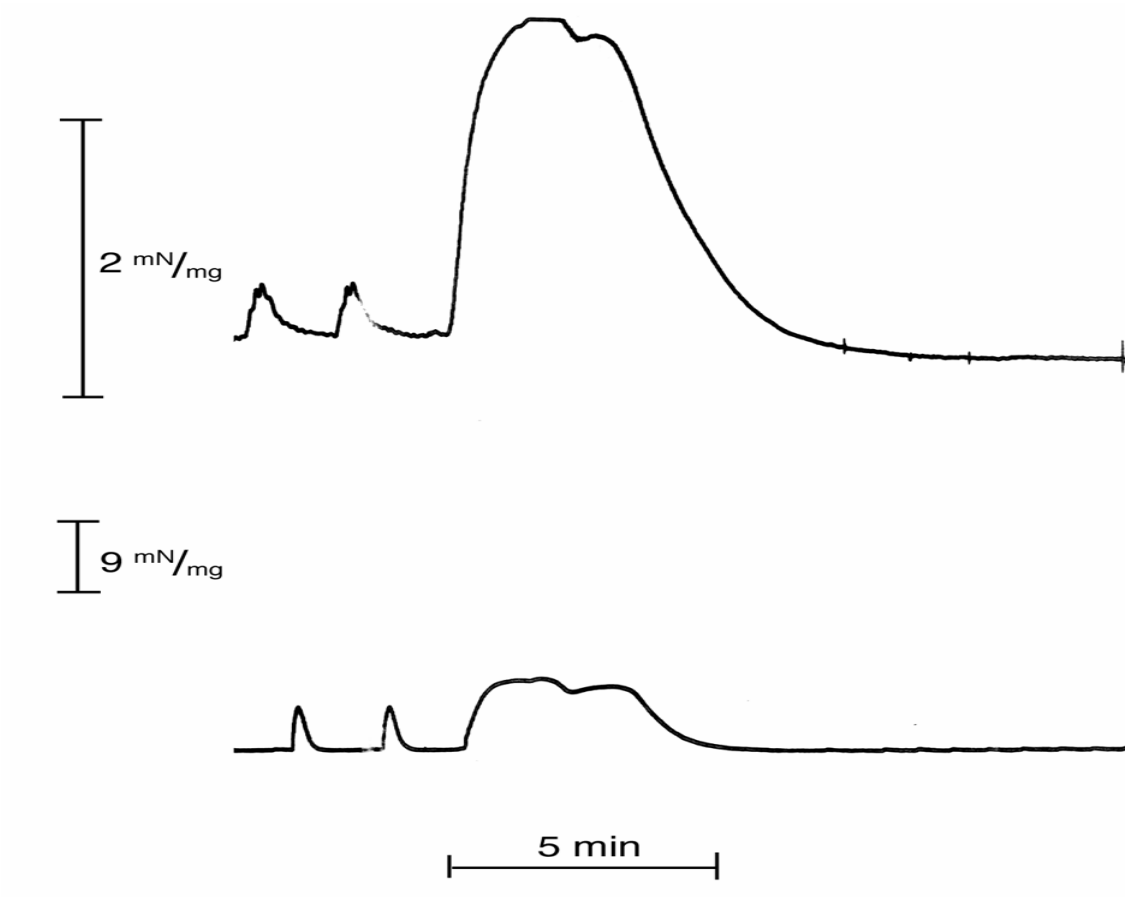


Figure 23. contractions generated by 16Hz frequency stimulation (T_{16Hz}) and spontaneous curve of the smooth muscle strip in the presence of carbachol. Ratio between obstructed (top curve) is significantly greater than in sham operated control fetuses (bottom curve)

5.6. Urinary bladder innervation is decreased in response to outlet obstruction

At 105 days gestation, sham fetal bladders were well-developed, with a urothelial layer covering the lamina propria; detrusor muscle bundles were apparent, with a trilaminar appearance, and with little connective tissue between individual bundles (Figure 24A). S100 (Figure 24B) and PGP 9.5 (Figure 24C) immunolocalised, most prominently, to nerve bundles between and within muscle fascicles and in the serosal layer. By contrast, obstructed bladders walls were thinner, their urothelium was flattened and the cellularity of the lamina propria appeared decreased (Figure 24D); furthermore, the muscular layer showed an increase of connective tissue between muscle bundles. In addition, there was an observed reduction of neuronal immunostaining with S100 (Figure 24E) and with PGP 9.5 (Figure 24F) in the detrusor muscle layers of the obstructed bladder. Finally, a semi-quantitative analysis of PGP 9.5 using western blot showed an appropriate band at 25 kDa in all bladders (Figure 25A), and densitometry confirmed a significant ($p<0.05$) reduction of signal intensity, factored for β actin protein, in obstructed compared to sham bladders (respectively, 81±11 versus 61±11 arbitary units, n=8 in each group) (Figure 25B).

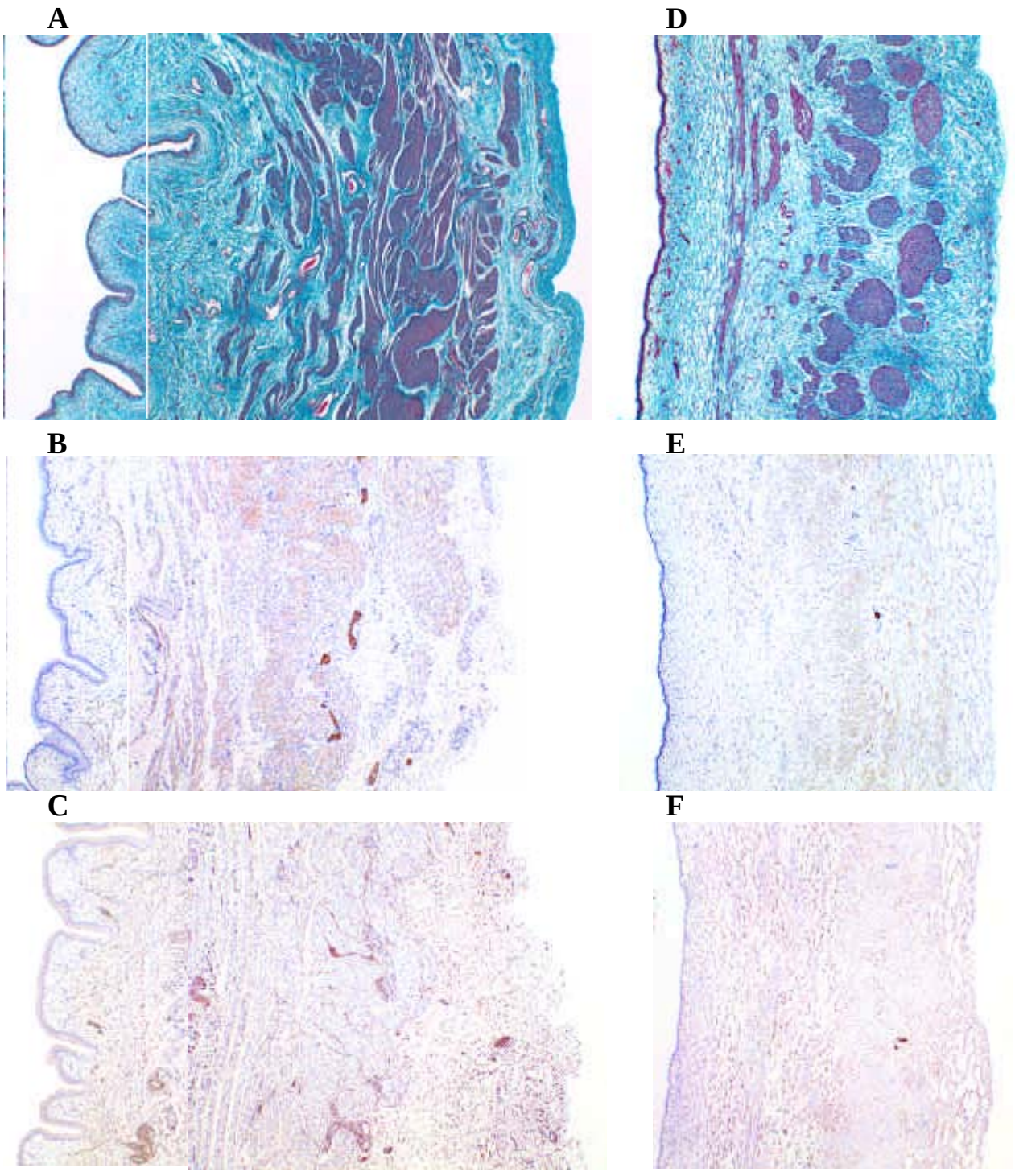


Figure 24.

Figure 24. Histology of sham and obstructed bladders **A-C** are sham bladders at 105 days gestation, and **D-F** are time-matched obstructed male organs. **A** and **D** were stained with Massons' trichrome; **B** and **E** were immunostained for S100 and counterstained with hematoxylin; **C** and **F** were immunostained for PGP 9.5 and counterstained with hematoxylin. In the sham bladder, note the urothelium, the lamina propria, the detrusor muscle bundles and associated nerves immunostained brown with S100 and PGP 9.5. In obstructed bladders, the urothelium is flattened and cellularity of the lamina propria appeared decreased and there is increased connective tissue deposition between detrusor muscle bundles; in addition, neuronal-pattern S100 and PGP 9.5 is diminished versus sham bladders.

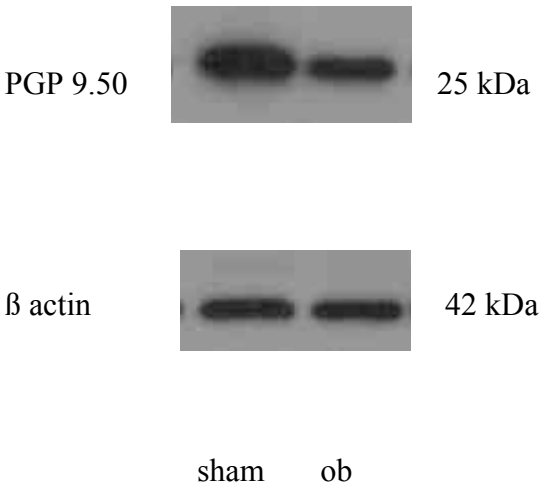


Figure 25A: PGP 9.5 western blot**:** Western blot for PGP 9.5 (25 kDa) and b-actin (42 kDa) in a representative sham (sh) and obstructed (ob) fetal male bladder.

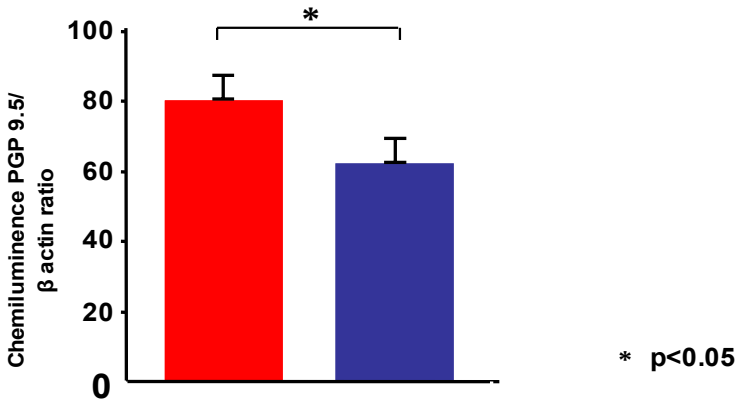


Figure 25B: Histogram showing PGP 9.5/b actin protein; closed bars from sham and open bars from obstructed male fetal bladders. Note significant ($p<0.05$) reduction in PGP 9.5 expression in the obstructed bladders.

5.7. Female fetuses

There was no significant difference between the fetal weights and bladder weights in the male and female fetuses 30 days post-operation (data not shown). In addition, none of the electrophysiological experiments distinguished any significant differences between male and female sham bladders (data not shown).

6. Discussion

The fetal sheep model used in this study generated bladder outflow obstruction at 75 days of gestation by ligation of the urachus and partial obstruction of the urethra by placement of an omega-shaped ring. After 30 days, experimental BOO had generated major changes in the gross structure of the urinary tract, associated with an increase of urinary bladder weight and disruption of nephrogenesis. Similar histological changes in the developing kidney have been documented after complete ureteric obstruction between 90 and 100 days gestation in the sheep (32,33), and resemble changes recorded

by other authors after experimental ovine fetal BOO (34,35). The focus of the current investigation, however, was the physiology of the obstructed fetal sheep bladder, especially in relation to contractility studies, cystometry and markers of bladder innervation. Following 30 days of BOO, the fetal bladder demonstrated reduced contractility, as assessed by force-frequency relationships; the pattern was consistent with denervation. The bladder also accommodated an increase in volume without any increase in storage pressure, was more flaccid and yet retained stress-relaxation features. Finally, we documented reduced innervation immunohistochemically and by western blot, consistent with the results from contractility studies. We now discuss these finding in more detail.

6.1. The normal fetal ovine bladder

The normal sham bladder at 105 days gestation was a well-developed compliant organ with a mean capacity of 10.3 ml associated with an end-fill pressure of 11.5 cm H_2O. The bladders exhibited partial stress-relaxation with intermittent filling but with a steady-state increase of pressure after each volume increment. The steady-state changes of pressure with volume occurred after an initial 'unfolding volume' of 0.4 ml in the sham bladders; thereafter, the steady-state pressure increased approximately linearly with volume. In order to quantify the elastic properties of the bladder wall, pressure values were converted to wall stress by use of the Laplace relation. Using this transform, wall stress increased linearly with increasing volume after the initial 'unfolding' phenomenon. This indicates that the steady-state properties of the bladder wall can be described purely as an elastic element. At wall stress values greater than approximately 10 kN.m -2 , there was deviation from this linear behaviour indicating that the elastic limit had been reached.

We also found that contractile responses to electrical and pharmacological stimulation were present in the sham bladder at 105 days gestation. This concurs with the observations reported by other investigators in the ovine bladder at 95 days gestation (36) and at 120 days gestation (37). These observations are consistent with our histological observations that these organs have a well-developed detrusor musculature at 105 days gestation; however, it is important to note that these fetal bladders were still growing, since 44% of detrusor myocytes were proliferative, as assessed by

immunostaining for proliferating cell nuclear antigen (NT and ASW, unpublished observations). In the current experiments, muscle preparations responded to electrical field stimulation, direct depolarisation by KCl and to muscarinic activation by carbachol. The field-stimulated responses were blocked by tetrodotoxin implying that the contraction was mediated by motor nerves embedded in the preparation. Furthermore, although ABMA, a purinergic receptor (P2X) agonist, produced a definite contractile response, atropine-resistant contractions were absent, except in one preparation. This may be explained by detrusor myocytes possessing surface P2X receptors but with any neurally-released ATP undergoing hydrolysis prior to reaching the smooth muscle cells. The same conclusions have been reached about the detrusor from adult sheep (38) and from stable human bladder (39). This contrasts with studies examining neuronal activation of bladder strips in animals such as guinea-pigs (40), rats and dogs (41) and cats (42) and in pathological human bladders (i.e. secondary to obstruction or instability) which have significant purinergic excitatory innervation (39). To our knowledge, ours is the first study to assess fetal sheep urinary bladder innervation, as assessed by S100 and PGP 9.5 immunohistochemistry; we documented the largest nerves around the detrusor layer. Further histochemical studies are required to define whether these are, for example, cholinergic, adrenergic or purinergic nerves. However, it is notable that Peters et al (43) found evidence for cholinergic receptors in the ovine fetal detrusor at 55-60 days gestation.

Finally, because human fetal BOO is almost exclusively a male disorder, the reported experiments addressed male sheep fetuses. However, we found no differences in bladder size or contractility between male and female ovine fetuses and this observation may be useful when planning further studies of normal fetal sheep physiology.

6.2. The fetal ovine bladder subjected to severe BOO

In response to EFS at different frequencies, obstructed urinary bladder strips were hypocontractile. Similar findings were recorded with all the stimulating agonists used; carbachol, an acetylcholine agonist, KCl, a membrane depolarising agent, and ABMA, a purinergic receptor agonist. The hypocontractility was, in part, likely to have been a result of impaired detrusor muscle contractility because the responses to all interventions were reduced. These interventions ranged from those which: activated

the muscle directly by depolarisation (KCl); acted via surface receptors (carbachol and ABMA); or via motor innervation (EFS). Furthermore, there was differential reduction of response in relation to EFS and by addition of carbachol, whereby the electrically stimulated response was affected more than direct muscarinic receptor activation. This could be interpreted as a denervation of the muscle tissue. In addition, the contractions elicited by ABMA or KCl were reduced to an equivalent extent as that to EFS. This implies that an additional activating pathway common to ABMA and KCl was also attenuated, namely membrane L-type Ca 2+ channels (44). A reduction of L-type Ca 2+ channel density in detrusor from obstructed adult human bladders has been previously reported, and is consistent with this explanation (45). Very recently, Levin and colleagues (36) reported results of short-term (three to five days) ovine BOO at 90 days gestation in a protocol involving placement of a urethral ring and urachal catheterization. The obstructed detrusor muscle produced a hypocontractile response to EFS, but the absolute change from normal was small. However, no reduction in contractile response was observed with ATP, carbachol and KCl. Furthermore, the protocol did not result in a significant increase of bladder weight, and no comment was made about the state of the upper urinary tract, including kidney histology. The results suggest that the model used by Levin et al (36) constitutes a less severe BOO than that used in our current study. The developing rabbit bladder has also been studied after BOO. Rohrmann et al (35), in their model of fetal lagomorphic BOO (by urethral ligation at day 23 of a 31 day gestation), recorded reduction in the contractility of obstructed bladder strips to EFS; however, there was an increased response to bethanechol and KCl suggesting a reduced innervation with a heightened reaction to stimuli. Filling cystometry showed that compared with sham bladders, the obstructed bladders had increased capacity, six-fold, to 61 ml for the same low pressure, 12 cm H2O, as the sham group. Further, during the initial stages of filling, the obstructed bladder had a ten-fold increase in ‘unfolding volume’ of approximately 5 ml. After this volume, steady-state pressure increased approximately linearly with volume and stress-relaxation was observed during intermittent filling. Pressure versus volume plots demonstrated an increased compliance (DV/DP) in the obstructed bladder that was mirrored as a decreased elasticity in the wall stress versus volume plots. These observations are consistent with a large, flaccid organ that, not surprisingly, is

consistent with the electrophysiology results showing hypocontractility. It is of interest that the wall-stress versus volume plots showed that both the sham and obstructed bladders attained elastic limits but in obstructed bladders, it was lower and was achieved at a considerably greater volume. It may be proposed, therefore, that exceeding the elastic limit may result in irreversible changes to the properties of the bladder wall, although this will occur at greatly different volumes in the normal and post-obstructed bladder. This may have occurred in the obstructed bladders in utero either chronically throughout the obstructive period or following the acute onset of BOO, and may have contributed to the hypocontractility observed. It is important to emphasise that these elastic properties were obtained from bladders with no neuronal input and thus reflect the physical properties of the bladder wall. Interestingly, Peters et al (35) also observed stress-relaxation during cystometry in their obstructed ovine fetal bladders. They noted that stress-relaxation was delayed in obstruction and they suggested this was due to decreased compliance of the bladder wall. Whilst there were similarities in the experimental model, the difference in results may be explained by differences in cystometry methodology and in the methodological derivation of bladder wall compliance. The estimate of half-relaxation time used by Peters et al (35) was a feature of the viscous elements of the bladder wall; however, this current study assessed bladder wall compliance by considering the elastic, rather than viscous, elements. Analysis of key neuronal proteins showed how denervation contributed to the hypocontractility in the obstructed bladder. Immunohistochemistry showed a marked reduction in the obstructed bladders in staining of neuronal proteins, S100 and PGP 9.5, as compared to sham bladders. Western blot confirmed this reduction in PGP 9.5 to be statistically significant. Peters et al (35) noted an increase in cholinergic receptor number (when normalized to smooth muscle cell number) in their obstructed fetal sheep model; a finding consistent with increased accumulation of muscarinic receptors in denervated muscle, as originally described by Brockes and Hall (47). Bladder innervation is decreased in response to outlet obstruction in the adult model in other species. A reduction of cholinergic and adrenergic innervation of human obstructed bladders was observed by Gosling et al (48); Malkowicz et al (49) found a similar reduction in the obstructed rabbit. Cumming (50) found that infravesical obstruction in human adults caused a significant decrease in detrusor innervation compared to

controls. In addition, following relief of obstruction, there was an increase in innervation back to pre-obstruction levels; explained by axonal impairment without neuronal cell body damage, thereby leaving the potential for axonal regeneration. Collectively, by functional, anatomical and molecular analyses, we have described that the obstructed fetal bladder becomes denervated resulting in a hypocontractile bladder. It is possible that other factors contribute to this hypocontractility. A change in detrusor muscle composition may occur as described by Peters et al (51) who found increased smooth muscle cell myosin with maintenance of fetal ratios of myosin heavy chain isoforms following BOO in ovine fetuses. Connective tissue elements may be important; we have observed an increase in connective tissue around obstructed muscle bundles (see results) and Peters et al (52) found increased extra cellular matrix with a decrease in collagen degradation in obstructed fetal sheep bladders. In addition, more speculatively, hypoxia may have a role in causing hypocontractility; but as far as we know, investigators have only studied adult models (53,54).

6.3. Comparison with human fetal BOO

As reviewed in the introductory section, urodynamic data collected postnatally suggests that the human bladder undergoes a temporal change in response to fetal BOO. In general, PUV bladders are small, thick-walled and overactive around the time of valve resection in the first months of postnatal life. Over the next decade, bladder capacity can increase, greater than expected for age, and eventually the detrusor fails, leaving a large, atonic organ (55,56,57). However, PUV are a clinical spectrum, with a subset of affected boys having dilated, poorly-functioning bladders at birth (57); it could be speculated that such patients have more severe degrees of fetal BOO. The fetal sheep model described in the current study is associated with a large and hypocontractile bladder prior to term. We therefore speculate that this model represents the severe end of the human PUV spectrum.

7. Conclusion

1. We were able to develop a reproducible fetal animal model for investigating of developmental, physiological and histological changes of partial infravesical obstruction as a model of posterior urethral valve.
2. We were capable to monitor prenatally sham operated and obstructed fetuses with US and we could record obvious difference between them. In addition we registered a relatively fast effect to urinary outflow obstruction.
3. We documented a significant improvement of bladder compliance and calculated bladder wall tension was found less by the same amount of intravesical volume in obstructed fetuses compared to control ones.
4. We characterised the electromechanical properties of sham and obstructed fetal bladders and we found that obstructed bladder strips were hypocontractile exposed to both electrical and pharmacological stimulation. However, the electrically stimulated response was affected more than direct muscarinic receptor activation. This should be interpreted that the obstructed fetal bladder becomes denervated.
5. With immunohistochemistry we could proved a marked reduction in the obstructed bladders in staining of neuronal proteins, S100 and PGP 9.5 as compared to sham.

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10. Summary

Congenital urinary bladder outflow obstruction (BOO) caused by posterior urethral valves is a common cause of end-stage renal failure in boys. We hypothesised that fetal BOO perturbs detrusor contractility and innervation and bladder storage volume-pressure relationships. Severe BOO was induced in male fetal sheep by placement of a urethral ring and urachal ligation midway through gestation, at 75 days. These fetuses were examined 30 days after surgery when urinary tract dilatation, enlarged urinary bladders and cystic renal dysplasia were documented.

As assessed by force-frequency relationships, obstructed fetal bladder strips were significantly hypocontractile versus sham-operated controls in response to electrical field stimulation and specific agonists carbachol, KCl and a-β methylene-ATP. Hypocontractility was greater with nerve-mediated stimulation than with carbachol, suggesting denervation. Reduced innervation was confirmed by S100 and PGP 9.5 immunohistochemistry, and by measuring a significant reduction in PGP 9.5 protein expression using western blotting. In addition, ex vivo filling cystometry established that the obstructed fetal bladders were more compliant, with a larger capacity, more flaccid and retained stress relaxation. Hence, in response to severe experimental fetal BOO, the urinary bladder becomes hypocontractile, and this is associated with aberrant innervation.

10. Összefoglalás

Fiúkban a hátsó húgycső billentyű okozta veleszületett alsó húgyúti szűkület a leggyakoribb oka a végstádiumú veseelégtelenségének. Hipotézisünk az, hogy az intrauterin kialakult alsó húgyúti obstrukció gátolja a hólyag detrusor simaizom összehúzódási képességét, beidegzését és a hólyag térfogat- nyomás kapcsolatát.

Hím bárány magzatokban az urachus elzárásával és a húgycső, gyűrűvel való szűkítésével súlyos fokú alsó húgyúti obstrukciót hoztak létre a 75. intrauterin napon. A műtétet követő 30. napon húgyúti tágulatot, megnagyobbodott húgyhólyagot és cystás vese elégtelenséget találtak. Az obstruált magzatok húgyhólyag simaizom nyalábjai elektromos ingerlésre, specifikus agonisták jelenlétében, mint carbachol, KCl és α-β metilén-ATP az erő-frekvencia kapcsolatában szignifikáns hipokontraktilitást mutatott a sham operált kontroll állatokéhoz képest. Az összehúzódási képesség csökkenése az ideg közvetített ingerlésre nagyobb mértékű volt, mint a carbachol hatására kiváltotté, mely beidegzési sérülést sugall. Immunohisztokémiai vizsgálatok, mint az S100 és a PGP 9.5, valamint a western blott módszerrel vizsgált PGP 9.5 protein kiválasztódás szignifikáns kisebb mennyisége a denervációt csökkenését igazolták. Továbbá az alsó húgyúti szűkített magzati húgyhólyagok az ex vivo töltéses cystometriás vizsgálaton növekedett kapacitást, fokozott petyhüdtséget, emelkedett compliancet és megőrzött stressz relaxációt mutatott. Így a súlyos alsó húgyúti szűkület a húgyhólyagot hipokontraktilissé teszi, és beidegzése rendellenessé válik.

11. Articles and chapter connected to Ph.D. thesis

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