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ANALYSIS OF THE MODIFICATIONS IN THE COMPOSITION OF BLADDER GLYCOSAMINOGLYCAN AND COLLAGEN AS A CONSEQUENCE OF CHANGES IN SEX HORMONES ASSOCIATED WITH PUBERTY OR OOPHORECTOMY IN FEMALE RATS

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ABSTRACT

Purpose: The effects of female sex hormones on rat vesical extracellular matrix were evaluated by analyzing glycosaminoglycan (GAG) and collagen composition under different hormonal conditions.

Materials and Methods: Bladders were obtained from Wistar rats, including young prepubertal females at age 30 days (YF), and adult intact females (AF), adult oophorectomized females (AOF), adult males and adult sham operated females at age 120 days. Oophorectomy and sham operation were performed at age 30 days. Bladders were analyzed for total GAG and collagen concentration per mg dry tissue and for the contents of GAG species, as determined by agarose electrophoresis and reported as the percent of total sulfated GAG.

Results: Collagen concentration in AF (54.80 ± 4.60 μg/mg) was different from that in YF (34.52 ± 5.29 μg/mg, $p < 0.001$) and AOF (63.25 ± 3.51 μg/mg, $p < 0.001$). GAG concentration in AF (0.71 ± 0.18 μg/mg) was different from that in YF (0.45 ± 0.07 μg/mg, $p < 0.001$) and males (0.46 ± 0.10 μg/mg, $p < 0.001$). The GAG species detected were dermatan sulfate and heparan sulfate. Dermatan sulfate content in AF (90.9% ± 2.8%) was different from that in YF (86.6% ± 2.4%, $p < 0.005$), AOF (87.9% ± 2.1%, $p < 0.005$) and males (87.7% ± 4.7%, $p < 0.005$). Heparan sulfate content in AF was 9.1% ± 2.8%, which differed from that in YF (13.4% ± 2.4%, $p < 0.025$) and AOF (11.2% ± 2.9%, $p < 0.025$).

Conclusions: Extracellular matrix of the female rat bladder undergoes marked remodeling during normal growth up to early adulthood with important consequences for vesical viscoelastic properties. Also, oophorectomy performed at a prepubertal age may lead to greater vesical wall stiffness.

KEY WORDS: bladder; extracellular matrix; menopause; puberty; rats, Wistar

Sex hormones can variously affect extracellular matrix (ECM) molecule turnover by mesenchymal cells such as fibroblasts and smooth muscles cells in vivo[1–3] and in vitro.[4] Although this effect usually has a normal regulatory role, ECM may also be abnormally affected, which has been associated with many diseases, including urinary tract disorders. Of these disorders urinary incontinence, vesical instability[5] and interstitial cystitis[6] have a higher incidence in women and/or are associated with female sex hormones. However, the effects that these hormones would have on ECM components of the vesical wall, such as collagen and proteoglycans, are little known to date.

Because of their involvement in several vesical disorders, urothelial surface glycosaminoglycans (GAG) have received much attention from investigators.[6] However, ECM components, including proteoglycans and their constituent GAG, are also distributed elsewhere in the bladder wall.[7] They have been implicated in other urinary disorders such as vesical instability[5] that are associated with alterations in mechanical functions of the urinary tract wall. Such functions are more often attributed to smooth muscle cell contractility and its regulation, which are adversely affected by estrogen deprivation.[8] However, vesical ECM also has a noticeable role in these properties.[9] Since the synthesis of proteoglycans and collagen by mesenchymal cells can be modulated by estrogens,[4,10] changes in these hormones could lead to ECM remodeling and, thus, to alterations in bladder function.

Although experimental oophorectomy increases vesical collagen concentration,[2,5] to our knowledge there are no data yet on how it would affect bladder wall proteoglycans/GAG. An early investigation demonstrated that oophorectomized rabbits and dogs are more prone to vesical infection[11] and it has been suggested that this result might be related to a decrease in urothelial surface GAG. However, there is no direct evidence showing actual changes in bladder wall GAG as a result of sex hormone deprivation. Likewise, information on the effects that growth, especially the onset of puberty, would have on the overall composition of the vesical ECM is rather limited[12] and little is known on whether this composition differs between mature females and males. In the current study these issues were addressed by evaluating the effects of female sex hormones on the biochemical composition of vesical GAG and collagen in rats under different hormonal conditions.

MATERIALS AND METHODS

Animals. Wistar rats were divided into groups and sacrificed at certain ages, including young females at age 30 days

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(prepubertal), and intact adult females (non-old adult), oophorectomized adult females, adult males and sham operated adult females at age 120 days. Bilateral oophorectomy and sham operation were performed at age 30 days using ketamine general anesthesia. All bladders from these animals were analyzed individually. The number of specimens per group varied according to the experiment.

Bladder preparation. Animals were sacrificed under ether anesthesia. Immediately afterward the whole bladder, excluding the urethra and ureters, was removed, fixed in cold acetone and maintained in this fixative for 24 hours at 4C. The bladders were then finely minced and submitted to 2 changes of 24 hours each in 40 ml chloroform-methanol (2:1 volume per volume) at room temperature. The solvent was decanted and after incubation at 60C for 30 minutes a preparation of whole dry and defatted bladder was obtained and weighed.

GAG extraction, identification and quantitation. All procedures for the extraction, purification, identification and quantitation of GAG were done as previously described.[13] Briefly, about 15 to 25 mg dry vesical tissue samples were digested with twice crystallized papain (Sigma Chemical Co., St. Louis, Missouri). Free GAG chains in the supernatant were isolated by precipitations with cetylpyridinium chloride and ethanol. The amount of GAG in this preparation was assessed by hexuronic acid assay. Identification of the different GAG species was accomplished by agarose gel electrophoresis combined with analysis of the susceptibility of individual GAG bands to degradation by chondroitin AC and ABC lyases (Sigma Chemical Co.) and nitrous acid.

To quantitate the sulfated GAG species, including chondroitin sulfate, dermatan sulfate (DS) and heparan sulfate (HS) stained gel slabs were digitized on a flatbed scanner. Band density was measured with Scion Image, version 4.0.2 (Scion Corp., Frederick, Maryland) software.

Determination of collagen concentration. About 10 to 15 mg dry, defatted tissue sample were hydrolyzed in 1 ml 6 N HCl for 18 hours at 118C in sealed test tubes. The hydrolysate was then diluted by adding 5.7 ml distilled water and neutralized with 1 ml 6 N NaOH. Insoluble residues were removed by centrifugation at 3,000 × gravity for 15 minutes. Hydroxyproline was assayed in this hydrolysate as previously described.[13]

Statistics. Statistical procedures were done according to Sokal and Rohlf[14] using an in-house developed C++ program. Variations among groups for each quantitative parameter were analyzed by 1-way ANOVA. When significant differences between biochemical parameters were detected, pairwise planned comparisons were done for intact adult females vs young females, oophorectomized adult females, adult males and sham operated females. These comparisons were evaluated using the Bonferroni method for multiple comparisons. Whole bladder dry weight was analyzed only in terms of how it would be affected by hormonal deprivation. Therefore, oophorectomized females were compared with intact adult females only and the differences were assessed by 1-way ANOVA. All results are presented as the mean ± SD with statistical significance considered at $p < 0.05$.

RESULTS

The concentration of collagen in the bladder wall of the rat was significantly affected by female sex hormones using the young female, adult female, oophorectomized adult female and male groups (ANOVA $p < 0.001$, fig. 1). In addition, procedures to locate individual divergent groups indicated that the collagen concentration was 58.8% higher in adult than in young females (54.80 ± 4.60 vs 34.52 ± 5.29 μg/mg, $p < 0.001$), while in oophorectomized adult females the concentration of this molecule increased by 15.4% compared with adult females (63.25 ± 3.51 μg/mg, $p < 0.001$, fig. 1). On the other hand, the collagen content of male bladders (54.69 ± 6.57 μg/mg) was almost identical to that of adult females (fig. 1).

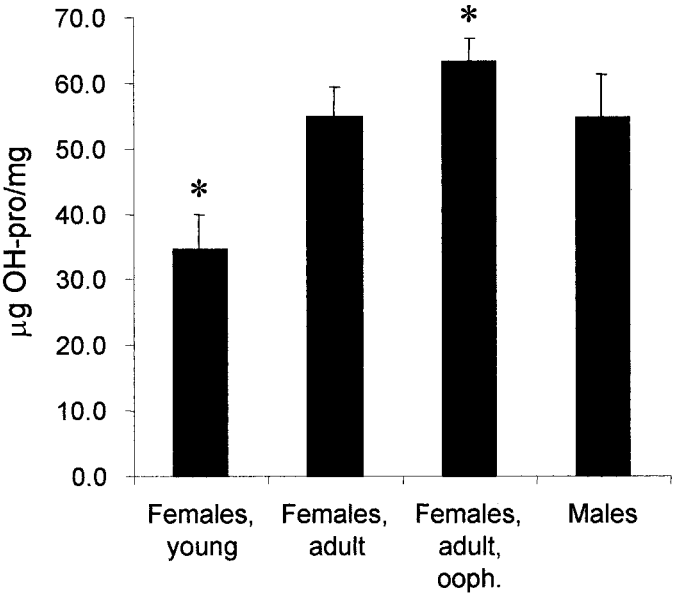

FIG. 1. Concentration of vesical collagen in rats under different sex hormone conditions. Dry, defatted tissue samples were submitted to acid hydrolysis, followed by hydroxyproline (*OH-pro*) assay to estimate collagen concentration. Results are shown as μg hydroxyproline per mg dry tissue. Bars represent mean ± SD of 25 young females, 30 adult females, 15 adult oophorectomized (*ooph.*) females and 15 males. Asterisk indicates significant differences vs adult females (Bonferroni test pairwise multiple comparisons $p < 0.001$).

As for collagen, vesical GAG concentration was also affected by female sex hormones (1-way ANOVA $p < 0.001$, fig. 2). In the adult female bladder GAG concentration was 55.3% higher than in young females (0.71 ± 0.18 vs 0.45 ± 0.07 μg/mg, $p < 0.001$, fig. 2). However, compared with the adult female group oophorectomy did not significantly change vesical GAG content (0.70 ± 0.11 μg/mg, fig. 2). Therefore, since oophorectomy increased collagen concentration by 15.4%, this procedure decreased the GAG-to-collagen ratio in the bladder wall by a similar amount. Also in contrast to what was verified for collagen, adult female bladder had 52.5% more GAG than adult male bladder (0.46 ± 0.10 μg/mg, $p < 0.001$, fig. 2).

Agarose gel electrophoresis of total extracted GAG revealed that the rat bladder wall contained DS and HS with little or no chondroitin sulfate (data not shown). Therefore, we focused quantitative analyses on these 2 prevailing GAGs. Overall the relative content of DS varied little but significantly among the groups (1-way ANOVA $p < 0.001$, fig. 3). Adult females had a DS content that was 5.0% greater than that of young females (90.9% ± 2.8% vs 86.6% ± 2.4%, $p < 0.005$, fig. 3), whereas oophorectomy decreased this content by 3.3% with regard to that of adult females (87.9 ± 2.1%, $p < 0.005$, fig. 3). Adult females also had slightly more (3.7%) DS than males (87.7% ± 4.7%, $p < 0.005$, fig. 3). On the other hand, relative changes in the proportion of HS among the groups were more pronounced (1-way ANOVA $p < 0.001$, fig. 4). Thus, adult females had 32.4% less HS than young females (9.1% ± 2.8% vs 13.4% ± 2.4%, $p < 0.025$, fig. 4), while in oophorectomized adult females the relative content of this GAG was 23.7% higher than in adult females (11.2% ± 2.9%, $p < 0.025$, fig. 4). However, the relative content of HS in male bladders (10.2% ± 2.8%) was not significantly different from that in adult females (fig. 4). Oophorectomy also decreased whole bladder dry weight by 9.8% in relation to values of adult females (16.60 ± 2.65 vs 18.40 ± 2.55 mg, p

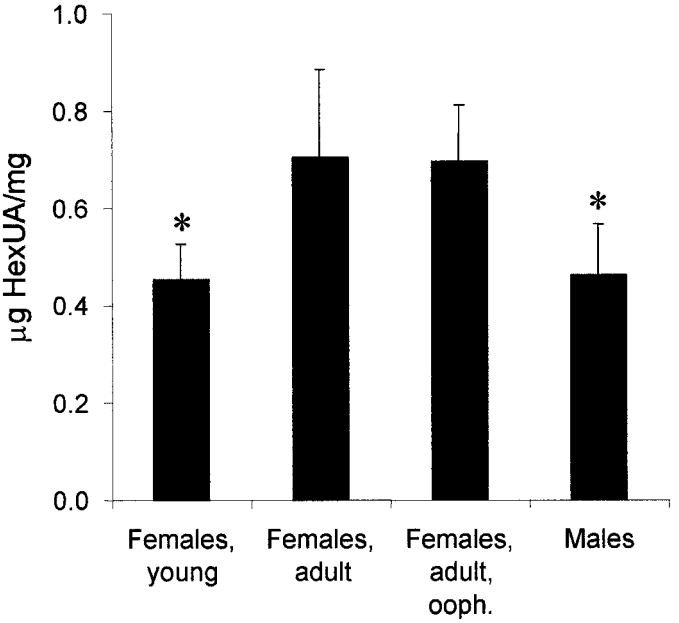

FIG. 2. Concentration of vesical GAG in rats under different sex hormone conditions. Dry, defatted tissue samples were submitted to papain digestion and precipitation with cetylpyridinium chloride, followed by hexuronic acid (*HexUA*) assay to estimate GAG concentration. Results are shown as μg hexuronic acid per mg dry tissue. Bars represent mean ± SD of 25 young females, 27 adult females, 21 adult oophorectomized (*ooph.*) females and 24 males. Asterisk indicates significant differences vs adult females (Bonferroni test pairwise multiple comparisons $p < 0.001$).

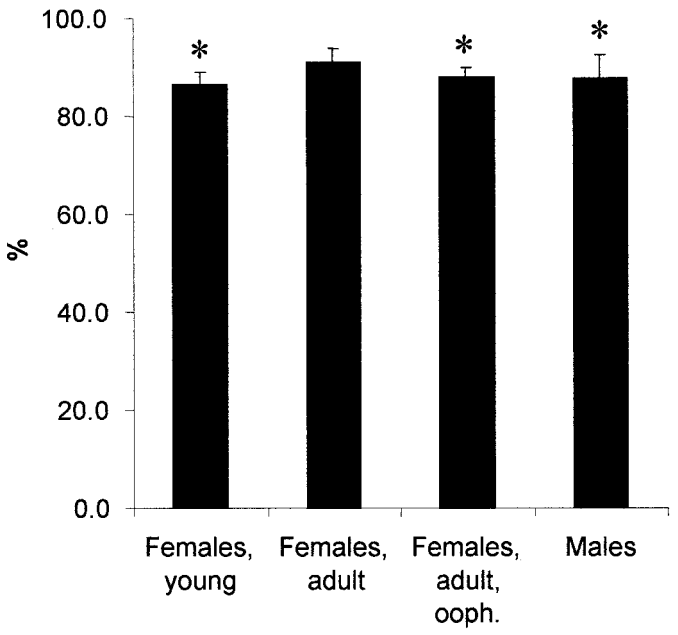

FIG. 3. Relative bladder DS content in rats under different sex hormone conditions. Total GAG was extracted from defatted tissue samples and submitted to agarose gel electrophoresis, from which relative DS content as percent of total sulfated GAGs was determined. Bars represent mean ± SD of 25 young females, 24 adult females, 18 adult oophorectomized (*ooph.*) females and 27 males. Asterisk indicates significant differences vs adult females (Bonferroni test pairwise multiple comparisons $p < 0.005$).

<0.05, fig. 5). In sham operated adult females the results of these biochemical parameters for vesical GAG and collagen as well as for whole bladder dry weight were not significantly different from those of adult females (data not shown).

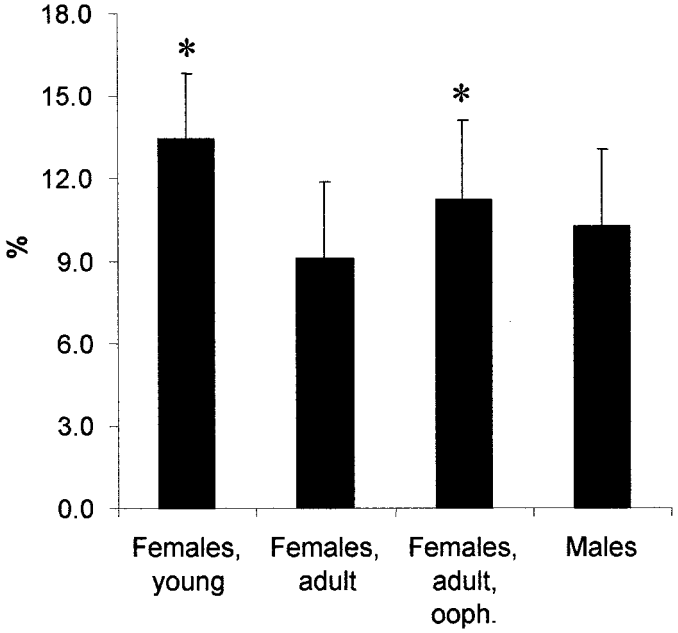

FIG. 4. Relative bladder HS content in rats under different sex hormone conditions. Relative contents of individual GAG species were determined by agarose gel electrophoresis. Results are shown as percent of total sulfated GAG. Bars represent mean ± SD of 25 young females, 24 adult females, 21 adult oophorectomized (*ooph.*) females and 21 males. Asterisk indicates significant differences vs adult females (Bonferroni test pairwise multiple comparisons $p < 0.025$).

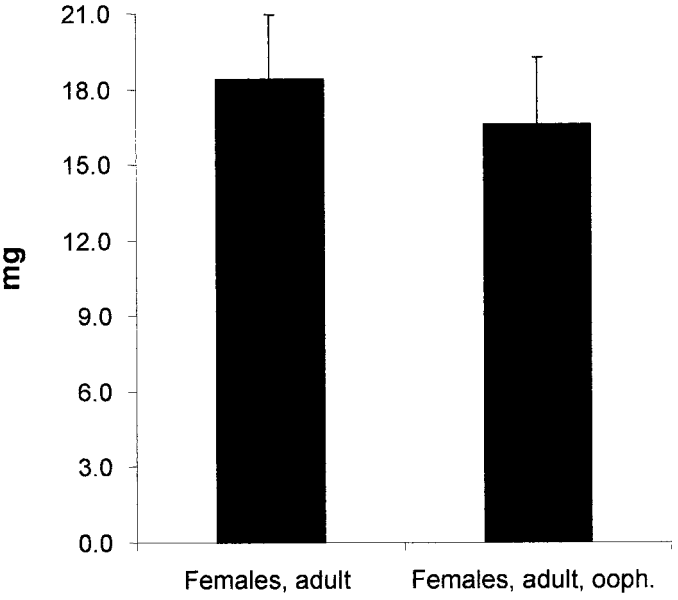

FIG. 5. Effects of oophorectomy (*ooph.*) on bladder dry weight. Whole bladders excluding urethra and ureters were removed, fixed in acetone, delipidated in chloroform-methanol, dried at 60C and weighed. Bars represent mean ± SD of 19 adult females and 21 adult oophorectomized females (1-way ANOVA $p < 0.05$).

DISCUSSION

Experimental studies of postmenopause urinary disorders are usually done in animal models in which oophorectomy is performed after puberty and often late in adulthood.[1,2,5,11] The experimental model used in the current study differs in this regard, in that oophorectomy was performed at a prepubertal age, thereby, allowing us to investigate how sex hormones would affect vesical ECM composition in the normal postnatal growth of the female rat.

Our results show that variations in plasma levels of female sex hormones paralleled different changes in ECM composition of the rat bladder wall. During normal growth of the female rat from a prepubertal age to early adulthood there were marked increases in total GAG and collagen concentrations as well as a slight increase in DS and a more important decrease in HS. Compared with intact adult females, bladders from oophorectomized adult females had a slightly higher collagen concentration but showed no change in total GAG, whereas DS and HS content was decreased and increased, respectively. Bladders from adult males differed from those of females of comparable age in that they had less total GAG and, hence, a higher collagen-to-GAG ratio and slightly less DS.

Since GAG and collagen have important structural functions in tissues subjected to stretching forces, these modifications have direct consequences for the viscoelasticity of the bladder wall. In the arterial wall resistance to deformation is decreased by up to 10 times when GAG or collagen is enzymatically removed[15] and stiffness correlates positively with collagen concentration.[16] Thus, even if we consider structural differences between the arterial and vesical walls, our results suggest that the bladder wall of a non-old adult female is more resistant to deformation than that of a prepubertal female, estrogen deprivation before puberty leads to a slightly stiffer bladder wall in non-old adult females and the male bladder is slightly stiffer than that of the female.

Although the concentration of skin collagen is decreased in oophorectomized rats,[1] in general estrogen inhibits collagen synthesis by several types of mesenchymal cells.[4] In the absence of estrogen the expected opposite effect occurs. Thus, in experimentally induced liver fibrosis Xu et al noted that oophorectomy enhanced collagen accumulation.[3] They also found that the fibrotic response of the female liver was less than that of the male, which implies an inhibitory effect of estrogen on collagen synthesis. These facts are in agreement with our findings showing a 15% increase in vesical collagen concentration in the oophorectomized rats.

However, a seemingly stimulatory effect of estrogen underlies the 60% increase in collagen concentration in adult females compared with young prepubertal animals. This finding may be explained if one considers that trophic factors that cause the bladder to increase in size during normal animal growth would also stimulate collagen synthesis, especially in the growth spurt after puberty. In fact, there is an increase in steady state mRNA levels for type I collagen shortly after puberty.[12] Because estrogen levels also increase with the onset of puberty, inhibitory action of the sex hormone on collagen would be antagonized by this stimulatory effect of the trophic factors. Consequently, if estrogen deprivation is performed, the stimulatory effects of the trophic factors on collagen would be enhanced. These results also indicate that since type I is the most abundant collagen type in the majority of tissues, including the rat bladder,[17] the marked increase in vesical collagen in the normal growth of the female rat from ages 30 to 120 days is due at least in part to type I collagen. Notably in this period there are also 2 important GAG changes that bear on the mechanisms proposed. 1) There is a slight but significant increase in DS, a GAG derived mostly from decorin, which is a proteoglycan typically associated with type I collagen in interstitial connective tissue.[18] 2) Prepubertal female bladders have noticeably greater content of HS, a GAG that corresponds among others to cell surface proteoglycans that act as co-receptors for growth factors.[18] The rat bladder also contains type III collagen,[17] which imparts tissue flexibility, whereas a tissue that is more enriched in type I collagen would be less compliant.[16] Thus, growth related greater expression of type I collagen also supports the proposition that the vesical wall of non-old adult females is less distensible than that of a prepubertal female.

The alterations that we found in the bladder wall of the oophorectomized group are similar to those that the paraurethral ECM of women undergoes after menopause.[19] Accordingly there is an increase in collagen concentration and a decrease in the GAG-to-collagen ratio. However, it should be mentioned that in the rat the increase in collagen concentration was much less pronounced, while the relative content of DS was decreased and that of HS was increased, implying changes in proteoglycan composition. However, in paraurethral tissue proteoglycans were unchanged. Also in contrast to our findings, in the rat uterine tissue estrogen increases the overall contents of galactosaminoglycans as well as the message for syndecan-3, a cell surface HS proteoglycan.[10] In the sheep uterus the message for decorin, a DS proteoglycan, is also enhanced by estrogen,[20] which is in line with the slight but significant decrease in vesical DS content that we detected in the oophorectomized group. In addition, although estrogen has an inhibitory effect on vascular smooth muscle cell proliferation,[4] our results showed that oophorectomy decreases overall bladder weight, as also reported by Eika et al.[2] Together these results imply that with respect to proliferation as well as to collagen and proteoglycan synthesis bladder wall cells respond to estrogen in ways that are different from those of other tissues.

CONCLUSIONS

Our results indicate that ECM of the female rat bladder undergoes marked remodeling during normal growth up to early adulthood with important consequences on vesical viscoelastic properties. If oophorectomy is performed at a prepubertal age, less pronounced but significant ECM alterations occur and contribute to greater vesical wall stiffness.

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