Structural Study of Gubernaculum Testis in Fetuses with Prune Belly Syndrome

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Abbreviations and Acronyms

CGRP = calcitonin gene-related peptide

PBS = prune belly syndrome

Vv = volumetric density

WPC = weeks of gestation

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Purpose: We compared and contrasted the structure of the gubernaculum testis in fetuses with prune belly syndrome and normal controls.

Materials and Methods: We studied a total of 6 gubernacula from 3 male fetuses with prune belly syndrome and a total of 14 from 7 male fetuses without an anomaly. Gubernacular specimens were cut into 5 μm sections and stained with Masson trichrome to quantify connective tissue and smooth muscle cells, with Weigert stain to observe elastic fibers and with picrosirius red with polarization to observe collagen. Immunohistochemical analysis was done with tubulin to observe the nerves. Images were captured with a BX51 microscope and DP70 camera (Olympus®). Stereological analysis was done with Image-Pro and ImageJ (MediaCybernetics®) using a grid to determine volumetric density. Means were statistically compared with the Mann-Whitney test. All tests were 2-sided with $p < 0.05$ considered statistically significant.

Results: Prune belly syndrome fetuses were at 17 to 31 weeks of gestation and control fetuses were at 12 to 35 weeks of gestation. Quantitative analysis showed no difference in the volumetric density of smooth muscle cells in prune belly syndrome vs control gubernacula (mean 15.70% vs 19%, $p = 0.2321$). Collagen fiber analysis revealed a predominance of green areas in prune belly syndrome gubernacula, suggesting collagen type III, and a predominance of red areas in control gubernacula, suggesting collagen type I. Elastic fibers were significantly smaller in prune belly syndrome gubernacula than in control gubernacula (mean 14.06% vs 24.6%, $p = 0.0190$). Quantitative analysis demonstrated no difference in the volumetric density of nerves in prune belly syndrome or control gubernacula (mean 5.200% vs 3.158%, $p = 0.2302$).

Conclusions: The gubernaculum in fetuses with prune belly syndrome had altered concentrations of collagen and elastic fibers. These structural alterations could be one of the factors involved in cryptorchidism in prune belly syndrome.

Key Words: testis, prune belly syndrome, cryptorchidism, collagen, elastic tissue

Prune belly syndrome is a disorder characterized by abdominal muscle deficiency or hypoplasia, urinary tract malformation such as a large, hypotonic bladder and dilated, tortuous ureters, and bilateral cryptorchidism.[1] Urethral obstruction is present in a third of patients with PBS, which could be the primary cause of the malformations in this syndrome.[2,3] To our knowledge the cause of cryptorchism in this syndrome is unknown.

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However, it is speculated that anatomical changes in the anterior abdominal wall hinder an increase in intra-abdominal pressure, which is one of the factors needed for testicular descent. It was also speculated that the large bladder in this syndrome makes the inguinal canal extraperitoneal so that the gubernaculum and its contained processus vaginalis cannot develop normally in the inguinal canal.[2,3]

Various factors have been proposed as the causative agent of testicular descent in humans, including increased intra-abdominal pressure,[4,5] development of the epididymis, spermatic vasa, deferential ducts and inguinal canal,[6] stimuli from the genitofemoral nerve,[7] hormonal stimulus originating in placental gonadotrophin and testosterone produced by the fetal testes,[8] and gubernacular development.[6]

The gubernaculum seems to be the most important anatomical structure in the process of testicular descent. The gubernaculum is an elongated, cylindrical structure that connects the inferior pole of the testis and the tail of the epididymis to the inguinal canal and scrotum.[6,9] It is composed of an abundant and often loose extracellular matrix and mesenchymal cells such as fibroblasts and smooth muscle cells.[10] The role of the gubernaculum during testicular descent has been explained mainly by its capacity for dilatation and contraction.[6,10]

Previous groups assessed gubernacular structure in human fetuses and in patients with cryptorchidism.[9–12] However, to our knowledge there is no morphological study in the literature of the gubernaculum testis in patients or fetuses with PBS. We hypothesized whether the structure of the gubernaculum in fetuses with PBS is similar to that in normal fetuses and whether increased intra-abdominal pressure in PBS causes alterations in gubernacular structure. Thus, we compared and contrasted the structure of the gubernaculum testis in fetuses with PBS and in normal controls.

MATERIALS AND METHODS

The experimental protocol was approved by the ethical committee for human experimentation at our university. This study was performed in accordance with the ethical standards of the hospital institutional committee on human experimentation.

We studied 6 gubernacula from a total of 3 male fetuses with PBS and 14 from a total of 7 male fetuses without an anomaly. The fetuses were macroscopically well preserved. Fetal gestational age was determined in WPC according to the foot length criterion, which is currently considered the most acceptable parameter to calculate gestational age.[13–15] Fetal crown-rump length and body weight were also evaluated immediately before dissection. The same observer analyzed the measurements.

After measurements the fetuses were dissected using a stereoscopic lens at 16/25× magnification. The abdomen and pelvis were opened to identify and expose the urogenital organs and inguinal canal, and reveal testicular position.

Testicular position was classified after dissection as 1) abdominal when the testis was proximal to the internal ring, 2) inguinal when the testis was found between the internal and external inguinal rings, and 3) scrotal when the testis had passed beyond the external inguinal ring and was in the scrotum. We observed proximal and distal insertions of the gubernaculum and the structure of the inguinal canal (fig. 1). The relationship between the testis and the epididymis was also evaluated.

The testis and gubernaculum were separated from the other structures, fixed in 10% buffered formalin and routinely processed for paraffin embedding. Sections (5 μm) were obtained at 200 μm intervals. Smooth muscle and connective tissue, elastic system fibers and collagen were studied by histochemical and immunohistochemical methods.

Sections were stained with hematoxylin and eosin to assess tissue integrity. Other staining methods were also used, including Masson trichrome to quantify connective and smooth muscle tissue, Weigert resorcin fucsin with previous oxidation to observe elastic system fibers and picrosirius red with polarization to observe different collagen types. Gubernacular nerves were analyzed immunohistochemically with β III tubulin (mouse monoclonal antibody).

Connective and smooth muscle tissues, nerves and elastic system fibers were quantified by a stereological method.[16–18] For quantitative analysis we studied 5 microscopic fields chosen at random for a total of 25 test areas per gubernaculum. We used ImageJ, version 1.46r, loaded with a plug-in (http://rsb.info.nih.gov/ij/). All sections were photographed with a DP70

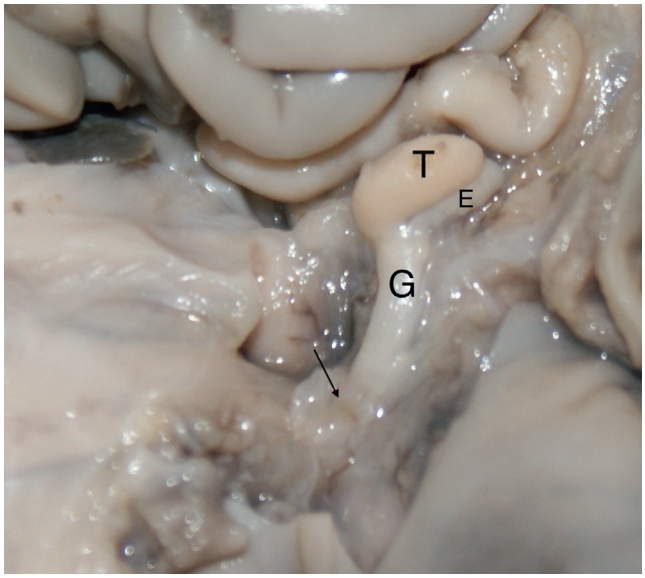

Figure 1. Control fetus at 21 WPC. Anterior abdominal wall was extirpated and each testis was in abdomen. Note relationship between left testis (*T*), epididymis (*E*) and gubernaculum (*G*). Arrow indicates internal inguinal ring.

digital camera under the same conditions at a resolution of 2,040 × 1,536 pixels. The camera was directly coupled to the BX51 microscope and data were saved as a TIFF file. To quantify smooth muscle tissue we used ImageJ color segmentation, which selects structures of different colors and calculates the amount of each component (fig. 2, *a* and *b*).

To quantify elastic fibers and nerves we used ImageJ to determine the Vv of each component (fig. 2, *c*). The results of each field were obtained using the quantification assessment method by superimposing a 100-point test grid (multipurpose test system) on the video monitor screen. We determined the arithmetic mean of the quantification of 5 fields per section. We subsequently determined the mean quantification value of the 5 sections per gubernaculum for a total of 25 test areas.

Means were statistically compared with the Mann-Whitney test for all categorical variables and the Wilcoxon rank sum test for continuous variables. All tests were 2-sided with $p < 0.05$ considered statistically significant.

RESULTS

After dissection the 3 PBS fetuses had a typical aspect of the anterior abdominal wall, prostatic urethra obstruction, an enlarged bladder and bilateral hydronephrosis. These fetuses were at 17 to 31 WPC and weighed 240 to 2,150 gm. Control fetuses were at 12 to 35 WPC and weighed 210 to 2,860 gm (see table). All 3 PBS fetuses had abdominal testes. In the control group 12 testes (85.71%) were abdominal and 2 (14.29%) were scrotal.

We observed no structural alteration in the inguinal canal in the 10 study fetuses. In the 6 PBS testes and 12 abdominal control testes the proximal gubernacular insertion was attached to the testis and epididymis, and the distal insertion was fixed in the inguinal canal. For the scrotal testis the proximal gubernacular insertion was fixed to the testis and epididymis, and the distal insertion was in the scrotum. We observed no epididymal anomaly in our sample. In control

Male fetus age and parameters

Subject No.	Age (WPC)	Wt (gm)	Crown-Rump Length (cm)
PBS:			
1	17	240	18
2	23	1,100	25
3	31	2,150	43
Control:			
4	12	210	18
5	16	430	22
6	18	250	18
7	19	380	21
8	21	401	21
9	21	450	22
10	35	2.860	34

fetuses at 21 WPC or greater we observed the processus vaginalis developing in the gubernaculum. We did not see the processus vaginalis developing in the gubernaculum in the 3 PBS fetuses (fig. 3).

Quantitative analysis demonstrated that elastic fibers were significantly smaller in PBS than in control gubernacula (mean ± SD 14.06% ± 3.316% vs 24.60% ± 4.370%, $p = 0.0190$). Figure 4, *a* and *b* shows the arrangement of elastic fibers in normal and PBS gubernacula. Quantitative analysis revealed no difference in smooth muscle cell Vv in PBS and control gubernacula (mean 19% ± 1.686 vs 15.70% ± 2.672%, $p = 0.2321$). Figure 4, *c* and *d* shows the smooth muscle cell arrangement in normal and PBS gubernacula. Quantitative analysis also demonstrated no difference in nerve Vv in PBS and control gubernacula (mean 5.2% ± 2.02% vs 3.158% ± 1.251%, $p = 0.2302$, fig. 5, *a* and *b*).

Picrosirius red with polarization photomicrographs revealed a difference in colors between the groups, which suggested changes in the collagen fiber organization of PBS gubernacula. Analysis showed a predominance of green areas in PBS fetal gubernacula, suggesting collagen type III, and a predominance of red areas in control gubernacula, suggesting collagen type I (fig. 5, *c* and *d*).

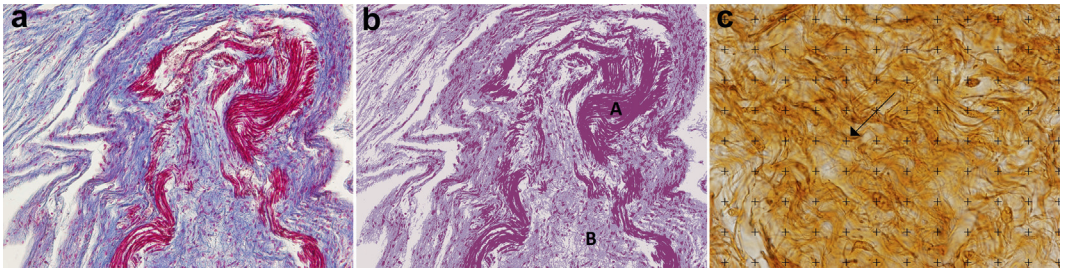

Figure 2. Fetal gubernacular morphometric analysis. *a* and *b*, muscle tissue (*A*) color in control fetus at 16 WPC before and after quantification by color segmentation to show muscle cells. *B*, connective tissue. *c*, elastic fiber (arrow) quantification in control fetus at 21 WPC using test grid software. Masson trichrome stain, reduced from ×400.

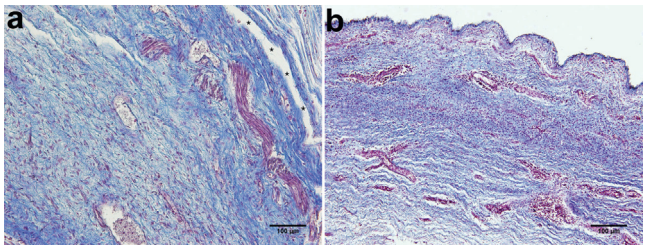

Figure 3. Distal gubernaculum. *a*, control fetus at 21 WPC with processus vaginalis (asterisks) developing in gubernaculum. *b*, in fetus at 27 WPC with PBS no processus vaginalis development was noted. Masson trichrome stain, reduced from ×200. Scale bars indicate 100 μm.

DISCUSSION

Testicular descent is a complex process mediated by endocrine and mechanical factors.[4–7] The testes start to migrate from the abdomen during the second trimester after 17 WPC.[19] The gubernaculum increases in volume mainly during the second trimester when the testis passes through the inguinal canal, most likely due to increased glycosaminoglycan synthesis.[11] It is supposed that the increase in gubernacular volume is important to facilitate testicular passage through the inguinal canal.[6]

Bilateral cryptorchidism is characteristic of PBS.[1–3] Contraction of the abdominal wall muscles, liver and intestine growth, and meconium accumulation increase pressure in the fetal abdomen. According to some groups this favors testicular migration.[4,20] Abdominal musculature contraction is impaired in PBS. Mechanical obstruction due to bladder distention is another factor believed to hinder testicular migration in this syndrome.[1–3] Another theory to explain bilateral cryptorchidism in PBS is structural alteration of the inguinal canal, which hampers passage of the testis.[1,2] However, in the 3 fetuses with PBS in our study we observed no anatomical change in the inguinal canal. We also did not observe the processus vaginalis developing in the gubernaculum in the 3 PBS subjects.

Growth of the vaginal process during testicular descent divides the gubernaculum into 3 parts, including 1) the main gubernaculum, which corresponds to the portion covered by the visceral layer of the peritoneum of the vaginal process, 2) the vaginal

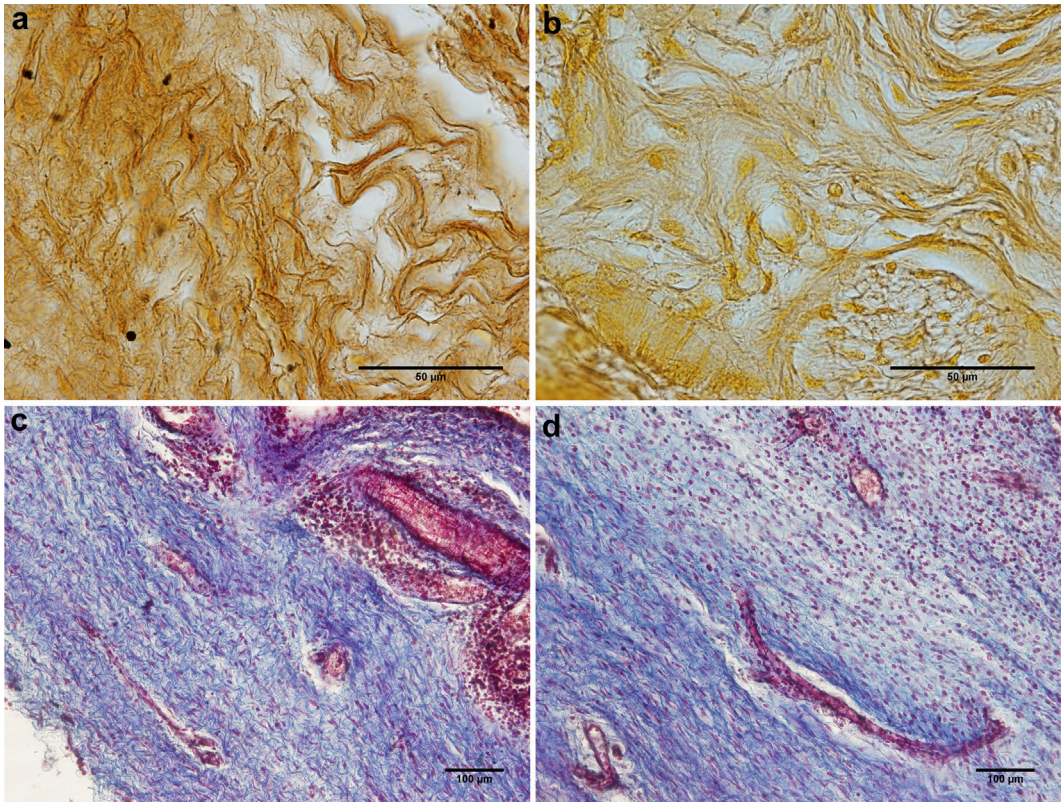

Figure 4. Gubernaculum. *a*, elastic system fibers (brown areas) in control fetus at 21 WPC. *b*, elastic system fibers in fetus at 23 WPC with PBS. *a* and *b*, Weigert resorcin fucsin stain, reduced from ×1,000. Scale bars indicate 50 μm. *c*, smooth muscle in control fetus at 18 WPC. *d*, smooth muscle in fetus at 17 WPC with PBS. *c* and *d*, Masson trichrome stain, reduced from ×200. Scale bars indicate 100 μm.

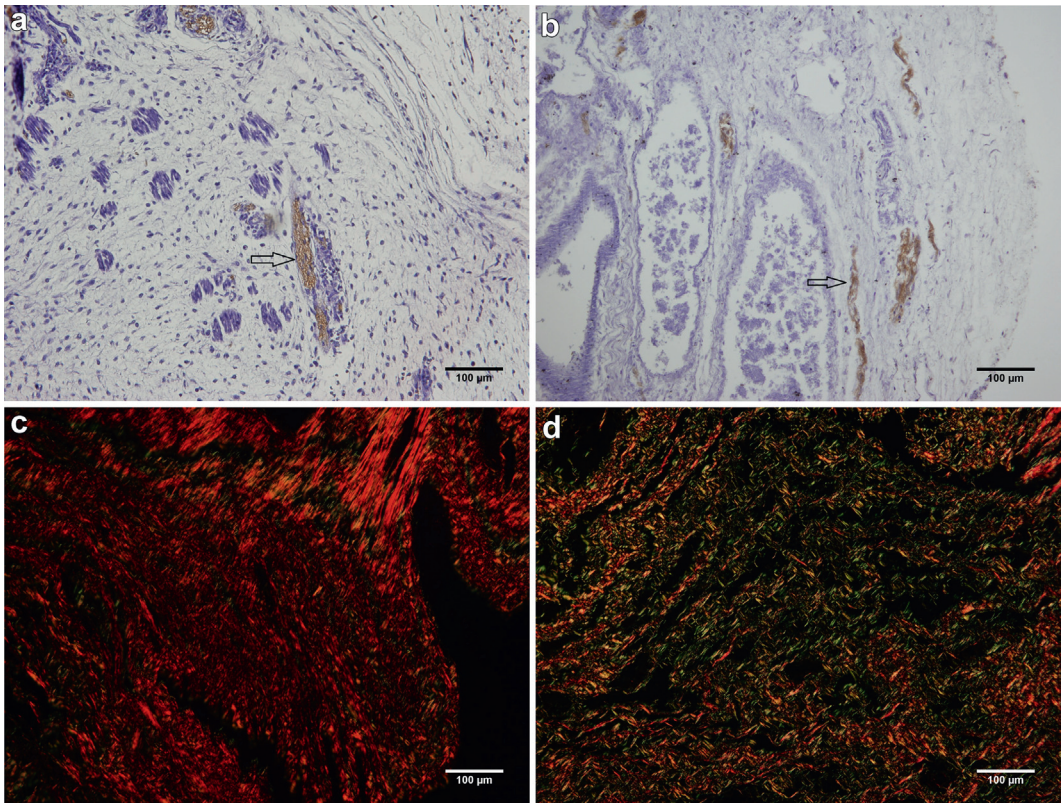

Figure 5. Gubernaculum. *a*, nerves (arrow) in control fetus at 35 WPC. *b*, nerves (arrow) in fetus at 31 WPC with PBS. *a* and *b*, tubulin stain, reduced from ×200. *c*, in control fetus at 35 WPC predominance of red areas suggests collagen type I. *d*, in fetus at 31 WPC with PBS predominance of green areas suggests collagen type III. *c* and *d*, Picrosirius red with polarization, reduced from ×400. *a* to *d*, scale bars indicate 100 µm.

gubernaculum, which corresponds to the portion that externally surrounds the parietal portion of the vaginal process, and 3) the infravaginal gubernaculum, corresponding to the caudal region of the gubernaculum, which has not been invaded by the vaginal process.[6,21] Maintenance of this undifferentiated mesenchyma along the inguinal canal and scrotum is essential for downward extension of the vaginal process to occur, during which it follows the pathway created by gubernacular dilatation to form the canal through which the testis will reach the scrotum.[6,21] The absence of a peritoneal tunnel to allow the testis to descend to the scrotum may be one of the causes of undescended testis in PBS. Is altered intra-abdominal pressure in PBS the cause of this vaginal process anomaly? Is this one of the causes of cryptorchidism in PBS? These are good questions for future research in testicular descent in humans and in experimental models.

The gubernaculum has a small amount of musculature that is mainly located in the distal portion independent of fetal age.[10] These findings suggest that unlike rats, the human gubernaculum should not be capable of significant contraction.[6,22] We observed no difference in the distribution or quantity of gubernacular muscular fibers in fetuses with PBS and control fetuses.

Previous studies of human fetuses demonstrated that elastic fibers appear only later in testicular descent.[10] During the testicular migration period gubernacular connective tissue undergoes extensive remodeling and ultimately becomes an essentially fibrous structure rich in collagen and elastic fibers.[10] In our sample elastic fibers were significantly smaller in PBS gubernacula even in fetuses at greater than 25 WPC.

During testicular descent the gubernacular extracellular matrix is remodeled. This develops at the start of the migration period between 15 and 20 WPC when the gubernaculum has a low collagen content. This composition is typical of looser, more hydrated connective tissue.[10,11] Gradual remodeling then occurs, which by 30 WPC leads to an extracellular matrix with a higher collagen content that is altogether indicative of denser connective tissue.[10,11,22,23] In our sample we observed a predominance of collagen type III in PBS gubernacula. In earlier phases of the remodeling and repair of connective tissues the synthesis of type III collagen is enhanced.[24,25] Thus, based on these findings the

results of the collagen structure suggest that the collagen matrix of the PBS gubernaculum is disrupted or degraded. We speculate that mechanical obstruction or the altered intra-abdominal pressure in PBS hinders gubernacular remodeling.

The gubernacular nerve supply (the genitofemoral nerve) descends on the anteromedial surface of the psoas muscle from the L1-L2 segments.[7] The second phase of testicular descent is regulated by androgens and CGRP released from the sensory nucleus of the genitofemoral nerve. In rodents active proliferation of the gubernacular tip and cremaster muscle, its rhythmic contraction and the chemotactic gradient provided by CGRP result in eventual migration of the testis into the scrotum. The importance of this mechanism was corroborated in an experimental model in which genitofemoral nerve sectioning led to cryptorchidism.[6]

Increased CGRP levels lead to rhythmic contraction of the testicular gubernaculum, which induces testis migration to the scrotum.[7] The CGRP action site is the neuromuscular junction. In experimental animals such as rats the large musculature strengthens this hypothesis.[26,27] However, the human gubernaculum is basically composed of an abundant extracellular matrix with a large concentration of glycosaminoglycans.[10,12] Thus, it is questionable to extend this theory of traction induced by CGRP to humans.

We observed a small quantity of nerves in control and PBS gubernacula, which did not statistically differ. To our knowledge this is the first study to assess and quantify the distribution of the nerves of the human testicular gubernaculum. The small quantity of nerves in the gubernacula could confirm the theory that rhythmic contraction of the gubernaculum, mediated by stimulus from the genitofemoral nerve,[10,12,27] has little importance in humans. However, future research is needed to clarify this topic.

Our study has some limitations. 1) Sample size was small. Fetuses with PBS are rare so that observations in a small sample may be important, although the small number is a weakness. 2) There was an unequal WPC distribution between PBS and control subjects. 3) We did not quantify levels of hormones such as CGRP in the fetuses or testosterone receptors in the gubernaculum because it was technically impossible.

CONCLUSIONS

Analyzed gubernacula from fetuses with PBS showed altered concentrations of collagen and elastic fibers. We observed no processus vaginalis development in the gubernaculum in fetuses with PBS. These structural alterations could be factors involved in cryptorchidism in PBS.

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