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REGIONAL DIFFERENCES IN THE EXTRACELLULAR MATRIX OF THE HUMAN SPONGY URETHRA AS EVIDENCED BY THE COMPOSITION OF GLYCOSAMINOGLYCANS

E. ALEXSANDRO DA SILVA, FRANCISCO J. B. SAMPAIO,* VALDEMAR ORTIZ AND LUIZ E. M. CARDOSO

From the Urogenital Research Unit, State University of Rio de Janeiro, Rio de Janeiro, Brazil

ABSTRACT

Purpose: Despite the concept that the spongy urethra is a unique entity clinical evidence suggests the existence of segmental structural differences. The spongy urethra has a vascular nature, its cells may express different phenotypes and the extracellular matrix that they synthesize should reflect these differences. Glycosaminoglycans are components of the extracellular matrix that have key roles in the normal physiology and pathology of several tissues. Although total collagen content of the urethra was determined, we also analyzed urethral glycosaminoglycans (GAGs).

Materials and Methods: Fresh, macroscopically normal cadaveric urethral samples were obtained from 15 men who died at a mean age of 25.4 years. The urethra was divided into glanular, penile and bulbar segments, which were then analyzed separately. Total GAG concentration was assessed by hexuronic acid assay and expressed as μg. hexuronic acid per mg. dry tissue, while the proportions of sulfated GAGs were determined by agarose gel electrophoresis. Hyaluronan concentration was determined by ion exchange chromatography and total tissue collagen was estimated as hydroxyproline content.

Results: Total GAG concentration was heterogeneous along the spongy urethra ($p < 0.001$). Mean values plus or minus standard deviation in the glanular, penile and bulbar segments were 2.53 ± 0.42, 2.11 ± 0.47 and 1.47 ± 0.4 μg./mg., respectively. The most predominant GAG was hyaluronan and its highest mean concentration of $50.1\% \pm 3.7\%$ was found in the glanular urethra. The most predominant sulfated GAG in the male urethra was dermatan sulfate, followed by chondroitin sulfate and heparan sulfate. Total collagen content and the GAG-to-collagen ratio varied along the spongy urethra and were lowest in the bulbar segment.

Conclusions: The extracellular matrix of the human spongy urethra shows regional differences, as evidenced by biochemical analysis of GAG and collagen. This heterogeneity implies functional adaptations in the various segments and may affect the physiology and segmental incidence of urethral diseases.

KEY WORDS: urethra, extracellular matrix, glycosaminoglycans, proteoglycans, collagen

The epithelium of the human male urethra has regional differences and may be squamous, stratified or pseudostratified columnar, or transitional cell epithelium.[1] Beneath the basement membrane a layer of connective tissue contains the vascular sinusoids of the corpus spongiosum and smooth muscle fibers as well as numerous mucous glands.[2,3] The connective tissue includes cells, mainly fibroblasts and an extracellular matrix that contains collagen, proteoglycans, elastic fibers and glycoproteins.[4] The extracellular matrix has important roles in tissue and organ development, remodeling and wound repair.[5,6] Although some components of the urethral extracellular matrix have been studied previously,[7,8] to our knowledge there are no data on glycosaminoglycan (GAG) composition in the different segments of the male urethra.

GAGs are heteropolysaccharide chains composed of disaccharide repeating units, in which 1 sugar is a hexosamine and the other is a neutral sugar or hexuronic acid.[9] Except for hyaluronan, also known as hyaluronic acid, which is not sulfated and occurs as free chains in tissues, each GAG is covalently linked to a protein, forming proteoglycans. Proteoglycans are important components of cell surface and extracellular matrixes, and individual proteoglycans interact specifically with other matrix components, such as collagen, laminin and fibronectin, as well as with growth factors and cytokines.[10] These interactions are responsible for structural organization of the extracellular matrix, regulation of cell-cell and cell-matrix interaction, and modulation of the cytokine effect. Thus, GAGs have a key role in the extracellular matrix and may affect cell growth, migration, adhesion and differentiation.

Despite the general belief that the spongy urethra has a uniform structure clinical evidence suggests that structural differences may be present along it. Biomechanical properties required for normal functioning of the spongy urethra differ significantly among the glanular, penile and bulbar segments. Also, some diseases often affect specific segments of the male urethra, for example balanitis xerotica obliterans,[11] and the molecular mechanism of anterior urethral development has not yet been completely elucidated.[12] These facts suggest a differential composition and structural organization of cellular and stromal components along the male

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* Requests for reprints: Urogenital Research Unit-UERJ, Av. 28 de Setembro, No. 87, Fundos-FCM-térreo, 20551–030, Rio de Janeiro, RJ, Brazil.

urethra. The spongy urethra can be considered a vascular organ that contains fibroblasts as the major cellular component.[13] It has been shown that the composition of the extracellular matrix varies along the arterial tree and different vascular cells may express different phenotypes, which should be reflected in the extracellular matrix that they synthesize.[14–16] We performed biochemical quantification of major components of the extracellular matrix in 3 segments of the spongy urethra of young men. Although total collagen content was determined, emphasis was placed on the analysis of GAGs.

MATERIALS AND METHODS

Fresh, macroscopically normal cadaveric urethras were obtained from 15 men 16 to 38 years old (mean age 25.4) who died of causes not related to the urogenital tract. The local committee on human research approved the investigation. None of the subjects was circumcised. Time between death and specimen extraction was 4 to 10 hours (mean 6).

After liberation of all skin around the penis a small H-shaped fragment of the pubic bone was removed. The urethra and penile shaft were completely removed and stored at −20C. Specimens were thawed after 24 to 48 hours and the spongy urethra was completely mobilized from adjacent structures. It was divided into anatomical segments, including the glanular (glans penis limited by the coronal sulcus), penile (from 1.5 cm. proximal to the urethral junction with the coronal sulcus to 1.5 cm. distal to the median insertion of bulbospongiosus muscle) and bulbar (the following 1.5 cm. proximal to the median insertion of the bulbospongiosus muscle to urethral insertion in the rhabdosphincter) urethra. Blood residues and eventual secretions of the intraurethral glands were removed with saline solution. The whole samples were immersed immediately in 10 volumes of acetone, finely minced and defatted in chloroform-methanol, 2:1 volume per volume.

Total tissue collagen was estimated as hydroxyproline content.[17] Total GAGs were extracted by a previously described protocol.[14] Briefly, approximately 155 mg. dry tissue were rehydrated for 24 hours at 4C in 0.1 M. sodium acetate buffer, pH 5.0, containing 5 mM. cysteine and 5 mM. ethylenediaminetetraacetic acid. Twice crystallized papain (Sigma Chemical Co., St. Louis, Missouri) was added and the mixture was incubated at 60C for 24 hours. After centrifugation cetylpyridinium chloride was added to the supernatant to precipitate GAGs. The GAG-cetylpyridinium chloride complex in the pellet was dissolved in 2 M. NaCl and GAGs were precipitated by adding absolute ethanol. Precipitates were collected by centrifugation and washed twice with 80% ethanol and once with absolute ethanol. The final pellet was dried and dissolved in distilled water. This material was then used for all subsequent analyses.

The concentration of total GAGs was assessed by a hexuronic acid assay[18] and expressed as μg. hexuronic acid per mg. dry tissue. Total GAGs were submitted to agarose gel electrophoresis in 0.05 M. 1,3-diaminopropane: acetate buffer, pH 9.0.[19] The relative concentration of sulfated GAGs (chondroitin sulfate, dermatan sulfate and heparan sulfate) was determined by densitometry of toluidine blue stained gel using Scion Image 4.0.2 software (Scion Corp., Frederick, Maryland). Total GAGs were fractionated by ion exchange chromatography on DEAE-Sephacel (Pharmacia, Piscataway, New Jersey) columns eluted with a linear gradient of 0.1 → 0.9 M. NaCl. The 3 peaks obtained were identified as hyaluronan, heparan sulfate and chondroitin sulfate plus dermatan sulfate using previously described procedures.[14] Differences among groups were determined by 1-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparisons test with $p < 0.05$ considered significant. All numerical data are expressed as the mean plus or minus standard deviation (SD).

RESULTS

Total GAG concentration varied significantly among all 3 analyzed segments of the spongy urethra ($p < 0.001$, fig. 1). Mean total GAGs in the glanular, penile and bulbar urethras were 2.53 ± 0.42, 2.11 ± 0.47 and 1.47 ± 0.4 μg./mg., respectively. The predominant sulfated GAG in the human spongy urethra was dermatan sulfate, followed by chondroitin sulfate and lesser amounts of heparan sulfate (fig. 2). The concentration of chondroitin sulfate showed no significant changes along the spongy urethra ($p = 0.091$), while dermatan sulfate and heparan sulfate showed a heterogeneous distribution. The highest concentration of heparan sulfate (13.5% ± 3.3%) was present in the bulbar portion and was significantly different ($p < 0.05$) from that in the penile and glanular urethras (10.6% ± 2.9% and 9.0% ± 2.8%, respectively). The relative concentration of dermatan sulfate in the glanular urethra (58.5% ± 8.9%) was higher than in the penile and bulbar urethral segments (51.3% ± 5.0% and 50.1% ± 8.7%, respectively).

Ion exchange chromatography separated urethral GAGs into 3 peaks (fig. 3). Hyaluronan was the predominant GAG ($p < 0.01$). Glanular hyaluronan concentration (50.1% ± 3.7%) was significantly higher than that in the penile and bulbar urethral segments (34.8% ± 2.4% and 40.7% ± 5.0%, respectively, fig. 4).

The distribution of total collagen content along the spongy urethra was similar to that of total GAG content. The highest concentration of total collagen, expressed as μg. hydroxyproline per mg. dry tissue, was present in the glanular segment (60.88 ± 8.26), and was significantly different from the concentration in the bulbar urethra (44.63 ± 7.5, fig. 5, *A*). The same difference was found in regard to the GAG-to-collagen ratio (fig. 5, *B*).

DISCUSSION

The mammalian external genitalia are highly developed structures that permit efficient internal fertilization. The human male urethra is structurally unique with functional specialization not similar to that of other mammals.[20,21] Therefore, mechanisms of genital tubercle formation and

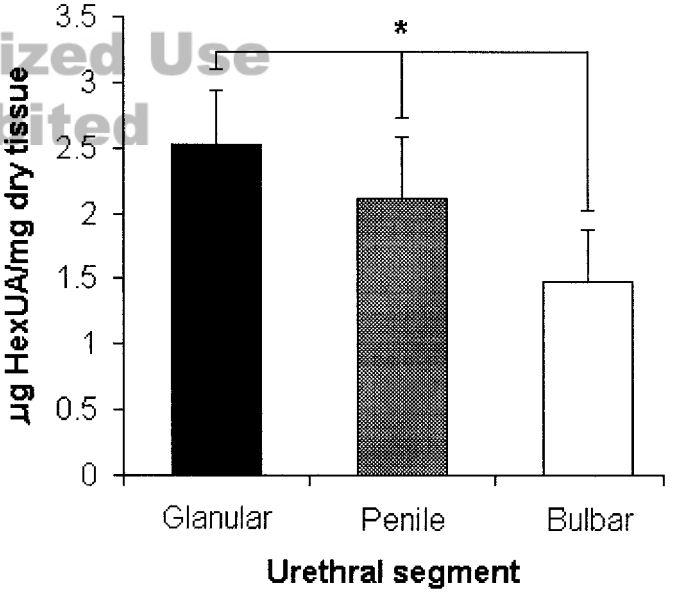

FIG. 1. Concentration of total GAGs in segments of normal human male spongy urethra as assessed by hexuronic acid (*HexUA*) assay varied significantly along urethra (ANOVA $p < 0.001$) and was different in all segments analyzed. Results represent mean of 15 individuals. Error bars indicate 1 SD. Asterisk indicates Bonferroni multiple comparisons test $p < 0.05$.

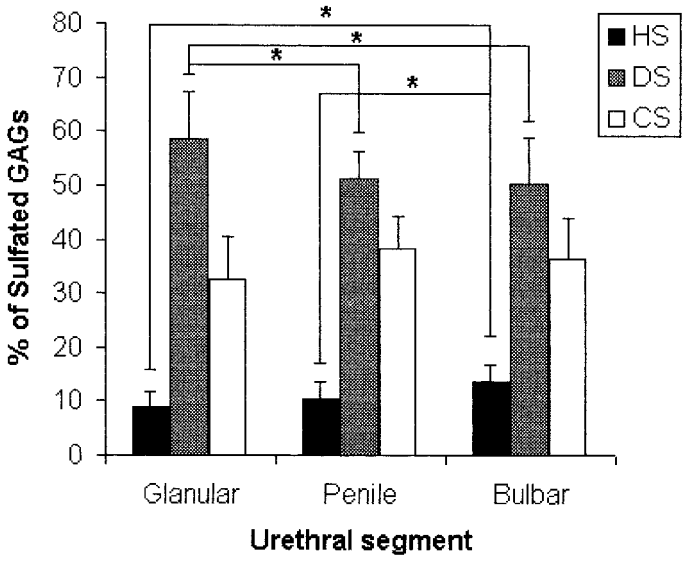

FIG. 2. Relative concentration of sulfated GAGs in segments of spongy urethra determined by agarose gel electrophoresis. Predominant sulfated GAG in all segments was dermatan sulfate (*DS*), followed by chondroitin sulfate (*CS*) and heparan sulfate (*HS*). Heparan sulfate and dermatan sulfate relative concentrations varied among urethral segments (ANOVA $p < 0.001$ and 0.01, respectively). Results represent mean of 15 individuals. Error bars indicate 1 SD. Asterisk indicates Bonferroni multiple comparisons test $p < 0.05$.

urethral disease have been little studied by molecular analysis due to the lack of a suitable ideal experimental model. The extracellular matrix presents significantly structural differences in the phylogenetic scale. For example, proteoglycans are some of the largest, varied and most complex molecular structures in mammalian cells.[10] Thus, it is imperative that studies of normal human male urethra tissues should be performed to gain better knowledge of the major biosynthetic pathways by which cells produce matrix components. After normal structures have been determined molecular alterations in several urethral diseases may be precisely identified. Because matrix molecular changes also occur with aging, we performed biochemical quantification of collagen and GAGs from 15 normal fresh urethras of young men, consisting of a homogenous and significant group.

Anatomically and functionally urethral connective tissue components may serve different purposes in different regions of the male urethra.[7,22] In our study the glans differed markedly from the other spongy segments (bulbar and penile) in total collagen content and GAG composition with a higher concentration of hyaluronan and smaller concentration of heparan sulfate. These differences suggest that since tissue physicochemical characteristics partially depend on the extracellular matrix, different compliance must be present in the segments of the male urethra and different segmental responses to injury may also occur. For example, although balanitis xerotica obliterans, a form of lichen sclerosus et atrophicus, affects the glans and penile urethra by causing stricture, it rarely extends into the bulbar urethra.[11] Lichen sclerosus can be classified as an interface dermatitis characterized by epidermal, basement membrane zone and dermal pathological changes.[23] The epithelial basement membrane zone is a scaffold for tissue organization and regeneration that serves as a physical and chemical barrier, and a template for cell attachment. Although to our knowledge the exact function of GAGs in the lichen sclerosus is not defined, they can have some role. A reasonable explanation of hyaluronan accumulation in the superficial dermis of lichen sclerosus et atrophicus is not available.[24] Heparan sulfate, a basement membrane GAG with heterogeneous distribution along the male urethra, showed the lowest concentration in the glanular urethra and the highest concentration in the bulbar urethra. This curious distribution may be associated with the urethral incidence of balanitis xerotica. Also, the bulbar urethra had the lowest concentration of total collagen, while the glanular urethra showed the highest content. Ultrastructurally the most prominent abnormality in lichen sclerosus is the variation in diameter and alignment of the collagen fibers.[25] Decorin and biglycan are small proteoglycans that contain dermatan sulfate chains. Dermatan sulfate was the predominant sulfated GAG in the spongy urethra and its highest concentration was found in the glanular urethra. Dermatan sulfate proteoglycans inter-

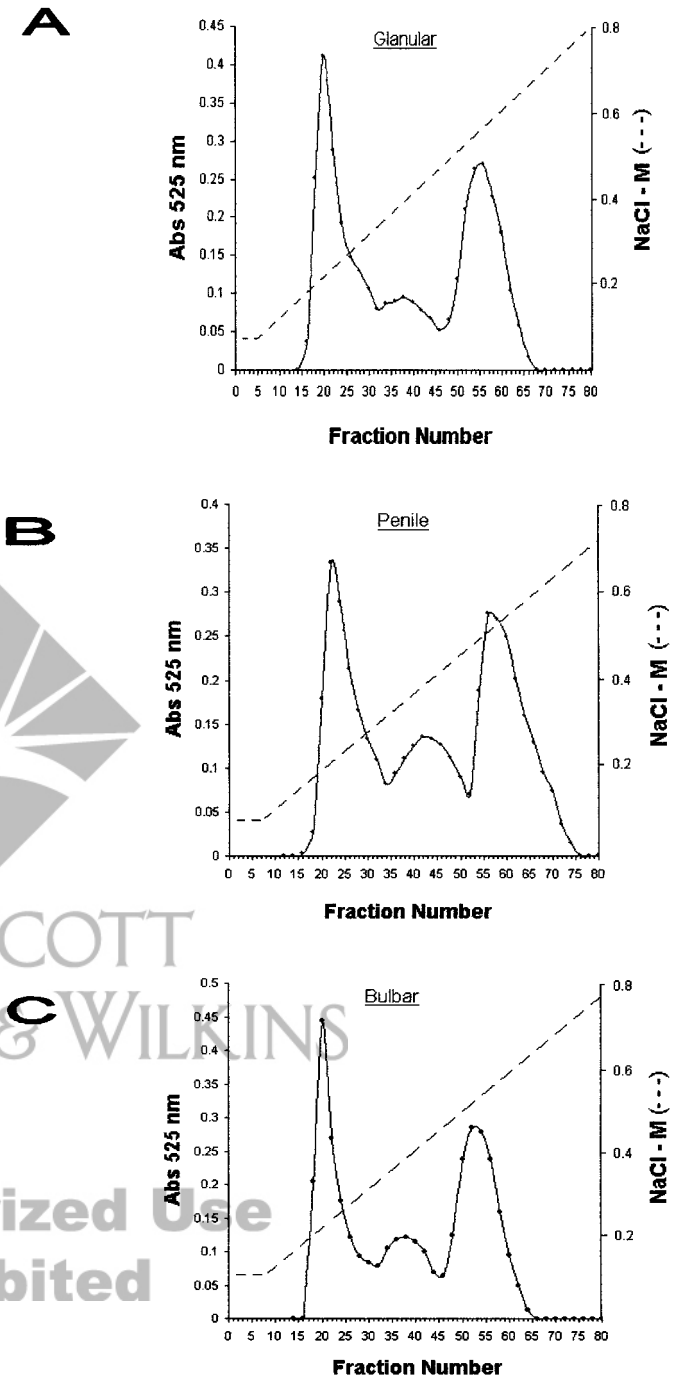

FIG. 3. Ion exchange chromatography of GAGs from 3 segments of 1 representative normal urethra. Fractions were assayed for hexuronic acid (circles) and NaCl (dashed lines) concentrations. Hyaluronan was predominant GAG in all 3 segments. *A*, glanular urethra. *B*, penile urethra. *C*, bulbar urethra. Left peak represents hyaluronan. Middle peak represents heparan sulfate. Right peak represents chondroitin sulfate plus dermatan sulfate. *Abs*, absorbance.

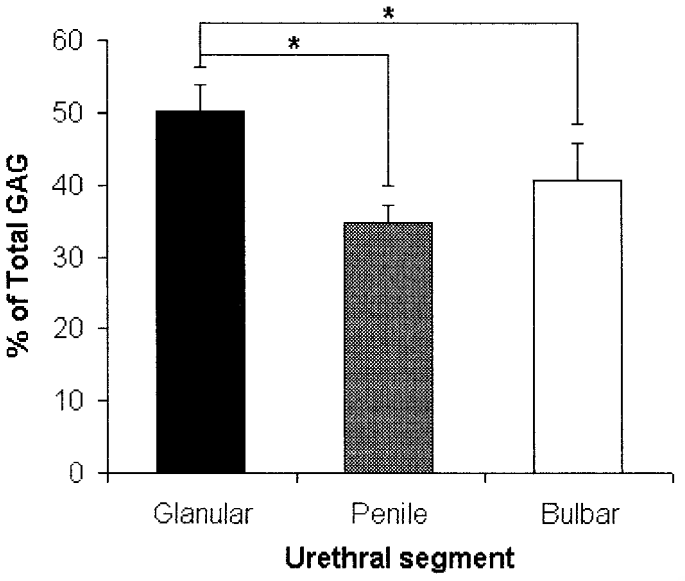

FIG. 4. Relative concentration of hyaluronan in segments of normal human spongy urethra determined by ion exchange chromatography varied significantly along urethra (ANOVA $p = 0.001$) with highest concentration in glanular urethra. Results represent mean of 4 individuals. Error bars indicate 1 SD. Asterisk indicates Bonferroni multiple comparisons test $p < 0.05$.

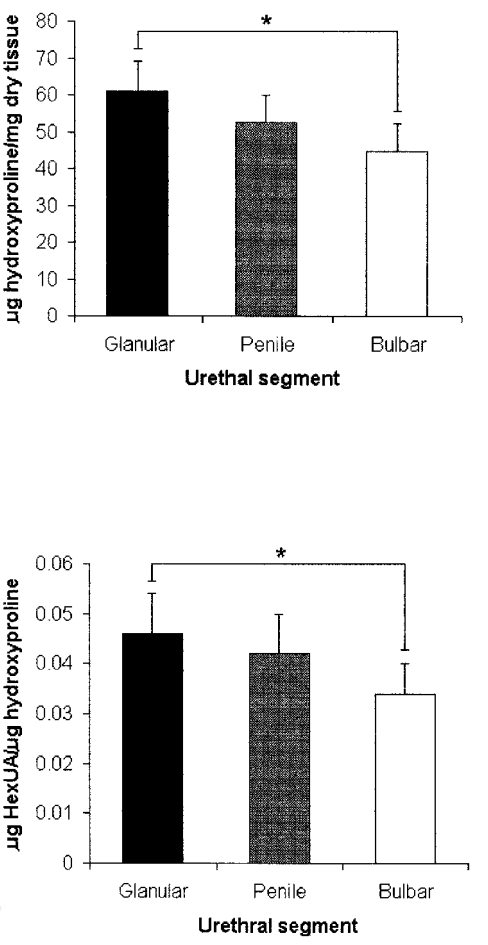

FIG. 5. Total collagen content in 3 segments of spongy urethra of young men as determined by hydroxyproline assay. *A*, total collagen concentration varied significantly along spongy urethra (ANOVA $p < 0.001$). Concentration in glanular urethra was higher than in bulbous portion. *HexUA*, hexuronic acid. *B*, GAG-to-collagen ratio showed significant changes along urethra (ANOVA $p < 0.01$). Results represent mean of 9 individuals. Error bars indicate 1 SD. Asterisk indicates Bonferroni multiple comparisons test $p < 0.05$.

act with collagen and cause increased stability of collagen fibrils and orient fibrillogenesis.[10] Moreover, decorin is important for controlling cell adhesion and proliferation, and it may also inhibit cell migration.[26]

The human spongy urethra has significant collagen content.[8] Due to interaction of GAGs with collagen it was suggested that GAGs have a key role on urethral compliance, although to our knowledge this matter has not been yet studied. The regulation of extracellular matrix deposition is a key event in many physiological and pathological conditions, and altered proteolytic balance may favor accumulation of extracellular matrix and, therefore, decreases tissue compliance characteristics, for example fibrosis.[27] This scenario can be applied to urethral injury, in which final compliance of the strictured urethra determines the clinical presentation and urodynamic findings.

The urethra is a target of several kinds of injury. In addition to trauma, urethral strictures may also be idiopathic, congenital or due to infection, such as gonococcal urethritis.[3] Bacterial invasion of mucosal cells of the human urethra is considered a primary event in the pathogenesis of a gonococcal infection. Cell surface heparan sulfate proteoglycans may have a role in establishing infection by functioning as receptors for the invasion promoting gonococcal opacity protein.[28] Furthermore, physicochemical properties of the human urethral lining, which depend on GAGs composition, permit interaction with another microorganism, such as Escherichia coli and Staphylococcus saprophyticus.[29]

Chondroitin sulfate proteoglycan has been implicated in the regulation of cell migration and others tissue specific functions. Also, it may interact with hyaluronan and, therefore, may have a role in homeostasis.[10] In contrast to the distribution of the other urethral GAGs, we found that chondroitin sulfate has homogeneous distribution along the spongy urethra. The role of chondroitin sulfate in the human urethra remains only speculative.

High hyaluronan content has been reported in the rat penis.[30] However, quantification of corpus spongiosum hyaluronan was not performed separately from that of the other penile structures, such as the corpora cavernosa penis, tunica albuginea and os penis.[31] GAG composition differs in the human spongy urethra and corpora cavernosa penis or tunica albuginea.[32] The significantly greater concentration of hyaluronan, total GAGs, collagen and GAG-to-collagen ratio in the glanular urethra suggests that this segment is subjected to higher tensile strength under stressful functional conditions compared with the bulbar segment. While the bulbar segment has a key role in the ejaculation process, it does not participate actively during intercourse. Hyaluronan may act as a structural element and due to its physicochemical properties hyaluronan has an important hydrodynamic role in tissues.[33] When subjected to compressive forces, water is displaced from individual molecules. This tissue swelling is readily dissipated when compressive forces are removed.[10] Considering that viscoelasticity of the hyaluronan molecule is important for the structural integrity and functioning of the connective tissues, it may well be involved in the biomechanical properties of the penis. The glans must remain supple and yet stable during thrusting, while the penile shaft must be rigid with high buckling pressure.[7] Also, organs that change shape under varying physiological circumstances, for example the uterus, bladder and penis, show more hyaluronan between the epithelial cells or smooth muscle cell layers than organs that do not change shape to any great extent.[30]

Hyaluronan may also act as a signaling molecule.[33] While the literature on hyaluronan in several tissues is abundant, to our knowledge no study has yet shown hyaluronan signaling functions specifically in the urethra. Hyaluronan and cell surface heparan sulfate proteoglycans appear to have modulatory roles,

such as presenting growth factors to their primary receptors.[34,35] Fibroblast growth factors are essential signaling molecules for embryogenesis and events involving repair. It has been demonstrated that the fibroblast growth factor system is an essential element for orchestrating genital tubercle development, particularly in the glanular urethra.[36]

The male urethra is an androgen sensitive organ.[37,38] Deficiency or inhibition of the enzyme 5α-reductase, which converts testosterone into its 5-fold more potent metabolite dihydrotestosterone, is a known cause of hypospadias.[39] Some members of the fibroblast growth factor family are a heparin/heparan-binding growth factor[40] and may have a role in hormonal modulation of the urethral tissue. We noted the highest concentration of heparan sulfate in the bulbar urethra, which is the site of the urethral meatal opening in the most severe type of hypospadias.

CONCLUSIONS

The concentration of collagen and GAGs varies significantly along the human spongy urethra. Moreover, GAG composition showed a different distribution in the 3 segments analyzed. Therefore, the extracellular matrix of the normal spongy urethra of young men has regional differences. This heterogeneity suggests segmental and functional adaptations of this tissue and may be related to the incidence and localization of urethral disease. Our results of GAG composition in the normal human urethra also provide a basis for studies of GAG-proteoglycan metabolism in pathological processes of the male urethra.

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