Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 23
Sofia ● 2016

Morphology

The Importance and Application of Testicular Angiotensin Converting Enzyme (tACE) as a Marker for Evaluation of Mammalian Spermiogenesis and Fertility

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Two isoforms – somatic (sACE) and testicular (tACE) are known. The aim of the present study is to reveal the role of tACE as a marker for germ cell differentiation, in particular for spermatid development. The specific pattern of tACE protein expression was studied in normal and experimental condition (spontaneous hypertension, androgen withdrawal, hyperglycaemia). Normally, tACE could serve as a marker for developmental stage of germ cell differentiation. In experimental pathological conditions that lead to male infertility, tACE was proven as a useful marker for evaluation of spermiogenesis. Testicular ACE provides precise evaluation for retention of the first spermatogenesis and spermatid depletion in adulthood. The summary of our data obtained so far characterized tACE as a proper cellular marker for elongating spermatids and mature spermatozoa. Our review provides new vision for tACE as unique tool for evaluation for stage-specific changes in spermiogenesis and in particular stage in spermatid elongation.

Key words: angiotensin converting enzyme (ACE), testis, spermatogenesis, spermatids, germ cells.

Introduction

Angiotensin I-converting enzyme (ACE, kininase II, CD 134) is well-known component of renin-angiotensin system (RAS) and kallikrein-kinin system (KKS), both playing an important role in male reproduction [25, 29]. ACE is membrane bound zink metallopro-

teinase dipeptidase that removes 2 residues from C terminus of certain peptides. ACE is responsible for the conversion of angiotensin (Ang) I to the potent vasoconstrictor Ang II and for inactivation of vasodilator peptide bradykinin. Therefore, ACE has been implicated in the control of blood pressure and fluid-electrolyte balance [23]. ACE acts through two G protein coupled receptors, AT I and AT II. Activation of AT I receptors is responsible for vasoconstriction and aldosterone release while AT II receptors are proposed to mediate antagonizing effects and apoptosis [11].

There is another form of ACE named ACE 2 in humans and mammals. This enzyme is Zink metalloproteinase with carboxypeptidase activity that shares approximately 42% identity with the catalytic site of somatic ACE [34]. ACE 2 is involved in the generation of alternative angiotensin peptides in particular in the conversation of Ang II to Ang (1-7), which is vasodilatator and Ang (1-9) [35]. This data suggested that ACE2 can be viewed as a counterbalancing tissue-specific mechanism within the activated RAS.

ACE exists in two isoforms – somatic and testis-specific (germinal) and both are encoded by one and the same gene having 26 exons. Somatic ACE (sACE) is the longer variant having MW of 170 kDa. This isoform generates Ang II and it is blocked by ACE inhibitors [12, 13]. In contrast, the shorter variant – testicular ACE (tACE), having MW 110 kDa, does not generate Ang II and the substrate is unknown. The isoform is not blocked by ACE inhibitors. Testicular ACE is transcribed by alternative promoter in intron 12 only during spermatogenesis being localized in developing post meiotic germ cells (spermatids) [18]. As a result N-domain is unique in this isoform due to translated exon 13. The C-domain is identical in both, sACE and tACE.

Somatic ACE is produced by endothelium and several other somatic tissues [22]. The same enzyme is localized in male reproductive system mostly in endothelia cells and Leydig cells of the testis as well as in epithelial cells of the epididymis and prostate. The sACE is expressed in human germ cells during fetal life and is constant feature of germ cell cancer, analyzed by monoclonal antibodies.

Studies on human males revealed time-related changes in the cell specific expression of sACE. Switch of both ACE isoforms in human germ cells was reported: sACE occurred in foetal gonocytes but only tACE is exclusively expressed in spermatids and spermatozoa. Sertoli cells show generally only a weak and markedly diffuse immunoreactivity for sACE. This labelling disappears towards the end of gestation but may be maintained in some seminiferous tubules for months after birth. Both of Leydig cell populations – fetal and adult expressed sACE [26].

Both of ACE isoenzymes play an essential role in male reproduction system. Main function of sACE is local production of Ang II that modulates Leydig cell steroidogenesis and regulates tubular contractility in the prostate. Somatic ACE and generated AngII may contribute to sperm motility, capacitation and acrosome reaction via the angiotensin II type (AT II) receptor [14]. Furthermore the ACE product - angiotensin II is supposed to be involved in the regulation of fluid- and electrolyte transport in the epididymis. Ang II secreted from the basal cells in the epididymis may exert its effect on electrolyte transport by acting on Ang II receptors on the basolateral membrane of the principal cells [36]. Somatic ACE is localized on apical portion of epididymal epithelial cells. Acting as peptidase, ACE was suggested to participate in remodeling of seminal fluid as well as in detoxification. In addition, sACE cleaves and inactivates LHRH (Luteinizing hormone-releasing hormone) and substance P, both neuropeptide hormones involved in testosterone production by Leydig cells. Somatic ACE is expressed mainly in human germ cell during fetal development and it is constant feature of intratubular germ cell neoplasia (CIS), being oncofoetal marker [12]. Served as peptidase, the sACE cleaves and inactivates the tetra peptide

goralatide (N-acetyl-seryl-aspartyl-lysil-proline – AcSDKP), which is a natural and circulating inhibitor of proliferation of hematopoietic stem cell and other progenitor cells. AcSDKP blocks the S phase entry of normal but not of neoplastic cells and thus promotes survival and resistance of stem cells to chemotherapy and radiation. Therefore, inhibition of sACE may open new strategies in the prevention of side-effect during cancer therapy [13]. In KKS, sACE is responsible for degradation of bradykinin which stimulates germ cell proliferation [2] suggesting negative role of sACE for germ cell mitotic division.

Recent studies by Akman et al. [1] indicated that treatment with an ACE inhibitor (Ramipril) play protective role against the toxic effect of doxorubicin, used as chemotherapy agent for cancer therapy. However, combined application of Ramipri and Erythropoietin hormone (Darbepoetin) provided remarkable protective effects for testicular macro-parameters (area of seminiferous tubules, lumen and seminiferous epithelium) and sperm parameters (sperm count, motility, live-dead sperm rate and total abnormal sperm rate).

The peptidase ACE2 was localized primary into Leydig cells of the rat testis and in both Leydig and Sertoli cells of the human testis demonstrated by immunohistochemistry. ACE2 was not present in germ cells or endothelial cells, thereby showing a different cellular distribution to the homologous peptidase tACE, but overlapping with the distribution sACE [15].

Testicular ACE is germ cell specific isoform that is essential for male fertility. This isoform is expressed in germ cells during spermiogenesis and tACE is localized only in elongating spermatids and spermatozoa. In contrast to sACE, tACE does not generate vasoconstrictor peptide AngII and substrate for tACE has not been identified. Acting as dipeptidase tACE is responsible for release of GPI proteins from sperm membrane that is important for sperm-zona pellucida binding, necessary for fertilization. Acting like a GPI-anchored protein releasing factor, tACE shed various GPI-anchored proteins, mostly PH-20 and Tesp5 from the cell surface of germ cells [20]. Therefore, tACE may serve as marker for fertilizing ability of spermatozoa and tACE contributes to fertilization as a dipeptidase at least in the epididymis [10].

ACE activity in the testicular complex is possibly linked with androgens and is involved with spermatogenesis and sperm maturation. The testicular ACE is expressed at high level by developing germ cells and is present in mature sperm. Using of indirect immunofluorescence and immunoperoxidase method, tACE was found in elongating spermatids in the testicular seminiferous tubules as well as in spermatozoa within the epididymal tubular lumen in sexually mature, but not in immature rabbits, suggesting that the presence of tACE is dependent on sexual maturation on stage-specific manner [25]. The same results were observed in mice and human tACE in the testis. Species-specific expression of tACE was demonstrated in human testis where ACE was found only in adluminal membranes of post meiotic germ cells later than step 3 round spermatids corresponding to step 7 round spermatids in rat [13, 26]. The same cellular distribution was described in mice.

For better understanding of the role of tACE and sACE in the male reproduction, an insertional disruption of the somatic but not the testicular ACE gene was generated. Males homozygous for this mutation have normal amounts of testicular ACE mRNA and protein but completely lack of somatic ACE (equal to complete knockout of sACE) is responsible for severe kidney pathology. Nevertheless, homozygous for sACE mutation males have normal fertility, proving conclusively that somatic ACE in males is not essential for their fertility [16]. ACE null mice lacking both somatic and testicular ACE are infertile suggesting that only tACE has critical importance for male fertility by acting differently compared to sACE [25]. Infertility, independently

of normal testis weight, sperm count and morphology, is due to altered sperm migration in the oviduct and their ability to bind zona pellucida [31]. Mutants exhibit also low blood pressure and renal dysfunction. Experiments with transgenic expression of testicular ACE in ACE null mice restored fertility, whereas transgenesis of somatic ACE in ACE mutants does not and mice are infertile. Therefore sACE cannot substitute tACE in male reproduction.

Materials and Methods

The role of tACE as a marker for evaluation of mammalian and human spermatogenesis in normal and pathological condition was established by application of proper experimental approach involving treatments that lead to disturbance of spermatid development and inappropriate testosterone support. We use three experimentals as follow:

1) Spontaneously Hypertensive Rats (SHR) - model for genetic hypertension – testes were sampled at 4^{th} month [5];

2) Androgen withdrawal after selective ablation of Leydig cells by injection of ethane dimetanesulfonate (EDS) at dose of 75 mg/bw in adult rats. Testes were sampled at days 7^{th} , 14^{th} , 21^{st} , 35^{th} post EDS [8];

3) Streptozotocin (65 mg/kg) induced diabetes mellitus – neonatally on pnd 1, prepubertally on pnd 10 where testes were sampled on pnd 50. Diabetes mellitus in adulthood was induced on pnd 60 and testes were sampled at 1 and 2 months after Streptozotocin injection [21].

Testicular ACE was visualized by ABC-HRP immunohistochemistry using a polyclonal antibody in dilution of 1:500 (Santa Cruz) [5]

In all the three models measurement of Plasma T levels was performed by RIA.

Results and Discussion

Detailed immunohistochemical analysis in our previous studies [4, 6] revealed stage-specific pattern of tACE expression in post meiotic germ cells in rat testis. The cycle of the seminiferous epithelium in the rat comprises fourteen stages and spermiogenesis involved 19 steps. Schematic and semi-quantitative expression of tACE was shown in **Fig. 1** and **Table 1**, respectively.

Table 1. Semi-quantitative evaluation of tACE immunoexpression at the stages of the seminiferous epithelium and steps of spermiogenesis in adult rats

Stages of the seminiferous epithelium								
Steps of spermiogenesis								
VII		VIII		IX	X–XI	XII–XIV	I–III	IV–VI
7	19	8	19	9	10–11	12–14	15–16	17–18
–	++++	–/+	++++	+	++	++/+++	+++	++++

First faint immunoreactivity appeared in the cytoplasm of round spermatids step 8 (stage VIII of the cycle) in a round shape manner (**Fig. 2A**). Weak intensity was found in elongating spermatids step 9 at stage IX of the cycle of seminiferous epithelium. Later that stage, the immunostaining progressively increased and was located in cau-

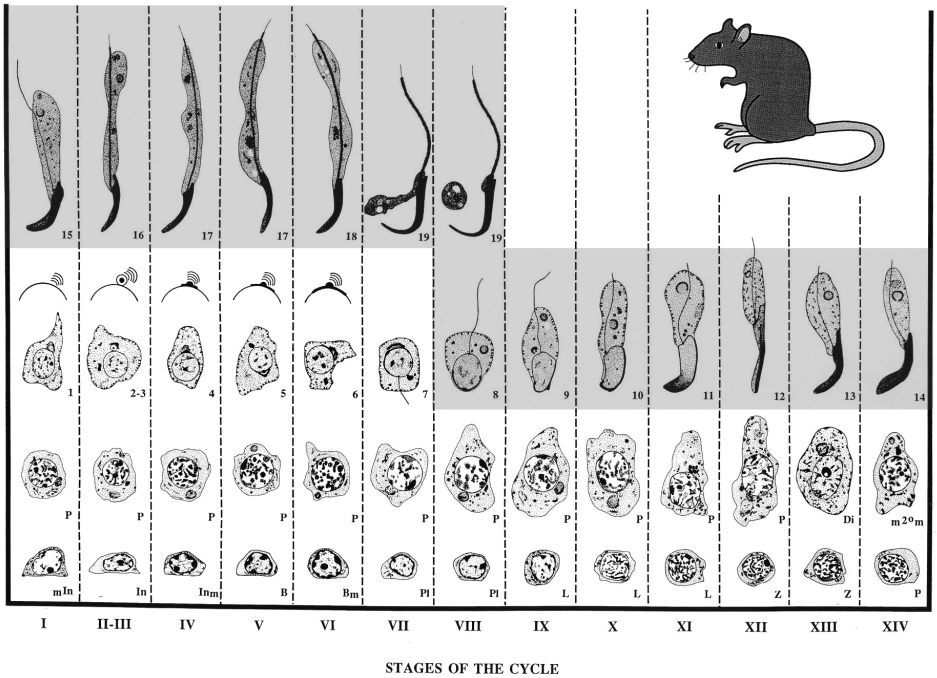

Fig. 1. Schematic presentation of expression of ACE during stages of seminiferous epithelium. On the scheme of the rat spermatogenesis all germ cell types and fourteen stages of the spermatogenesis are illustrated. Expression of tACE spanned from step 8 to step 19 of spermiogenesis (marked with grey)

dally organized cytoplasm of elongating spermatids. Immunoexpression became strong later than steps 12 of spermiogenesis (stage XII of the cycle) and reached maximum in steps 17–19 (stages IV–VIII of the cycle). No immunoexpression was observed in other germ cell types (spermatogonia, spermatocytes) as well as in somatic cells (peritubular cells, Leydig and Sertoli cells).

With one exception our results are consistent with the data by Sibony et al. [30] and Langford et al. [22]. The difference between these author groups could be explained by using different protocols and antibodies against the portion common to the testicular and somatic ACE.

Our data for stage specificity of tACE localization during spermatogenic cycle characterizes tACE [5] as a good marker for the stage of spermatid differentiation. In the rat testis expression of tACE starts and reaches maximum in androgen dependent stage VIII of spermatogenic cycle that implies androgen regulation of enzyme production in germ cells. Localization pattern of tACE revealed the importance of elongation phase of spermatids in male germ cell differentiation with respect to gene expression and not only to morphological modifications. Expression of tACE in post meiotic germ cells is an example for specific gene activation and translation during spermiogenesis.

In the course of the first spermatogenesis tACE appeared in stage-specific manner. Lack of tACE expression in the testis is due to absence of corresponding type of spermatids. Mid-pubertal testis (28-day-old) is negative for tACE as germ cell development proceeds to stage round spermatids 1-3 step. In late pubertal testis (42-day-old) spermatogenesis is not completed and proceeds to elongating spermatid 16 step in stage III. Immunoreactivity is observed in all the stages with an exception of stages IV–VI.

Lack of reaction in these stages is due to that elongating spermatids step 17-19 did not appear yet. Therefore, in the course of the first spermatogenic wave tACE is a marker for developmental stage of germ cell differentiation [4].

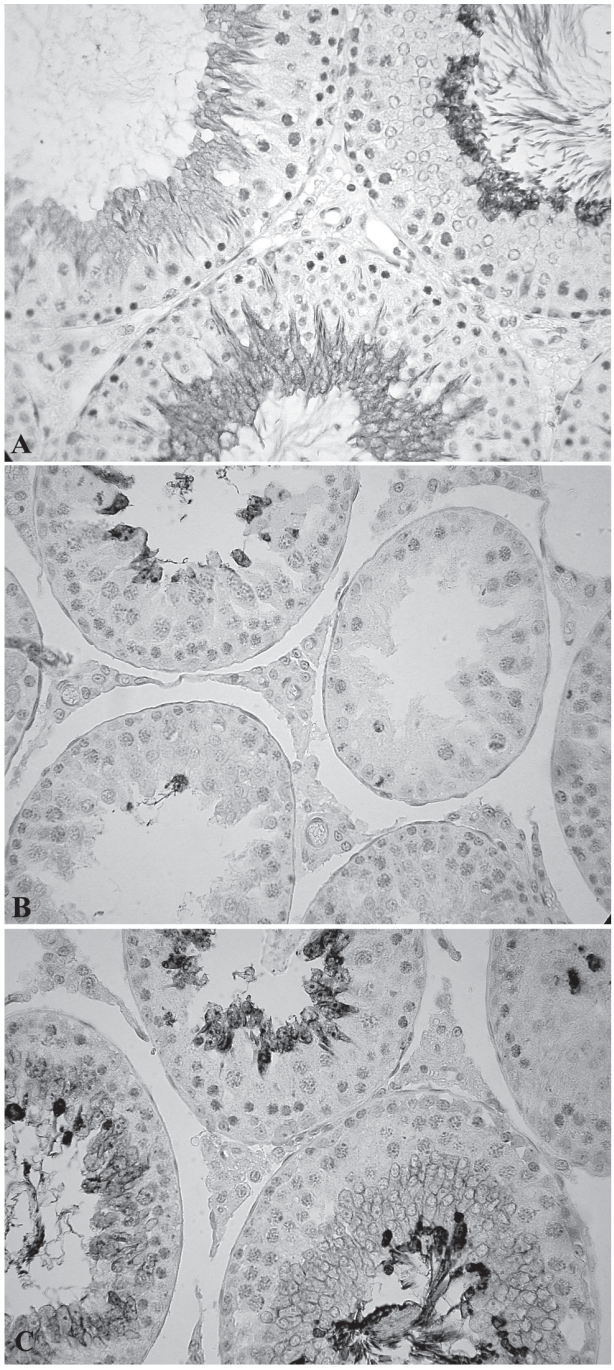

Fig. 2. Immunohistochemistry for tACE in adult rat testis. Localization of tACE in developing spermatids later step 8 (×400): **A**) Testis from control rat; **B**) Testis from SHR rat with depletion of elongating spermatids; **C**) Testis from SHR rat with stronger expression of tACE in elongating spermatids steps 9–14

Changes in the expression of tACE were reported in some pathological conditions such as hypertension and cancer. Advantages of genetic model for spontaneous hypertension rats (SHR) were well described by Azu [7]. Our previous data [5, 6] in SHR suggested relationship between hypertension, disturbance of spermatogenesis and elevated androgen production. In 20% of adult SHR (4-month-old) destructive changes in testicular histology were seen manifested by germ cell depletion, and reduced diameter of seminiferous tubules. Loss of elongated spermatids was proven by lack of immunopositive cells (**Fig. 2B**). In experimental group immunoexpression of tACE in spermatids steps 9-14 were more intensive than corresponding steps of the controls. As a result stage-specificity in SHR was not as prominent as in control (**Fig. 2C**). Plasma testosterone levels were obviously higher in SHR rats but significance was not reached due to highly variable values. High blood pressure development in SHR is suggested to be androgen-modulated [19].

Loss of tACE expression in germ cell depleted tubules in SHR is due to absence of corresponding stages of spermatid differentiation. Therefore, tACE can be used as a marker for germ cell depletion due to hypertension. Expression of tACE in post meiotic germ cell, specifically altered by SHR, suggested possible involvement of component of RAS in the process of spermiogenesis.

EDS experimental model is widely applied as a useful tool for investigation of androgen dependent events of spermatogenesis. Our previous data [3] revealed that expression of rat tACE started and reached maximal expression in androgen dependent stage VIII of spermatogenic cycle suggesting androgen regulation of the enzyme and EDS model was used for that case. By day 7th post EDS the Leydig cell completely absent from testicular interstitium as validated by negative reaction of marker enzyme, 3β-HSD and that is associated by drop in testosterone levels below 0.1 ng/ml [8]. Moreover, we previously found that androgen withdrawal induced profound germ cell apoptosis especially spermatocytes and round spermatids, demonstrated by maximal value of apoptotic index on day 7th [9]. In addition, lack of testosterone production coincides with loss of androgen receptor in Sertoli cells. Compromised cross-talk between Sertoli cells, Leydig cells and germ cells was suggested.

Morphological observations on testicular section from rats by 7th day after EDS injection showed total loss of elongating spermatids, steps 9-14 in corresponding stages IX–XIV of the cycle (**Fig. 3A**). Immunohistochemical study of tACE revealed that some seminiferous tubules do not contain positive cells whereas in some tubules single immune-reactive cells are seen (data not shown). Therefore, not all of the tubules in stages IX–XIV were completely depleted from elongating spermatids steps 9-14 (data not shown). Intensity of immune reaction in EDS testes is normal and stage-specific pattern of tACE expression was not different from that in controls. As far as, early (I–VI) and middle (VII–VIII) stages, they contained normal location of elongating spermatids steps 15-19. On day 7th post EDS we found stronger immunoexpression of tACE in round spermatids at stage VIII compared with the control (**Fig. 3B**). Our finding could be explained as a compensatory mechanism to androgen withdrawal due to absence of Leydig cells.

By day 14th post EDS single Leydig cells appeared in testicular interstitium proved by positive immunoreactivity for 3β-HSD and it is associated with considerable rise in plasma testosterone levels but significantly quite lower than controls [3]. Androgen receptor reappeared in Sertoli cells with no evidence for stage specific pattern. Histological observations revealed loss of elongating spermatids in all the stages of spermatogenic cycle. Immunohistochemistry for tACE details extent of loss of elongating spermatids. We found seminiferous tubules in all the stages of the spermatogenic cycle that were immunonegative for tACE, indicative completely depleted from elonga-

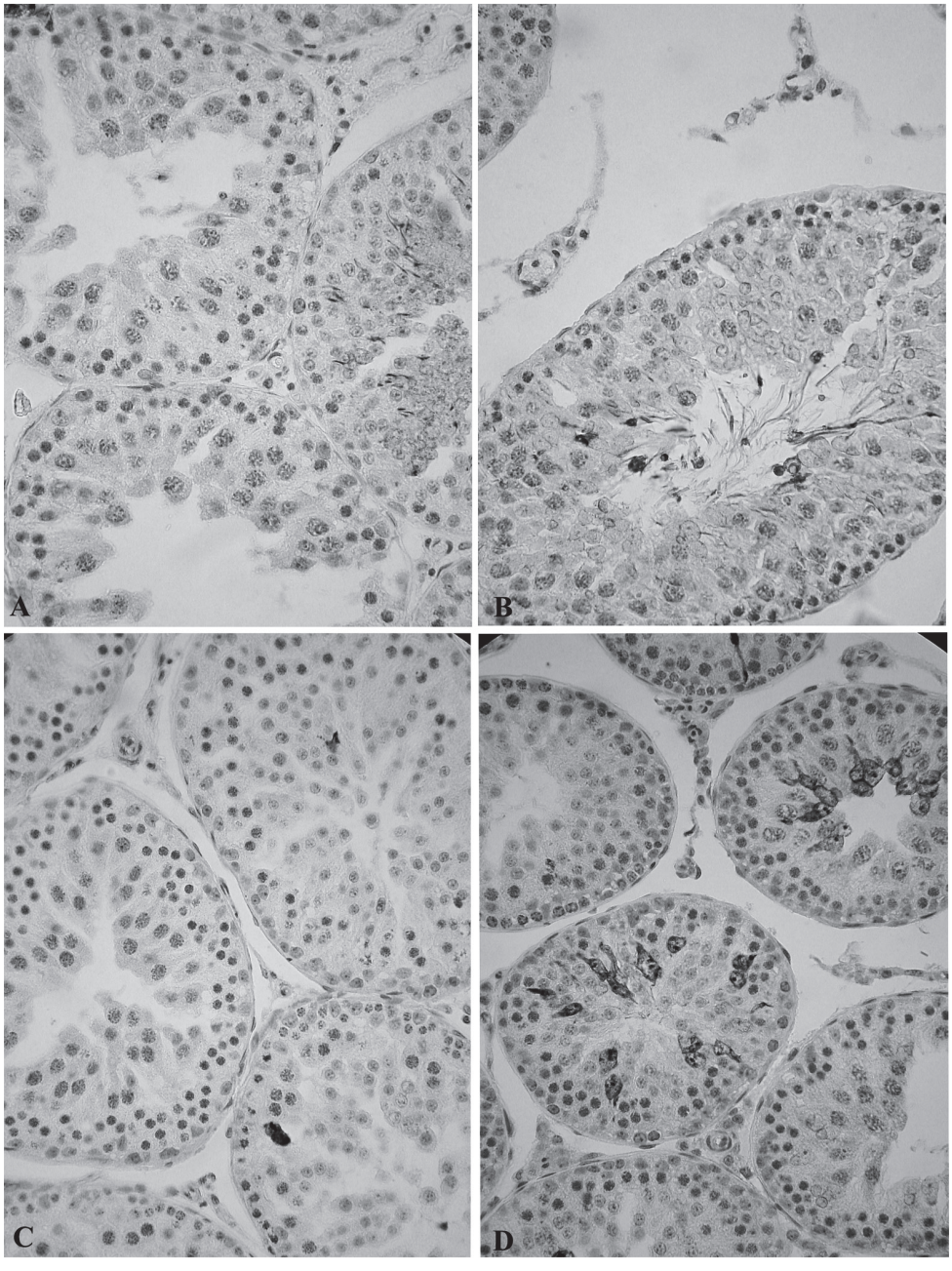

Fig. 3. Immunohistochemistry for tACE in adult rat testis after treatment with EDS (×400): **A**) Rat testis at 7th day after EDS treatment with loss of elongating spermatids steps 9-14; **B**) Rat testis at 7th day after EDS treatment with stronger expression of tACE in elongating spermatids step 8; **C)** Rat testis at 14th day after EDS treatment with complete loss of elongating spermatids; **D**) Rat testis at 14th day after EDS treatment with incomplete loss of elongating spermatids

ting spermatid (**Fig. 3C**). Moreover, tubules with single immunoreactive cells were also seen that suggested for incomplete loss of elongated spermatids (**Fig. 3D**). Depletion of elongating spermatids could be explained as a result of germ cell apoptosis that affected spermatocytes and round spermatids. Moreover germ cell apoptotic index still has significantly higher value compared to the controls [9]. The role of androgen signaling in Sertoli cells for post meiotic differentiation/spermatid development is demonstrated in knockout model for targeted disruption of androgen receptor in Sertoli cells (SCARKO) [33].

By day 21st post EDS more Leydig cells were found that is associated with higher testosterone levels compared to 14th post EDS even lower than normal value [8]. First signs for recovery of spermiogenesis we observed by histological and immunohistochemical studies. We found immunoreactive elongating spermatids step 9-14 in late stages of the cycle, IX–XIV. Occasionally, spermatids step 15-16 can be seen in early stages I–III (data not shown).

In conclusion, our data from immunohistochemical studies on tACE in EDS treated testes suggested that depletion and recovery of elongating spermatids occurred in stage-specific pattern. Therefore, application of tACE could be considered as a new tool for evaluation of post meiotic stages of spermatogenesis under conditions of hormonal/androgen deprivation. In particular, tACE can be recommended for precise visualization and evaluation of spermatid loss that is not optional by routine histological technique.

Reproductive dysfunction is a consequence of diabetes mellitus (DM), but the underlying mechanisms are poorly understood. Abnormal sperm production and failure of reproduction is a long time recognised consequence of DM, and it is accepted that infertility is a common complication in diabetic men [32]. In rats DM induces decreased testicular weight, sperm number and motility, testosterone levels that are an expression of abnormal spermatogenesis [28]. Nevertheless, the cellular alteration of testes has never been deeply studied, and the molecular mechanisms underlying this dysfunction are poorly understood.

We developed experimental approach for diabetes mellitus (DM) induced neonatally on pnd 1 (NDM) or prepubertally on pnd 10 (PDM) or in adulthood (ADM) [21]. In that study we aimed to evaluate which of three types of hyperglycaemia is the most severe risk factor for male fertility.

Our data reported recently [21] revealed that hyperglycaemia in adulthood revealed significant reduction of body weight by 20-30% whereas the 10% decrease in testis weight was not significant. The similar tendency for testis weight was found in animals with induced DM neonatally (NDM) or prepubertally (PDM) (data not shown). Blood glucose levels were strongly elevated up to 4 times in ADM and they were higher by 20-30% in NDM and PDM compared to the controls. Plasma testosterone levels were not significantly lower than controls in all the experimental groups.

Immunohistochemistry for tACE showed intact stage specific pattern of expression of tACE on day 50th in all the experimental group spermatogenesis starting in step 8 round spermatid at stage VIII of the spermatogenic cycle and reaching maximum in step 19 mature elongated spermatids at the same stage. Gross morphology of the testis in ADM rats is relatively normal. Histology of the testes from 50-day-old NDM rats is more altered than in ADM rats. Differential response to the hyperglycaemia was found as in some animals ST with enlarged lumen were seen and in other rats shrinkage of seminiferous tubules in stage VIII was found (**Fig. 4A**). Comparative analysis revealed that testicular morphology was most affected in 50-day-old PDM. Spermatogenesis is not completed and different degree of delay in spermatid development was observed (**Fig. 4B**). It was indicated by lack of elongating spermatids in stages IV–VII or in all the early stages (I–VII), or total loss of elongating spermatids in all the fourteen stages.

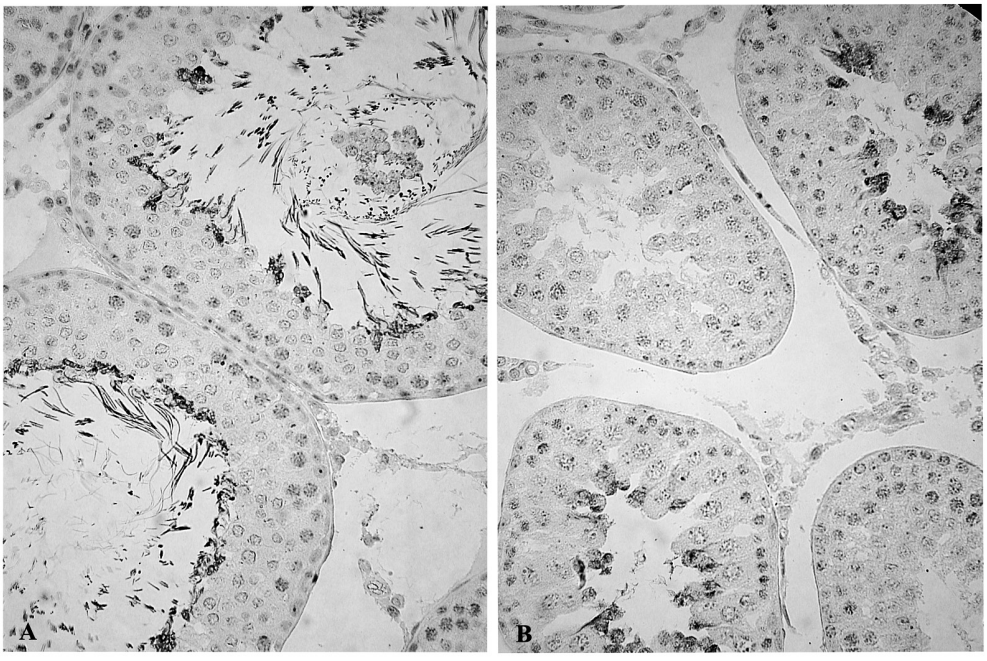

Fig. 4. Immunohistochemistry for tACE in 50-day-old rat testis after Streptozotocin induced diabetes mellitus (DM) in developing rats (×400): **A)** Adult testis from rat after NDM; **B)** Adult testis from rat after PDM with incomplete spermatogenesis

Alteration in spermatogenesis in diabetic state is mainly due to Leydig cell functional impairment caused by oxidative stress. As a consequence decreased testosterone production is responsible for the suppression of germ cell development [27].

The application of immunohistochemistry for tACE in diabetic condition demonstrated the dynamics of spermatid population, in particular the delay in development of post meiotic stages of spermatogenesis after PDM. Testicular ACE also could be considered as a marker for assessment of developmental stage of delayed spermatogenesis. Moreover, our comparative analysis provided the first evidence that prepubertal testis is more affected by hyperglycaemia than adult testis. Induction of DM on d10, when the first proliferative waves have started, affects germ cell development in a stronger extent compared to DM induced on day 1 when gonocytes are still quiescent [17, 24]. Our data indicate that the spermatogenesis is more vulnerable to DM at the time of proliferative phase of spermatogonia (4.5^{th} -12^{th} day) rather than the time of their mitotic arrest/quiescent period before 4.5^{th} day p.p. Long-term diabetes with sustained hyperglycemia leads to significant testicular dysfunction associated with decreased fertility potential.

Conclusion

The summary of our data obtained so far characterized tACE as a proper cellular marker for elongating spermatids and mature spermatozoa. Application of tACE in other experimental condition leading to subfertility or male infertility could provide better understanding for cellular and molecular mechanisms involved in regulation of sper-

matogenesis. For example, metabolite syndrome often associated with overweight, hypertension, DM type II, poor male reproduction and hence tACE could be used for detailed investigations of spermatogenesis applying proper experimental design as developmental studies under condition of hyperglycaemia and application of high fat diet on rats. Comparative investigations on the expression of other germ cell and Sertoli cell markers will develop broad view on the role of tACE in cell-cell interactions and such studies are in progress.

Our review provides new vision for tACE as a unique tool for evaluation for spermiogenesis, in particular stage in spermatid elongation. Assessment of germ cell depletion during experimental and pathological conditions could open future perspectives for development of new strategies for treatment of male infertility as well as for development of male contraception program.

Acknowledgements: This study was supported by grants from Medical University of Pleven No 1/2008, No 7/2009, No 2/2010, and Program for Carreer Development of Young Scientists – Grant DFNP – 72/2016.

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