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Original Article

Stage Specific Expression of Angiotensin-Converting Enzyme and Thickened Lamina Propria in Relation to Male Fertility

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Abstract

Introduction: The testis is an immune privileged organ that provides a specific environment for germ cell development. Various factors responsible for inflammatory changes can lead to deterioration of the immune tolerant model found in the testis. As a result, the thickness of the proper membrane of seminiferous tubules changes and the process of spermatogenesis is disturbed.

Aim: The purpose of the present study was to find the connection between the changes in the level of testis-specific isoform of angiotensin-converting enzyme (tACE) expression and the morphological changes of the seminiferous tubule wall of the testis in patients with infertility.

Materials and methods: The study included 24 infertile men who underwent a testicular biopsy. Routine histological techniques, immunohistochemical reactions for tACE, α-smooth muscle actin, and morphometric analysis were performed to examine the biopsy preparations.

Results: By using testicular biopsy to diagnose patients with infertility, a stage-specific pattern of the processes associated with thickened proper membrane of seminiferous tubules was established and a decreased or absent spermatogenic activity was observed.

Conclusions: A significant increase in the proper membrane thickness of the seminiferous tubules in the testis was found in patients with infertility. This finding shows that the processes take place gradually over time, correlating with the degree of pathology, and that changes do not depend on the factors causing them. We also found that the degree of proper membrane thickening correlated with disturbances of spermatogenesis, using tACE expression as a marker for spermatogenic epithelium differentiation.

Keywords

angiotensin-converting enzyme, infertility, membrana propria, myofibroblasts, spermatogenesis

INTRODUCTION

Most of the testicular parenchyma is made up of the seminiferous tubules where the process of spermatogenesis occurs. The seminiferous tubules develop during puberty with the formation of the tubular lumen. The seminiferous tubule consists of the spermatogenic epithelium and the proper membrane, membrana propria (MP). The MP of the seminiferous tubules, also known as the peritubu-

lar lamina, surrounds the cellular elements. Differentiated epitheliocyti sustentans (Sertoli cells, ES) and incessantly proliferating spermatogenic cells are placed inside the tubular lumen.[1] Myofibroblasts located in the testis are the main component of MP of the seminiferous tubules. Thickened MP is found in patients with various fertility disorders and in the seminiferous tubule wall in the ageing testis.[2]

Human MP is composed of several layers of myoid cell and extracellular matrix between them.[3-5] The myoid cells seemed to develop into myofibroblasts for defense against testicular damage, and positively secreted extracellular matrix (ECM).[3] The biological functions of MP are determined by the myofibroblasts and their contractile elements which take part in the transportation of sperm and seminal fluid in the seminiferous tubules.[2] Another feature of MP is the barrier function providing permeability of substances, penetrating the seminiferous tubules from the interstitium to the spermatogenic epithelium. In addition, these substances act like growth factors, regulatory factors, interleukins, influencing the ES activity.[6-8]

The myoid cells provide structural integrity of the tubules and participate in the regulation of spermatogenesis.[6,9] These cells also control cellular growth, differentiation and migration, and influence tissue enlargement and remodelling.[10,11]

The steroid producing cells, endocrinocyti interstitiales (Leydig cells, EI) prominent in the inter-tubular space, produce and secrete one of the essential male reproductive hormones, the testosterone.[6] Androgens are essential for myoid cell differentiation and testicular development, especially for blood-testis barrier formation and germ cell development.[12-14] The mature peritubular myoid cells express smooth muscle actin (α-SMA), which is a marker for smooth muscle cells.[15] The myoid cells interact with ES[16] and this interaction is crucial to the structural formation of the blood-testis barrier and the stimulation of the ES cells to produce biologically active substances.[17]

The testicular angiotensin-converting enzyme (tACE) is a shorter isoform of ACE, which is specifically expressed only in the testes. It has been found that this enzyme plays an essential role in spermatogenesis and the stages of spermatid differentiation in the testes.[18,19] The manifestation of tACE can be registered after the completion of meiosis as the maximum expression is being processed during the acrosomal stage and continues to be expressed in the acrosomal region until the end of sperm maturation.[20,21] It takes part in all processes and the development of mature sperm, their maturation in the epididymis, capacitation, acrosome reaction, i.e., its expression is of particular importance to male fertility.[20-22] Therefore, it can be used as a marker for spermatogenic activity.

Immunochemical analysis of adult rat testis also revealed a stage-specific pattern of tACE expression in the cytoplasm of postmeiotic germ cells. tACE could serve as a marker for germ cell depletion in experimental and pathological conditions.[18,22]

AIM

The purpose of the present study was to find a connection between the changes in the level of tACE expression and the morphological alterations in the seminiferous tubule wall in patients with infertility.

MATERIALS AND METHODS

Testicular biopsy

Testicular biopsies from patients with history of infertility and azoospermia were included in this study by testicular sperm extraction (TESE) and using a standard open surgical biopsy technique which is performed at the same time as ART. The testicular tissue obtained was provided by Malinov Specialized Surgical Hospital in Sofia. The study was conducted between 2018 and 2019, and included 24 infertile men (age range 21-42 years) with azoospermia (obstructive azoospermia [n=12], non-obstructive azoospermia [n=3], varicocele [n=6], and cryptorchidism [n=3]). All study patients gave their written informed consent to undergo a testicular biopsy. Tissue samples were removed until spermatozoa were identified or 3–4 biopsy pieces were extracted from each testis. The samples were fixated in Bouin's solution after being washed and placed in 70% alcohol, embedded in paraffin blocks, cut into slices using an automatic paraffin microtome (Leica 2055), dewaxed, and rehydrated in descending series of ethanol (100%, 95%, 70%) and distilled water.

Formation of biopsy material groups

The morphological analysis of the biopsy material based on the MP morphology and spermatogenic activity allowed each biopsy preparation to be assigned to one of four groups showing increasing pathology. The four groups the biopsy material was allocated to according to Volkmann et al.[23] were: intact MP and preserved spermatogenesis (group 1), reduced expression of tACE and increased amount of ECM between the layers of myofibroblasts in the MP (group 2), reduced expression of tACE and presence of two layers of myofibroblasts and thickened ECM between them (group 3), and lack of spermatogenic activity and thickened MP with missing inner myofibroblast layer (group 4).

Histology and histochemistry

The testicular biopsies were fixed at room temperature in Bouin's solution and embedded in paraffin. Five-μm-thick paraffin sections were stained with haematoxylin and eosin (H&E) and Heidenhain's azan[24] and analysed immunohistochemically.

Hematoxylin-eosin staining

The sections were stained with Mayer's hematoxylin (5 min) and differentiated in tap water (20 min). After completion of the differentiation, the sections were stained with water-soluble eosin for 5 minutes. The subsequent stained sections were dehydrated again in ascending grades of ethanol (70%, 95%, and 100%), cleared in xylene (2×10 min) and covered with Canadian balm.

Azan staining

For Azan staining, the slides were incubated in aniline alcohol (0.1 ml aniline dissolved in 100 ml 90% ethanol) for 5 min and in prewarmed azocarmine G (0.1% azocarmine G, 1% glacial acetic acid) for 10 min at 60°C. The specimens were chilled for 10 min, washed with H_2O, differentiated with aniline alcohol (for 5 to 10 minutes), briefly washed with acetic alcohol and H_2O, and incubated in 5% phosphotungstic acid for 2 hours. The next steps were incubation in aniline blue/orange G solution (0.25% aniline blue, 1% orange G, 4% glacial acetic acid) for 2 hours and then differentiation using 96% alcohol.

Immunohistochemistry

Sections were deparaffinised, then subjected to antigenic detection of the epitopes with citrate buffer, and an endogenous peroxidase blockade was made with hydrogen peroxidase (3%), a kit (ref: No. BBK 120, Scy Tek, USA) was used to block the endogenous biotin and a reagent to block non-specific binding (Superblock, Scy Tek), followed by incubation for 24 hours at 4°C with anti-goat ACE - 1:300 (sc-12187, Santa Cruz Biotechnology Inc. USA) and monoclonal anti-α smooth muscle actin (A-2547, Sigma) 1:5000 - NRS/TBS/BSA, next incubated with secondary antibody: biotinylated anti-goat (No. AGL015 Scy Tek., USA) for 10 min. The reaction was visualized with 3,3′-diaminobenzidine tetrachloride (DAB, ScyTek Lab. Inc., USA); counterstaining was performed with Mayer's hematoxylin. As negative controls, sections in which the primary antibodies were replaced by a buffer solution (PBS) were used. Microphotographs were performed with Nikon Microphot SA microscope (Japan), combined with Camedia-5050Z digital camera (Olympus, Japan) at ×100 and ×400 magnification.

Morphometric examination

The measurements were performed using Olympus DP - Soft 4.1 software, Japan. Morphometric measurement was performed on paraffin sections measuring the wall thickness of an average of 50 seminiferous tubules per patient. An average of six measurements of the seminiferous tubule wall thickness were carried out at ×200 magnification on Azan staining slices and on a positive immunohistochemical reaction for actin (α-SMA). The entire wall thickness - BM + MP was measured, as well as its components. The groups were determined based on the seminiferous tubule thickness, according to the classification of Volkmann et al.[24] and not according to the diagnosis. The percentage of the distribution of tACE in the seminiferous tubules in the testis was determined. Using a measuring grating (19×25 fields) at ×200 magnification, the value of the relative percentage distribution of tACE immunopositive cells was calculated according to the formula $x=(n/475)\times100$, where n is the number of squares with positive cells for tACE, and 475 is the total number of squares. The results obtained were analysed statistically.

Statistical analysis

The data were analysed using SPSS 19.0. Statistical significance between experimental groups was determined by the independent samples *t*-test and Mann-Whitney U test, the differences were considered significant at $p<0.05$. Correlative analysis was performed. Data are presented as mean± SEM.

Ethical approval

All methods used in the study were approved by the Scientific Ethics Committee at the Medical University of Plovdiv with decision No. P-1166/15.04.2016.

RESULTS

Following the H&E and Heidenhain's azan staining of paraffin sections, the histological analysis of group 1 revealed clearly distinguishable structures - seminiferous tubules containing approximately three layers of myofibroblast cells in MP, ES, and normal spermatogenesis with all types of germ cells - spermatogonia, primary spermatocytes, round and elongated spermatids, mature sperm. The immunohistochemical analysis of group 1 revealed expression of the tACE protein only in the spermatogenic epithelium of the seminiferous tubules. Heidenhain's azan staining was performed to visualize myofibroblast cells (red nuclei and light pink cytoplasm) forming MP. We found a change in the type of MP - as thickness was increased, the relief was changed, and the irregular outline contour was increased. In the testicular interstitium, clusters of EI and blood vessels were well distinguished.

In the comparative analysis of the preparations in groups 2, 3, and 4, the MP thickening was pronounced, accompanied by deposition of extracellular matrix between the myofibroblast layers, which was well visualized by H&E and azan staining, as well as with immunoreactivity for α-SMA.

In group 2, we found MP thickening accompanied by deposition of extracellular matrix between the myofibroblast layers, which was visualized by H&E and azan staining and immunoreactivity for α-SMA. Decreased tACE expression in individual seminiferous tubules was reported. Disorganization and exfoliation of the spermatogenic

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epithelium and its location in the lumen were observed in some of the tubules. In the testicular interstitium, disintegration of the characteristic arrangement of EI was observed, accompanied by lymphocyte infiltration.

In group 3, the two layers of myofibroblasts could be distinguished, an inner row of myofibroblasts and several outer layers of myofibroblasts with ECM deposited between them, with variable thickness in the individual tubules. Using α-SMA, the thickness of the seminiferous tubule wall was measured, and we found significant thickening in individual tubules probably due to the larger amount of ECM between the myofibroblast layers. Single EI and lymphocyte infiltration were observed in the interstitium.

In group 4, tACE was not visualized in seminiferous tubules with strongly thickened MP, in which only ES were present in groups 3 and 4. Immunoreactivity for α-SMA was visualized only in the outer layer, composed of several layers of myofibroblasts which were in contact with the cells in the interstitium, a dense layer of ECM of variable thickness was located inside it, and in certain tubules the lumen was strongly narrowed due to the deposition of ECM. The spermatogenic epithelium was in direct contact with the ECM in the seminiferous tubules. Only blood vessels were visualized in the testicular interstitium **(Fig. 1)**.

Fig. 2A presents the statistical analysis of morphometric data on the MP thickness in azan staining. The difference in the MP thickness when comparing the individual groups is noticeable and there is a gradual increase from group 1 to group 4. The data from the group comparison reveal statistically significant differences between groups 2, 3, and 4 compared to group 1 ($p<0.05$).

Statistical analysis of morphometric data on immunoreactivity for α-SMA is presented in **Fig. 2B**. The results showed a statistically significant difference between the values measured in the groups in ascending order. Comparisons between group 1 and group 2 showed a significant difference ($p=0.003$, $p=0.004$), with an increase in tubular wall thickness between group 1 and group 2 ($p=0.004$). Comparison of the data from group 3 ($p=0.003$) and group 4 ($p=0.004$) showed a tendency towards an increase of the average values of the seminiferous tubule wall thickness, compared to group 1.

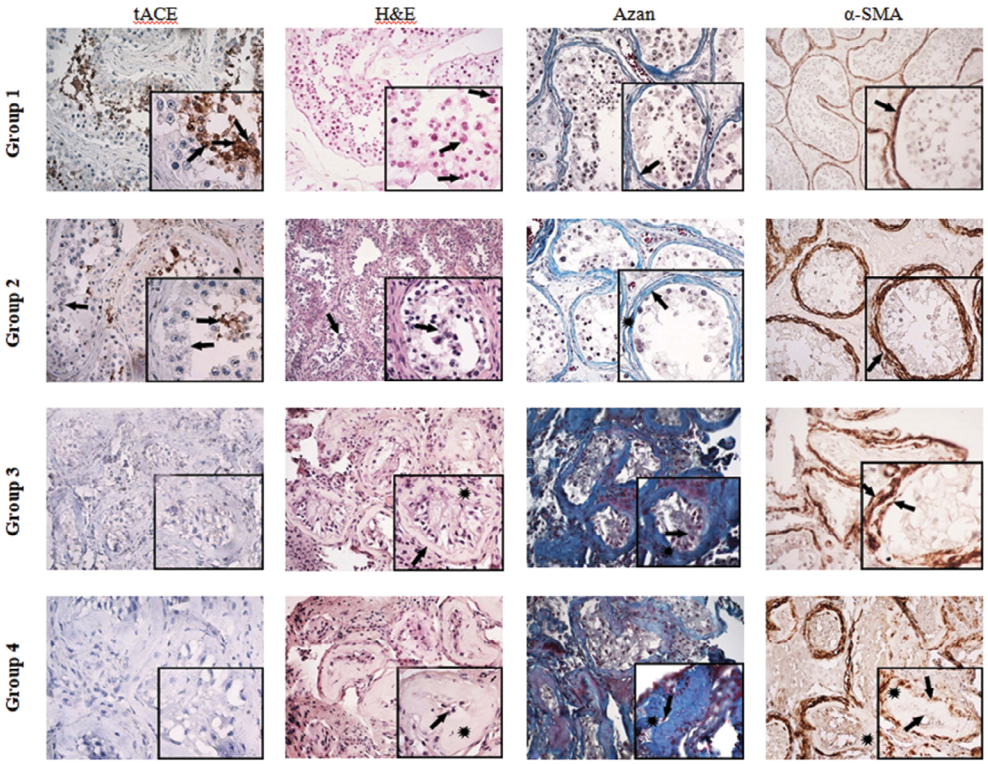

Figure 1. Seminiferous tubules (groups 1-4) – immunoreactivity for tACE and α-SMA, haematoxylin and eosin (H&E), and azan (A) staining; ×100, ×400.
Seminiferous tubules of group 1 - (tACE), (H&E), and (A) show normal MP thickness and preserved spermatogenesis; (→) immunoreactivity for α-SMA visualizes myofibroblast cells in MP. By group 2 - reduced tACE expression in individual seminiferous tubules accompanied by disorganization and exfoliation of the spermatogenic epithelium in the lumen (→). (A) and α-SMA show an increase in MP thickness. Lymphocytic infiltration in the testicular interstitium is found. Group 3 - low tACE expression in single round spermatids, (H&E) and (A) outline distinct ES. α-SMA in MP clearly distinguishes two layers of myofibroblasts - outer and inner (→); ECM (*) is deposited between them. There is no specific organization of EI, presence of lymphocyte infiltration. Group 4 - tACE is not visualized; (H&E) and (A) show a reduced lumen with single ES; α-SMA visualizes the absence of an inner layer of myofibroblasts, and only the presence of an outer layer, composed of several myofibroblast layers.

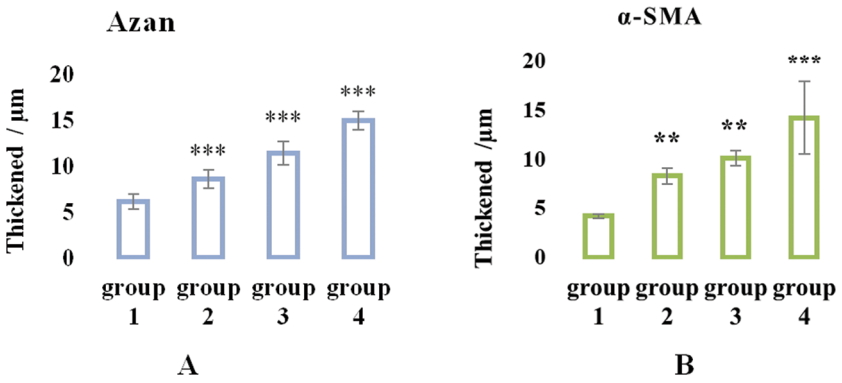

Figure 2. Analysis of the MP thickness of the seminiferous tubule wall. **A.** Azan staining; **B.** α-SMA immunoreaction; ** $p<0.05$ when compared to group 1.

Fig. 3 presents the analysis of the results of the mean distribution of tACE immunopositive cells per tubule. It showed a significantly higher number of spermatogenic cells per tubule in group 1 compared to group 2 ($p=0.007$) and group 3 ($p=0.003$). There is a tendency towards a decrease in the average percentage of tACE immunopositive cells, as their number in group 4 is equal to 0.

The statistical analysis showed a very strong negative correlation between MP thickness and the percentage of tACE-positive cells in the seminiferous tubules in both types of measurements - tACE-azan ($r=-0.714$, $p=0.01$) and α-SMA-tACE ($r=-0.529$, $p=0.01$). The higher value in the correlation between tACE-azan is probably due to the methodology and the non-specific deposition of azan on collagen fibrils in adjacent structures **(Figs 4, 5)**.

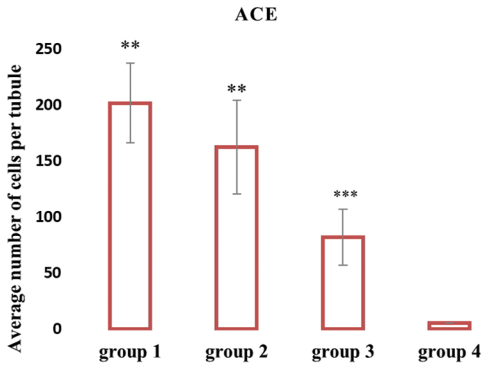

Figure 3. Analysis of the percentage of tACE immunopositive cells in the seminiferous tubule wall; ** $p<0.05$ when compared to group 1.

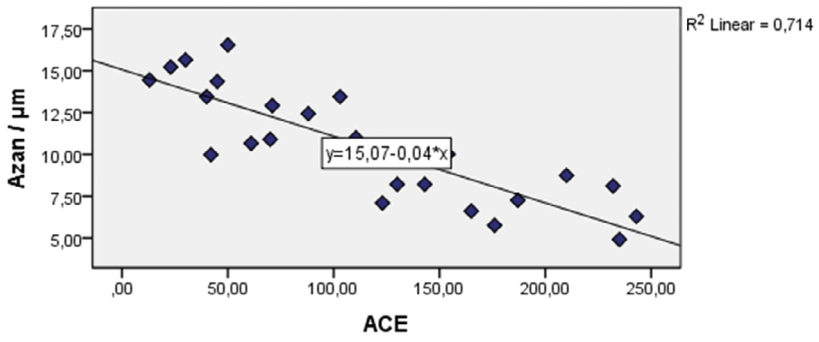

Figure 4. Correlation analysis: MP (Azan) morphology and tACE-immunopositive spermatids.

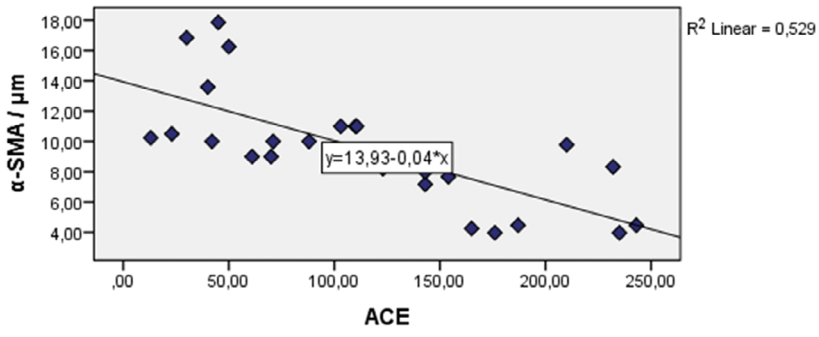

Figure 5. Correlation analysis: MP (α-SMA) morphology and tACE-immunopositive spermatids.

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DISCUSSION

One of the most common causes of male infertility is a past medical history of inflammatory processes in the testis and the epididymis. Inflammation of the testis leads to changes in the seminiferous tubule wall and impairment of spermatogenesis. In case of pathological changes, MP cells increase the secretion of various molecules of the extracellular matrix. This results in impaired communication between the myofibroblasts and the spermatogenic epithelium, which is essential for maintaining spermatogenesis. This association has been demonstrated in in-vitro studies of ES and peritubular cells of rats, which indicates that MP cells are closely related to cell populations in the walls of the seminiferous tubules and the interstitial cells.[25] The immune cells in the epididymis are in direct contact with the sperm in the epididymal lumen in contrast to the testis, which has a blood-testis barrier that provides an immune privileged environment for sperm development. These differences in structure are believed to underlie the spread of inflammation from the epididymis to the testis.[26] When inflammatory processes in the epididymis are registered as acute bacterial epididymo-orchitis, despite adequate therapies, in a large percentage of cases, immune infiltrates are found during control tests of the testis.[27]

Inflammation leads to lymphocyte infiltration and reactive T-cells activation in the testicular interstitium adjacent to the myofibroblasts,[6,10,27] accompanied by local production of cytokines with a pro-inflammatory profile, which leads to impaired immune tolerance in the testis.

The results of the present study support the above-mentioned data and are indicative for the crucial role of the inflammation processes of the testis and the epididymis in the etiology and pathogenesis of male infertility. It is important to note that in our classification of biopsy materials, the stages of the pathological changes in the seminiferous tubules can be traced, regardless of the etiology. In the present study, group 1 included biopsies in which variations of intact tubules with preserved spermatogenic activity were observed. The histopathological changes we found in the second group preparations were characterized by massive lymphocyte infiltration in both the testicular interstitium and the seminiferous tubules, these observations corresponded to the results reported by previous studies.[28] Disorganization and exfoliation of the spermatogenic epithelium and its location in the lumen were observed in some of the tubules, most likely due to the action of several different factors that lead to disintegration and disruption of the cellular contacts between ES and developing spermatogenic cells. In a study of such disorders, O'Bryan et al.[29] reported a lack of Sox 8-protein production by ES, which was a major factor in providing and maintaining these contacts. The biological functions of MP are determined mainly by myofibroblast cells which have been shown to possess contractile elements (α-SMA) involved in the transport of sperm and testicular fluid into the seminiferous tubules.[8] In our study, we found that the wall of the seminiferous tubules of infertile men with impaired spermatogenesis is reorganized, leading to the development of tubular fibrosis as evidenced by ECM deposition and is generally one of the hallmarks of male infertility. Fibrosis and morphological changes in myoid cells, including hypertrophy, have been reported in several studies, finding abnormalities in myoid cell-specific functions, involving paracrine and contractile abilities.[30,31] Our investigations revealed that group 1 included biopsies in which there were variations of intact tubules with preserved spermatogenic activity while in groups 2 and 3, specific thickening of MP and an increase in the ECM between the layers of myofibroblast cells were observed, which in some tubules was accompanied by impaired spermatogenesis. Our results for the group 1 MP thickness (between 3-5 μm) are in accordance with the values cited in the literature, but according to some other authors up to 7 μm are taken for being with normal thickness of MP.[3,32] Complete absence of spermatogenic epithelium and loss of adluminal space was observed in group 4. The measurements in groups 1-4 are in accordance with the results reported by Volkmann et al.[23], reflecting the change in the thickness of MP. Thickening of MP was found to be independent of the clinical background of testicular alterations and can be suggested as a general predictive factor for disturbed spermatogenesis in agreement with other investigetions.[23,32] Many studies have been focused on the influence of immune cells as a possible factor affecting MP and male infertility. It has been found that infiltration of immune cells (mainly lymphocytes, macrophages, and mast cells) into the wall of the seminiferous tubules leads to disturbances in spermatogenesis. Immune cells affect the function of peritubular myoid cells through factors that stimulate their phenotype and secretory activity. In patients with infertility, the deposition process is accompanied by thickening of the seminiferous tubules.[31] This is believed to disrupt both paracrine signalling in the testes and spermatogenesis[30,31] and these data are in accordance with our results.

In the present study, the immunoreactive expression for tACE was used to report the stage-specific sperm differentiation because it is well known that tACE is exclusively expressed in differentiating haploid spermatids, with a significant role in the stages of spermiogenesis.[19,20] This enzyme is involved in all processes of sperm maturation - capacitation, movement in the female reproductive tract, and acrosome reaction.[21,22] Our analysis showed a very strong negative correlation between the MP thickness (by azan staining and α-SMA immunoreactivity) and the percentage of tACE-positive cells in the seminiferous tubules. In this relation, our results provide additional evidence for the important role of tACE in the process of spermatogenesis and support the previous data characterizing tACE as a suitable marker for sperm differentiation and male fertility.[22,33]

CONCLUSIONS

The data analysis showed an increase in the MP thickness corresponding to the degree of pathology; these changes developed gradually over time following a certain pattern which did not depend on the causes. It was also found that the degree of MP thickening correlated with the disturbance in spermatogenesis, using tACE expression as a marker for spermatogenic epithelial differentiation.

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Стадийно-специфическая экспрессия ангиотензинпревращающего фермента и утолщённая lamina propria в связи с мужской фертильностью

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Резюме

Введение: Яичко – это иммунно-привилегированный орган, который обеспечивает специфическую среду для развития зародышевых клеток. Различные факторы, ответственные за воспалительные изменения, могут привести к ухудшению иммунорезистентной модели яичка. В результате изменяется толщина оболочки семенных пузырьков и нарушается процесс сперматогенеза.

Цель: Целью настоящего исследования было установление взаимосвязи между изменениями уровня экспрессии тестикулярной изоформы ангиотензин-I-превращающего фермента (tACE) и морфологическими изменениями стенки семявыносящих протоков у пациенток с бесплодием.

Материалы и методы: В исследование включены 24 мужчины с бесплодием, которым выполнена биопсия яичка. Были выполнены обычные гистологические методы, иммуногистохимические реакции на tACE, α-актин гладких мышц и морфометрический анализ.

Результаты: Биопсия яичка для диагностики бесплодия у пациентов выявила стадийность процессов, связанных с утолщением оболочки семенных пузырьков, снижением или отсутствием сперматогенной активности.

Заключение: Выявлено достоверное увеличение толщины оболочки семенных пузырьков яичка у пациентов с бесплодием. Полученные данные показывают, что процессы протекают постепенно во времени, коррелируют со степенью патологии и эти изменения не зависят от вызвавших их факторов. Мы также обнаружили, что степень утолщения мембраны коррелирует с нарушением сперматогенеза посредством экспрессии tACE как маркера дифференцировки сперматогенного эпителия.

Ключевые слова

ангиотензинпревращающий фермент, бесплодие, membrana propria, миофибробласты, сперматогенез