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ORIGINAL ARTICLE

Extracellular matrix remodeling in the human gubernaculum during fetal testicular descent and in cryptorchidic children

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Abstract
Purpose We evaluated extracellular matrix remodeling in human fetal and cryptorchidic gubernacula.
Methods Gubernacula were obtained from 40 normal human fetuses aged 15–29 weeks postconception (WPC) and from 39 children aged 1.3–10 years who had been submitted to orchiopexy. Total collagen and glycosaminoglycan (GAG) concentrations were assessed as μg hydroxyproline and μg hexuronic acid per mg dry gubernacular tissue, respectively, and proportions of sulfated GAG were determined by agarose gel electrophoresis.
Results Fetal age correlated with collagen ($r = 0.77$, $P < 0.001$) and GAG ($r = -0.83$, $P < 0.01$) concentrations, which varied from 10 to 50 μg/mg and 7.1–2.5 μg/mg, respectively. Collagen and GAG concentrations in cryptorchidic gubernacula varied from 80.0 to 120.0 μg/mg and from 0.8 to 1.0 μg/mg, respectively. These values did not correlate with patient's age, but when the testis was located more proximally, collagen content was lower. At 15–18 WPC, GAG consisted of 57.3% ± 13.5% chondroitin sulfate and 28.2% ± 10.1% dermatan sulfate, and at 25–28 WPC, 42.7% ± 8.7% and 71.8% ± 11.3%, respectively. GAG in postnatal gubernacula consisted mostly of dermatan sulfate.
Conclusions From the 15th to the 29th WPC, the extracellular matrix of the gubernaculum undergoes extensive remodeling and this may contribute to testicular descent. Cryptorchidic gubernacula are markedly more fibrous than the corresponding fetal tissue, change little after birth, and have a lower collagen concentration when the undescended testis is abdominal in position.

Keywords Cryptorchidism · Collagen · Glycosaminoglycan · Testes migration · Gubernaculum

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Introduction

Testicular descent in humans occurs between the 15th and 30th week postconception (WPC) when the testes migrate from the abdomen to the scrotum [1]. The mechanisms that underlie this process are not yet well understood, even though they may be associated with important disorders such as cryptorchidism and other testicular abnormalities [2, 3]. Various factors have been proposed as the causative agent of testicular descent in humans, including an increase in intra-abdominal pressure, stimuli from the genito-femoral nerve and from active peptides, androgens and their receptors, and the development and site of attachment of the gubernaculum [2–8].

The gubernaculum is an elongated, cylindrical mesenchymal structure that connects the inferior pole of the testis and the tail of epididymis to the scrotum [1, 8]. It is composed of an abundant, often loose extracellular matrix, and mesenchymal cells such as fibroblasts and smooth muscle cells [5]. Although experimental data indicate that during testicular migration the gubernaculum undergoes extensive remodeling [5, 9], the composition of its extracellular matrix is still poorly known, especially in the human tissue.

The role of the gubernaculum in testicular migration has been explained mainly in terms of its capacity to dilate and contract [4, 8, 9]. For example, the increase in gubernacular

volume, which is probably due to an enhanced expression of proteoglycans and their glycosaminoglycan (GAG) side chains [9], is thought to facilitate the passage of the testis through the inguinal canal [4, 8]. Although the mechanisms that regulate these pronounced gubernacular modifications are not well understood, experimental evidences suggest that hormones and other factors may be involved [2–4, 8, 10, 11].

Most of these data have been obtained from experiments using animal models [4, 9]. However, it has been suggested that these models may not be suitable for studies on testicular migration as far as humans are concerned [4]. Besides marked anatomical differences [12], testicular descent in such animals may take place after birth and/or under different physiological conditions and regulatory mechanisms [4].

Few studies on testicular migration have used human fetuses [5–7, 13], and no investigation has been carried out yet on the biochemical composition of gubernacular connective tissue using a sample of human fetuses spanning a broad range of gestational ages. Thus, it is not clear whether the pronounced structural modifications the human gubernaculum undergoes during testicular descent [5] are associated with significant changes in extracellular matrix components. Likewise, little is known on the composition of this tissue when the testis fails to migrate [14].

In the present study, we addressed these issues by analyzing collagen and GAG compositions in gubernacula from human fetuses and cryptorchidic children at various ages.

Materials and methods

The experimental protocol described herein was approved by the Ethical Committee for Human Experimentation of the State University of Rio de Janeiro, Brazil. Written informed consents were obtained from the parents of all the children enrolled in this study.

Tissue samples

Fetal gubernacula were obtained from a sample of 40 fresh, macroscopically normal male human fetuses aged 15–29 weeks postconception (WPC), which corresponds to 17–31 menstrual weeks. All fetuses were macroscopically well preserved, showed no signs of malformations, and their ages were determined using the foot length method [15]. Once age was estimated, the abdomen, inguinal canal, and scrotum were opened to identify and remove the gubernaculum, as previously described [1, 5, 6, 13]. Postnatal gubernacula were obtained from 39 children aged 1.3–10 years who had been submitted to surgery for undescended testes and who had received no hormonal treatment. These gubernacula were classed according to the position of the undescended testis as abdominal, inguinal, and suprascrotal [2]. Only one gubernaculum was used per individual, and the analyzed segments excluded the proximal and distal endings and were free from macroscopically visible muscular tissue.

Immediately after removal, the gubernacula were fixed in cold acetone and kept in this fixative for 24 h at 4°C. The gubernacula were then finely minced and submitted to two changes of 24 h each in 40 mL of chloroform/methanol (2:1, volume/volume) at room temperature. The solvent was then decanted, and after an incubation at 60°C for 30 min, a preparation of dry and defatted gubernaculum was obtained and weighed.

Because of the relatively small size of the tissue samples and of the different sensitivities of the assay methods, the actual number of gubernacula used in the analyses (N) varied according to the experiment.

Analysis of glycosaminoglycans

All methods for the extraction and analysis of GAG have been described in detail elsewhere [16]. Briefly, dry gubernacula were digested with twice crystallized papain (Sigma, St. Louis, MO, USA), and GAG chains were isolated from the supernatant by precipitations in cetylpyridinium chloride and ethanol. The final pellet, consisting of total gubernacular GAG, was then dissolved in distilled water and stored at −20°C. The amount of GAG in this preparation was assessed by a hexuronic acid assay, and GAG concentration in gubernacula was expressed as μg hexuronic acid per mg dry, defatted tissue.

Identification of the different sulfated GAG species was made by agarose gel electrophoresis of the total GAG preparation coupled with specific degradations. GAG bands on the gel were quantitated using the ImageJ v.1.43 software (NIH, Bethesda, Maryland, USA), and the relative content of the individual GAG species was expressed as percent of total sulfated GAG. Means for these values were calculated for fetal age groups 15–18 WPC and 25–28 WPC.

Analysis of collagen

The concentration of total collagen in the gubernacular tissue was determined by a colorimetric hydroxyproline assay after hydrolysis [17] of approximately 10–15 mg dried, defatted gubernacula. Results were expressed as μg hydroxyproline per mg dry, defatted tissue.

Statistics

Correlation between patient's or fetal age and total GAG or collagen concentration in gubernacula was determined by linear regression followed by a *t* test for the correlation

coefficient. Differences between two groups were assessed by the Wilcoxon two-sample test or Student's *t* test. Results are shown as mean ± SD, and statistical significance was considered when $P < 0.05$.

Results

GAG concentration in fetal gubernacula decreased sharply during fetal growth as it was significantly and negatively correlated ($r = -0.8543$, $P < 0.001$) with gestational age (Fig. 1a). Conversely, collagen concentration increased during fetal growth, as indicated by a significant and positive correlation ($r = 0.7485$, $P < 0.001$) with gestational age (Fig. 1b). Gubernacula from cryptorchidic children, on the other hand, behaved differently. The results showed no noticeable pattern with regard to the overall composition of the extracellular matrix, as patient's age was correlated neither with GAG (Fig. 1c; $r = 0.1311$) nor with collagen (Fig. 1d; $r = 0.1310$) concentrations.

A comparison was also made between the overall compositions of fetal and postnatal gubernacular samples. On average, collagen concentration in postnatal gubernacula (90.24 ± 17.96 μg/mg, $N = 19$) was much higher (*t* test, $P < 0.001$) than that in the fetal samples (29.17 ± 11.04 μg/mg, $N = 23$). In contrast, fetal gubernacula were much more enriched (*t* test, $P < 0.001$) in GAG (4.73 ± 1.97 μg/mg, $N = 11$) than the postnatal ones (0.60 ± 0.38 μg/mg, $N = 17$).

Because the concentrations of collagen and GAG in inguinal ($r = 0.1171$ and $r = 0.5116$, respectively) and suprascrotal ($r = 0.1583$ and $r = -0.0793$, respectively) gubernacula were not significantly associated with patient's age, we investigated whether these concentrations varied as a function of the position of the undescended testes (Fig. 2). GAG concentration (Fig. 2a) showed no significant variation as indicated by the values for the inguinal (0.65 ± 0.32 μg/mg, $N = 7$) and suprascrotal (0.53 ± 0.40 μg/mg, $N = 6$) samples. Collagen concentration (Fig. 2b) in the suprascrotal samples (107.91 ± 11.67 μg/mg, $N = 6$), on the other hand, was significantly different (Wilcoxon two-sample test, $P = 0.005$) from that of the inguinal ones (87.74 ± 10.14 μg/mg, $N = 11$). Therefore, the higher the position of the undescended testes, the lower the concentration of collagen in the gubernaculum.

Determination of the relative contents of GAG species in fetal gubernacula indicated that, in the 15–18 WPC age group ($N = 7$), chondroitin sulfate consists of 57.3% ± 13.5% and dermatan sulfate of 28.2% ± 10.1% of total sulfated GAG. Later in gestation, there was a marked reversal of these proportions. Thus, in the 25–28 WPC age

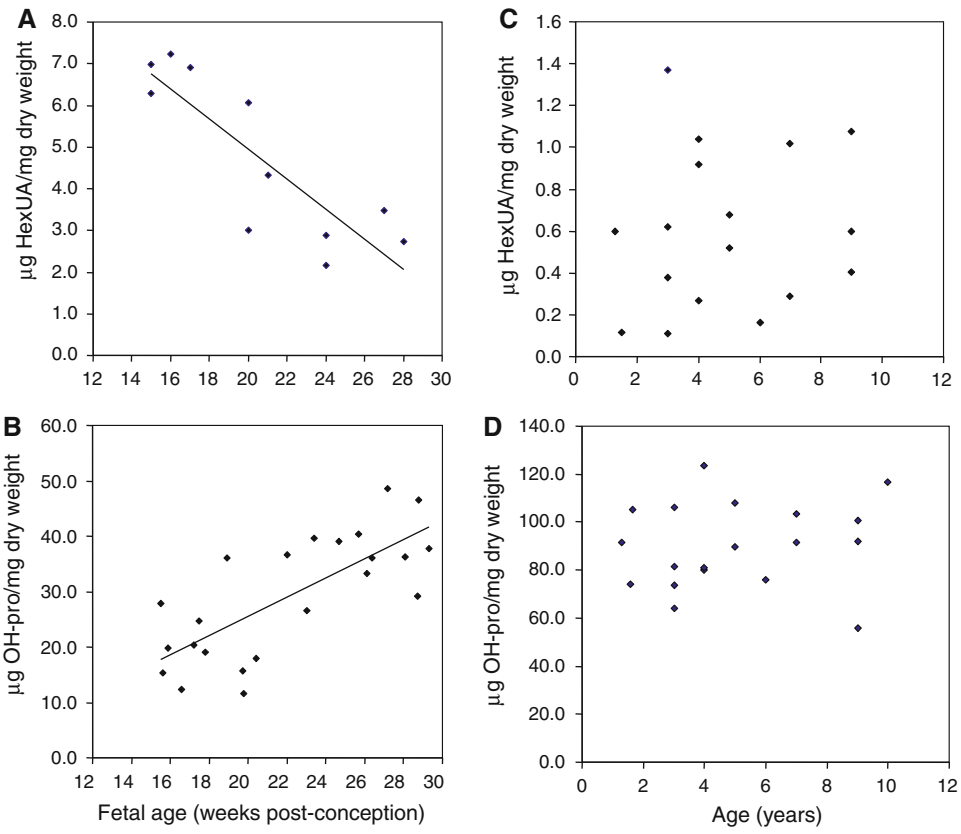

Fig. 1 Concentration of total GAG (**a, c**) and collagen (**b, d**) in gubernacula from human fetuses (**a, b**) and cryptorchidic children (**c, d**). Dry, defatted gubernaculum samples were submitted to papain digestion and a hexuronic acid (HexUA) assay to estimate GAG concentration, and acid hydrolysis followed by a hydroxyproline (OH-pro) assay to estimate collagen concentration. Linear regression and a *t* test for the correlation coefficient indicate that fetal age is negatively correlated with GAG ($r = -0.8543$, $P < 0.001$, 11 fetuses) and positively correlated with collagen ($r = 0.7485$, $P < 0.001$, 23 fetuses) concentrations, whereas patient's age is correlated neither with GAG ($r = 0.1311$, 17 patients) nor with collagen ($r = 0.1310$, 19 patients) concentration. Individual concentration values for fetuses and children are shown as diamonds

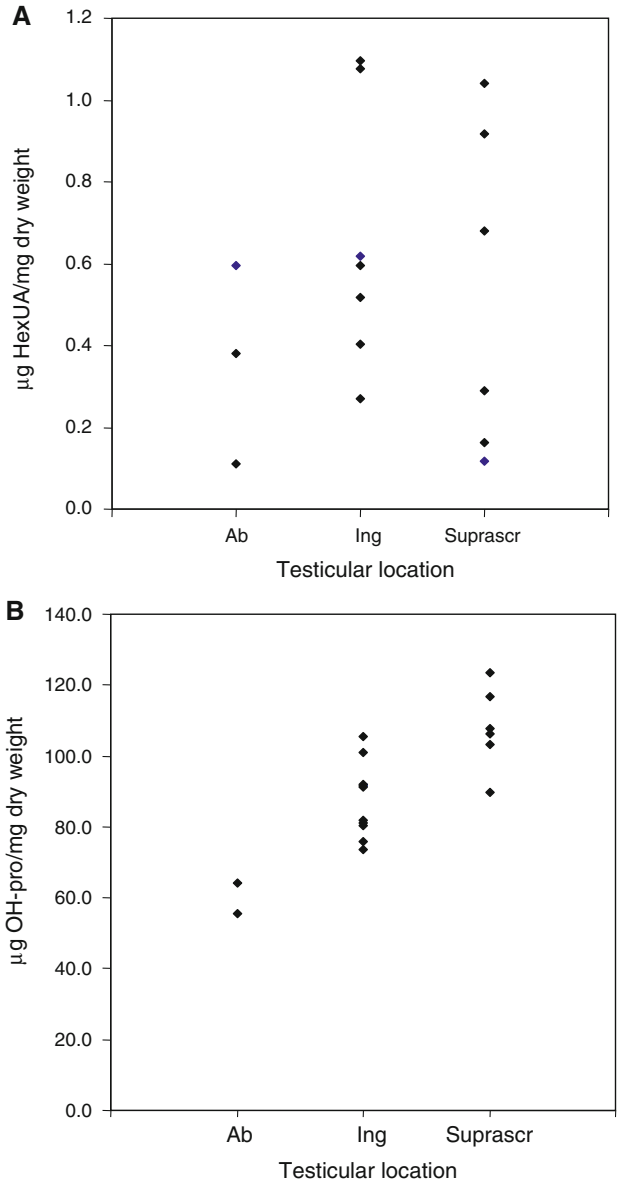

Fig. 2 Concentration of total GAG (**a**) and collagen (**b**) in 17 and 19 gubernacula, respectively, as a function of the position of the undescended testes. After removal from cryptorchidic children, gubernacula were classed as abdominal (Ab), inguinal (Ing), and suprascrotal (Suprascr). GAG and collagen were then assayed as hexuronic acid (HexUA) and hydroxyproline (OH-pro), respectively. A trend can be perceived towards less collagen in higher locations, and the concentration in suprascrotal samples is significantly different (Wilcoxon two-sample test, $P = 0.005$) from that in inguinal ones. Individual concentration values are shown as diamonds

group ($N = 5$), the contents of chondroitin sulfate and dermatan sulfate were 42.7% ± 8.7% and 71.8% ± 11.3%, respectively. In gubernacula from cryptorchidic children aged 1–10 years, sulfated GAG consisted mostly of dermatan sulfate, whereas chondroitin sulfate was either absent or present in minor amounts (not shown).

Discussion

We have previously shown that between the 15th and the 30th WPC, when testes migrate from the abdomen to the scrotum [1], there is a marked morphological reorganization of the gubernacular extracellular matrix [5]. Thus, by the 15th WPC, the gubernaculum is made up of a loose extracellular matrix with few collagen fibers. The latter then increase gradually in quantity, so that, by the 30th WPC, the gubernaculum is essentially a dense fibrous tissue.

The results of the current biochemical study are consistent with these morphological findings. Thus, earlier in gestation, the gubernaculum has a higher concentration of GAG, a comparatively low collagen content, and more chondroitin sulfate than dermatan sulfate. This composition is typical of a looser and more hydrated connective tissue. A gradual remodeling then occurs, leading, by the 30th WPC, to an extracellular matrix with a high collagen content, a low GAG concentration, and a predominance of dermatan sulfate over chondroitin sulfate, which altogether are indicative of a denser connective tissue [18]. For example, dermatan sulfate derives mostly from decorin, a proteoglycan that is often associated with dense bundles of type I collagen in the interstitial connective tissue [19]. These data are also in agreement with previous findings showing that the wet/dry mass ratio of the gubernaculum decreases after testicular descent [9]. A decrease in the concentration of GAG has also been demonstrated to take place in the gubernaculum during testicular migration in pigs [9].

Our previous morphological findings also showed that, in the human fetal gubernaculum, muscular cells are present in small amounts and are located at its distal end only [5]. Further, these cells are mostly oriented transversely with regard to the long axis of the gubernaculum. Based on these data, we hypothesized that an active contraction of the gubernaculum as a causative agent of testicular descent in humans would be unlikely.

One of the fundamental physiological roles of proteoglycans and their GAG side chains is the retention of water and electrolytes in tissues via ionic interactions [19]. Thus, if the GAG concentration of a tissue is experimentally reduced, a decrease in tissue hydration and turgescence ensues, which in turn leads to a decrease in tissue volume [20]. This alteration may also occur as a consequence of normal processes, such as during dermal aging, in which a normal decrease in GAG content is associated with wrinkling, loss of elasticity, and diminished turgidity [21]. The extracellular matrix remodeling that the fetal gubernaculum undergoes from the 15th to the 30th WPC, as shown by our results, should produce approximately these effects, namely, a reduction in its volume and, possibly, in its

length. The extent to which this structural change results in a traction force that contributes to testicular descent is not yet possible to ascertain. However, it should at least act synergistically with other factors, as discussed above, that cause the testes to migrate.

The changes that the fetal gubernaculum undergoes during testicular descent are thought to be mediated by several stimulatory and inhibitory factors. Thus, the testicular paracrine factor descendin is considered to cause a dilation of the gubernaculum, thereby facilitating the passage of the testes through the inguinal canal [4, 8]. This swelling corresponds to the loose matrix with high GAG and low collagen contents that we found in fetal gubernacular samples by the 15th WPC. Estrogens would counter this effect, either directly or by downregulating the expression of the insulin-like growth factor 3 [10], itself an enhancer of gubernacular growth [11]. Interestingly, while it has been shown that degradation and shortening of the gubernaculum is dependent on androgens [4], others have demonstrated that these hormones may also stimulate gubernacular outgrowth [11]. Therefore, it is not yet clear how these factors regulate the enhanced breakdown and expression of extracellular matrix molecules that occur during the normal remodeling of the fetal gubernaculum.

Even though our postnatal samples were from cryptorchidic children, the differences they presented in relation to fetal gubernacula suggest that the conspicuous changes that occur during the gestational period, i.e., a trend towards a fibrous tissue, do continue after birth. Accordingly, the contents of collagen and GAG in postnatal samples were much higher and much lower, respectively, than those in the fetal tissue. This is consistent with data showing that, in cryptorchidic children treated with human chorionic gonadotrophin, the relative volume of collagen and elastic fibers in the gubernaculum are increased, even though this therapy is not always effective in stimulating testicular descent [14]. In addition, dermatan sulfate was the only sulfated GAG detected in postnatal gubernacula, which, together with the other findings, imply a fibrous tissue [18]. However, and unlike what was found in the fetal samples, collagen and GAG concentrations in gubernacula from cryptorchidic children showed no significant correlation with age. This indicates that the gubernaculum attached to the undescended testis does not undergo appreciable remodeling after birth.

Data from postnatal gubernacula, when combined with the original position of the undescended testes, also provide information that might be related to the pathophysiology of cryptorchidism. Thus, in testes located in the abdomen, the gubernacular collagen concentration was comparatively low. This concentration tended to be higher in samples of inguinal location, which was in turn significantly lower than the concentration from suprascrotal locations. We have argued above that a gradual remodeling of the fetal gubernaculum, leading to a less hydrated and more dense and fibrous tissue, may be involved in normal testicular descent. If that is so, then a lower collagen concentration when the cryptorchidic testis is more abdominal in position suggests that an underactivity of the factors that mediate this remodeling is associated with failure to migrate. Also, because the composition of gubernacular collagen and GAG remains essentially unchanged after 1 year of age, hormone therapy for cryptorchidism should have a limited effect on gubernacular extracellular matrix, especially when the testis is more distally located. This is in fact a common position of the testis in cryptorchidic boys [22], and hormone treatment does have a low success rate [23]. However, it is not yet possible to ascribe this low efficiency to a lack of effect on the gubernaculum.

In conclusion, earlier in testicular descent the gubernaculum is more hydrated and less fibrous and subsequently undergoes extensive remodeling that results in a denser tissue. This should lead to a decrease in gubernacular volume and length, which should contribute with other factors that cause the testis to migrate. Cryptorchidic gubernacula are markedly more fibrous than the corresponding fetal tissue, change little after birth, and have a lower collagen concentration when the undescended testis is abdominal in position.

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Conflict of interest The authors declare no conflict of interest.

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