folia medica

Folia Medica 65(6):933-939
DOI: 10.3897/folmed.65.e101144

Original Article

The Testicular Form of Angiotensin-Converting Enzyme as a Marker for Human Sperm Quality Assessment

Mina Pencheva[1], Pavel Rashev[2], Yvetta Koeva[3], Nina Atanassova[4], Donka Keskinova[5]

[1] *Department of Medical Physics and Biophysics, Faculty of Pharmacy, Medical University of Plovdiv, Plovdiv, Bulgaria*

[2] *Academician K. Bratanov Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences, Sofia, Bulgaria*

[3] *Department of Human Anatomy, Histology and Embryology, Faculty of Medicine, Medical University of Plovdiv, Plovdiv, Bulgaria*

[4] *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Sofia, Bulgaria*

[5] *Department of Applied and Institutional Sociology, Faculty of Philosophy and History, Paisii Hilendarski University of Plovdiv, Plovdiv,*

Corresponding author: Mina Pencheva, Department of Medical Physics and Biophysics, Faculty of Pharmacy, Medical University of Plovdiv, 15A Vassil Aprilov Blvd., 4002 Plovdiv, Bulgaria; Email: mina.pencheva@mu-plovdiv.bg

Received: 28 Jan 2023 ♦ **Accepted:** 5 Apr 2023 ♦ **Published:** 31 Dec 2023

Citation: Pencheva M, Rashev P, Koeva Y, Atanassova N, Keskinova D. The testicular form of angiotensin converting enzyme as a marker for human sperm quality assessment. Folia Med (Plovdiv) 2023;65(6):933-939. doi: 10.3897/folmed.65.e101144.

Abstract

Introduction: Spermatozoa are rapidly changing cellular structures that are highly dependent on their interaction with the environment. These interactions cause fundamental changes in the spermatozoa's cells and membrane.

All physiological changes that a spermatozoon goes through are required for fertilization. One of the proteins that are essential for the physiological processes in the spermatozoon membrane is the testicular angiotensin-converting enzyme (tACE). In human ejaculated spermatozoa, tACE is found on sperm plasma membrane in the head, neck, and midpiece of the tail having an active role in the capacitation and acrosome reaction.

Aim: Immuno-histochemical and fluorescent testing of the testicular isoform of the angiotensin-converting enzyme during spermiogenesis and acrosome membrane of spermatozoa.

Materials and methods: Testis biopsies from infertile males have used immunohistochemical testing and fixed spermatozoa for the immunofluorescence assay of tACE.

Results: The immunohistochemical test showed tACE expression during spermiogenesis and its participation in the stages of spermatid differentiation in the testis. The immunofluorescent test follows the manifestation of tACE in untreated, capacitated, and acrosome-reacting spermatozoa. In the process of capacitation and acrosome reaction, we found considerable dynamics accompanied by a change in the expression of tACE on the sperm membrane.

Conclusions: tACE expression during spermiogenesis and its visualization in the acrosome region confirms the active role of the enzyme in the processes of maturation, capacitation, and acrosome reaction, which determines the enzyme as a reliable marker for the selection of quality spermatozoa in assisted reproduction.

Keywords

acrosome, fertilization, sterility, spermatozoa, tACE

Copyright by authors. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC-BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

PENSOFT.

INTRODUCTION

Infertility is a worldwide reproductive health problem. It is estimated to affect about 15% of couples globally, with men being responsible for approximately half of the cases.[1] Male infertility is frequently caused by problems with the quality of the spermatozoon, which are most commonly manifested by low sperm motility and morphological deviations in the sperm, which prevent spermatozoa from carrying out the required movements and penetrating the ovum.[2] One of the enzymes participating in all processes of maturation and realization of spermatozoa is the testicular isoform of angiotensin-I-converting enzyme (tACE). In humans, ACE is isolated in two isoforms which are coded by one gene, somatic ACE and germinal or testicular ACE (tACE).[3]

Fertility was reduced in experimental animals with genetically manipulated tACE, demonstrating the enzyme's role in the male reproductive system and its importance for fertilization.[3]

AIM

The aim of this study was to assess tACE immunohistochemical expression during spermiogenesis and tACE participation in the stages of spermatid differentiation in biopsy specimens from infertile men's testes, as well as immunofluorescent analyses of the enzyme expression in untreated, capacitated, and acrosome-reacted spermatozoa in the studied groups.

MATERIALS AND METHODS

Group formation

Testicular biopsies from patients with a history of reproductive disorders and azoospermia were included in this study by testicular sperm extraction (TESE). The study included 24 men with reproductive disorders (age range 21–42 years).

The three groups into which the biopsy material was distributed were as follows: group 1: intact membrane propria (MP) and preserved spermatogenesis, group 2: reduced expression of tACE and increased amount of extracellular matrix (ECM) between the layers of myofibroblasts in the MP, and group 3: reduced expression of tACE and presence of two layers of myofibroblasts and thickened ECM between them.[4]

Semen collection and analysis

Semen samples were taken from the men with reproductive disorders. Part of the semen sample was used for the assessment of sperm parameters with computer-assisted sperm analysis (CASA system, Microptic, Barcelona, Spain) according to the World Health Organization (WHO) criteria: normozoospermic (n=31 men), teratozoospermic (n=24 patients).[5]

Processing of ejaculate

Ejaculated sperm was processed using the method described previously.[6] The ejaculated spermatozoa were washed with phosphate buffered saline (PBS) to a concentration of 5×10^6 cells/mL. One part from untreated ejaculated sperm was used immediately after the procedure: 20 μL of this sperm was placed on slides which were allowed to air-dry. The capacitation medium (Ferti Cult, Ferti Pro, Belgium) was added to another part of the sample from untreated sperm. This was then placed in an incubator for 1 hour at a temperature of 37°C and 5% CO_2. 20 μL was taken from the surface fraction using the swim-up method and dropped on slides and left to air-dry. The protocol described by Gianzo et al. was applied for the sperm capacitation from normal and pathological semen samples.[7] Calcium ionophore A23187 (sc-3591, Santa Cruz Biotechnology, Inc., Texas, USA) was added to a final concentration of 20 μL to stimulate acrosome reaction and placed back in an incubator for 1 hour. Then the samples were taken out of the incubator, some material was taken from the surface layer, dropped 20 μL from it on glass slides and let them air-dry. For fixation, ice methanol was applied for 20 minutes and then air-dried. The fixed material was stored in a refrigerator at 4°C for up to 3 months.

Immunohistochemistry

Immunohistochemical staining was performed on formalin fixed, paraffin-embedded 5-μm sections following antigen retrieval, endogenous biotin, and peroxidase block, followed by incubation with anti-goat ACE - 1:300 (sc-12187, Santa Cruz Biotechnology Inc. USA), then incubated with secondary antibody: biotinylated anti-goat (No. AGL015 ScyTek., USA) for 10 minutes. The reaction was visualized with 3,3′-diaminobenzidine tetrachloride (DAB, ScyTek Lab. Inc., USA); counterstaining was performed with Mayer's hematoxylin.

Immunofluorescence

Sperm samples were fixed in 4% paraformaldehyde and treated with 0.5% Triton X-100 for 10 minutes. The sperm was blocked with 10% BSA for 1 hour and incubated with primary antibody ACE (sc-12187, Santa Cruz Biotechnology, Inc) diluted to 1:500 in 1% BSA in PBS overnight at 4°C, which was followed by incubation with goat anti-rabbit IgG Alexa Fluor-568, (ab-175470, Abcam). After washing, the slides were mounted using Vecta shield mounting medium with DAPI (Vector Lab., Burlingame, CA, USA). The samples were examined with a laser scanning confocal microscope Leica TCS SPE (Leica Microsystems GmbH, 35578 Wetzlar, Germany).

Morphometric analysis of sperm

Intensity was measured in arbitrary units (AU) for tACE head sperm on at least 100 cells from each patient group. The measurements were performed using the DP–Soft v. 3.2 (Olympus, Japan).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (v. 25); *P* values of less than 0.05 were considered to indicate statistical significance. We used the Spearman's coefficient test, the independent *t*-test, and the paired samples test.

RESULTS

Cellular localization of tACE in human testes

The present tACE immunoreaction in the seminiferous tubules in the testis showed normal spermatogenesis; spermatogonium (white arrow), pre-leptotene spermatocytes (red arrow), pachytene spermatocytes (black arrow) **(Fig. 1A)**. The results from the immunohistochemical analysis confirmed that tACE is found mainly in round (blue arrow) and elongated spermatids (green arrow) **(Fig. 1A)**. In groups 2 **(Fig. 1B)** and 3 **(Fig. 1C)**, there is predominant visualization of tACE in round spermatids (black arrow, blue arrow).

In comparison to the intact MP and preserved spermatogenesis (group 1), the intensity of the tACE immunoreaction of the reduced expression of tACE and increased amount of ECM between the layers of myofibroblasts in the MP (group 2) was significantly lower ($p<0.001$) **(Fig. 2)**. The morphometric analysis revealed that the intensity of tACE immunoreaction in the reduced expression of tACE and presence of two layers of myofibroblasts and thickened ECM between them (group 3) was significantly lower, as compared to the one observed in the group of preserved spermatogenesis (group 1) ($p<0.001$).

Correlation analysis

The analysis of correlations between the sperm indicators and the intensity (AU) of tACE in spermatozoa **(Table 1)** shows that there is a strong negative correlation between morphology, vitality, and progressively motile spermatozoa (PR) ($p<0.01$) in the three studied groups (AU-ejaculated, AU-capacitated, AU-AR). In measuring the percentage of spermatozoa expressing tACE, a strong positive correlation was found between the indicators of immobile spermatozoa (IM) and the defects in the head and neck segment ($p<0.01$) in all three groups.

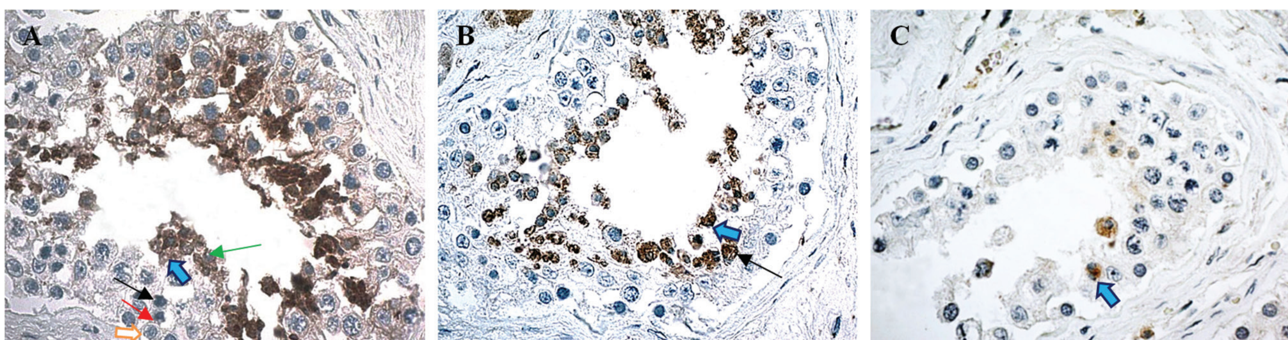

Figure 1. Photomicrographs of tACE immunoreaction in the testis.

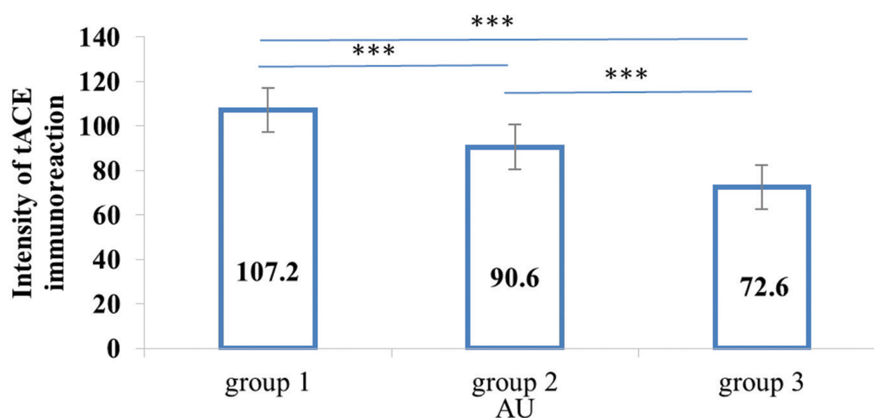

Figure 2. Intensity of tACE in the testis. *** $p<0.001$

Table 1. Correlation analysis between sperm indicators and intensity (AU) of tACE and the percentage of spermatozoa expressing tACE for normozoospermic and teratozoospermic patients (n=5 patients)

	AU-ejaculated	AU-capacitated	AU-acrosome reacted	% ejaculated	% capacitated	% acrosome reacted
Concentration	−0.287*	−0.269*	−0.254	0.394**	0.421**	0.297*
PR%	−0.583**	−0.483**	−0.458**	0.684**	0.525**	0.574**
NP%	−0.191	−0.349**	−0.229	0.327*	0.296*	0.274*
IM%	0.611**	0.556**	0.518**	−0.724**	−0.581**	−0.595**
Morphology	−0.752**	−0.724**	−0.703**	0.842**	0.667**	0.728**
Head defect	0.660**	0.767**	0.653**	−0.837**	−0.701**	−0.705**
Neck	0.693**	0.723**	0.597**	−0.836**	−0.782**	−0.705**
Vitality	−0.617**	−0.706**	−0.559**	0.813**	0.656**	0.715**
Age	0.059	0.103	0.054	−0.177	−0.090	−0.013

Note: The table shows Spearman's rho. *$p<0.05$ and **$p<0.01$

Taking into account the performed correlation analysis between the sperm indicators and the percentage of spermatozoa expressing tACE **(Table 1)**, we found that there is almost the same correlational strength among them, but it is of a different type, i.e., with reverse direction, from the correlation between the sperm indicators and the intensity (AU).

Determination of ACE intensity in sperm

The immunofluorescent analysis of the intensity of tACE expression shows that the intensity in the immunofluorescent signal is stronger with the spermatozoa from the normozoospermic group in comparison to those with teratozoospermia. The results from the tACE expression can likely be accounted for by the enzyme in the infertile groups being located predominantly in certain areas, while in those with normozoospermia, it is evenly distributed in the acrosome membrane **(Fig. 3)**.

DISCUSSION

Spermatozoa develop in the testes undergoing a series of genetic and morphological transformations, processes such

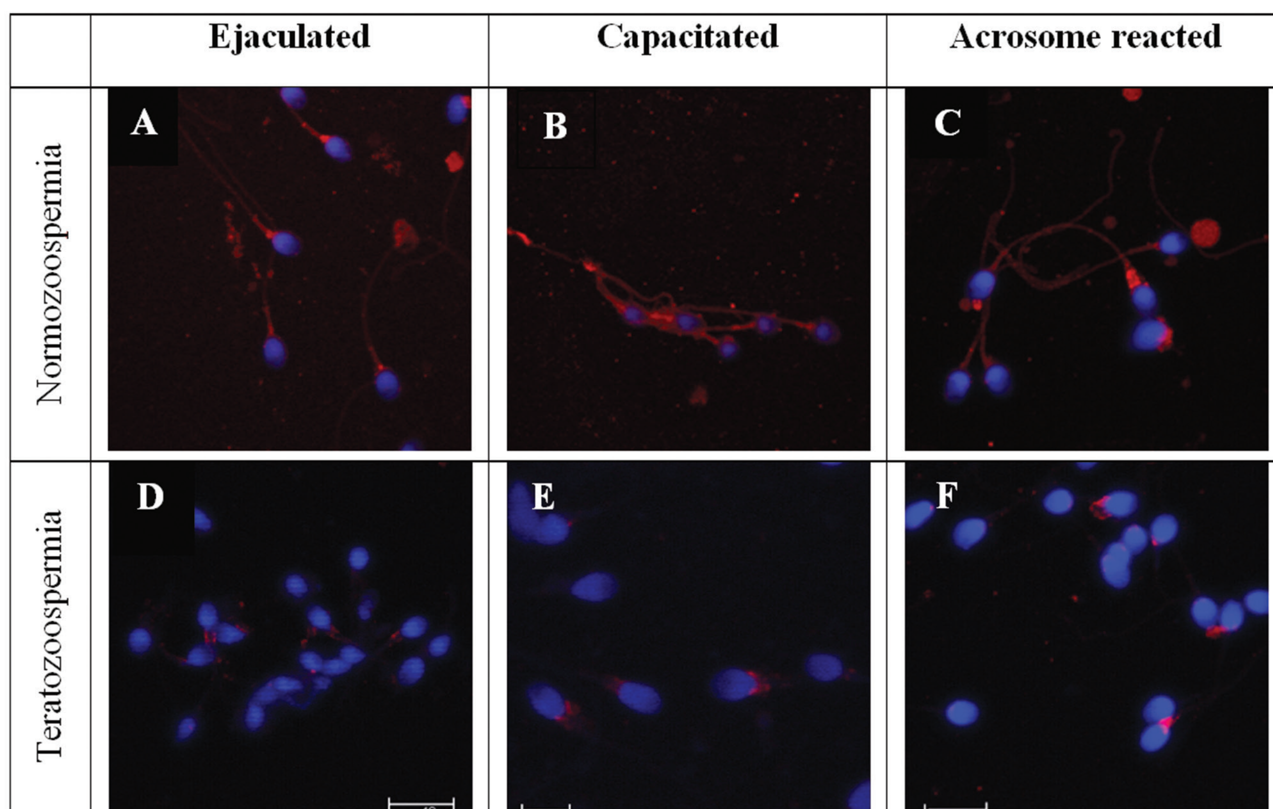

Figure 3. Comparative measurement of intensity (AU) in sperm (scale bar, 5 μm and 10 μm).

as maturation, capacitation, acrosome reaction, and, of course, movement.[8] The working hypothesis of the present study is that the expression of essential proteins in the sperm membrane and their relocation during the capacitation process and their release during the acrosome reaction underlies the ability of spermatozoa to participate in fertilization followed by the development of a quality embryo.

In the present study, we analyzed tACE expression in the sperm membrane of spermatozoa classified as normozoospermic and teratozoospermic as compared to the one of ejaculated, capacitated spermatozoa and those with acrosome reaction and in correlation with the fertile status of man.

The immunohistochemical analysis conducted by us confirms the established tACE expression in testes but also shows an expression pattern correlating with the degree of thickening of the canal walls and the change in the expression intensity in the groups with impaired reproduction **(Table 1)**.

The change in the intensity of tACE expression may be due to various factors such as reduced androgen regulation, the influence of environmental factors causing elevated levels of free radicals, and oxidative stress expressed in morphological changes including reduced spermatogenesis and impaired organization of germ cells.[9]

The research done proves tACE and a positive relationship between sperm motility and fertilization success.[6,10] The statistical analysis found that the reduced expression of tACE in humans is associated with reproductive disorders, abnormal sperm morphology, which can result in reduced fertility.

The percentage of spermatozoa expressing tACE in the processes of capacitation and acrosome reaction is also reported as higher in normozoospermic spermatozoa in comparison to those in the groups with reduced fertile ability. tACE is not found in the acrosome and postacrosome region of normozoospermic AR spermatozoa but is still visible in the equatorial region and neck.

Analyzing immunofluorescence expression, we discovered that it was found primarily in a specific area of the membrane in pathological spermatozoa, as opposed to normozoospermic spermatozoa, which had a uniform distribution of the signal in the acrosome of the spermatozoon. Nikolaeva et al. also report a uniform distribution (ejaculated and capacitated sperm) with the help of self-generated monoclonal antibodies.[11]

The role of tACE in the acrosome, neck, and mid-piece of the tail of the spermatozoa is well established – tACE participates there in the hydrolysis of Ang I into Ang II, Ang II expressing its activity through its receptors AT1 and AT2.[12]

The tACE/Ang II - AT1 axis exerts influence on the spermatozoa motility thus stimulating the processes of oxidative phosphorylation through the membrane NADPH oxidase which is the main source for ROS generating.[13]

The immunofluorescent tACE expression found by us presupposed appropriate positioning and participation of tACE in signaling which influences the secondary activation of Ca^{2+}, necessary for activation of key acrosome proteins.[14]

The established continuing tACE visualization in the equatorial region presumes its participation in the conversion of Ang I into Ang II, which is known to have a proliferative effect and stimulates growth factors necessary for the early development of the embryo.[15]

On the other hand, the poor expression or lack of enzyme expression is linked to early and late influence on the developing embryo by some authors. In a recent study, Gianzo et al. evaluated the relationship between tACE and the quality of the embryo and found that specimens with a lower percentage of tACE expression in spermatozoa are linked to poorer quality embryos.[16]

The location in a highly reactive area such as the equatorial one probably determines the tACE ability to participate in signal-transduction processes in both directions both on the cell membrane and on the inner side coming into contact with the proteins on the internal cellular membrane.[17]

Dynamic rearrangement of proteins and in particular tACE in capacitation, proves its role in the fertilization process.[14]

CONCLUSIONS

The main contribution of this study is in the summary of our data related to the role of tACE in spermatogenesis, in the process of capacitation and AR, and its role in male sterility.

These studies have also reported that tACE is a promising male infertility biomarker and the clinical assays for tACE have the potential to replace diagnostic testicular biopsies and help in the prediction of outcome of sperm retrieval procedures thereby increasing the reliability and success of assisted reproduction techniques.

Author contributions

Design: M.P, Y.K., and N.A.; Materials: M.P.; Data collection or processing: M.P., D.K., and P.R.; Analysis and interpretation: M.P., D.K., P.R., Y.K., and N.A.; Drafting of the manuscript: M.P.

Acknowledgements

This research is supported by the Bulgarian Ministry of Education and Science under the Young Scientists and Postdoctoral Students 2 National Program.

REFERENCES

1. Agarwal A, Mulgund A, Hamada A, et al. A unique view on male infertility around the globe. Reprod Biol Endocrinol 2015; 26:13–37.

2. Ortega C, Verheyen G, Raick D, et al. Absolute asthenozoospermia and ICSI: what are the options? Hum Reprod Update 2011; 17:684–92.
3. Howard T, Balogh R, Overbeek P, et al. Sperm-specific expression of angiotensin-converting enzyme (ACE) is mediated by a 91-base-pair promoter containing a CRE-like element. Mol Cell Biol 1993; 13(1):18–27.
4. Pencheva M, Koeva Y, Pavlova E, et al. Stage specific expression of angiotensin-converting enzyme and thickened lamina propria in relation to male fertility. Folia Med (Plovdiv) 2022; 64(1):41–8.
5. World Health Organization. Laboratory Manual for the Examination and Processing of Human Semen. 5th edition, 2010.
6. Pencheva M, Keskinova D, Rashev P, et al. Localization and distribution of testicular angiotensin I converting enzyme (ACE) in neck and mid-piece of spermatozoa from infertile men in relation to sperm motility. Cells 2021; 10(12):3572.
7. Gianzo M, Muñoa-Hoyos I, Urizar-Arenaza I, et al. Angiotensin II type 2 receptor is expressed in human sperm cells and is involved in sperm motility. Fertil Steril 2016; 105(3):608–16.
8. Bernabò N, Sanchez MR, Valbonetti L, et al. Membrane dynamics of spermatozoa during capacitation. In: Ahmed RG, editors. New Insight in Germ Cells Signalling. Chapter 4; Germ Cell 2018:156.
9. Welsh M, Saunders PT, Atanassova N, et al. Androgen action via testicular peritubular myoid cells is essential for male fertility. FASEB J 2009; 23(12):4218–30.
10. Ball BA, Gravance CG, Wessel MT, et al. Activity of angiotensin-converting enzyme (ACE) in reproductive tissues of the stallion and effects of angiotensin II on sperm motility. Theriogenology 2003; 59(3-4):901–14.
11. Nikolaeva MA, Balyasnikova IV, Alexinskaya MA, et al. Testicular isoform of angiotensin I-converting enzyme (ACE, CD143) on the surface of human spermatozoa: revelation and quantification using monoclonal antibodies. Am J Reprod Immunol 2006; 55(1):54–68.
12. Vinson GP, Puddefoot JR, Ho MM, et al. Type 1 angiotensin II (AT1) receptors in rat and human sperm. J Endocrinol 1995; 144:369–78.
13. Garrido AM, Griendling KK. NADPH oxidases and angiotensin II receptor signaling. Mol Cell Endocrinol 2009; 302(2):148–58.
14. Visconti PE. Understanding the molecular basis of sperm capacitation through kinase design. Proc Natl Acad Sci 2009; 106:667–8.
15. Pijacka W, Hunter MG, Broughton PF, et al. Expression of renin-angiotensin system components in the early bovine embryo. Endocr Connect 2012; 1:22–30.
16. Gianzo M, Arenaza IU, Hoyos IM, et al. Human sperm testicular angiotensin converting enzyme helps determine human embryo quality. As J Androl 2018; 20(5):498–504.
17. Inoue N, Ikawa M, Okabe M. The mechanism of sperm-egg interaction and the involvement of IZUMO1 in fusion. J Asian Androl 2011; 13(1):81–7.

Тестикулярная форма ангиотензинпревращающего фермента в качестве маркера оценки качества спермы человека

Мина Пенчева[1], Павел Рашев[2], Ивета Коева[3], Нина Атанасова[4], Донка Кескинова[5]

[1] Кафедра медицинской физики и биофизики, Факультет фармации, Медицинский университет – Пловдив, Пловдив, Болгария

[2] Институт биологии и иммунологии размножения „Акад. К. Братанов", Болгарская академия наук, София, Болгария

[3] Кафедра анатомии человека, гистологии и эмбриологии, Факультет медицины, Медицинский университет – Пловдив, Пловдив, Болгария

[4] Институт экспериментальной морфологии, патологии и антропологии с музеем, София, Болгария

[5] Кафедра „Прикладная и институционалная социология", Философско-исторический факультет, Пловдивский университет „Паисий Хилендарски", Пловдив, Болгария

Адрес для корреспонденции: Мина Пенчева, Кафедра медицинской физики и биофизики, Факультет фармации, Медицинский университет – Пловдив, бул. „Васил Априлов" № 15А, 4002 Пловдив, Болгария; Email: mina.pencheva@mu-plovdiv.bg

Дата получения: 28 января 2023 ♦ **Дата приемки:** 5 апреля 2023 ♦ **Дата публикации:** 31 декабря 2023

Образец цитирования: Pencheva M, Rashev P, Koeva Y, Atanassova N, Keskinova D. The testicular form of angiotensin converting enzyme as a marker for human sperm quality assessment. Folia Med (Plovdiv) 2023;65(6):933-939. doi: 10.3897/folmed.65.e101144.

Резюме

Введение: Сперматозоиды представляют собой быстро меняющиеся клеточные структуры, которые сильно зависят от их взаимодействия с окружающей средой. Эти взаимодействия вызывают фундаментальные изменения в клетках и мембранах сперматозоидов.

Все физиологические изменения, которые претерпевает сперматозоид, необходимы для оплодотворения. Одним из белков, необходимых для физиологических процессов в мембране сперматозоида, является ангиотензинпревращающий фермент семенников (tACE). В эякулированных сперматозоидах человека tACE обнаруживается на плазматической мембране сперматозоидов в головке, шее и средней части хвоста, играя активную роль в капацитации и акросомной реакции.

Цель: Иммуно-гистохимическое и флуоресцентное исследование тестикулярной изоформы ангиотензинпревращающего фермента в процессе спермиогенеза и акросомной мембраны сперматозоидов.

Материалы и методы: В биоптатах яичек бесплодных мужчин использовали иммуногистохимическое исследование и фиксированные сперматозоиды для иммунофлуоресцентного анализа tACE.

Результаты: Иммуногистохимический тест выявил экспрессию tACE в процессе спермиогенеза и его участие в стадиях дифференцировки сперматид в семеннике. Иммунофлуоресцентный тест отслеживает проявление tACE в необработанных, капацитированных и реагирующих на акросомы сперматозоидах. В процессе капацитации и акросомной реакции мы обнаружили значительную динамику, сопровождающуюся изменением экспрессии tACE на мембране спермия.

Заключение: Экспрессия tACE в ходе спермиогенеза и его визуализация в акросомной области подтверждает активную роль фермента в процессах созревания, капацитации и акросомной реакции, что определяет фермент как надёжный маркер отбора качественных сперматозоидов при вспомогательной репродукции.

Ключевые слова

акросома, оплодотворение, стерильность, сперматозоиды, tACE