Testicular Hypoplasia Is Driven by Defective Vascular Formation

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OBJECTIVE To determine if vanishing testis could result from a fault in embryological development as a result of an arrest in endothelial cell migration rather than secondary to just a random physical torsion/twist. A testicular nubbin or vanishing testis is considered to be secondary to a neonatal torsion and is usually associated with a hemosiderin deposit.

MATERIALS AND METHODS Cases of vanishing testis excision were compared with age-matched controls from cadaveric testes without known genitourinary pathology. To assess the testis microvasculature, we performed immunohistochemistry using an automated staining platform with controlled and standardized conditions and positive and negative controls. We used cluster of differentiation (CD) 34 to stain blood vessel endothelium, stem cells, and interstitium; CD31 (all endothelium); and D2-40 for lymphatic endothelium. Morphometric analysis was carried out, % of the total tissue with CD31 and CD34 positive stain was assessed, and the number of the lymphatic vessels (D2-40) per mm^2 was counted.

RESULTS Of the 10 cases, 7 had evidence of hemosiderin deposit and calcification. The % distribution of CD34 in controls was higher, 13.4 ± 3.1 (mean ± standard deviation), compared to nubbin cases, 4.5 ± 2.9 ($P \leq .001$). The % distribution of CD31 was 2.8 ± 0.83 in controls compared to 1.31 ± 0.60 in cases ($P \leq .001$). The lymphatic distribution was similar in both groups, cases (6.4 ± 4.3 n/mm^2) and controls (6.4 ± 1.7 n/mm^2) ($P = .99$)

CONCLUSION This histopathological study suggests that disturbances in endothelial development may be a contributing factor leading to testicular hypoplasia and a resultant nubbin testis, independent of a physical torsion event. UROLOGY ■■: ■■–■■, 2016. © 2016 Elsevier Inc.

Testicular development is a complex process involving differentiation and intricate vascular formation. The process starts with a bipotential gonad, and an expression of Y-linked male sex-determining gene, *Sry* gene, induces differentiation into testis.[1]

Vascular development in the testis is a complex process involving vascular networks. It is thought that the testis vasculature forms from a combination of angiogenesis (sprouting off of the major vessels) and vasculogenesis (de novo vessel formation from inherent vascular progenitors).

Once detached from the mesonephric vascular plexus, under the influence of vascular endothelial growth factor, the migrating endothelial cells rearrange to form the main testicular artery bordering the coelomic endothelium.[2] This sets off branching into the testicular parenchyma, promoting morphogenesis and patterning of the organ.[3,4] Venous development mostly follows the arterial mapping.[2] Lymphangiogenesis, on the other hand, is a late process,[2] initiated from the lymphatics associated with the vas and epididymis.[5]

Vanishing testis is considered to be driven by a neonatal and/or perinatal torsion event and is usually associated with hemosiderin deposits.[6] The role of microvascular development in vanishing testis is unknown. Our preliminary data[7] suggest that there are alterations in endothelial cells that may be driving the pathological process independent of torsion. Endothelial cell migration and testicular cord formation are interdependent processes involving rearrangement of migrating endothelial cells from the mesonephric vascular plexus.

We hypothesize that vanishing testis can be driven by developmental vascular defect as a result of arrest in endothelial cells migration rather than secondary to a random physical torsion or twist. We therefore sought to determine whether the testicular microvasculature is underdeveloped in vanishing testis compared with age-matched healthy testicles from cadaveric specimens.

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MATERIALS AND METHODS

Subjects

After an institutional review board approval (PRO14120520), retrospective review of patients who underwent unilateral orchidectomy (December 2008-August 2010) for diagnosis of nonpalpable testis was performed. Patients were identified based on the Current Procedural Terminology code (54520) for orchidectomy with the help of the Center for Assistance in Research using the eRecord system. Patients who underwent unilateral orchidectomy with presumptive diagnosis of testicular nubbin secondary to neonatal or perinatal testicular torsion were included in the analysis. Of the 10 patients, 6 patients had clinically palpable nubbin on the ipsilateral side. Of the 6 patients, 2 patients had contralateral hypertrophy on clinical examination. Of the remaining 4 patients, 2 were palpable but small in the inguinal canal and 2 were nonpalpable. All patients underwent inguinal exploration and testicles found surgically with this approach.

Patients who underwent unilateral orchidectomy for acute testicular torsion in newborn period were excluded from the analysis. Cases of testicular nubbin excision (average age 9.5 [range 2-12] months) were compared with age-matched controls (5.14 [2-12 months]) from cadaveric testes without known genitourinary pathology (n = 10 for both groups).

The pathology slides for the cadaveric testis were retrieved from the pathology database.

We used Lambert equation (LxWXHX0.71) to calculate the testicular volume; the measurements were obtained from the pathology reports following orchidectomy.

Based on the inclusion and exclusion criteria, 10 cases and controls were randomly selected for the pilot study.

Histology

Testicular samples were serially sectioned and sampled appropriately at selected levels for vascular labeling. Specimens were fixed immediately in 10% buffered formalin solution and embedded in paraffin. Cut sections (5 μm thick) were mounted on slides and stained with hematoxylin and eosin for routine clinical morphological assessment. To assess the testis microvasculature, we performed Benchmark XT immunohistochemistry automated staining platform (Ventana Medical Systems, Tucson, AZ) following the manufacturer's instructions, with controlled and standardized conditions and positive and negative controls.

We used primary antibodies for cluster of differentiation (CD) 34 that in the testis stains blood vessel endothelium, stem cells, and interstitium; CD31 or platelet endothelial cell adhesion molecule (PECAM-1) that stains all endothelial cells; and Podoplanin (D2-40) to stain lymphatic endothelium (Table 1).

Table 1. Primary antibodies and dilution for CD31, CD34, and D2-40 markers

Primary antibodies	Dilution
CD31-, anti-CD31 antibody clone (JC70A)	DAKO M0823 dilution at 1:200
CD34-, anti-CD34 antibody clone (QBEnd-10)	DAKO M7165 at 1:1:250 dilution
D2-40 monoclonal antibody (Covance)	1:50

Image Capture and Morphometric Analysis

Whole slide images were scanned using Aperio Scanscope at X20 (scan resolution at 0.499 μm/pixels). After scanning, digital images at 20× magnification were captured (image dimension 1024 × 768 pixels) and technical artifact areas (section folds, knife marks, etc.) were excluded. The morphometric analysis was carried out on the whole testicular area available for analysis using Image J software and the technique described at the NIH website (http://rsbweb.nih.gov/ij/docs/).[8]

The percentage of tissue with CD31 and CD34 positive stain was assessed and the number of the lymphatic vessels (D2-40) per mm^2 was counted.

Statistical Analysis

Continuous variables were summarized as mean ± standard deviation. Statistical analyses including Student's paired *t* tests were two sided and $P \leq .05$ was considered significant. Statistical analyses were performed using SPSS 23.0 (SPSS, Chicago, IL).

RESULTS

The average age for cases was 9.5 (range 2-12) months and for age-matched controls it was 5.14 (range 2-12) months. All 10 cases had clinical diagnosis of nonpalpable testis, of which 8 (80%) were left sided. Histologic examination of the cases revealed evidence of hemosiderin-laden macrophage deposits and dystrophic calcification in 7 cases; the other 3 cases only revealed the presence of testicular atrophy. The average testicular volume for the 10 cases was 0.34 ± 0.31 mL (0.049-1.14). In 3 cases with testicular atrophy, the average volume was 0.52 ± 0.55 (0.049-1.14) mL compared to 0.27 ± 0.17 (0.039-0.59) mL in those with hemosiderin deposits and calcification ($P = .25$).

Vanishing Testis Had Less Vascular Density Than Controls

Our results revealed significant underexpression of CD31 and CD34 endothelial, stem cells and interstitial markers in cases with vanishing testis. CD31 showed a marked diminution in the staining pattern of the endothelial cells in cases compared to controls (Fig. 1A,B). The normal distribution of the tubules is distorted in cases with pronounced interstitial fibrosis. The seminiferous tubules are dysmorphic with hyalinized basement membrane. Normal architectural pattern of the seminiferous tubule is distorted (Fig. 1). Likewise CD34 expression was significantly decreased in cases compared to controls (Fig. 1C,D).

D2-40 stains not only lymphatic endothelium but also sertoli cells as seen in both cases and controls (Fig. 1E,F). The lymphatic vessels with arrows show similar distribution in both cases and controls, although surrounded by a significant amount of interstitial fibrosis in cases even between the tubules.

The percentage distribution of CD34 in controls was higher, 13.4 ± 3.1 (mean ± standard deviation), compared to cases, 4.5 ± 2.9 (Table 2, $P < .001$). The % distribution of CD31 was 2.8 ± 0.83 in controls compared to 1.3 ± 0.6 in cases (Table 2, $P < .001$). The numbers of

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