Elastic fibres in the vesicourethral junction and urethra of the guinea pig: quantification with computerised image analysis

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ABSTRACT

Elastic fibres, which are intimately associated with collagen, a major component of the urethra, have been assumed to contribute to the resting urethral closure pressure. The Miller stain for elastin was used to demonstrate elastic fibres in cryostat sections of guinea pig bladder base, vesicourethral junction (VUJ) and urethra. Computerised image analysis was employed to objectively quantify these fibres. Both male and female guinea pigs showed significantly greater amounts of circularly disposed elastic fibres in the VUJ than in the other 2 regions examined. This particular disposition of fibres may be responsible for imparting resiliency and plasticity to the VUJ, allowing it to distend and recoil repeatedly in response to urine outflow. Furthermore, the elastic fibres may be partly responsible for the passive occlusive force in this region. Elastic fibres in the distal urethra were not quantified because of their relative paucity. Sagittal sections of the urethra revealed a mass of longitudinally arranged elastic fibres localised almost exclusively within the mucosa, submucosa and longitudinal smooth muscle layer. Functionally, this arrangement may exist to facilitate urethral length changes that occur in micturition.

Key words: Bladder neck; elastin; urinary incontinence.

INTRODUCTION

Elastic fibres are a major constituent of connective tissue. They confer the property of elastic recoil to sites whose ability to function effectively involves reversible extensibility; such sites include the lungs, skin and blood vessels. The existence of several pathological processes involving elastic fibres has prompted research into changes in these fibres in pulmonary emphysema (Pierce et al. 1995), skin ageing (West, 1994) and atherosclerosis (O’Brien, 1984).

Elastic fibres are also present in structures of the urinary tract but have been studied less extensively except by Escala et al. (1989), who examined the development and distribution of elastic fibres in the upper urinary tract of the human fetus and by Augsburger (1997), who investigated the elastic fibre system of the female canine urethra. Prior to these studies, the distribution and probable role of elastic fibres in the urethra itself had received only brief and passing mention in anatomical studies of the cat (Cullen et al. 1983), dog (Augsburger et al. 1993) and human (Lapides, 1958; Woodburne, 1961; Young, 1965; Nyo, 1969; Gosling et al. 1983; Huisman, 1983) urethra.

It is well established that the resting urethral closure pressure is important in preserving continence and is maintained through the combination of several factors: the integrity of the vesicourethral junction (VUJ), urethral and pelvic floor musculature, hydrostatic pressure in the submucosal blood vessels and tension of urethral fibroelastic elements. The fibroelastic tissue has been assumed by some authors to be a major factor in the generation of the resting urethral and VUJ closure pressure. This was first addressed by Awad & Downie (1976) who, after eliminating the neural factors responsible for intraurethral pressure in bitches, observed a residual urethral closure pressure. This finding was confirmed by Bump et al. (1988), who demonstrated that the fibroelastic elements exerted an effect on the urethral pressure profile equivalent to that exerted by the vascular bed. Elliot (1954) concluded that postoperative incontinence in men was due to excessive removal of smooth muscle and connective tissue in the vicinity of the prostatic...
apex and membranous urethra. Pennington & Lund (1960) described a dense ring of elastic tissue in the aforementioned regions and concluded that elastic fibres play a role in generating the ‘primary internal urethral resistance’ and, furthermore, that incontinence may result through damage to this region following prostatectomy. Similarly, Ho et al. (1997) reported an abundance of elastic fibres amongst the smooth muscle bundles associated with the human male membranous urethra. Gosling et al. (1983) suggested that the innumerable elastic fibres observed by Lapides (1958) in the VUJ of the human female urethra were important in providing passive occlusion of the urethral lumen. The presence of elastic fibres in the VUJ and their relevance in maintaining continence was also observed and commented upon by Woodburne (1961). In contrast, some investigators have viewed elastic fibres in the urethra as minor contributors to the resting urethral closure pressure (Hickey et al. 1982; Huisman, 1983; Augsburger et al. 1997), claiming that their presence serves only to anchor and support muscle fibres.

Morphometric studies exist quantifying elastic fibres in the dog (Cullen et al. 1981a), bitch (Cullen et al. 1981b), cat (Cullen et al. 1983) and human (Ho et al. 1997) urethra. The present study sought to objectively quantify the percentage area of elastin stain (and hence elastic fibres) in tissue sections of the guinea pig bladder base, VUJ and proximal urethra using computerised image analysis. Elastic fibre arrangement and distribution in the VUJ and along the urethra are also described, and their possible physiological role discussed.

MATERIALS AND METHODS

Tissue preparation

Young male and female Dunkin Hartley guinea pigs (500 g) were killed by carbon dioxide overdose followed by cervical dislocation. The abdomen was opened and the pubic symphysis resected, allowing access to the underlying bladder and urethra. The bladder and urethra were carefully removed en bloc, together with the distal part of the vagina in the female; in the male, the penile urethra was excluded. On removal, bladders were sliced transversely at the level of the ureteric orifices and the bladder bodies discarded.

For transverse sections, the tissue was placed in Tissue-Tek O.C.T. embedding medium (BDH Laboratory Supplies, Poole, UK) such that the longitudinal axis of the urethra or tissue block was oriented vertically. Conversely, sagittal sections were obtained by orienting the tissue so that its longitudinal axis ran horizontally. The tissue was snap frozen in isopentane cooled in liquid nitrogen. Serial sections (10 μm) were then cut on a Bright Microtome 5030 (Bright Instrument Company, Huntingdon, UK) in a cryostat chamber maintained at −30 °C, and mounted on Vectabond (Vector Labs, Peterborough, UK) treated slides. Once cutting was complete, every 16th section was selected for the demonstration of elastic fibres. A neighbouring section was stained with Masson trichrome for muscle and connective tissue content to assist with the spatial localisation of the elastic fibres.

Elastin stain

Tissue sections were fixed for 20 min in 10% formol saline followed by washing twice in 70% ethanol for 2 × 10 min. They were then placed in Miller stain (R. A. Lamb, London, UK; Miller, 1971) for a fixed duration of 2 h, which proved optimal for good specific with low nonspecific staining. All tissue sections from all guinea pigs used were stained simultaneously to eliminate any between batch variation of staining intensity. The sections were counterstained lightly with neutral red solution for 3 min; thereafter they were dehydrated through a graded series of alcohols, cleared in Histo-Clear and coverslipped, using Histomount as a sealant.

Quantitative morphometric analysis

A normal objective lens (× 2.5) was used to capture whole histological cross-sections. This involved constructing a montage of the tissue section from several restricted subfields. Images were captured using a standard 625-line charge coupled device (CCD) camera attached to a Leitz Laborlux D light microscope (Leica Instruments, Milton Keynes, UK). On construction of the montage, it was noticed that fluctuations in light intensity from the microscope, combined with the variable sensitivity of the camera’s photo diodes, gave rise to images of different light intensities and patterns. This was accounted for by subtracting a blank reference image taken from a section-free region of the microscope slide. Real time images were grabbed, digitised and displayed directly on a monitor. Montages were constructed using the image editing program Adobe Photoshop. Image analysis and processing were performed using Optilab; image processing was used to optimise the signal to noise ratio. First, quantification of elastic fibres entailed thresholding, i.e. separating pixels (picture units) into groups of similar characteristics. In this case the similar characteristic was the colour of
The elastin stain, which fell within a narrow range of pixel shades (160 ± 15 blues shades). Secondly, it was necessary to define the section area. The area occupied by elastic fibres was expressed as a percentage of the entire cross-sectional area. This included the luminal area which was considered negligible as the lumen was always collapsed and the urothelium closely apposed (see Fig. 1A).

Statistical analysis of data

Data were expressed as the mean ± the standard error of the mean (S.E.M.). The statistical significance of differences was tested for using an unpaired t test. P values less than 0.05 were considered statistically significant. N represents the number of animals used in the study and n equals the number of observations.

RESULTS

Female guinea pig VUJ and urethra

Morphology. Two smooth muscle layers were identifiable in the bladder base: an outer layer consisting of predominantly circularly arranged broad interlacing smooth muscle bundles and an inner layer of longitudinally arranged smooth muscle bundles. Sagittal sections of the VUJ revealed that the broad interlacing smooth muscle bundles from the outer layer of the bladder base did not pass into the proximal urethra. However, the narrower bundles from the inner layer, which were highly infiltrated with connective tissue, were observed to blend imperceptibly with the longitudinal smooth muscle of the proximal urethra.

The urethral lumen was bordered by a prominent mucosa and submucosa which possessed an extensive vascular plexus. The submucosa was least developed in the proximal urethra but increased in thickness distally. The longitudinal smooth muscle layer of the urethra appeared to be in direct continuity with the longitudinal muscle of the bladder base and completely encircled the submucosa. Distally, this muscle layer only occupied the ventral and dorsal aspects of the urethra.

The circularly arranged smooth muscle was sandwiched between the longitudinal smooth muscle and the outer striated muscle sheath. The circular smooth muscle layer was much narrower ventrally than dorsally, the width of the former region consisting of just a few cells. This layer was only present over the proximal two thirds of the urethra, although faint wisps of circular smooth muscle were occasionally evident in the distal urethra. Changes in the musculature of the dorsal urethral wall were observed where the distal urethra fused with the underlying vagina. This was seen as a progressive interruption of the outer striated muscle sheath with increased intermingling of smooth and striated muscle, which was interspersed greatly with connective tissue.

A thick outer sheath of striated muscle was the most prominent layer. This layer was complex, as it underwent a transition in its orientation, from being circularly arranged in the proximal urethra, to obliquely arranged in the mid-urethra and finally, longitudinally arranged in the distal urethra. Distally, the longitudinally arranged striated sheath was shown to consist of an inner and an outer longitudinal component with a relatively thin, horse-shoe shaped circularly arranged striated muscle layer sandwiched between the 2 longitudinal layers.

Elastic fibres. Light microscopic examination of transverse sections showed that elastic fibres were sparse in the bladder base. Those present were located in the mucosa, submucosa, between smooth muscle bundles, within blood vessel walls, and surrounding the insertion point of the ureters. These fibres were very fine, displaying no defined orientation. Caudally, towards the VUJ, an increase of elastic fibres was observed, particularly in the dorsal region. The sparsely scattered fibres in this region developed into dense circularly arranged bands which eventually encircled the internal urethral meatus (Fig. 1A).

Computerised image analysis by means of thresholding (Fig. 1B) demonstrated that on average, elastic fibres constituted 1.4 ± 0.4%, 6.4 ± 0.5% and 2.1 ± 0.42% of the cross-sectional area in representative sections taken from the bladder base, VUJ and proximal urethra, respectively. The increase of
fibres were tightly coiled and rippled in appearance (Fig. 4A). A modicum of circularly arranged elastic fibres was associated with the circular smooth muscle layer. In contrast to the submucosa, mucosa and longitudinal smooth muscle layer, individual fibres associated with this layer were linear in appearance (Fig. 4B). Elastic fibres were also present in blood vessel walls. In general, intramuscular bundles and the epithelium were devoid of elastic tissue throughout the length of the urethra.

**Male guinea pig VUJ and urethra**

*Morphology.* The musculature of the bladder base in the male guinea pig was similar to that in the female. As in the female, the striated muscle was the most predominant component of the urethra, although it was arranged differently. Striated muscle fibres originating at the VUJ occupied the ventral and lateral aspects of the proximal urethra. Medially, the striated muscle was circularly arranged while laterally, it was longitudinal in nature. Distal to the ejaculatory ducts, these muscle fibres completely encircled the urethra and ran the full course of the membranous urethra. This arrangement of striated muscle in the guinea pig is considered analogous to the human external urethral sphincter.

The epithelium of the membranous urethra was rich in mucous secreting simple acinar glands, more so than in the proximal urethra. The ejaculatory ducts, which fused with the proximal urethra dorsally also, possessed these glands. The ducts comprised the ductus deferens, seminal vesicle and coagulating gland (Cooper & Schiller, 1975). The dorsal urethra, which the ejaculatory ducts pierced, was devoid of all muscle but possessed an abundance of dense irregular connective tissue rich in blood vessels.

The submucosa was surrounded by a longitudinal smooth muscle layer. This layer was continuous with the longitudinal smooth muscle layer of the bladder base, and formed a compact band of muscle in the proximal urethra. Here, this layer was less dense dorsally and laterally. Distally, this muscle was strongly interspersed with connective tissue and was confined to the ventral and dorsal regions of the urethra. Circular smooth muscle was present only proximally, where it completely encircled the urethra. Unlike the ventral region, where the circular smooth muscle fibre bundles were narrow and just a few cells wide, the dorsal region exhibited broad smooth muscle fibre bundles. Circularly arranged smooth muscle was virtually absent from the membranous urethra.

*Elastic fibres.* The distribution of elastic fibres in

![Graph illustrating the distribution of elastic fibres.](image)

**Fig. 2.** Graph illustrating the distribution of elastic fibres. The amount of elastin stain (i.e. elastic fibres) was quantified using computerised image analysis and expressed as a percentage of the cross-sectional area. Sections were taken from the male (●) and female (□) guinea pig bladder base, VUJ and proximal urethra. n = 6 and n = 3. ** Statistically different (P < 0.001) from the bladder base and proximal urethra, *P < 0.05.**

![Sagittal section of the female guinea pig bladder base, VUJ and proximal urethra stained with Miller’s stain.](image)

**Fig. 3.** Sagittal section of the female guinea pig bladder base, VUJ and proximal urethra stained with Miller’s stain, demonstrating the mass of longitudinally arranged elastic fibres in the lamina propria and between the longitudinal smooth muscle bundles. VB, ventral bladder; VUJ, vesicourethral junction; BB, bladder base; C St, circular striated muscle; C Sm, circular smooth muscle; L Sm, longitudinal smooth muscle; L P, lamina propria. Bar, 1 mm.

circularly arranged fibres in the VUJ in comparison with neighbouring regions was highly significant (P < 0.001) (Fig. 2). It was noted that elastic fibres in the proximal, mid and distal urethra were localised predominantly in the submucosa and longitudinal smooth muscle layer. Sagittal sections showed that these elastic fibres were arranged parallel to the longitudinal axis in 2 well defined layers lying ventrally and dorsally, and running the full length of the urethra (Fig. 3). Within this region, individual elastic
the male was very similar to that in the female. The only major difference was in the region of the ejaculatory ducts. Here, transverse sections revealed lumina from several ducts appearing within the submucosa. The submucosa at this location revealed a mass of apparently haphazardly arranged elastic fibres. Again, 2 layers of longitudinally oriented elastic fibres were discernible in the proximal urethra. The inner layer, which constituted the submucosa, was more noticeable and comprised numerous wave-like elastic fibres. The outer layer possessed a delicate meshwork of elastic fibres occupying the intercellular spaces between muscle cells of the longitudinal smooth muscle layer.

Quantification studies demonstrated that on average, elastic fibres occupied $2.3 \pm 0.7\%$, $9.5 \pm 0.2\%$ and $5.9 \pm 0.6\%$ of the cross-sectional area in representative sections taken from the bladder base, VUJ and proximal urethra, respectively. The increase of circularly arranged fibres in the VUJ in comparison with neighbouring regions was highly significant ($P < 0.001$) (Fig. 2). The male VUJ showed a significantly higher percentage of elastic fibres than the female ($P < 0.05$). The elevated percentage of elastic tissue in the male proximal urethra was probably accountable for by the presence of the ejaculatory ducts. Elastic fibres were scantily distributed in the membranous urethra, where they mingled diffusely with the striated muscle.

**DISCUSSION**

Elastic fibres possess unique physical and chemical properties and their abundance at particular anatomical sites has rejuvenated interest as to their role in the pathogenesis of certain diseases (Davidson et al. 1995). Mature elastic fibres are composed of 2 very different components, an amorphous protein called elastin and a microfibrillar structure (Ross & Bornstein, 1971), the former being the more abundant and giving rise to the characteristic wavy appearance of these fibres when viewed under a microscope. Unattached elastic fibres possess rubber like properties, they can stretch easily and rapidly, and can return to their prestressed state with minimal loss of energy, thereby providing elasticity and stretchability to tissue. In conjunction with collagen, elastic fibres can sustain higher stress than would otherwise be possible.

In the present study, elastic fibres were localised by light microscopy using the highly selective, high affinity Miller stain. The strong dye-fibre affinity of the Miller stain, which is thought to be due primarily to Van der Waals attraction forces (Horobin & Flemming, 1979) was reflected by the low levels of nonspecific background staining obtained. Traditionally, elastic fibres have been stained with well known histological dyes such as Gomori’s aldehyde-fuchsin, Weigert’s resorcin-fuchsin and orcein (Horobin & Flemming, 1979). More recently, polyclonal antibodies to human elastin have been used to demonstrate elastic fibres (Ewalt et al. 1992). The Miller stain is an amalgamation of the Weigert’s stain with crystal violet, new fuchsin and Victoria Blue 4R. Quantification of elastic fibres in this case, using computerised image analysis, was not a measure of the actual intensity of the elastin stain but a measure of the percentage occupation of whole cross-sectional areas by elastic fibres. As all tissue sections from all guinea pigs used were stained simultaneously, i.e. exposed to the Miller stain and ethanol washes for the same duration, it was considered quite feasible to compare the relative amount of stained elastic fibres between sections from different regions and between guinea pigs.
In both male and female guinea pigs, transverse sections progressing caudally from the ureteric orifices to the proximal urethra showed a marked increase of circularly arranged elastic fibres in the VUJ. A similar increased density of elastic fibres has also been observed in the VUJ of man (Lapides, 1958; Woodburne, 1961) and dog (Woodburne, 1961), although neither of these studies utilised quantification techniques. It seems plausible that the function of the circularly disposed elastic fibres in the guinea pig VUJ may function to assist in its closure, instilling resilience and compliance to a region that incessantly distends and recoils. Lapides (1958), Woodburne, (1961) and Gosling et al. (1983) firmly proclaimed that closure of the VUJ principally results from the presence of fibroelastic components in this region, since both elastic fibres and smooth muscle possess an inherent capability of generating tension. It is not known exactly why a greater percentage of elastic fibres was observed in the male VUJ other than that they may serve to maintain a competent VUJ capable of preventing retrograde ejaculation.

Median sagittal sections revealed a large array of longitudinally arranged elastic fibres lying predominately in the submucosa and in association with the longitudinal smooth muscle bundles. The bitch urethra has also been shown to possess this arrangement (Augsburger et al. 1993; Augsburger et al. 1997) with fine fibres in the mucosa and coarser fibres in the submucosa. Cullen and co-workers documented elastic fibres as being oriented obliquely in the submucosa of the dog (Cullen et al. 1981a), bitch (Cullen et al. 1981b) and female cat urethra (Cullen et al. 1983), further concluding that elastic fibres were less numerous in the dog than in the bitch urethra.

There are conflicting opinions as to the importance of elastic fibres in the urethra. Hickey et al. (1982) did not observe abundant elastic fibres in the female mouse or human urethra, claiming that their role in the passive restoration of urethral calibre after radial distension is overrated. Donker et al. (1972) asserted that the elastic tissue did not contribute to the urethral pressure profile because the pressure profile was highest in the mid-urethra away from the VUJ and proximal urethra where most of the elastic tissue was reported to be located (Woodburne, 1961). Huisman, (1983) and Tanagho & Smith, (1966) concluded from their observations, which showed a paucity of elastic fibres in the human urethra, that the contribution of elastic fibres to the urethral closure mechanism must be considered as negligible.

From this study of the guinea pig VUJ and urethra we subscribe to the idea that elastic fibres themselves may be partly responsible for the passive occlusive force in the VUJ but contribute very little to resting urethral closure pressure, thus agreeing with Augsburger (1997) and disagreeing with Awad & Downie (1976) and Bump et al. (1988), and further that they may be important in aiding urethral length changes. Considering the fact that tissues tolerate greater stress in the directions in which their collagen and elastic fibres are oriented (Hukins, 1982) and that the circumferential stress in a pipe containing fluid under pressure is shown to be twice the longitudinal stress as determined by rheological studies (Gordon, 1978) then a greater prevalence of circularly arranged elastic fibres in the urethral wall would be expected, particularly if elastic fibres are responsible for urethral closure. However, there was a scanty presence of circularly arranged fibres in the wall of the guinea pig urethra and a preponderance of longitudinally arranged elastic fibres.

Cystography studies have shown that the urethra shortens at the onset of voiding (Graber et al. 1974). This action has been attributed to contraction of the longitudinal smooth muscle bundles in the urethra (Lapides, 1958) which have also been implicated in ‘funnelling’ and consequently opening the VUJ. Recent biochemical (Hornebeck et al. 1986) and morphological studies (Murakumo et al. 1995) have shown elastic fibres to be intimately associated with smooth muscle cells. We consider the elastic fibres associated with the longitudinal smooth muscle bundles of the urethra suitable for both unifying the contractile force of the smooth muscle bundles to shorten the urethra, as well as augmenting passive recoil of the longitudinal smooth muscle bundles, hence restoring urethral length. This uniform transmission of contractile force by collagen and elastic fibres to muscle bundles was also commented upon by Paniagua et al. (1983) in a study investigating the distribution of elastic fibres in the human ductus deferens. They further ascribed the role of elastic fibres in the lamina propria as providing elastic recoil of the ductus following contraction and dilatation at ejaculation and as participating in peristaltic movement.

Structures of the lower urinary tract have evolved to provide an efficient mechanism for urine storage and expulsion. Although extrapolation of results from the guinea pig urethra to events in the human urethra must be made cautiously, we feel that the presence and organisation of elastic fibres in the guinea pig VUJ and urethra is consistent with the role these structures play in accomplishing both continence and micturition.
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REFERENCES