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Expression of extracellular matrix proteins and vimentin in testes of azoospermic man: an immunohistochemical and morphometric study

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Abstract **Aim:** To investigate the changes in the extracellular matrix protein expression and the morphology of seminiferous tubules in the testis of 88 azoospermic men. **Methods:** The patients were of the following categories: (1) 22 cases of Sertoli-cell-only syndrome, (2) 20 cases of spermatogenic arrest, and (3) 46 cases with hypospermatogenesis. Testicular sections were immunohistochemically stained for fibronectin, vimentin, laminin and collagen type IV. The seminiferous tubular diameter and the connective matrix zone (CMZ, the acellular zone between the basement membrane [BM] and the peritubular cells) thickness were measured. Seminiferous tubules were typed according to the thickness of the connective matrix in the lamina propria. The predominant tubule type and the Johnsen and Silber scores were determined. **Results:** The mean tubular diameter were 119 ¡À27, 117 ¡À20, and 140 ¡À38 μm for Groups 1, 2, and 3, respectively. Both the laminin and the type IV collagen were localized to the epithelial BM and peritubular cells. In most of the tubules, BM and peritubular cells were separated by a homogenous acellular layer, the CMZ, in which laminin, type IV collagen, fibronectin and vimentin were not present. It is perceived that the worse the testicular histology, the higher the thickness of the CMZ. **Conclusion:** In testis with no or low sperm production, the diameter of the seminiferous tubules is decreased, the thickness of the seminiferous tubular wall is increased and a CMZ is formed between the peritubular cells and the BM. The thickness of CMZ is increasing with the advancement of testiclar deterioration. The most important morphologic predictive factor for spermiogenesis is the predominant tubule type.*(Asian J Androl 2002 Mar: 4:55-60)*

1 Introduction

Seminiferous tubules are surrounded by lamina propria, which is composed of basement membrane (BM) and outer three to six layers of myoid cells, connective tissue, and fibroblasts, and the outmost one or two layers are composed of mainly fibroblasts [1]. Type I and IV collagens and laminin are present in the BM and the thickness of the BM increases by 50 % in the aged [2]. The type IV collagen and laminin are produced by the Sertoli cells, while type I and IV collagens and fibronectin are secreted by the myoid cells [3].

Fibronectin is present in the peritubular cells and absent in the Sertoli cells as identified by the immunofluorescence in the rat testicular cell culture [4]. Another study indicated that in monoculture, fibronectin and type IV collagen were positive in the rat peritubular cells, but laminin was negative; in Sertoli cells type VI collagen and laminin were positive and fibronectin was negative.

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Co-cultures of peritubular cells and Sertoli cells resulted in increased formation and deposition of ECM components [5]. In case of impaired spermatogenesis, the lamina propria was frequently thickened by an increase in the ECM components with differentiation of the inner myoid cells towards fibroblastic nature [6].

In the Sertoli-cell-only syndrome, the localization of laminin and type IV collagen is different from that in the normal testis. In the normal testis both laminin and type IV collagen are localized at the epithelial BM and around the myoid cells. However in the Sertoli-cell-only syndrome, the epithelial BM and the first myoid cell layer are separated by a wide homogenous layer negative for both laminin and type IV collagen, resulting in the appearance of two concentric rings around the tubular lumen: an inner ring representing the BM and the outer ring, the myoid cell layers [7]. This homogenous layer is composed of collagen fibres and deposits of amorphous substance [8] and contains collagen types I and III [9]. In the testes of cryptorchid men, two weakly stained layers of type IV collagen are present; the inner layer occupies the innermost part of the lamina propria and the outer layer surrounds the periphery of lamina propria [10]. A thickened BM is observed in atrophied testis [11]. Normal or pathologic, all testicular tissue demonstrates vimentin positivity in the Sertoli cells [12-14]. Immunostaining of myoid cells with anti-vimentin antibodies is weak in normal or near-to-normal tubules, and intense in hyalinised tubules [8,15].

The aim of this study was to investigate the expression of ECM proteins and vimentin and the morphology in the testis of Turkish azoospermic men.

2. Materials and methods

2.1 Testicular biopsy specimens

Testicular biopsy specimens, harvested from 88 Turkish males, aged 19-48 years, who had non-obstructive azoospermia and normal karyotype were used for this study. They were divided into three groups according to their histopathologic picture: Group 1 (Sertoli-cell-only syndrome) 22 cases, Group 2 (maturation arrest at spermatocyte level) 20 cases and Group 3 (hypospermatogenesis with the presence of elongated spermatids and/or spermatozoa) 46 cases. Testis biopsy materials were obtained by open biopsy and fixed for 2-12 hours in Bouin's solution before embedding in paraffin. Four μm sections were prepared for H & E, masson trichrome, PAS, and immunohistochemistry.

2.2 Immunohistochemistry for fibronectin, vimentin and collagen type IV

After deparaffinization and rehydratation, antigen retrieval was performed in a microwave oven. A water-filled container was heated to 90 ¡æand then slides in 10 mmol/L citrate buffer pH 6.0 were placed in the container. Subsequent procedures were carried by the MSIPE protocol by Dako Optimised Staining System and Techmate 500 Plus software (Dako, Glostrup, Denmark) using collagen type IV (1:50, monoclonal mouse anti-human, clone CIV 22, Dako, Glostrup, Denmark), vimentin (1:25, monoclonal mouse anti-human, clone V9, Dako, Glostrup, Denmark) and fibronectin (1:500, polyclonal rabbit anti-human, Ig fraction, Dako, Glostrup, Denmark) antibodies. After counterstaining with H & E, the sections were dehydrated in graded ethanol rinses, cleared in xylene, and coverslipped with Permount. Negative control tests run without primary or secondary antibody.

2.3 Immunohistochemistry for laminin

The 4 μm tissue sections were deparaffinized and rehydrated according to the standard protocol. Antigen retrieval was performed by incubating for 15 minutes with trypsin solution. After peroxidase treatment for 10 minutes, human serum was applied for five minutes. Then, sections were incubated for 30 min at room temperature with anti-laminin antibody (1:30, polyclonal rabbit anti-human, Dako, Glostrup, Denmark) and the antibody was visualized with an avidin-biotin horseradish peroxidase complex using a Universal LSAB kit (Dako, Glostrup, Denmark). After counterstaining with H&E, the sections were dehydrated in graded ethanol rinses, cleared in xylene, and coverslipped with Permount. Negative control tests were run without the primary or secondary antibody.

2.4 Microscopic examination and scoring

The slides were examined under a light microscope. The seminiferous tubular diameter and the connective matrix thickness in the tubular wall were measured using a vernier ocular and expressed as μm. The connective matrix zone (CMZ) thickness in the tubular wall is measured as the thickness of the zone between the BM and the peritubular cells of the seminiferous tubular wall. Seminiferous tubules were typed according to the thickness of their connective matrix in the lamina propria: (a) no apparent connective matrix, (b) the thickness of the connective matrix was 1-5 μm; (c) thickness of connective matrix was more than 5 μm, and (d) hyalinised tubule. This scoring system is modified from Santamaria et al. [8], who subdivided the stage of sclerosis into five types: (a) tubules with normal lamina propria thickness (¨Q7 μm), (b) tubules with slightly thickened lamina propria (7.1-10 μm), (c) tubules with initial sclerosis (10.1-

20 μm), (d) tubules with advanced sclerosis (20.1-35 μm), and (e) tubules with complete sclerosis (>35 μm). The reason of modifying this scoring system is our emphasis on the thickness of the connective matrix rather than the whole seminiferous tubular wall. The best two sections were selected for each case and at least 30 tubules per section were measured for determining the mean tubular diameter and the thickness of the connective matrix. Measured tubules were classified into above described four tubule types, in which the most frequent tubule type for each patient is defined as predominant tubule type. Scoring was performed according to Johnsen [16] and Silber et al. [17].

2.5 Statistics

Data were expressed in mean ¡ÀSD. Chi-square tests, Kruskal-Wallis variance analysis and Mann-Whitney U test were used for statistical analysis performed with the help of SPSS 10.0 software. Correlation tests and binary logistic regression analysis were also used. $P<0.05$ was set as significant.

3 Results

3.1 Age, tubular diameter and Johnson score

The data related to the age, the mean tubular diameter and the mean Johnsen score are summarized in Table 1.

3.2 Laminin immunohistochemistry

Immunostaining for laminin revealed that it was present in the BM and peritubular cells of seminiferous tubules. Two different staining patterns were observed in the BM of the tubules. In most cases laminin demonstrated a diffuse staining in BM, but in some testes, the laminin deposits formed invaginations into the seminiferous epithelium. A total of 22.5 % of the patients demonstrated laminin deposits and no significant difference was observed between the patient groups. The peritubular cells in Type a tubules were negative or weakly positive with laminin immunohistochemistry, but the peritubular cells in Type b and c tubules were usually positive. The Sertoli cells were uniformly negative. In all patients, most of the Leydig cells demonstrated a granular staining with laminin. In all the patients at least 50 percent of the Leydig cells were positive. The vascular walls were also positive.

3.3 Collagen type IV immunohistochemistry

With immunostaining, collagen type IV was present in the BM and peritubular cells of the seminiferous tubules. The collagen type IV expression in the peritubular cells disappeared in degenerated tubules. A granular staining is observed in 33 ¡À22 % of the Leydig cells.

3.4 Fibronectin immunohistochemistry

In all the patients the peritubular cells and the interstitial cells were positive with fibronectin immunohistochemistry. The Sertoli cells were faintly or weakly positive.

3.5 Vimentin immunohistochemistry

The peritubular cells, the Sertoli cells, the Leydig cells and the interstitial cells were positive with vimentin immunohistochemistry in all the patients.

3.6 Predominant tubule type

As can be seen from Table 2, the predominant tubule type was significantly different between the 3 groups (Group 1 vs. 2 $P<0.05$; Group 1 vs. 3 $P<0.01$; Group 2 vs. 3 $P<0.05$).

3.7 CMZ

Both laminin and type IV collagen were localized at the epithelial BMs and the peritubular cells. However, in most of the tubules, the epithelial BM and the first peritubular cell layer were separated by a wide homogenous layer (CMZ) negative for both laminin and type IV collagen, resulting in the appearance of two concentric rings around the tubular lumen, an inner ring representing the BM and the outer ring the peritubular cell layers. The peritubular cells were also positive for fibronectin and vimentin. The layer between the BM and the peritubular cell was also negative for fibronectin and

Table 1. Age (year), tubular diameter (μm) and Johnsen score in the patients. Group I: Sertoli-cell-only, Group II: Maturation arrest, Group III: Hypospermatogenesis.

	Group 1	Group 2	Group 3	*P*		
	n=22	*n*=20	*n*=46	1 vs 2	1 vs 3	2 vs 3
Age	33 ± 5	36 ± 5	36 ± 6	>0.05	>0.05	>0.05
Tubular diameter	119 ± 27	117 ± 20	140 ± 38	>0.05	<0.05	<0.01
Johnsen score	1.85 ± 0.23	2.64 ± 0.76	4.67 ± 2.02	<0.01	<0.01	<0.01

Table 2. Number of patients with different predominant tubule types. Predominant tubule type is the most frequent tubule type for each patient. Type a: Normal tubule wall, b: Slightly thickened tubule wall (1-5 μm thickness of connective matrix), c: Moderately thickened tubule wall (thickness of connective matrix is more than 5 μm). Group I: Sertoli-cell-only, Group II: Maturation arrest, Group III: Hypospermatogenesis.

	Type a	Type b	Type c	Total
Group 1	3 (13.6 %)	6 (27.3 %)	13 (59.1 %)	22 (100 %)
Group 2	10 (50.0 %)	4 (20.0 %)	6 (30.0 %)	20 (100 %)
Group 3	33 (71.7 %)	9 (19.6 %)	4 (8.7 %)	46 (100 %)
Total	46 (52.3 %)	19 (21.6 %)	23 (26.1 %)	88 (100 %)

vimentin. It appeared as a homogenous blue zone with masson trichrome, and as a homogenous red zone with PAS (Figure 1). Occasionally the peritubular cells were observed near the BM or in the CMZ. Table 3 lists the data related to the minimum and maximum thickness of the CMZ in the seminiferous tubules. When compared according to the presence of CMZ, Group 3 was better than Group 1 (P<0.01) and Group 2 (P<0.01). There was no significant difference between Group 1 and 2. There was a negative correlation between the minimum CMZ thickness and the Silber score (r=-0.288, P<0.01) and the Johnsen score (r=-0.431, P<0.01). The maximum CMZ thickness is higher in Group 1 than in Group 3 (P<0.01). There was a negative correlation between the maximum CMZ thickness and the Silber score (r= -0.320, P<0.01) and the Johnsen score (r=-0.493, P< 0.01).

Binary logistic regression analysis was performed for the presence of elongated spermatids and/or spermatozoa. Morphometric variables (mean tubular diameter, predominant tubule type, minimum CMZ thickness, maximum CMZ thickness) were entered into the equation and only the predominant tubule type revealed a significant association (P<0.01).

4 Discussion

In this study, we examined the relationship between the morphometric parameters and the state of spermatogenesis and the characteristics of laminin, type IV collagen, fibronectin, and vimentin expression in primary infertile azoospermic males.

The mean tubular diameter is 180 ¡À30 μm in normal testes [1]. In the present study, mean tubular diameter is 140 ¡À38 μm in Group 3. This finding supports the previous studies reporting a decreased tubular diameter in men suffering from hypospermatogenesis [10, 15]. The mean tubular diameter in Group 3 was higher than in other 2 groups, while it was not significantly different between Group 1 and 2. In certain cases, the simultaneous occurrence of complete hyalinisation in some tubules and presence of spermiogenesis in other tubules were observed. Fifty percent of Group 3 patients carried some tubules scored 1 according to Johnsen. These findings demonstrate that in case of infertility, one should not expect a all-encompassing disturbance in the histopathologic picture [18].

In the study of Skinner et al. [5], the cells in the rat peritubular cell culture are negative with laminin. In our study, the peritubular cells were negative with laminin immunohistochemistry when there was no connective matrix in the tubular wall. The tubules with connective matrix demonstrated negative, positive, partial positive or weak staining with laminin immunohistochemistry. The peritubular cells in the end stage tubules were also laminin negative. According to these findings, the peritubular cells in normal testis do not have active laminin synthesis, but through the direct or indirect effect of stimuli deteriorating the testicular structure, the peritubular cells starts laminin synthesis; this synthetic process diminishes in the end stage tubule.

Both the BM and the peritubular cells were positive with collagen type IV immunohistochemistry. The col-

Table 3. Number of patients as per minimum and maximum thickness of connective matrix zone. Group I: Sertoli-cell-only, Group II: Maturation arrest, Group III: Hypospermatogenesis.

		0 μm	1-3 μm	4-7 μm	7-10 μm	>10 μm	Total
Group 1	Minimum	9 (41%)	10 (45%)	3 (14 %)	-	-	22 (100%)
	Maximum	-	1 (5%)	1 (5 %)	2 (9 %)	18 (81 %)	22 (100%)
Group 2	Minimum	11 (55%)	6 (30%)	3 (15 %)	-	-	20 (100%)
	Maximum	-	1 (5%)	4 (20 %)	2 (10 %)	13 (65 %)	20 (100%)
Group 3	Minimum	40 (87%)	6 (13%)	-	-	-	46 (100%)
	Maximum	5 (11%)	7 (15%)	4 (9 %)	4 (9 %)	26 (56 %)	46 (100%)
Total	Minimum	60 (68%)	22 (25%)	6 (7%)	-	-	88 (100%)
	Maximum	5 (6 %)	9 (10 %)	9 (10 %)	8 (9 %)	57 (65 %)	88 (100%)

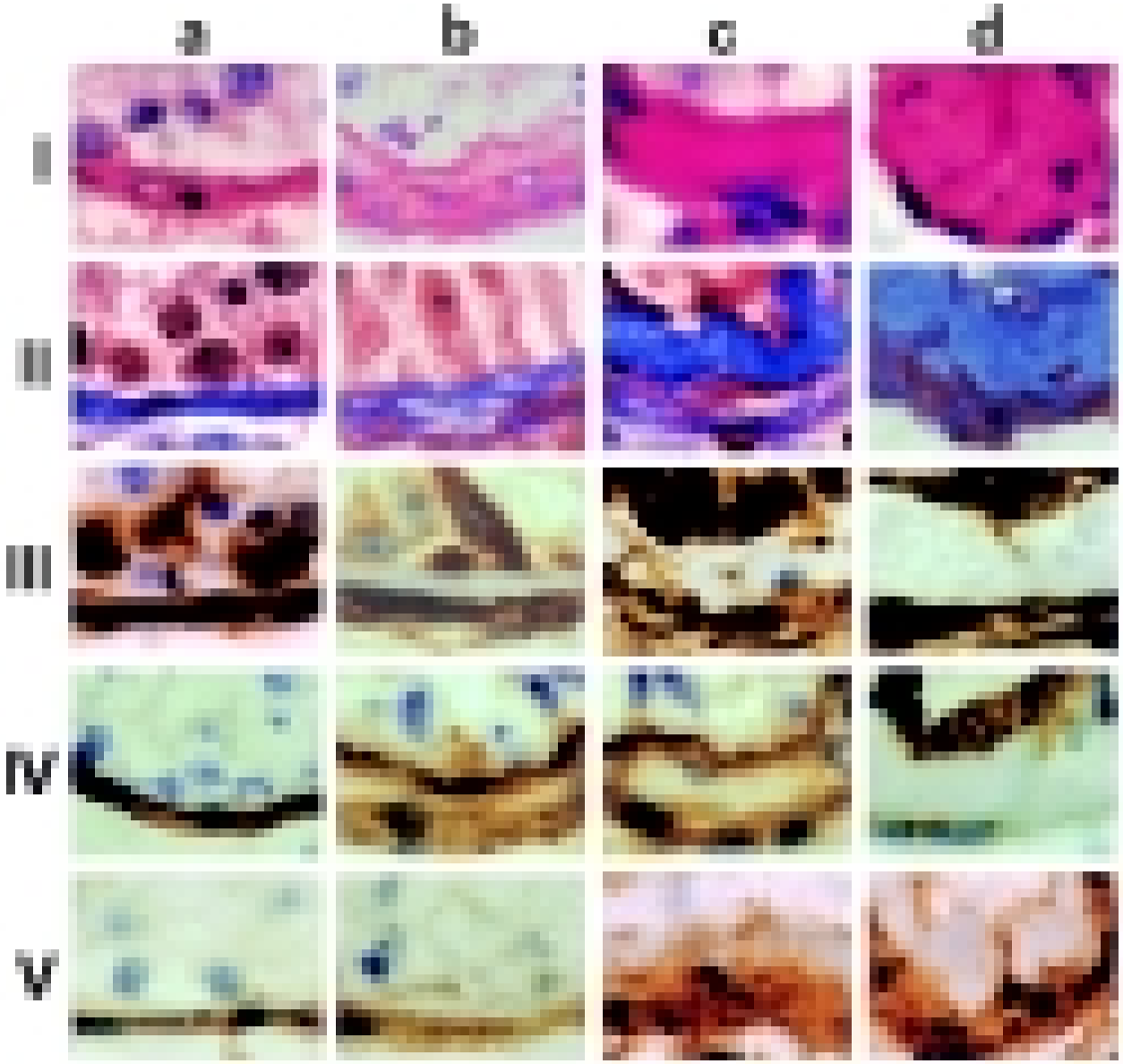

Figure 1. Photomicrographs (×1000) of seminiferous tubular wall. a-d: types of tubule; I: PAS; II: Masson trichrome; III: Vimentin immunohistochemistry; IV: Collagen type IV immunohistochemistry; V: Fibronectin immunohistochemistry.

lagen type IV expression in the peritubular cells disappeared in the degenerated tubules. A close relationship between spermatogenesis and the morphology of testes was observed. In testes with no or low sperm production, the diameter of the seminiferous tubules decreases, the thickness of the seminiferous tubule wall increases, a connective matrix is formed between the peritubular cells and the BM, and the thickness of this matrix increases with deteriorating testicular histology. In case of absence of spermatids, there were always tubules with CMZ. Possibly, the presence of the connective matrix in the tubule wall decreases the interchange between the spermatogenic epithelium and the blood and thus disturbs spermatogenesis; disturbed spermatogenesis will cause increased synthesis of connective matrix. The mechanism of this process is not completely defined up to date.

Santamaria et al. [19,20] performed experimental studies to enlighten the physiopathology of this process. In these studies, they created artificial testis ischemia with intra-scrotal injection of epinephrine. In the control testes, BrdU-labelled nuclei (proliferating cells) were observed only in spermatogonia and some primary spermatocytes, whereas testes from epinephrine-treated rats showed BrdU labelling in some of the spermatogonia and peritubular cells. The percentage of peritubular cells that were immunopositive for the proliferating cell nuclear antigen (PCNA) also increased. The absolute volume occupied by laminin and fibronectin immunostaining

decreased from the 3rd to the 8th week of treatment, and increased from the 8th to the 11th week. These changes, associated with germ cell depletion and tubular fibrosis, suggest that tubular ischemic atrophy caused by epinephrine alters the peritubular myoid cells, which change their immunophenotype and increase the secretion of the extracellular matrix components producing tubular fibrosis. The mechanism of this alteration may involve direct effects on the peritubular cells or the changes may be secondary to germ cell and/or Sertoli cell lesions.

The relationship between the morphometric data and the success of assisted reproduction awaits further study [21]. On the other hand, the molecular pathogenesis of diminished spermatogenesis in testes should be more clearly defined and possible early diagnostic and therapeutic approaches should be investigated.

In conclusion, a clear relation is present between the testicular morphology and its function. The Seminiferous tubular diameter decreases and the thickness of tubular wall increases in the testis of azoospermic men. The main component responsible of this increase is the CMZ, which is located between the BM and the peritubular cells. The CMZ does not contain laminin, fibronectin, collagen type IV or vimentin. The most important morphologic predictive factor for spermiogenesis is the predominant tubule type.

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