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Conscious voiding during bladder obstruction in guinea pigs correlates with contractile activity of isolated bladders

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ABSTRACT

Purpose: There are many hypotheses accounting for detrusor overactivity, however the exact mechanisms are still incompletely understood. We used a model of bladder outlet obstruction in male guinea pigs as a way to produce detrusor overactivity. The objective was to determine whether changes in voiding of obstructed guinea pigs correlates with specific changes in contractile activity of their isolated bladders *in vitro*.
Material and methods: Conscious voiding activity of sham-operated and obstructed animals was measured in metabolic cages. Contractile activity (spontaneous or evoked by distension, electrical field stimulation or cholinergic agonists) was recorded via a pressure transducer in the isolated bladders *in vitro*.
Results: The frequency of conscious voiding increased (while voiding volume decreased) in the obstructed group, compared with the sham-operated group, 4 weeks after surgical intervention. In comparison to the sham-operated animals, the bladders from the obstructed guinea pigs were enlarged and inflamed, their frequency of spontaneous contractions was higher, while the amplitudes of electrical field stimulation (EFS)-induced contractions and bladder compliance were lower. Changes in conscious voiding during obstruction were significantly associated with alterations in structural parameters (bladder weight, thickness and histological damage score) and functional contractile parameters (frequency of spontaneous contractions, amplitude of EFS-induced contractions and bladder compliance) of their isolated bladders.
Conclusions: Our findings revealed significant association between conscious voiding and structural and contractile activity changes of the isolated bladders in obstruction. The data suggest that change in contractile activity of the bladder itself is a major contributor to obstruction-induced bladder overactivity.

Keywords: bladder, obstruction, overactivity, contraction, smooth muscle

1. Introduction

Bladder outlet obstruction is a common problem for the aging men. In most cases it caused by enlarged prostate gland due to benign prostatic hyperplasia, results in the development of lower urinary tract symptoms (Nordling, 2002; Roehrborn, 2011). Patients with bladder outflow obstruction suffer from storage dysfunction, with symptoms similar to overactive bladder syndrome (OAB, such as urgency, frequency, nocturia and in some cases, urge incontinence) as well as voiding dysfunction, with symptoms such as straining, intermittency, dribbling, incomplete bladder emptying and a weak urinary stream (Levin et al., 2000; Nordling, 2002; Roehrborn, 2011). Recorded in human urodynamic diagnostic studies, detrusor overactivity (DO, i.e. non-voiding involuntary detrusor contractions during urine storage phase) is usually responsible for OAB with urge incontinence. In males, bladder outlet obstruction is the most likely cause of DO (Nordling, 2002; Mirone et al., 2007; Oelke et al., 2008).

The partially obstructed animal bladder, caused by mechanical obstruction induced by narrowing of the urethra, is a well-established model of the bladder outlet obstruction in men with benign prostatic hyperplasia and is also widely used as model for DO (Mirone et al., 2007; Parsons and Drake, 2011). As for benign prostatic hyperplasia patients, the resulting changes in urodynamic function in animal models of bladder obstruction depend on the extent and duration of obstruction. They range from bladder overactivity to bladder underactivity. In human patients with bladder outflow obstruction, as well as in animal models, bladder dysfunction develops through several stages: (i) an increase in bladder mass accompanied by smooth muscle hypertrophy, urothelial and fibroblast proliferation; (ii) a compensatory phase where bladder mass stabilizes, pressure either remains normal or increases; (iii) a decompensated fibrotic, high pressure bladder with progressive inability to empty (Levin et al., 2000; Metcalfe et al., 2010). In the early stages of bladder outlet obstruction, partially obstructed animal bladder models closely mimic human DO and OAB. Notwithstanding the interspecies variability in morphological and functional properties of the bladder, the increases in spontaneous myogenic activity, non-voiding contractions and voiding frequency have been demonstrated in many species (Sibley, 1987; Mostwin et al., 1991; Igawa et al., 1994; Drake et al., 2003; Kubota et al., 2008; Baker et al., 2010).

The mechanisms of DO and OAB symptoms such as urgency and frequency are still poorly understood. Several hypotheses have been put forward, including neurogenic, myogenic and autonomous bladder hypotheses which are not mutually exclusive. The neurogenic theory proposes that changes in the CNS micturition pathways including damage of descending inhibitory pathways, enhanced excitatory transmission in micturition reflex pathways, and/or sensitisation of bladder afferents could be involved (de Groat, 1997). The myogenic hypothesis proposes that detrusor muscle itself becomes more excitable and more spontaneously active (Brading, 1997). The overlapping autonomous (integrative) bladder hypothesis suggests that increased local micromotions distort small regions of the bladder wall prior to micturition, over-stimulating afferent nerves and thus giving rise to an increase sensation of urgency (Coolsaet et al., 1993; Drake et al., 2001).

It has been established in human studies that 74% of patients with urgency and urgency urinary incontinence (OAB wet) had DO (Hashim and Abrams, 2006) and symptoms of urgency incontinence strongly correlate with DO (Hyman et al., 2001). However, non-voiding contractions during storage phase have been recorded during ambulatory urodynamics in 38% of healthy volunteers and only few described urgency associated with the detrusor spontaneous activity (Robertson, 1999). In addition, no correlation was found between amplitude of involuntary detrusor contractions and subjective report of urgency in patients with OAB symptoms (Romanzi et al., 2001). DO is a common (50-75%) urodynamic occurrence in patients with bladder outflow obstruction due to benign prostatic hyperplasia (Robertson, 1999; Nordling, 2002; Oelke et al., 2008). It is still not completely understood which particular changes in contractile activity of the bladder during urine storage phase could be responsible for OAB symptoms in patients with bladder outflow obstruction. So far, this important question has not been addressed in animal models of bladder outlet obstruction. In the present study, we have used a model of gradual bladder obstruction (Mostwin et al., 1991) where a loose ring was placed around the proximal urethra of immature male guinea pigs and obstruction developed gradually over four weeks, as the guinea pig matured. We have compared conscious voiding pattern in the sham-operated and obstructed groups of guinea pigs and then have measured spontaneous, distension-, electrical field stimulation (EFS)- and cholinergic agonist-induced contractile activity of isolated bladders taken from the same animals. The main aim of the study was to determine whether changes in conscious voiding observed in obstructed guinea pigs correlates with specific changes in contractile activity of their isolated bladders *in vitro*.

2. Materials and methods

2.1 Bladder obstruction procedure

We used slightly modified method of gradual partial bladder outflow obstruction described in male guinea pigs (Mostwin et al., 1991). Briefly, under isoflurane (2%) anaesthesia, after locating distal bladder neck, a polyethylene catheter (1.52 mm external diameter) was used as a spacer, and placed alongside the junction of distal bladder neck and proximal urethra of the immature (around 5 weeks of age) male guinea pigs. A 3.0 silk thread was tied around both the junction and the catheter, giving a loop with a total diameter of around 2 mm. Then the catheter was removed, leaving the loop in place. Note that this procedure did not narrow the proximal urethra at the time of operation. This allows obstruction to develop gradually as the animal matures over 4 weeks. To determine whether damage to the bladder neck during surgical intervention contributed to changes in voiding, a control group of sham-operated animals underwent the same surgical operation and dissection as obstructed group, but with the ligature removed before closing the abdomen and skin. The weight of animals in the obstructed and sham-operated groups were not different (241 ± 3.2 g, n=35 and 247 ± 3.8 g, n=34, respectively) prior to surgery. Control untreated animals were matched by age (around 9 weeks) to the obstructed and sham-operated groups. All experimental procedures undertaken in this study were approved by the Animal Welfare Committee of Flinders University.

2.2 Conscious voiding in a metabolic cage

Spontaneous voiding of the sham-operated and obstructed guinea pigs was measured in metabolic cages over a 6 hour period under the lights prior to operation and 1-4 weeks after. Sham-operated guinea pigs served as a control to obstructed animals. No conscious voiding measurements were performed for the age-matched untreated control guinea pigs. The voided urine was collected in a cup connected to a force transducer (Grass Force-displacement transducer FT03, Grass Instruments, Quincy, Mass, USA) for measurement of volume and frequency of conscious micturition. Since specific gravity of the guinea pig urine is 1.015 (Hawkins et al., 2009), for volume measurement we assumed that 1g = 1ml for the sham-operated and obstructed groups.

2.4 Whole bladder in vitro

The day following measurements of conscious voiding the bladder was removed from the humanely killed animals in both the obstructed and sham-operated groups. Both ureters were ligated and a 2 mm o.d. stainless steel cannula was inserted into the urethra and tied securely in place. The bladder was flushed gently 2-3 times with Krebs solution and placed in an organ bath (150 ml volume) containing Krebs solution of following composition (in mM): NaCl, 118; KCl, 4.75; NaH_2PO4, 1.0; $NaHCO_3$, 25; $MgSO_4$, 1.2; $CaCl_2$, 2.5; glucose, 11; bubbled with 95 % O_2 - 5 % CO_2 and maintained at around 37° C. The cannula was attached to a T-piece adaptor, the left arm of which was connected to a syringe pump (SP200, WPI, USA) to allow the slow infusion of Krebs solution. The right arm of the T-piece was connected to a pressure transducer (Viggo-Spectramed model P23XL, Oxnard, CA, USA). Intravesical pressure was recorded on a Maclab/8s data acquisition system with Chart 7 software (ADInstruments, Castle Hill, NSW, Australia) using an iMac computer running OSX 10.7.1. To record spontaneous contractile activity, all isolated bladders were slightly pre-distended with 0.75 ml of Krebs solution (Zagorodnyuk et al., 2009) and bladders were allowed to equilibrate for at least 30 minutes before experiments. Bladder distensions (at 0.5 ml/min) were performed 4-5 times at 15 minutes interval until two last responses were similar (Zagorodnyuk et al., 2009). In the control and sham-operated groups, 2.2 ml infusions were performed, followed by manual emptying of the 2.2 ml of infusate, before the next infusion. So, in most cases, maximal intravesical volume was ~ 3ml (including residual 0.75ml volume) which was slightly less that average voiding volume (3.56 ± 0.24 ml, n=32) measured in control conscious guinea pigs. Using this protocol, minimal change in basal pressure during bladder distension was seen in control guinea pigs (see Fig. 2A). In 30% cases in the obstructed group, the infusion was stopped earlier at 0.5-1.4 ml since baseline pressures exceeded 20-50 cmH_2O. In all cases, bladder compliance *in vitro* was calculated as change in bladder volume (i.e. infused volume) divided by corresponding change in basal bladder pressure (in ml/cmH_2O).

The amplitude, frequency and area under the curve (AUC) of each individual phasic contractions were measured. A single phasic contraction was defined as a contraction with amplitude greater than 0.1 cmH_2O that returned to the baseline level. Since both frequency and amplitude of spontaneous contractions were increased with bladder distension, only AUC was calculated for phasic distension-evoked contractions *in vitro*. In majority of preparations, the frequency response curve, in response to EFS, was constructed by repetitive stimulation

(1-30 Hz for 3 s, 0.15 ms, 100V) applied via two platinum plates placed on either side of the bladder. Usually the bladder responses to a second frequency response curve, generated after an interval of 15 min, were similar to the first one; if not, a 3rd curve was constructed. In some preparations, bethanechol or physostigmine concentration-response curves, measured as AUC in units of $cmH_2O.s$ during 3 min periods at the end of each concentration-response, were obtained 30 min after EFS.

2.5 Alterations at histological level

At the conclusion of organ bath experiments, bladders were opened, weighed and transferred to a Petri dish, containing phosphate-buffered saline, where they were maximally stretched and pinned around the perimeter as an open, flat sheet. After overnight fixation in 4% paraformaldehyde, a small strip (~5x30 mm) of full thickness bladder wall was taken at the medial axis of the posterior bladder wall, extending just above proximal urethra and up to the bladder apex. The strip was embedded in Paraplast paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Photomicrographs were taken using a QImaging RTV 5 megapixel digital camera with an Olympus BX50 brightfield microscope using x4 or x40 objectives. The thickness of the mucosa (including urothelium and lamina propria), smooth muscle and adventitia layers of the bladders were calculated in three randomly selected fields from the middle part of the section from each guinea pig. Mean histological damage score was calculated as sum of scores for infiltration of white blood cells (WBCs), and presence of small hemorrhages (tissue red blood cells, RBCs) in the connective tissue of the lamina propria, smooth muscle and adventitia and for bladder edema in the lamina propria. The severity of lesions in the urinary bladder, analysed by a blinded observer using the x40 objective, was graded as followed: infiltration of WBCs defined as 0 (0-2 cells), 1 (2-10 cells) as mild, 2 (>10) as severe; presence of RBCs defined as 0 (0 cells), 1 (a few cells) as mild, 2 (abundant cells in the tissue) as significant; edema score: 0 (no), 1 (yes).

2.6 Drugs

Physostigmine (eserine) hemisulfate, betanechol, hyoscine [(-) scopolamine] hydrobromide, tetrodotoxin and PPADS were obtained from Sigma (St. Louis, MO, USA).

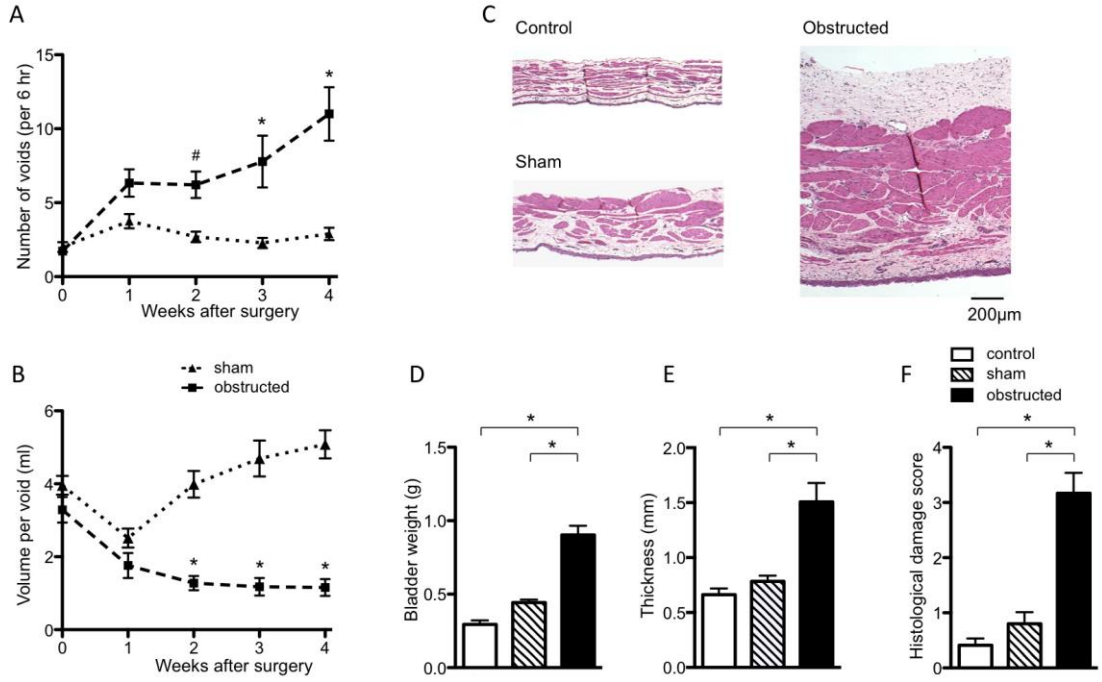
2.7 Data analysis

Results are expressed throughout as means ± standard error of the mean, with n referring to the number of animals. Statistical analysis was performed by analysis of variance (one way ANOVA) using Prism v.6 software (GraphPad Software, Inc., San Diego, CA, USA). To see whether any parameters of contractile activity correlate with each other and with conscious voiding, a series of partial correlations, factor analyses and discriminant analyses (Norusis, 1993; Tabachnick and Fidell, 1996) were carried out with SPSS v19. Differences were considered significant if $P<0.05$.

3. Results

3.1 Conscious voiding

Fig. 1



The voiding frequency of the obstructed guinea pigs (n=19) was significantly greater than control measurements taken from the same animals before surgery. When compared with the sham-operated animals, frequency of voiding was also significantly higher at 2, 3 and 4 weeks following obstruction. For example, at week 4, the number of voids during the 6 hour measuring period in the obstructed group was significantly higher than in the sham-operated group (11.0 ± 1.81, n=15 and 2.89 ± 0.42, n=19, P<0.0001 two way ANOVA, Tukey post test) (Fig. 1A). In contrast, voiding frequency of the sham-operated group was slightly increased only at the 1st week post surgery and then returned to control levels in subsequent weeks (Fig. 1A). The voiding volume was inversely proportional to the voiding frequency, decreasing significantly in the obstructed guinea pigs each week following obstruction. Conversely, in the sham group, a decrease was only seen in the 1st week after operation and by the 2nd week voiding volume recovered to the levels recorded before the sham operation (Fig. 1B). When compared to the sham group, voiding volume of the obstructed guinea pigs was significantly smaller at 2, 3 and 4 weeks following obstruction. For example, at week 4, the mean volume per void in the obstructed group was significantly smaller than shams (1.16 ± 0.23, n=15 and 5.08 ± 0.39, n=19, respectively, P<0.0001 two way ANOVA, Tukey post test) (Fig. 1B). It is worth mentioning that total voiding volume during the 6 hours period was not significantly different between sham and obstructed group in any week after surgery (eg: 13.1 ± 1.66, n=19 and 10.0 ± 1.37, n=15, NS, two way ANOVA, Tukey post test at week 4, comparing sham and obstructed groups).

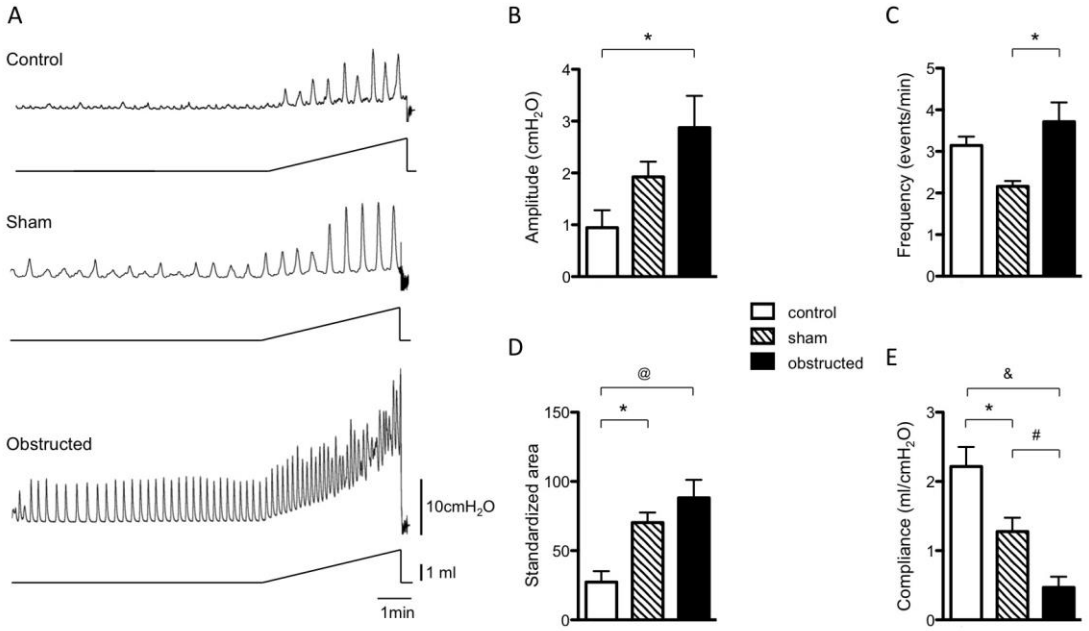
3.2 Changes in bladder weight and histology in obstruction

Bladders from the obstructed group (n=19) were significantly heavier (0.90 ± 0.06 g, n=19) than those from both the sham-operated group (0.44 ± 0.02, n=21, P<0.0001, one way ANOVA, Tukey post test) and untreated control group (0.29 ± 0.03, n=13, P<0.0001, one way ANOVA, Tukey post test) (Fig. 1D). Hematoxylin and eosin staining revealed significant muscle hypertrophy and hyperplasia of the adventitia in the obstructed group compared with shams and controls. Detrusor muscle thickness in the obstructed group (0.70 ± 0.05 mm, n=18) was significantly greater than that of shams (0.47 ± 0.03 mm, n=20, P<0.0001, one way ANOVA, Tukey post test) or controls (0.40 ± 0.02 mm, n=18, P<0.0001, one way ANOVA, Tukey post test). Similarly, the thickness of adventitia in the obstructed group (0.61 ± 0.14 mm, n=18) was significantly greater than that of shams (0.16 ± 0.02 mm, n=20, P<0.001, one way ANOVA, Tukey post test) or controls (0.14 ± 0.02 mm, n=18, P<0.0001, one way ANOVA, Tukey post test). There was no significant difference in the thickness of the mucosa between obstructed, sham and control groups (0.20 ± 0.02 mm, n=18, 0.15 ± 0.01 mm, n=20 and 0.16 ± 0.02 mm, n=18, respectively). Overall, the bladder wall was thicker in the obstructed group (1.51 ± 0.17 mm, n=18) than in shams (0.79 ± 0.05 mm, n=20, P<0.0001, one way ANOVA, Tukey post test) or controls (0.66 ± 0.06 mm, n=18, P<0.0001, one way ANOVA, Tukey post test) (Fig. 1E). Histological damage score (which includes infiltration of leukocytes in the bladder wall, edema and tissue red blood cells) in the obstructed group (3.17 ± 0.37, n=18) was also significantly higher than in shams (0.8 ± 0.21, n=20, P<0.0001, one way ANOVA, Tukey post test) or control groups (0.41 ± 0.12 (n=18, P<0.0001, one way ANOVA, Tukey post test) (Fig. 1F).

3.3 Spontaneous and distension-induced contractile activity in vitro

The frequency of spontaneous contractions of the bladder did not differ significantly between the age-matched control and sham-operated groups (Fig. 2A,C). The frequency of spontaneous contractions was higher in obstructed preparations (3.72 ± 0.46 events per min, n=19) when compared to the sham-operated animals (2.16 ± 0.13, n=21, P<0.01) but not compared to controls (3.14 ± 0.21, NS, n=18) (Fig. 2C). The area under the curve (AUC) of spontaneous phasic contractions was greater in obstructed preparations (278 ± 62.6 $cmH_2O.s$, n=19) than in the control group (111.3 ± 31.2 $cmH_2O.s$, n=18, P<0.05, one way ANOVA, Tukey post test) but not sham-operated animals (224.1 ± 30.7, NS, n=21). Similarly, the amplitude of spontaneous contractions was greater in obstructed preparations (2.88 ± 0.61 cmH_2O, n=19) when compared to the control group (0.95 ± 0.34, n=18, P<0.01, one way ANOVA, Tukey post test) but not the sham-operated animals (1.93 ± 0.29, NS, n=21) (Fig. 2B). The standardized amplitude (to the thickness of detrusor muscle layer) of spontaneous contractions was not different between the three groups (control: 2.94 ± 1.23 cmH_2O/mm, n=18; sham: 4.34 ± 0.72 cmH_2O/mm, n=21; obstructed: 4.17 ± 0.78 cmH_2O cmH_2O/mm, n=19).

Fig. 2

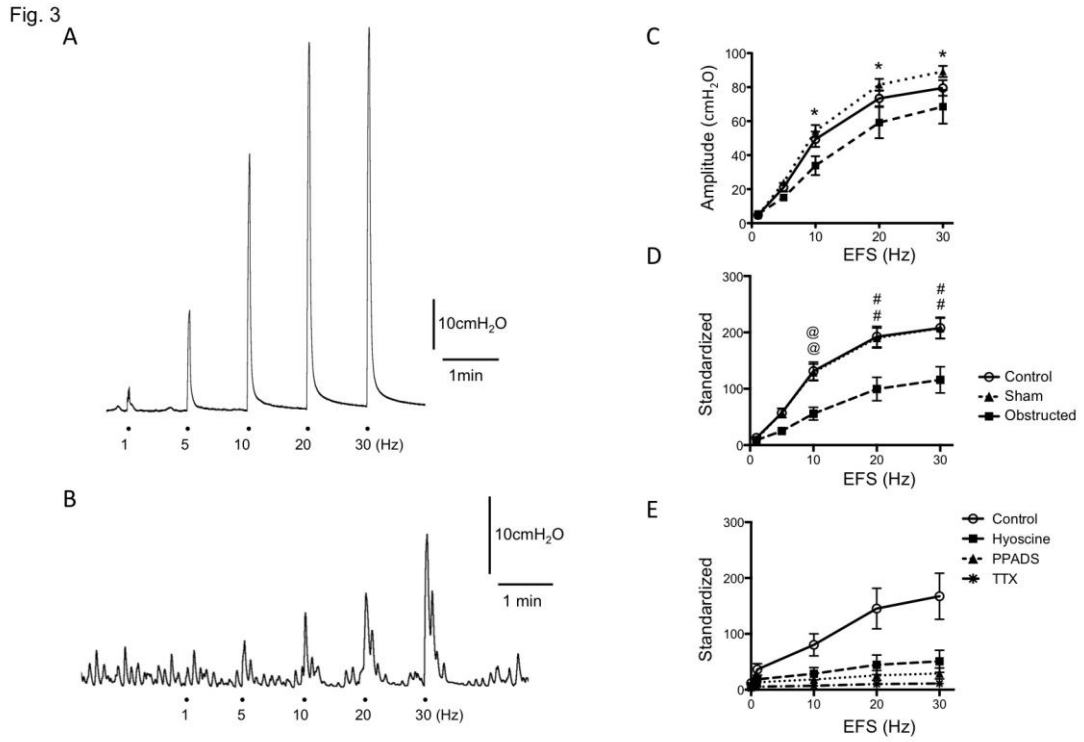


Slow ramp distension of the bladder by 2.2 ml evoked an increase in active phasic contractions (Fig. 2A). Overall, no significant difference was seen in the AUC (standardized to the duration of bladder distention in minutes) of distension-induced phasic contractions between the obstructed and sham groups, but AUCs for both the obstructed (88.2 ±12.9 cmH_2O.s/min, n=17, P<0.001) and the sham (70.3 ± 7.35 cmH_2O.s/min, n=21, P<0.01) groups were significantly greater than the control group (27.3 ± 7.91 cmH_2O.s/min, n=14) (Fig. 2D). Bladder compliance was significantly lower in obstructed bladders (0.47 ± 0.15 ml/cmH_2O, n=17) compared to both the sham-operated group (1.28 ± 0.19 ml/cmH_2O, n=21, P<0.05, one way ANOVA, Tukey post test) and the control group (2.21 ± 0.28 ml/cmH_2O, n=14, P<0.0001, one way ANOVA, Tukey post test). Bladder compliance was also significantly lower in the sham-operated group (n=21) compared with control group (n=14, P<0.01, one way ANOVA, Tukey post test) (Fig. 2E).

3.4 Electrical field stimulation of intramural nerves in vitro

The response of the isolated control bladders, *in vitro*, to repetitive EFS (1-30Hz) plateaued at about 30 Hz (Fig. 3A). EFS-induced responses in the sham group at 30Hz (89.3 ± 3.28 cmH_2O, n=20) were not different from age-matched controls (79.5 ± 4.58 cmH_2O, n=18) but were significantly higher than those seen in the obstructed group (68.5 ± 9.93 cmH_2O, n=18, P<0.01, two way ANOVA, Tukey post test) (Fig. 3A-C). In order to exclude the effects of hypertrophy (i.e. a greater muscle mass), we standardized the EFS-induced contractions to the thickness of the detrusor muscle layer. The standardized amplitude of responses of the bladders to EFS (30 Hz) in the obstructed group was significantly lower (116 ± 23.5 cmH_2O/mm, n=17) than both the sham (208 ± 18.1 cmH_2O/mm, n=19, P<0.0001, two way

ANOVA, Tukey post test) and control groups (208 ± 19.1 cmH_2O/mm, n=17, P< 0.0001, two way ANOVA, Tukey post test) (Fig.3D).



The EFS-evoked responses in the guinea pig isolated bladders have both cholinergic and purinergic components. The obstructed, sham-operated and control bladder did not differ in the relative contribution of either the cholinergic component, assessed after application of 3 µM hyoscine: 27 ± 4%, n=7, 28 ± 6%, n=5, and 27 ± 5%, n= 6, respectively (NS, one way ANOVA, Tukey post test) or the purinergic components, assessed after consecutive application of 30 µM PPADS: 17 ± 2%, n=7, 11 ± 3%, n=5, and 13 ± 3%, n= 6, respectively (NS, one way ANOVA, Tukey post test) (Fig. 3E). EFS-induced responses were abolished by tetrodotoxin (0.6 µM), leaving only very small contractions in response to high frequency stimulation (Fig. 3E).

3.5 Multiple correlation analysis of in vitro parameters in control, sham and obstructed groups

Multiple correlation analysis (partial correlations) was performed between the major *in vitro* parameters including frequency, amplitude and AUC of spontaneous and distension-induced phasic contractile activity, bladder compliance, amplitude of the EFS-induced responses at 30Hz, bladder weight, total thickness and histological damage score in three experimental groups, treating all data as one correlation matrix (all nine parameters were measured in 13 control, 19 sham and 14 obstructed bladders, total n=46). There were numerous significant correlations between the parameters (Table I). Some were unsurprising such as a high correlation between the amplitude of spontaneous contractions and the AUC of spontaneous

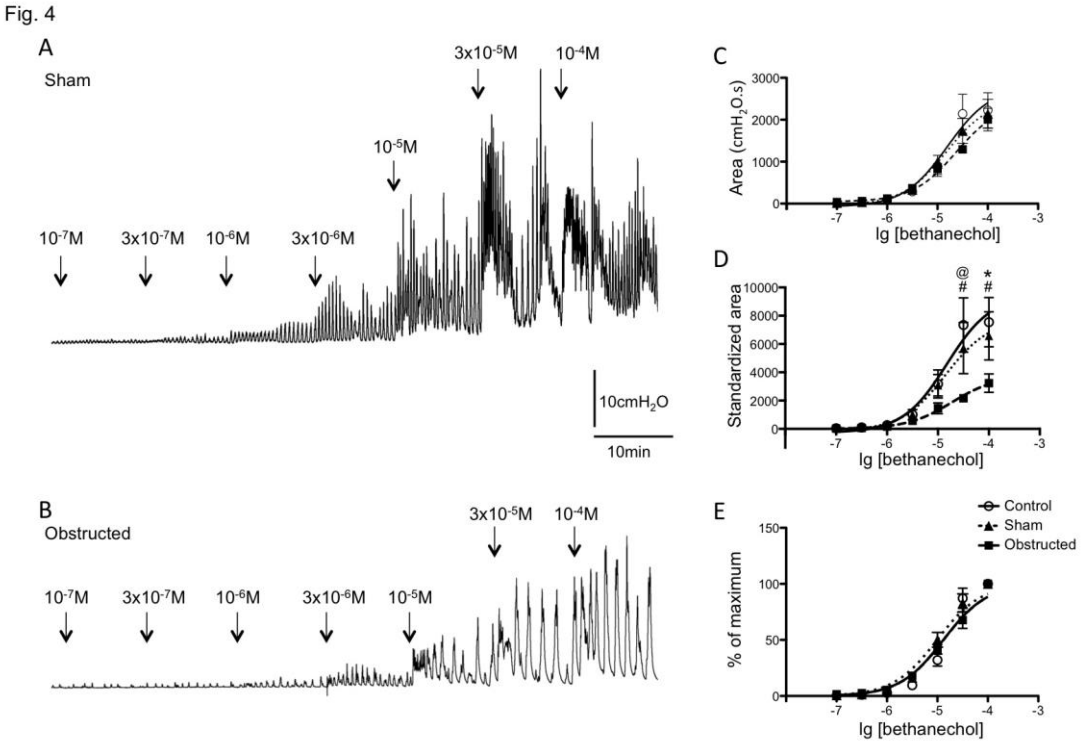
contractions (r=0.96, P<0.0001), AUC of distension-induced activity (r=0.64, P<0.0001), total wall thickness (0.63, P<0.0001) and bladder weight (r=0.57, P<0.0001).

Parameters	Weight	EFS	Spontaneous frequency	Spontaneous amplitude	Spontaneous AUC	Distension AUC	Compliance	Thickness
EFS	**-0.37 (P<0.005)**							
Spontaneous frequency	**0.32 (P<0.05)**	**-0.53 (P<0.0001)**						
Spontaneous amplitude	**0.57 (P<0.0001)**	0.02 (P=0.46)	0.1 (P=0.24)					
Spontaneous AUC	**0.5 (P<0.0001)**	-0.01 (P=0.47)	0.18 (P=0.11)	**0.96 (P<0.0001)**				
Distension AUC	**0.53 (P<0.0001)**	-0.19 (P=0.45)	-0.1 (P=0.25)	**0.64 (P<0.0001)**	**0.58 (P<0.0001)**			
Compliance	**-0.53 (P<0.0001)**	0.04 (P=0.4)	-0.19 (P=0.11)	**-0.58 (P<0.0001)**	**-0.56 (P<0.0001)**	**-0.71 (P<0.0001)**		
Thickness	**0.73 (P<0.0001)**	-0.11 (P=0.23)	0.14 (P=0.17)	**0.63 (P<0.0001)**	**0.51 (P<0.0001)**	**0.59 (P<0.0001)**	**-0.4 (P<0.005)**	
Damage score	**0.68 (P<0.0001)**	-0.22 (P=0.07)	0.14 (P=0.19)	**0.45 (P<0.001)**	**0.39 (P<0.005)**	**0.49 (P<0.0001)**	**-0.47 (P<0.0001)**	**0.69 (P<0.0001)**

In addition, the amplitude of spontaneous contractions was correlated with histological damage score (r=0.45, P<0.001) and was inversely correlated with the bladder compliance (r=-0.58, P<0.0001). Interestingly, the frequency of spontaneous contractions was correlated only with the bladder weight (r=0.32, P<0.05) and inversely correlated with the amplitude of EFS-induced responses (r=-0.53, P<0.0001) (Table I). The latter, in turn, was weakly inversely correlated with the bladder weight (r=-0.37, P<0.005). Bladder weight correlated significantly with both total thickness (r=0.73, P<0.0001) and histological damage score (r=0.68, P<0.0001). Significant positive correlations were also seen between bladder weight, total wall thickness and histological damage score with the parameters of contractile activity such as the amplitude and AUC of spontaneous and distension-induced phasic contractions and inversely with bladder compliance (Table I).

Factor analysis, using principal components, showed a high covariance between structural and functional variables. Most variability could be explained by two components: component 1 accounted for 51% and component 2 explained 18% of total variance. Component 1 contained covariance between structural parameters and functional parameters of contractile activity (r=0.7-0.8) except for the amplitude of EFS-induced responses and the frequency of spontaneous activity. The latter two parameters were highly inversely correlated in component 2 with r=0.84 and r=-0.78, respectively. When similar multiple correlation analysis was performed separately for the three groups of animals, highly significant inverse covariance between the amplitude of EFS-induced responses and the frequency of spontaneous activity occurred only in the obstructed group (r=0.68 and r=-0.88, respectively, n=14). There was

weak or no significant covariance of contractile parameters with bladder weight, total thickness and histological damage score for the control (n=13) and sham-operated (n=19) groups when data were analysed separately for each group. More importantly, factor analysis for the obstructed group alone (n=14) showed similar high covariance between structural and functional parameters as to those found for all three groups combined.



We also carried out a discriminant analysis (Norusis, 1993; Tabachnick and Fidell, 1996) on three experimental groups (n=46) in order to see well the nine parameters recorded in this study could predict the experimental group to which individual preparation belonged. The analysis converged on two canonical discriminant functions that explained all the variance seen in three groups. The first canonical discriminant function (*F1*) explained 80.5% of the variance and the second function (*F2*) explained the remaining 19.5% of the variance. The classification results showed that the *in vitro* parameters can be used to predict correctly the animal group from which the preparations came (control group membership: 11/13 correct; sham group 19/19 correct; obstructed: 13/14 correct).

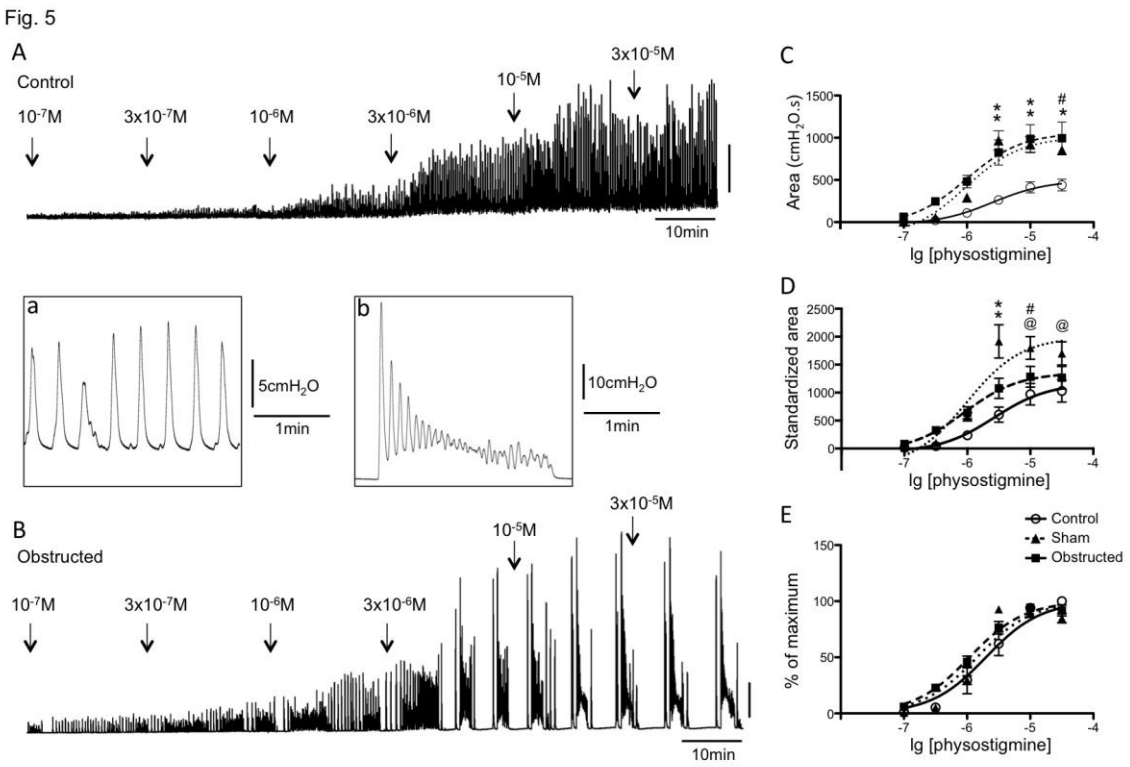
3.6 Effect of bethanechol and physostigmine in vitro

As shown above (subsection 3.4), EFS-induced responses in the obstructed group were significantly smaller compared with those in the sham and control groups. In a separate series of experiments, we investigated whether this was due to damage to autonomic nerves or was the result of altered postsynaptic mechanisms. In order to establish whether there is postsynaptic effect including cholinergic super-sensitivity or a decrease in acetylcholinesterase activity during obstruction, effects of the muscarinic agonist bethanechol

and physostigmine were compared in control, sham and obstructed groups. Bethanechol (10^{-7} - 10^{-4} M) evoked concentration-dependent contractions of whole bladders, increasing the amplitude of phasic contractions and elevating the baseline pressure, particularly at higher concentrations ($\geq 10^{-5}$ M) in all three groups of guinea pigs (Fig. 4). Concentration-responses curves were similar in all three groups without standardization (Fig. 4C). When responses were standardized to the thickness of the detrusor muscle, there was a significant decrease in the contractile response to bethanechol of the obstructed bladders at $3x10^{-5}$ M - 10^{-4} M (n=6), compared to sham (n=6, $P<0.05$ at 10^{-4} M) and control bladders (n=6, $P<0.01$ at 10^{-4} M, two way ANOVA, Tukey post test) groups (Fig. 4D). However, when contractile activity was normalized to the maximal contractile response (maximal contractile response, calculated as AUC, was achieved at 10^{-4} M for the control group and was 2293 ± 348 $cmH_2O.s$, n=6) no differences were detected. EC_{50} values for control [EC_{50}=14.2 µM (95% confidence intervals = 11.0 – 18.5 µM, n=6)], for sham [EC_{50}=10.4 µM (95% confidence intervals = 8.50 – 12.8 µM, n=6)] and for obstructed group [EC_{50}=13.3 µM (95% confidence intervals = 10.9 – 16.2 µM, n=6)] were not significantly different (one way ANOVA, Tukey post test) (Fig. 4E).

Physostigmine (10^{-7} - $3x10^{-5}$ M) evoked concentration-dependent contractions of isolated bladders. In controls, physostigmine (10^{-7} - $3x10^{-5}$ M) increased only the amplitude of phasic contractions (Fig. 5A and insert a). Maximal contractile response for physostigmine, calculated as AUC, was achieved at 10^{-4} M for the control group and was 440 ± 68 $cmH_2O.s$ (n=6). In contrast, in all preparations of the obstructed group, physostigmine ($\geq 3x10^{-6}$M), produced 2-3s bursts of contractile activity, consisting of an increase in baseline tension with superimposed phasic contractions (see Fig. 5B and insert b). In the sham-operated group, we observed a similar but less marked pattern: smaller bursts of contractions evoked by physostigmine ($\geq 3x10^{-6}$M) were observed in 5 out 8 preparations. There was significant increase in the contractile response to physostigmine for both obstructed (n=7, $P<0.001$ at 10^{-5} M, two way ANOVA, Tukey post test) and sham-operated bladders (n=8, $P<0.001$ at 10^{-5} M, two way ANOVA, Tukey post test) compared with control (n=6) at $3x10^{-6}$ M - $3x10^{-5}$ M (Fig. 5C). When standardized to detrusor muscle thickness, there was significant increase in overall contractile effect of physostigmine in the sham-operated group (n=8) compared with the control for $3x10^{-6}$ M - $3x10^{-5}$ M (n=6, $P<0.01$ at 10^{-5} M, two way ANOVA, Tukey post test) (Fig. 5D) and the obstructed group for $3x10^{-6}$ M - 10^{-5} M (n=7, $P<0.05$ at 10^{-5} M, two way ANOVA, Tukey post test). More importantly, physostigmine was slightly more potent in the obstructed group (EC_{50}=1.09 µM, 95% confidence intervals = 0.88 - 1.36 µM, n=7) compared with control (EC_{50}=2.0 µM, 95% confidence intervals = 1.38 - 2.88 µM, n=6, $P<0.05$, one way ANOVA, Tukey post test), when contractile activity was normalized to the maximal contractile response. The response in the sham-operated group was not different compared to the control or obstructed groups (EC_{50}=1.44 µM, 95% confidence intervals = 1.07 - 1.94 µM, n=8) (Fig. 5E). The effect of bethanechol and physostigmine on isolated bladders was not included in the multiple correlation analysis (see subsection 3.5) because sample size of these experiments was too small.

3.7 Multiple correlation analysis between frequency/volume of conscious voiding and contractile activity of isolated bladders in the sham and obstructed groups



We performed multiple correlation analysis between conscious voiding frequency/volume of the sham-operated (n=19) and obstructed animals (n=14) and the nine structural and functional parameters of isolated bladders recorded *in vitro* measured in the same guinea pigs (total n=33). As described above (subsection 3.5), significant correlations were revealed between various *in vitro* parameters (Table I). We also found significant correlations between *in vivo* voiding characteristics and some of *in vitro* parameters. As might be expected, there was significant inverse correlation between voiding frequency and voiding volume ($r=-0.57$, $P<0.0001$). Interestingly, average voiding volume correlated more strongly with *in vitro* parameters than the frequency of voiding. The voiding volume was significantly inversely correlated with wall thickness ($r=-0.46$, $P<0.003$), but there was only a weak correlation between the voiding frequency and wall thickness ($r=0.28$, $P<0.05$). There was a strong inverse correlation of the bladder weight with the voiding volume ($r=-0.62$, $P<0.0001$) and positive correlation with voiding frequency ($r=0.54$, $P<0.0001$). Both the voiding volume and voiding frequency were significantly correlated (negatively and positively) with histological damage score ($r=-0.52$, $P<0.001$ and $r=0.44$, $P<0.005$, respectively). In addition, there was significant inverse correlation between the voiding volume and the frequency of spontaneous contractions ($r=-0.39$, $P<0.01$). The voiding volume (or frequency) correlated directly (or inversely) with bladder compliance ($r=0.57$, $P<0.0001$ or $r=-0.33$, $P<0.05$, respectively). There was also weak correlation between the voiding volume and the amplitude of the EFS-induced responses ($r=0.33$, $P<0.05$). No other significant correlations were revealed with multiple correlation analysis of 11 (9 *in vitro* and 2 *in vivo*) parameters.

When all 11 variables were examined with factor analysis, using principal components, highly significant covariance was identified between *in vitro* and *in vivo* variables. When a

discriminant analysis was carried out (Norusis, 1993; Tabachnick and Fidell, 1996) on the sham-operated and obstructed groups (n=33), one canonical discriminant function (*F1*) could explain 100% of the variance. The classification results show that based on total 11 *in vitro* and *in vivo* parameters one can predict correctly 19/19 individual sham preparations and 13/14 individual obstructed preparations. This discriminant analysis strongly supports the results of the multiple correlation analysis and validates high correlation seen between *in vitro* and *in vivo* parameters.

4. Discussion

The present data revealed that changes in conscious voiding during obstruction were significantly associated with alterations in structural parameters (such as bladder weight, thickness and histological damage score) and functional contractile parameters (such as frequency of spontaneous contractions, amplitude of EFS-induced contractions and bladder compliance) of their isolated bladders. This strongly suggests that changes in contractile activity of the bladder itself contribute significantly to obstruction-induced bladder overactivity.

4.1 Bladder contractions in vitro and non-voiding contractions (NVCs) in vivo

Obstruction-induced increase in spontaneous bladder contractions *in vitro* as well as in NVCs and voiding frequency *in vivo* has been demonstrated in many species (Sibley, 1987; Mostwin et al., 1991; Igawa et al., 1994; Drake et al., 2003; Kubota et al., 2008; Baker et al., 2010). NVCs recorded *in vivo* during urine storage phase in animal models are widely used as a surrogate marker for DO in humans. In contrast to micturition contractions, NVCs are most likely of myogenic origin since tetrodotoxin failed to block them in rats and guinea pigs alike (Maggi et al., 1987; Igawa et al, 1994). Both the amplitude and frequency of NVCs recorded *in vivo* and spontaneous contractions recorded *in vitro* are increased with bladder filling in control guinea pigs (Drake et al., 2003; Zagorodnyuk et al., 2009; Biallosterski et al., 2011). In the present study, the amplitude of spontaneous contractions and AUC of distension-induced contractions of obstructed guinea pigs bladders *in vitro* were only slightly increased compared to shams. However, the frequency of spontaneous contractions was significantly greater. NVCs during urine storage phase in humans (i.e. DO) are more prevalent in bladder outlet obstruction patients compared with healthy volunteers, and their prevalence rise continuously with increasing grade of obstruction (Robertson, 1999; Nordling, 2002; Oelke et al., 2008). Localized NVCs were also more prevalent and sustained, and have a higher frequency in patients with urinary urgency compared to asymptomatic controls (Drake et al., 2005). Present data indicate that voiding volume in conscious obstructed guinea pigs was inversely correlated with frequency of spontaneous contractions of their isolated bladders. Thus our data suggest importance of the frequency, in addition to the amplitude, of NVCs in obstruction-induced bladder overactivity. Interestingly, in rats, the frequency of phasic contractions induced by distension of isolated obstructed bladders was lower compared to shams (Drake et al., 2003). This difference between rats and guinea pigs could be due to species, sex differences or to the amount of damage to the bladder neck area during surgery,

which would affect the degree of obstruction. Bladder outlet obstruction caused patchy denervation in the guinea pig bladder but not in rats (Gabella and Uvelius, 1990; de Jongh et al., 2009). In present study, cholinergic EFS-induced contractions were reduced in four weeks obstructed guinea pigs but were up-regulated in rats (Banks et al., 2005). This study revealed a strong inverse correlation between the frequency of spontaneous contractions and degree of damage to the autonomic nerves in the obstructed bladder. Taken together the data suggest that difference in changes of the frequency of bladder contractions in obstruction between these two species could be related to higher degree of obstruction-induced damage to autonomic nerves in the guinea pig bladder compared to rat.

It has been previously shown that sham operation itself changed contractile activity of isolated guinea pig bladders (de Jongh et al, 2007). Our results confirm this finding. In fact, both obstructed and sham-operated groups showed enhanced amplitude of spontaneous contractions, AUC of distension-induced contractions and decreased compliance compared to control untreated bladders. This indicates the importance of the bladder neck-proximal urethra region for normal bladder function: even slight damage to this region during sham surgical procedures significantly changes subsequent contractile behaviour of the isolated bladder.

4.2 EFS and effect of cholinergic agonists in obstruction

Electrical stimulation of the parasympathetic innervation of the bladder has both cholinergic and purinergic components; their relative contributions vary significantly between species. In healthy human bladder, the purinergic component is minor but is increased in obstruction (Bayliss et al., 1999), similar to the rabbit (Calvert et al., 2001). In the present study of guinea pigs, the relative contributions of the cholinergic and purinergic components did not change between control, sham and obstructed bladders. In contrast, in the obstructed rat bladder, the cholinergic component was up-regulated, while the purinergic component was unaffected (Banks et al., 2005).

Overall EFS-induced responses of the obstructed bladders were significantly smaller than those of the sham-operated animals, especially when standardized to the bladder wall thickness. This observation confirms previous findings that in animal models and in patients suffering from bladder outflow obstruction, there is damage to the autonomic innervation (Sibley, 1987; Williams et al., 1993; Brading, 1997; Levin et al., 2000; de Jongh et al., 2009). Oxidative stress due to ischemia followed by reperfusion, has been demonstrated in obstructed bladders and may underlie the changes in muscle function and patchy denervation of the detrusor (Conners et al., 2006; de Jongh et al., 2009; Scheepe et al., 2011). There was no inhibition of bethanechol-induced responses (up to 10^{-5} M) in obstructed guinea pig bladders compared to shams or controls. This indicates that a reduction in the amplitude of the EFS-induced responses in obstruction is rather due to presynaptic mechanisms, most likely reflecting damage to autonomic nerves within the detrusor.

In some studies in obstructed bladders from pigs and humans, super-sensitivity to cholinergic agonists has been described and loss of acetylcholinesterase activity has been postulated, since acetylcholine showed a higher increase in potency than the esterase-resistant agonist,

carbachol (Harrison et al., 1987; Sibley, 1987; Brading, 1997). In the present study supporting previous data from obstructed guinea pigs (Williams et al., 1993; de Jongh et al., 2009) and human patients (Bayliss et al., 1999), no super-sensitivity to stable cholinergic agonist was seen. Our data suggest a decrease in acetylcholinesterase activity in obstructed bladders compared with controls, revealed by the leftward shift and the higher peak value of the concentration-response curve for physostigmine, but not for the non-hydrolyzable muscarinic agonist, bethanechol. Interestingly, in control bladders, physostigmine increased only the amplitude of phasic contractions as previously reported (Zagorodnyuk et al., 2009). In sham, and especially, in obstructed bladders, physostigmine evoked repetitive and massive bursts of contractions which contribute to increased responses compared to controls. It has been previously shown that densities of interstitial cells (ICs) were increased in obstructed animals (Kubota et al., 2008; Grol et al., 2011; Kim et al., 2011). The difference in the pattern of the responses to physostigmine in the obstructed group may be due to the changes in the number of ICs and/or remodeling of IC networks.

4.3 Correlations between in vivo conscious voiding and in vitro parameters of contractile activity in obstruction

The present study revealed significant positive correlations between structural (bladder weight, wall thickness and histological damage score) and functional *in vitro* parameters of contractile activity (the frequency, amplitude and AUC of spontaneous and distension-evoked contractions) and negative correlations with amplitude of EFS-induced contractions and bladder compliance in obstructed animals. In particular, the data clearly demonstrated a significant correlation between the degree of bladder obstruction and damage to the autonomic innervation: the greater the bladder weight, wall thickness and histological damage score the smaller the amplitude of the EFS-induced responses. Damage to the autonomic innervation, in turn, correlated significantly with the frequency of spontaneous contractions *in vitro*. Changes in the frequency of contractions in obstructed bladders could be due to increased activity and/or density of ICs that was previously found in obstructed bladders (Kubota et al., 2008; Grol et al., 2011; Kim et al., 2011). In addition, obstructed bladders were less compliant that shams *in vitro*. This could be the result of increased frequency of spontaneous contractions, which summate to make the bladder wall actively stiffer. It has been previously shown a significant increase in connective tissue in obstructed bladders (Levin et al., 2000; Metcalfe et al, 2010), this will make them passively much stiffer than shams.

Present multiple correlation and discriminant analyses revealed that frequency of conscious voiding (and voiding volume) positively (and negatively for volume) correlated with major structural changes of the bladder such as bladder weight, wall thickness and histological damage score. In addition, the voiding volume of conscious animals inversely correlated with frequency of spontaneous contractions and positively associated with amplitude of the EFS-induced responses and bladder compliance. Overall, the data suggest that changes in the contractile activity of the bladder itself contribute to bladder overactivity observed during obstruction.

4.4 Other factors influenced obstruction-induced bladder overactivity

In addition to DO (i.e. involuntary detrusor contractions), an inefficient bladder emptying, which leads to increased residual volume, will result in increase in voiding frequency and reduction in voiding volume. It is well established that residual volume is significantly increased in bladder outflow obstruction in human patients and animal models (Rosier et al., 1995; O'Connor et al., 1997; Robertson, 1999; Pandita et al., 2000). The mechanism responsible for increased residual volume and bladder capacity in obstruction is still unclear. Oxidative damage to the autonomic innervation and to the detrusor muscle, demonstrated in animal models of bladder obstruction (Conners et al., 2006; de Jongh et al., 2009; Scheepe et al., 2011) could be involved. It has been recently shown that distension-sensitive bladder afferents had higher threshold volumes and lower tension sensitivity in obstructed rats (Zeng et al., 2012). This would lead to a weaker afferent drive of the micturition reflex, resulting in increased residual volume in obstruction. We did not measure residual volume in this study since we used a non-invasive method of determining the frequency and voiding volume in conscious male guinea pigs. However, the present data support inefficiency in bladder emptying in obstructed guinea pigs: conscious voiding volume was significantly correlated with the amplitude of the EFS-induced responses (i.e. smaller voiding volume was associated with greater damage to autonomic nerves). Thus, it is likely that obstructed guinea pigs had increased residual volume, which would contribute to reduced voiding volume and increased voiding frequency.

Our study revealed a greater inflammatory response in the obstructed bladders than in the shams and a significant correlation between both the voiding frequency and the voiding volume (positive and negative, respectively) with histological damage score. Enhanced release of pro-inflammatory mediators in the bladder wall in obstruction may activate and/or sensitize the nerve endings of some classes of bladder afferents leading to excessive urge and more frequent voiding, without necessarily causing large changes in bladder motor characteristics. Future studies are needed to test directly whether there are changes in excitability of sensory nerves in the obstructed bladder.

5. Conclusion

The data revealed significant association between conscious voiding of guinea pigs and structural and contractile activity changes of their isolated bladders in obstruction. Notwithstanding multifactorial causes of DO and OAB, the data suggest that change in contractile activity of the bladder itself is a major contributor to obstruction-induced bladder overactivity.

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Conflict of interest: the authors declare no conflict of interest

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Figure legends

Fig. 1. Conscious voiding characteristics of the sham-operated and obstructed guinea pigs and changes in their isolated bladders weight, wall thickness and histology. **A**: average number of voids recorded over 6 hr in metabolic cages 1-4 weeks after sham operation (n=19) or partial bladder outlet obstruction (n=15). **B**: averaged volume per void recorded during 6 hr in metabolic cages after sham operation (n=19) or partial bladder obstruction (n=15). **C**: examples of hematoxylin and eosin staining from the three groups of animals studied. **D**: bladder weight in the control (n=13), sham-operated (n=21) and obstructed groups (n=19). **E**: significant increase in thickness of the bladder wall was seen in the obstructed bladders (n=18) compared to controls (n=18) and shams (n=20). **F**: histological damage score blindly determined for control (n=18), sham (n=20) and obstructed bladders (n=18). $^{\#}$ $P<0.05$; * $P<0.0001$.

Fig. 2. Spontaneous and distension-induced bladder contractions in the control, sham-operated and obstructed groups. **A**: typical tracings of spontaneous contractions and contractions induced by the slow ramp distension to 2.2 ml at 0.5 ml/min in the control, sham and obstructed bladders. **B**: amplitude of spontaneous contractions in the control (n=18), sham-operated (n=21) and obstructed group (n=19). **C**: frequency of spontaneous contractions in the control (n=18), sham-operated (n=21) and obstructed group (n=19). **D**: area under the curve (standardized to the duration of distension in minutes) of the phasic distension-induced contractions for the control (n=14), sham-operated (n=21) and obstructed (n=17) groups. **E**: bladder compliance in the control (n=14), sham-operated (n=21) and obstructed (n=17) groups. $^{\#}$ $P<0.05$; * $P<0.01$; $^{@}$ $P<0.001$; $^{\&}$ $P<0.0001$.

Fig. 3. EFS-induced contractions of the isolated bladders in the control, sham-operated and obstructed groups. **A**: typical tracings showing large amplitude responses to EFS (1-30 Hz for 3 s, 0.15 ms, 100V) observed in the control bladder. **B**: smaller responses to EFS (1-30 Hz) were seen in obstructed guinea pigs. **C**: averaged data of the EFS (1-30 Hz)-induced contractions in the control (n=18), sham-operated (n=20) and obstructed group (n=18). EFS-induced contractions differed significantly between the sham-operated and obstructed groups. **D**: EFS (1-30 Hz)-induced contractions in the control (n=17) and sham-operated (n=19) groups were significantly different from obstructed group (n=17), when amplitudes were standardized to the thickness of the detrusor muscle layer. **E**: averaged data of the effects of hyoscine, PPADS and tetrodotoxin (TTX) on the amplitude of EFS-induced contractions of the obstructed bladders, standardized to the thickness of the detrusor muscle layer (n=7). * $P<0.01$; $^{@}$ $P<0.001$; $^{\#}$ $P<0.0001$.

Fig. 4. Concentration-response curves of bethanechol in isolated bladders from the control, sham-operated and obstructed groups. **A**: typical tracings showing the effects of increasing concentrations of bethanechol in the sham-operated guinea pig. **B**: typical tracings showing the effect of increasing concentrations of bethanechol in the obstructed bladder. **C**: concentration-response curves of bethanechol in control (n=6), sham (n=6) and obstructed (n=6) bladders. **D**: concentration-response curves of bethanechol in control (n=6), sham (n=6) and obstructed (n=6) bladders, standardized to the thickness of the detrusor muscle layer. **E**:

concentration-response curves of bethanechol in the control (n=6), sham (n=6) and obstructed (n=6) bladders, standardized to the maximal contractile response. $^{\#}$ P<0.05; * P<0.01; $^{@}$ P<0.001.

Fig. 5. Concentration-response curves of physostigmine in isolated bladders from the control, sham-operated and obstructed guinea pigs. **A**: typical tracings showing the effect of increasing concentrations of physostigmine in control bladder. **B**: typical tracings showing the effect of increasing concentrations of physostigmine in obstructed bladder. Calibration bar for the intravesical pressure traces in **A** - 5 cmH_2O, and in **B** - 10 cmH_2O. Inserts **a** and **b** show physostigmine ($3x10^{-5}$ M)-induced contractile activity at the expanded time scale for the control and obstructed bladders, respectively. **C**: concentration-response curves of physostigmine in the control (n=6), sham (n=8) and obstructed (n=7) bladders. **D**: concentration-response curves for physostigmine in the control (n=6), sham (n=8) and obstructed (n=7) bladders, standardized to the thickness of the detrusor muscle layer. **E**: concentration-response curves for physostigmine in the control (n=6), sham (n=8) and obstructed (n=7) bladders, standardized to the maximal contractile response. $^{@}$ P<0.05; $^{\#}$ P<0.01; * P<0.001.

Table I. Partial correlations between nine *in vitro* parameters in control, sham and obstructed groups.
Pearson correlation coefficients and significance levels (in brackets) are indicated.