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Partial urethral obstruction of rabbit urinary bladder: stereological evidence that the increase in muscle content is mostly driven by changes in number, rather than size, of smooth muscle cells

Tais H. de Castro Sasahara,[1] Terry M. Mayhew,[2] Sheila C. Rahal,[3] Emerson T. Fioretto,[1] Júlio C. C. Balieiro[4] and Antonio A. C. M. Ribeiro[1]

[1]*Laboratory of Stereology and Chemical Anatomy, Department of Surgery, College of Veterinary Medicine, University of São Paulo (USP), São Paulo, Brazil*
[2]*Centre for Integrated Systems Biology and Medicine, Queen's Medical Centre, University of Nottingham, UK*
[3]*Department of Veterinary Surgery and Anaesthesiology, Veterinary Medicine and Zootechnical College São Paulo State University (UNESP), Botucatu, São Paulo, Brazil*
[4]*Department of Basic Science, College of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, Brazil*

Abstract

The effects of partial urethral obstruction on the detrusor muscle of rabbit urinary bladder were investigated using stereological sampling and estimation tools. Twelve female Norfolk rabbits (2.5–3.0 kg body weight) were divided into four groups: 3, 7 and 12 weeks after surgical intervention to produce a standard partial obstruction and unobstructed controls. Following removal, bladder axes (craniocaudal, dorsoventral and laterolateral) and organ weights were recorded. Bladders were prepared for light microscopy by multistage random sampling procedures. Stereological methods were used to estimate the volume of muscle and the packing density and total number of myocyte nuclei in each bladder. We also estimated mean myocyte volume and the mean cross-sectional area and length of myocytes. Group comparisons were made by one-way analysis of variance. Changes in bladder axes were mainly laterolateral and craniocaudal. Mean bladder weight increased roughly six-fold by 3 weeks and 17-fold by 12 weeks and was accompanied, on average, by 12- and 33-fold increases in total muscle volume. These variables did not differ at 3 and 7 weeks post-obstruction. Increases in muscle content were not accompanied by changes in packing densities but were associated with increases in the total numbers of myocyte nuclei (13-fold by 3 weeks, 28-fold by 12 weeks). Mean myocyte volume did not vary significantly between groups but cells in obstructed groups were shorter and wider. These findings support the notion that partial outflow obstruction leads to an increase in the number, but not mean volume, of myocytes. If due solely to myocyte mitosis, the total of 43×10^8 cells found at 12 weeks could be generated by the original complement of 15×10^7 cells if an average of only 2.1×10^6 new cells was produced every hour. In reality, even this modest proliferation rate is unlikely to be achieved because myocyte proliferation rates are very low and it is possible that new myocytes can arise by differentiation of mesenchymal or other precursor cells.

Key words bladder; obstruction; rabbit; smooth muscle cells; stereology.

Introduction

Urethral obstruction is of considerable interest in human and veterinary medicine and may arise for various reasons, e.g. as a consequence of benign prostatic hypertrophy/hyperplasia (Krawiec, 1989; Coffey & Walsh, 1990). It results in urine retention, urinary bladder distension and abnormal growth of the detrusor muscle.

Correspondence
Dr Antonio A. C. M. Ribeiro, Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (USP), Ave. Prof Dr Orlando Marques de Paiva, 87 São Paulo, Brazil; CEP: 05508-000. F: +55 11 30917805; E: guto@usp.br

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Prostate tumours and lower urinary tract disease in humans and cats are also related to urethral stenosis (Houston et al. 2003). Experimental partial urethral obstruction is an established model with which to evaluate the morphological and functional consequences for the bladder. Although the rabbit bladder has been used extensively for studying obstructive dysfunction (Kato et al. 1990; Buoro et al. 1993; Roelofs et al. 1998), other animal models have also been investigated (Mattiasson & Uvelius, 1982; Gabella & Uvelius, 1990; Hanai et al. 2002).

In these models, a sequence of changes begins with a rapid increase in bladder mass and some degree of contractile dysfunction (Tong et al. 1992). The urothelium displays an early phase of hyperplasia whilst the connective tissue undergoes reorganization, neovascularization and collagen deposition. The smooth muscle layers enlarge and are surrounded by a thickened and well-vascularized serosa which supports myofibroblast differentiation (Kato et al. 1990; Tong et al. 1992; Buoro et al. 1993; Roelofs et al. 1998; Hanai et al. 2002). It has been reported that the detrusor muscle exhibits both hypertrophy and hyperplasia, which are, to some degree, time-dependent (Hanai et al. 2002). In part, evidence for myocyte hypertrophy has been adduced by examining cross-sections of cell samples some of which were biased towards the nucleus (Gabella & Uvelius, 1990). Myocytes are capable of division and, during this process, cytokinesis is followed by elongation of the daughter cells (Gabella, 1990). Various indices of proliferation have been investigated including mitotic index, total DNA and incorporation of tritiated thymidine (H^3-thymidine). The incidence of mitotic figures in smooth muscle cells appears to be very low (Gabella & Uvelius, 1990). By contrast, total DNA and incorporation of H^3-thymidine are increased in urethral obstruction although most labelling is observed in the urothelium and fibroblasts (Monson et al. 1992, 1994). *In vitro* studies have shown also that uptake of H^3-thymidine is greater in the body than at the base of the bladder (Tong et al. 1992). Stretching cultured detrusor cells increases the incorporation of H^3-thymidine and shifts cycling cells into S, G2 and M phases (Yu et al. 2003). It also induces cell hypertrophy and inhibits apoptosis (Galvin et al. 2002).

Cell hypertrophy is best estimated directly as cell volume. Using mean cross-sectional area alone as a measure of cell hypertrophy is potentially misleading as cell shape might have altered. Biasing towards nuclear sections could add to errors and uncertainties. In addition, many so-called cell proliferation indices are, in fact, phase-labelling indices. For example, mitotic index is a measure of the relative extent of M phase whilst labelling with bromodeoxyuridine (BrdU) and H^3-thymidine estimate the relative extent of S phase. Consequently, their ability to predict the true impact of proliferation is hampered by failure to determine the size of the pool of cycling cells (Mayhew et al. 2003) or to balance indices of proliferation against indices of loss (e.g. by apoptosis).

These possible sources of experimental uncertainty may be circumvented by a stereological approach in which total volumes and numbers of myocytes are estimated and used to calculate mean myocyte volume. At any given point in time, total cell number is the net outcome of cell proliferation and loss. Therefore, the aim of this study was to apply design-based stereological techniques to compare rabbit urinary bladders before and after partial outflow obstruction. Particular attention is given to the question of cell hypertrophy vs. hyperplasia in the detrusor muscle.

Materials and methods

Animals and surgical procedures

Twelve 3-month-old female Norfolk rabbits (body weights 2.5–3.0 kg) were obtained from the Animal House of São Paulo State University (UNESP), Botucatu, São Paulo, Brazil. Surgical procedures were conducted in the Experimental Surgery Centre of the Veterinary Medicine and Zootechnical College at UNESP. Animals were divided into four groups of equal size ($n = 3$), one of which (unobstructed or control group) did not undergo surgical intervention. For the other three groups, there was surgical intervention to produce outlet obstruction of 3-, 7- and 12-weeks duration. The operative technique has been described previously (Mattiasson & Uvelius, 1982).

Animals were sedated intravenously (i.v.) with 0.25 mg kg^{-1} Acepromazine 0.2% (Univet, São Paulo, Brazil) and with 0.25 mg kg^{-1} Butorphanol (Fort Dodge, KS, USA) and anaesthetized i.v. with a combination of 5 mg kg^{-1} Tiletamine and Zolazepan (Fort Dodge). Anaesthesia was maintained subsequently with a reduced dose (2.5 mg kg^{-1} i.v.). A retro-umbilical abdominal incision was undertaken to expose the urinary bladder, and the dorsal wall of the urethra was separated from

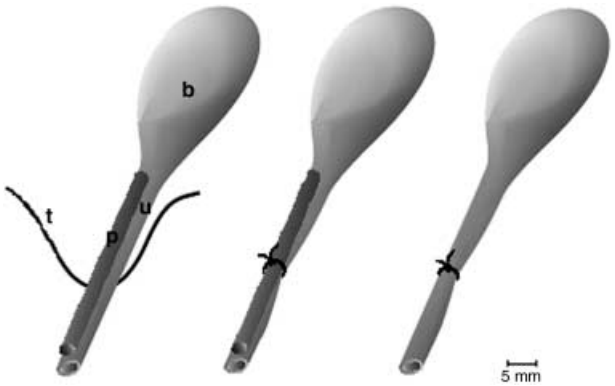

Fig. 1 Schematic illustration of the operative procedure to induce partial urethral obstruction: (b) bladder, (u) urethra, (p) 3-mm Steinmann pin, (t) 2-0 nylon silk used to tie the ligature around the pin. Scale bar, 5 mm.

its immediate relations. A 3-mm Steinmann-pin was positioned on the urethra to produce a standard degree of obstruction and a ligature was tied around it using 2-0 nylon silk (Fig. 1).

Post-operative antibiotic therapy comprised 2 weeks of subcutaneous (s.c.) 5 mg kg^{-1} enrofloxacin 2.5% (Schering-Plough, NJ, USA). Flunixin meglumine (10 mg, Schering-Plough) (1 mg kg^{-1} s.c.) was administered for 3 days and buprenorphine hydrochoride (0.02 mg kg^{-1} s.c., Schering-Plough) was applied every 12 h for 1 week. Three, 7 and 12 weeks after surgery, rabbits were killed via an overdose of pentobarbitone (150–200 mg kg^{-1} i.v.).

At appropriate stages, the bladder was localized and its craniocaudal (CC), dorsoventral (DV) and laterolateral (LL) axes were measured using digital callipers. The CC axis was taken as the largest distance between the apex and neck of the bladder (similar to Uvelius & Gabella, 1980), the DV axis as the largest distance between dorsal and ventral surfaces, and the LL axis as the largest transverse distance between the lateral borders of the bladder.

Following removal of urine by cystocentesis, bladders were filled *in situ* with, on average, 20 mL (control group) or 60 mL (obstructed groups) of phosphate-buffered saline solution (PBS; Sigma, St Louis, MO, USA; 0.1 M, pH 7.4). The contents were removed and the exercise repeated until the bladder content became clear. Thereafter, the bladder was fixed *in situ* by filling with 20 or 60 mL of a modified Karnovsky solution of 5% glutaraldehyde (Merck) and 1% formaldehyde (Sigma) in sodium cacodylate buffer (EM Sciences, Hatfield, PA, USA; pH 7.4, 0.125 M). After 10 min, the fixative was removed and the bladder excised and weighed. After blotting excess fixative, the wet weight (in g) was recorded and then converted into bladder volume (in cm^3) by dividing wet weight by tissue density, which was taken to be 1.06 g cm^{-3} (Ercan et al. 2002; Brüel & Nyengaard, 2005).

Bladder sampling for microscopy

After weighing, each bladder was cut with a ventromedian incision and opened out. A multistage sampling scheme based on systematic uniform random (SUR) sampling was applied. SUR sampling was chosen because of its efficiency (Gundersen et al. 1999; Nyengaard, 1999). To begin, a quadratic test lattice of squares was positioned randomly over the bladder mucosa and an SUR sample of 2-mm^2 pieces of tissue was obtained. In probability terms, this sample represented half of the entire organ. About 12 tissue pieces were obtained per control bladder. Corresponding totals for the 3-, 7- and 12-week obstructed groups were 17, 25 and 35 tissue pieces. For each bladder, randomly chosen pieces were used to estimate tissue shrinkage by comparing their volumes before and after processing and embedding. The rest of the tissue pieces from each bladder were processed for stereological analysis of key structural quantities.

Bladder samples were immersed in a modified Karnovsky solution in sodium cacodylate buffer for 72 h. They were post-fixed in 2% osmium tetroxide in cacodylate buffer (EM Sciences), block-stained with a solution of uranyl acetate (EM Sciences), dehydrated in graded ethanol concentrations and propylene oxide (EM Sciences) and block-embedded in Araldite (502 Polyscience, EM Sciences). Blocks were cured at 60–70 °C for 3 days. For light microscopy, 0.5-μm-thick sections were cut with glass knives, collected onto glass slides, dried on a hot plate, stained with toluidine blue and mounted under a coverslip with a drop of Araldite. SUR samples of microscopical fields of view were obtained by scanning sections and moving to new fields at fixed spacings determined by the *x*- and *y*-controls on the microscope stage. Section images were acquired using a Leica DMR microscope coupled with a DFC 300 FX Leica digital camera. They were recorded and analysed at a linear magnification of ×2900 (control bladders) or ×1200 (obstructed bladders). Magnifications were calibrated using stage micrometer scales as external standards.

Stereological estimations

The stereological aim was to obtain estimates of the volumes and numbers of key bladder compartments (the detrusor muscle and its smooth muscle cells). To this end, SUR sampling was employed in order to generate tissue sections which were random in terms of position. Relative volumes and particle numbers were estimated unbiasedly using design-based stereology with random test point probes and random volume probes (Howard & Reed, 2005).

Volume density of muscle fibres, V_V

The fractional volume of bladder occupied by muscle fibres was determined by point counting (Ercan et al. 2002). An SUR sample of fields was selected and test points randomly superimposed. We counted the total number of points falling within the bladder reference space ($\sum$ Pref) and the total falling on the muscle ($\sum$ Pmus). Volume density was then estimated simply as $V_V = \sum \text{Pmus}/\sum \text{Pref}$. Error variances for V_V ratios were estimated according to Gundersen et al. (1999) and Nyengaard (1999).

Total muscle volume, Vmus

The total volume of muscle in the bladder was estimated indirectly by multiplying muscle volume density by bladder volume.

Numerical density of myocyte nuclei, N_V

The two-way physical disector method (Sterio, 1984; Howard & Reed, 2005) was used to estimate the numerical density of myocyte nuclei within the musculature. The method consists of counting the number of particles (in this case nuclei) that are present in unbiased counting frames on reference sections but do not touch either the forbidden borders of the frames or their extensions or appear on parallel look-up sections. This number is contained in a volume given by the product of the tissue area bounded by the frame and the mean distance between section planes. The method is two-way because counts from reference to look-up may be repeated in the reverse (look-up to reference) direction. A convenient way of estimating muscle sectional area is to count test points, each of which is associated with a known area given on the scale of the specimen, i.e. taking into account areal magnification. Nuclear and test point counts were summed over SUR samples of counting frames from each bladder.

The relationship for determining N_V is

$$N_V = \sum Q^- / \sum V_{dis}$$

(Sterio, 1984) where $\sum Q^-$ represents the nuclear count and $\sum V_{dis}$ is the volume of all disectors sampled. The latter is estimated as $\sum P \cdot a_p \cdot h$ where P is the number of test points, a_p the area associated with each point and h the distance between disector planes. Depending on the bladder under investigation, h had the value of 1 or 2 μm. The important consideration is that h should be smaller than the length of a myocyte nucleus in the direction of sectioning. This avoids a nucleus being lost from counting in the space between the reference and look-up sections. As all sections were cut on the same microtome, discrepancies between nominal and true section thicknesses are likely to produce similar relative biases in the value of h and so the group estimates of N_V should allow valid comparisons.

A total of 140–180 nuclei were counted in control bladders. Corresponding numbers in obstructed bladders were 170–279 (3 weeks obstruction), 187–250 (7 weeks obstruction) and 197–271 (12 weeks obstruction) nuclei.

Total number of myocyte nuclei, N

The total number of myocyte nuclei per bladder was estimated by multiplying N_V by the volume of muscle in the corresponding bladder:

$$N = N_V \cdot \text{Vmus}.$$

This number is equal to the number of myocytes if each cell contains, on average, one nucleus. Examination of tissue sections confirmed the validity of this assumption. Regardless of the direction of sectioning, no cell profile contained more than one nuclear profile. Again, precision of estimation was calculated (Gundersen et al. 1999; Nyengaard, 1999).

Mean myocyte volume, Vmyo

This was calculated (μm^3) from estimates of the volume density and numerical density of myocytes (Korsgaard et al. 1993) which, because the reference volume (total

Table 1 The craniocaudal (CC), dorsoventral (DV) and laterolateral (LL) dimensions (in mm) and wet weight (g) of the bladder in unobstructed (control) and 3-, 7- and 12-week obstructed groups. Values are group means (CV)

Variable	Control	3 weeks	7 weeks	12 weeks
CC axis (mm)*	40.7 (0.44)	66.7 (0.17)	72.0 (0.13)	78.8 (0.02)
DV axis (mm)	16.7 (0.50)	41.7 (0.40)	48.0 (0.26)	48.1 (0.30)
LL axis (mm)***	23.0 (0.08)	41.7 (0.31)	83.0 (0.14)	85.7 (0.12)
Wet weight (g)**	2.17 (0.22)	13.52 (0.33)	12.52 (0.05)	36.08 (0.50)

Group differences: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The DV axis did not show significant group differences.

muscle volume) is common, is equivalent to dividing the total volume of muscle cells by their total number.

Monitoring myocyte shape

In order to test whether myocyte shape was affected, we examined cell profiles in regions where the muscle layers were sectioned favourably. In these regions (at least two per bladder), we applied unbiased counting frames (Gundersen, 1977), sampled myocytes sectioned transversely, and estimated cell profile areas by test point counting. The mean cross-sectional area (Amyo, in μm^2) of cells was estimated by dividing total cell profile area by the number of cell profiles. Having obtained this mean area, and knowing Vmyo, we computed mean cell length (Lmyo, in μm) using the relationship:

$$Lmyo = Vmyo/Amyo.$$

This method of estimating length and area is sensitive to the angle of sectioning and, hence, to departures from true cross-sectioning. Therefore, these measures are different from the design-based estimators used to calculate muscle volume, myocyte number and mean myocyte volume. Fortunately, the theoretical errors are modest for small departures from transverse sectioning and the relative biases are likely to be the same across groups. Consequently, and given the magnitudes of changes observed, these estimates retain their comparative worth.

Tissue shrinkage

The method for estimating tissue shrinkage effects was based on one described previously (Wulfsohn et al. 2004; Brüel & Nyengaard, 2005). Tissue samples taken at five random locations from each bladder were weighed and their wet weights converted into volumes assuming a tissue density of 1.06 g cm^{-3}. All samples were taken through processing to Araldite embedding, cut serially at 2 μm thickness and stained with toluidine blue. The volume of each sample was then estimated using the Cavalieri principle (Howard & Reed, 2005) with a section sampling interval of 1 in 20. Sectional areas were estimated by point counting. By comparing the two volume estimates for each bladder, the estimated volumetric shrinkage of organs amounted to 8–13% (within-group CV values 0.12–0.15) and we found no significant differences between groups. Consequently, final estimates were not corrected for shrinkage effects. Studies on bladders from minipigs have also indicated no differential shrinkage effects between control and unobstructed bladders (Nielsen, 1997).

Statistical analyses

For each variable, we calculated group means and coefficients of variation (CV = standard deviation divided by group mean). CV affords a convenient measure of the observed variation between organs within a group. Between-group comparisons were drawn using one-way analysis of variance (one-way ANOVA) in order to test the null hypothesis of no group differences (Sokal & Rohlf, 1981). Hypotheses were rejected at a probability level of $P < 0.05$. When differences were considered significant, *post-hoc* testing by the Tukey test helped to identify where the main differences between groups were located.

Results

Macrostructure

Findings are summarized in Table 1. In control (unobstructed) rabbits, the group mean (CV) for bladder wet weight amounted to 2.17 g (0.22) with a CC axis of 40.7 mm (0.44), a DV axis of 16.7 mm (0.50) and an LL axis of 23.0 mm (0.08). Except for the DV axis,

significant group differences in all these variables were detected. By 3 weeks, mean bladder weight had increased 6.2-fold but this had not increased further by 7 weeks. However, between 7 and 12 weeks, there was a further 2.9-fold increase in mean weight. These findings indicate that changes in mean bladder volumes were associated with alterations in bladder axes and, in particular, with alterations in the CC and LL axes (Fig. 2).

Microstructure

Qualitative inspection revealed changes in muscle arrangement, content and cellularity. The arrangement of muscle bundles varied between groups and, in control, 3-week and 7-week obstructed groups, the extracellular spaces between myocytes were enlarged. Myocyte morphology also differed between control and obstructed bladders. In the latter, cells appeared to be larger and more irregular in shape, with a significant proportion being wider but shorter. This was most evident in 7- and 12-week obstructed bladders (Fig. 3).

Stereological estimates

Estimates of the key structural quantities are summarized in Table 2.

Volume density and total muscle volume

In the control group, the volume density of muscle ranged from 0.237 to 0.295 (mean = 0.263, CV = 0.11) and there were significant differences between groups (variance ratio, $F = 7.48$; d.f. = 3,8, $P = 0.01$). Myocytes accounted for a higher proportion of bladder volume in all obstructed groups, the mean (CV) values ranging from 0.520 (0.19) at 3 weeks post-obstruction, to 0.412 (0.15) at 7 weeks and 0.491 (0.17) at 12 weeks.

Error variances for volume densities (expressed as coefficients of error, CE) were 0.04 (control bladders) and 0.07, 0.08 and 0.06, respectively, for the 3-, 7- and 12-week obstructed bladders. These estimates indicate that sampling and estimation errors contributed only 12–28% to the total observed variance between organs within a group.

After taking into account differences in bladder size, it was clear that the changes in muscle volume density were due to disproportionate increases in volume of muscle following obstruction. In controls, muscle

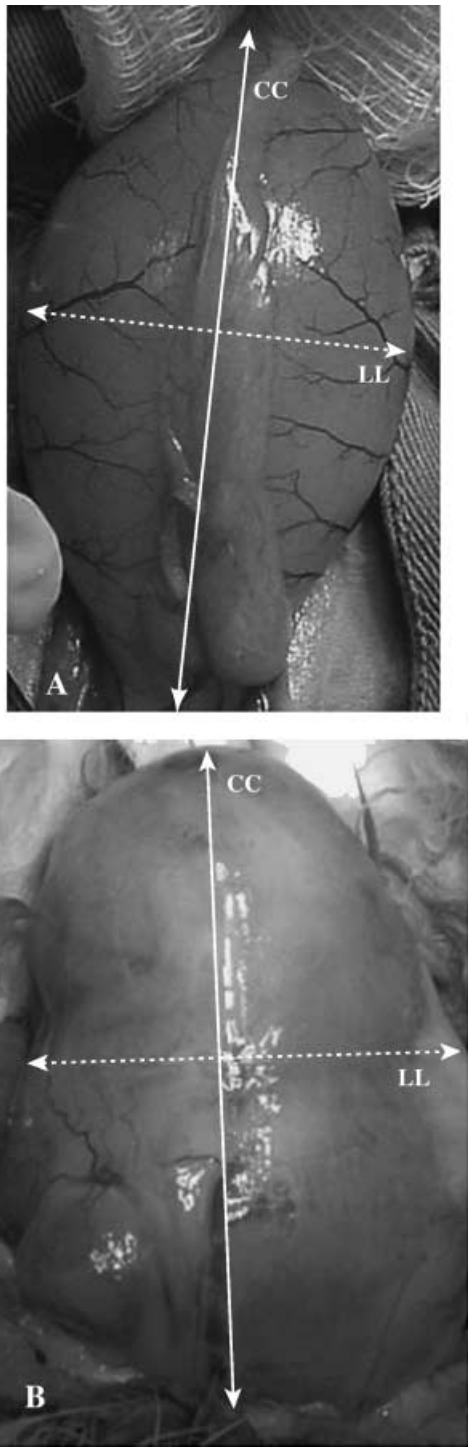

Fig. 2 Macroscopic appearance of rabbit urinary bladder. (A) unobstructed and (B) 12 weeks post-obstruction. Craniocaudal (CC) and laterolateral (LL) axes are indicated. In unobstructed bladders, group mean CC was 40.7 mm and LL was 23.0 mm. Corresponding values at 12 weeks post-obstruction were 78.8 mm and 85.7 mm, respectively (see text).

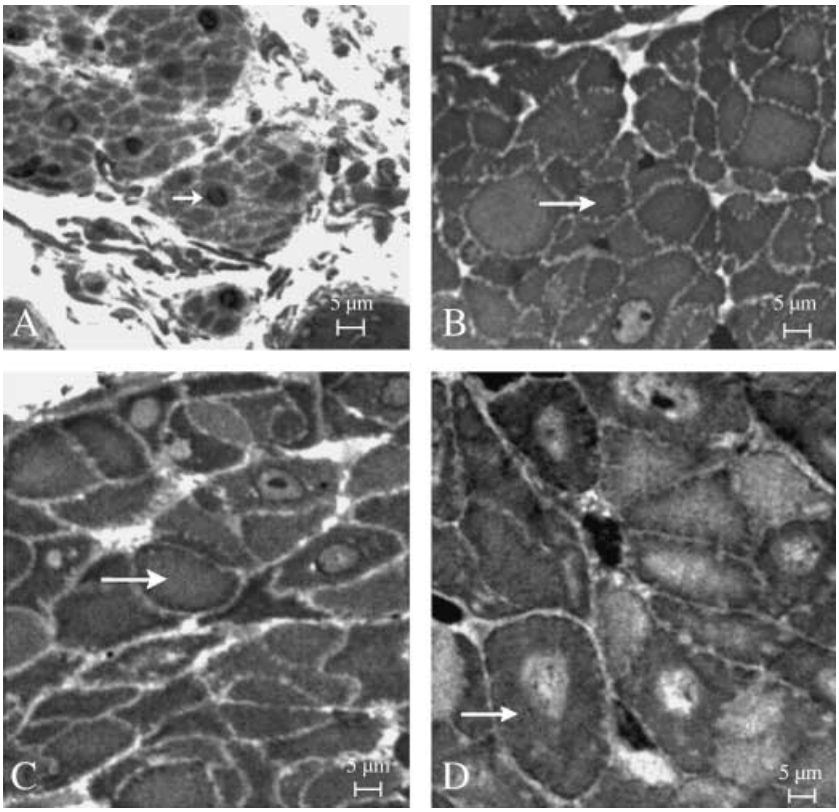

Fig. 3 Microscopical appearance of the detrusor muscle in resin sections: (A) unobstructed, (B) 3 weeks post-obstruction, (C) 7 weeks post-obstruction and (D) 12 weeks post-obstruction. Profiles of smooth muscle cells are indicated by arrows. Toluidine blue. All scale bars: 5 μm.

Table 2 Stereological estimates for the detrusor muscle (volume density and total volume) and myocytes (numerical density, number, mean cell volume, cross-sectional profile area and length) in the bladder of unobstructed and 3-, 7- and 12-week obstructed groups. Values are group means (CV)

Variable	Control	3 weeks	7 weeks	12 weeks
Muscle V_V**	0.263 (0.11)	0.520 (0.19)	0.412 (0.15)	0.491 (0.17)
Volume (mm^3)*	571 (0.24)	6900 (0.28)	5130 (0.10)	18 720 (0.60)
Cell N_V (mm^{-3})	262 500 (0.53)	280 200 (0.15)	275 900 (0.76)	259 800 (0.31)
Number ($\times 10^8$)*	1.54 (0.59)	19.84 (0.42)	14.79 (0.86)	42.99 (0.43)
Volume (μm^3)	1150 (0.38)	1870 (0.19)	2100 (0.66)	2110 (0.50)
Area (μm^2)***	8.78 (0.39)	49.48 (0.07)	46.71 (0.22)	71.05 (0.24)
Length (μm)**	134 (0.27)	37.6 (0.12)	45.4 (0.57)	26.5 (0.41)

Group differences: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. The numerical density and mean cell volume of myocytes did not show significant group differences.

volume was in the range 427–697 mm^3 (mean = 571 mm^3, CV = 0.24). Values were substantially greater in all treated groups ($F = 5.42$, d.f. = 3,8, $P < 0.05$). By 12 weeks post-obstruction, the volume had risen to a range of 6726–29 240 mm^3 (mean = 18 720 mm^3, CV = 0.60).

Numerical density and total numbers of myocytes

The disector method is illustrated on two obstructed groups in Fig. 4. In unobstructed bladders, the mean (CV) numerical density of myocyte nuclei amounted 262 500 mm^{-3} (0.53), range = 151 200–420 100 mm^{-3}. These packing densities did not alter significantly following obstruction. However, because of the greater total muscle content per bladder, the constant numerical density represented real increases in total complements of myocytes ($F = 6.21$, d.f. = 3,8, $P < 0.05$). In the control group, mean myocyte number was in the range $0.64–2.5 \times 10^8$ (mean = 1.54×10^8, CV = 0.59). Numbers were in the range $12.42–28.80 \times 10^8$ (mean = 19.84×10^8, CV = 0.42) by 3 weeks post-obstruction but was not greater at 7 weeks. By contrast, a further net increase in number occurred between 7 and 12 weeks when the final complement varied from 21.90 to 57.14×10^8 (mean = 42.99×10^8, CV = 0.43) myocytes.

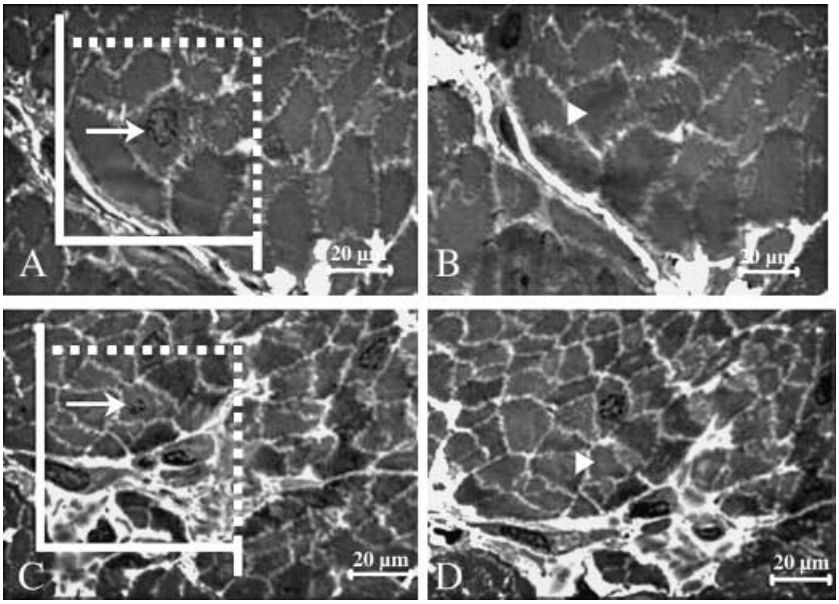

Fig. 4 Disectors (pairs of physical sections) used for counting myocyte nuclei in 7-week (A,B) and 12-week (C,D) obstructed bladders. The reference (A,C) and look-up (B,D) sections are separated by a spacing of 1 μm. Arrows show nuclei in reference sections which do not appear (arrowheads) in the partner look-up sections. Scale bars: 20 μm.

CE values for these estimates of total myocyte number were 0.07 for control bladders and 0.09, 0.10 and 0.11 for the three groups of obstructed bladders. Thus, the precision of number estimation contributed just 1–7% to the total observed variation between bladders within a group.

Mean myocyte volume

Mean cell volume in control bladders ranged from 702 to 1567 μm^3 (mean = 1150 μm^3, CV = 0.38) and we detected no significant differences between groups.

Shape changes: myocyte area and length

Although we detected no differences in mean cell volume, we were able to confirm changes in cell shape. The mean cross-sectional area of myocytes in control bladders (mean = 8.78 μm^2, CV = 0.39) was considerably different in obstructed bladders ($F = 18.77$, d.f. = 3,8, $P < 0.001$). At 3 and 7 weeks post-obstruction, the mean area was 5–6 times larger and, by 12 weeks, it was about eight times larger.

As mean cell volumes did not vary significantly between groups, it was to be expected that changes in cell mean cross-sectional areas would be accompanied by changes in mean length. The mean length of myocytes in unobstructed bladders ranged from 104 to 174 μm (mean = 134 μm, CV = 0.27) and decreased dramatically following obstruction ($F = 13.72$, d.f. = 3,8, $P < 0.01$). Mean length varied from 33 to 42 μm (mean = 37.6 μm, CV = 0.12) by 3 weeks, 17 to 69 μm (mean = 45.4 μm, CV = 0.57) by 7 weeks and 17 to 39 μm (mean = 26.5 μm, CV = 0.41) by 12 weeks.

Discussion

The present study has confirmed that partial outlet obstruction of the rabbit urinary bladder for a period of 12 weeks leads to organ hypertrophy associated with differential growth in bladder axes. The lateral (transverse) and craniocaudal axes, but not the dorsoventral axis, displayed significant increases in length. The hypertrophy is partly attributable to an increase in muscle volume which expanded to 3 weeks post-obstruction and then rose further between 7 and 12 weeks. Muscle growth was explained principally by changes in numbers of myocytes: differences in mean myocyte volume between control and 3-week obstructed bladders failed to attain statistical significance and mean cell volume did not alter up to 12 weeks post-obstruction. However, cell shape did alter progressively given that mean cross-sectional areas increased whilst mean lengths decreased. Given their greater subject-to-subject variation (expressed as CV), detecting significant differences in length of the dorsoventral axis and mean myocyte volume may require larger sample sizes.

Similar changes in bladder size and muscle content have been reported in rabbits and other mammalian

models of partial outlet obstruction (Gabella & Uvelius, 1990; Kato et al. 1990; Nielsen, 1997). Our results indicate disproportionate changes along the tranverse and craniocaudal axes of the bladder, suggesting regional variation in tissue remodelling. *In vitro* studies on bladder strips from different bladder regions have identified differences in pharmacological and physiological responses before and after obstruction (Levin et al. 1980; Longhurst et al. 1995; Schroder et al. 2002). The contractile properties of hypertrophic detrusor muscles after partial urethral obstruction have been studied and the active force reached considerably higher values than those found in control (unobstructed) organs. Moreover, the active force produced by the hypertrophic muscle decreased more rapidly at shorter lengths and the maximum force was reached at a considerably greater radius (Mattiasson & Uvelius, 1982). Changes are associated with differences in contractility and the expression of myosin isoforms (Austin et al. 2004).

Bladder hypertrophy following outlet obstruction does not involve simply an increase in muscle cell volume. Rather, it is a process accompanied by complex structural remodelling of the stroma and musculature, resulting in increases in bladder diameter and surface area. In the case of myocytes, their mean cross-sectional area increased following obstruction, a finding consistent with earlier studies (Gabella & Uvelius, 1990). However, in our study, this increase in mean area was not indicative of any change in mean cell volume. We conclude that mean cross-sectional area is not, in general, a valid estimator of mean cell volume. Enlarged cell profiles were polygonal, crescent-like, very flattened, indented by other cells or wedge-shaped, in contrast to the oval or rather simple polygonal shape of muscle profiles in the control group. In obstructed groups, the average cell altered shape by becoming shorter and wider. Irregularity in muscle cell shape has been reported also for 10-week obstructed rats (Gabella & Uvelius, 1990) and for the human bladder with urothelial carcinoma (Burkhard et al. 2005).

Our mean cell volume of 1150–2110 μm^3 is comparable with that of a vascular smooth muscle cell in subcutaneous resistance arteries (1530 μm^3; see Korsgaard et al. 1993). Our estimates of mean myocyte length (100–180 μm in control bladders and 17–70 μm in obstructed bladders) are consistent with textbook estimates for smooth muscle cells (20–500 μm depending on anatomical site) and for pig bladder (*c.* 200 μm; see Van Asselt et al. 1993). However, they are at the lower end of values (160–700 μm) reported for rabbit and guinea-pig bladders (Uvelius & Gabella, 1980; Hashitani et al. 2001).

An interesting outcome of the present study is the extent of the increase in number of smooth muscle cells. These increases are consistent with those found by Nielsen (1997) in a stereological study on the outlet-obstructed bladder of the minipig. Despite little evidence of mitosis within the myocyte population (Brent & Stephens, 1975; Nielsen, 1997; present study), it is clear that, from an initial complement of 1.54×10^8 myocytes, there was a net production of 41.45×10^8 additional cells by 12 weeks post-obstruction. On average, this is equivalent to a net increase of about 2.1 million cells per hour. By 3 weeks post-obstruction, there were 18.30×10^8 additional cells and this equates to roughly 3.6 million cells per hour. Although the mean total number did not differ between 3 and 7 weeks, a further rise by 28.20×10^8 cells occurred between 7 and 12 weeks. This equates to a net production of 3.4 million cells per hour. Although these two increases in cell number seemed to occur at roughly equal rates of production, further studies are required to check whether changes occurring between the selected timepoints have been missed.

In the minipig bladder, Nielsen (1997) noted a three-fold increase in total number of muscle cells after 9 weeks of outflow obstruction. In male adult and growing rabbits, three-fold increases in cell numbers were reported after 4 weeks of obstruction (Brent & Stephens, 1975). In rabbits, we found an almost ten-fold increase after 7 weeks and 28-fold after 12 weeks. In addition, Nielsen demonstrated a six- to eight-fold increase in the bladder weight by 9 weeks. Our findings are five- to six-fold by 7 weeks and 16- to 17-fold by 12 weeks. Interestingly, Nielsen also reported a 2.5-fold increase in mean cell volume whereas our comparable increases (about 1.8-fold) were not statistically significant.

We have been careful not to ascribe these increases in myocyte number solely to myocyte mitosis, partly because of the low rates which are reported (Brent & Stephens, 1975; Gabella & Uvelius, 1990; Nielsen, 1997). However, it is reasonable to conclude that at least some of this increase is due to myocyte proliferation given that these cells can divide and are known to incorporate H^3-thymidine (Monson et al. 1992, 1994). It has been proposed that tissue hypoxia might be an outcome of partial bladder outflow obstruction (Ghafar et al.

2002) and, *in vitro*, hypoxia inhibits myocyte proliferation possibly via the cell cycle inhibitor $p27^{kipl}$ (Galvin et al. 2004). In order to confirm myocyte proliferation, further *in situ* immunohistochemical studies are needed to localize cell-cycle phase markers (e.g. Ki67, BrdU, H^3-thymidine) specifically to smooth muscle cells.

In addition to myocyte hyperplasia, there are grounds for believing that part of the increase in number of myocytes is due to differentiation of mesenchymal cells or transdifferentiation of fibroblasts (Johnson, 1962; Brent & Stephens, 1975; Monson et al. 1992, 1994). Indeed, in the rabbit urinary bladder, mesenchymal cells in the thickened bladder serosa are capable of expressing a myofibroblast immunophenotype by 2 weeks post-obstruction and an immature myocyte immunophenotype by 4 weeks (Buoro et al. 1993). During this period, there was little or no uptake of BrdU by smooth muscle cells. Transforming growth factor-1 may play a tissue-dependent role in the conversion of myofibroblasts to myocytes (Roelofs et al. 1998).

In conclusion, by applying stereological sampling and estimation tools, we have confirmed that partial outflow obstruction in the rabbit model leads to an increase in the number of smooth muscle cells in the expanded bladder detrusor muscle. The amplification of cell number was associated with changes in cell morphophenotype but not in mean cell volume. It is likely that the increase in myocyte number is due largely to stimulation of the mesenchymal cell–myofibroblast–immature myocyte pathway but further studies are required in order to clarify this and any complementary contribution made by proliferation of myocytes themselves. As a further cautionary note, it would be interesting to analyse sets of end-control animals in order to assess the extent to which changes in structural quantities might be influenced by age effects.

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