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Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase ACTIVITY IN SPERMATOZOA OF INFERTILE MEN WITH DIFFERENT FORMS OF PATHOSPERMIA

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Ion-exchanging ATPases play an essential role in biology of spermatozoa, including their motility, hyperactivation, chemotaxis, acrosome reaction etc. The aim of present study was to analyze Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase activities in spermatozoa of the infertile men with different forms of pathospermia and to explore a possible role that they may play in male infertility. A significant reduction in ouabain-sensitive Na^+, K^+-ATPase activity in sperm cells of infertile men with oligozoo-, asthenozoo-, oligoasthenozoo- and leucocytospermia was shown. The results show that asthenozoo-, oligoasthenozoo- and leucocytospermic patients have significantly impaired thapsigargin-sensitive and thapsigargin-insensitive Ca^{2+}, Mg^{2+}-ATPase activity compared to healthy men. However, Ca^{2+}, Mg^{2+}-ATPase activity has a tendency to increase in patients with oligozoospermia. The depressed ATPase activity in the infertile men could be due to a reduction in intracellular adenosine triphosphate level and damage of spermal membranes caused by lipid peroxidation products. The most significant decrease in Ca^{2+}, Mg^{2+}-ATPase activity was observed in patients with leucocytospermia which could be explained by an excessive formation of the reactive oxygen species by the leucocytes. It is suggested that a decrease in the ion-exchanging ATPase activity may damage sperm functions and be one of possible causes of male infertility.

Keywords: Na^+, K^+-ATPase, Ca^{2+}, Mg^{2+}-ATPase, male infertility, pathospermia

INTRODUCTION

Infertility is a widespread complex problem affecting approximately 15–20 % of couples. Studies indicate that more than 40 % of infertility is related to male factor. In recent years, a steady tendency is traced: the number of the infertile men increases [18]. Data on structure of the infertility in men are rather contradictory. According to different authors the main causes of male infertility are: genital infection, endocrine pathology, varicocele, genetic factors, obstructive azoospermia, idiopathic oligo-, astheno-, teratozoospermia [11]. Defective sperm function is the most common cause of male infertility. Abnormal sperm characteristics, such as asthenozoospermia oligoasthenozoospermia are the most common cause of male infertility [28]. Study of functioning of sperm cells, which play a crucial role in the reproductive function, is crucially important.

Upon ejaculation, sperm cells pass a long distance throughout constantly changing environment. That is why they must have special mechanism to maintain ion homeostasis. A variety of ion channels, exchangers and pumps are present in spermatozoa. Na^+, K^+-ATPase (ouabain sensitive Ca^{2+}- independent, Na^+, K^+-activated, Mg^{2+}, ATP-dependent hydrolase, EC 3.6.1.37) is a plasma-membrane enzyme which catalyzes the active transport of Na^+ and K^+ across the cell membrane, using the energy of ATP hydrolysis.

Two isoforms of the catalytic subunit of the Na^+, K^+-ATPase, α1 and α4 which have different biochemical properties, coexist in spermatozoa. Spermatozoa from the knockout mice lacking α4 were unable of fertilizing eggs *in vitro* indicationg an essential role of Na^+, K^+-ATPase for sperm fertility [19]. Besides contributing to sperm membrane potential, Na^+ and K^+ transport catalyzed by Na, K-ATPase α4 is indirectly involved in maintaining sperm pH and intracellular calcium levels [6].

Calcium ions play a crucial role in regulation of spermatogenesis and fertilization including capacitation and acrosome reaction [7, 21]. Ca^{2+} gradients across the sperm plasma membrane, which is required for Ca^{2+} signaling, are maintained in superposition of different ion transporting systems. Ca^{2+}, Mg^{2+}-ATPase (EC 3.6.1.38) plays a pivotal role in Ca^{2+} extrusion from cytoplasm maintaining its concentration in the low nanomolar range (10-100 nM). Total Ca^{2+}, Mg^{2+}-ATPase activity of subcellular fraction consists of thapsigargin insensitive plasmatic membrane (PMCA) and thapsigargin sensitive ATPase of internal Ca^{2+}-stores. Two isoforms of PMCA, PMCA1 and PMCA4, are expressed in spermatozoa. PMCA4 is the most abundant isoform in spermatozoa localized in the principle piece of the sperm tail [15]. The presence of a thapsigargin-sensitive Ca^{2+} store in human spermatozoa was shown earlier [27]. The primary candidate for a sperm internal Ca^{2+} store is the acrosome since human spermatozoa do not contain endoplasmic reticulum [5].

Most functional studies of Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase have been conducted on sperm cells from rat, mouse and bull; little experimental evidence exists for human ATPase systems and their alteration in pathology. Studying of ion exchanging ATPases in the pathogenesis of disorders of male reproductive function is promising as it will enable improving diagnosis and contribute to the development of correct tactics for treatment. Since the Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase plays an essential role in biology of spermatozoa, the present study was designed to analyze Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase in sperm cells of infertile men with different forms of pathospermia to explore a possible role which they may play in male infertility.

MATERIALS AND METHODS

Patients. 34 infertile men with different forms of pathospermia were involved in this study. They were recruited between September 2016 and February 2017. A detailed medical history was performed in all cases. Exclusion criteria: subjects currently on any medication or antioxidant supplementation were not included. In addition, subjects with infertility over 10 years, azoospermia, testicular varicocele, genital infection, chronic illness and systemic diseases, smokers and alcoholic men were excluded from the study because of their well-known high seminal reactive oxygen species levels and decreased antioxidant activity [28] which may affect ATPase activities.

Control group consisted of 13 healthy men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and having 1–3 healthy children). Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' of abstinence from sexual activity. After liquefaction at 37 °C with 5% CO_2 in air, semen samples were examined for volume, sperm concentration, pH, morphology

and motility according to World Health Organization guidelines [26]. Before turning to study, all men were aware of patient information leaflets and gave informed an consent to participate in study. Terms of sample selection met the requirements of the principles of Helsinki Declaration on protection of human rights, Convention of Europe Council on human rights and biomedicine and the provisions of laws of Ukraine. Approval for study was taken from the Ethics Committe of Danylo Halytsky Lviv National Medical University. All patients and healthy donors gave written informed consent to participate in study (Ethical Committee Approval, protocol No 2 from February 16, 2015).

According to semen analysis, oligozoospermia was found in 8 patients, asthenozoospermia was detected in 10 patients, oligoasthenozoospermia was observed in 6 patients. These infertile men had leukocytes content in semen lower than $1.0 \cdot 10^6$ ml^{-1}. Leukocytospermia was noted in 10 patients (leukocytes content ranged from $1.0 \cdot 10^6$ ml^{-1} to $3.0 \cdot 10^6$ ml^{-1}) which indicates inflammation in this group of patients.

Cell preparation. Sperm cells were washed from semen plasma by 3 times centrifugation at 3000 *x*g for 10 min in media which contained (mM): 120 NaCl, 30 KCl, 30 Hepes (pH 7.4). The content of total protein in the samples was determined by Lowry method using a kit from "Simko Ltd", Ukraine. Determination of ATPases activities was carried out in the permeabilized spermatozoa. The detergent saponin in final concentration of 0.5% was added to sperm suspension for permeabilization of sperm membranes [22].

Assay of ATPases activity. The total Na^+, K^+-ATPase activity was assayed with the following incubation medium (mM): 120 NaCl, 30 KCl, 5 $MgC1_2$, 3 ATP, 1 EGTA, 0.01 thapsigargin (specific inhibitor of SERCA), 1 NaN_3 (specific inhibitor of mitochondrial ATPase), 20 Hepes-Tris (pH 7.4; at 37 °C). Protein concentration did not exceed 50 mg/ml. The reaction was started by addition of aliquot of permeabilized sperm cells. After 5 min incubation, 1 ml of stop solution containing (mM) 1.5 M sodium acetate, 3.7% formaldehyde, 14% ethanol, 5% trichloroacetic acid (pH 4.3) acid was added. P_i was determined by Fiske-Subbarow method [23] using assay kit "Simko Ltd" (Ukraine). Ouabain-sensitive Na^+, K^+-ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of 1 mM ouabain, which is known to block completely the Na^+, K^+-ATPase. Activity was expressed as nmoles of released P_i mg of protein per min.

Ca^{2+}, Mg^{2+}-ATPase activity was assayed using the following incubation medium (mM): 150 KCl, 5 $MgC1_2$, 5 ATP, 0.05 $CaCl_2$, 1 ouabain, 1 NaN_3, 20 Hepes-Tris (pH 7.4; at 37 °C). Ca^{2+}, Mg^{2+}-ATPase activity was calculated as the difference between ATPase activity in Ca^{2+} containing media and Ca-free medium (1 mM EGTA). Specific inhibitor of SERCA thapsigargin (0.01 M) was used to evaluate thapsigargin-sensitive ATPase.

Statistical analysis. The results are presented as the mean ± standard deviation of the mean. One-way ANOVA was performed to detect statistical significance. Differences were considered significant at $P < 0.05$ as the minimum significance level.

RESULTS AND DISCUSSION

Many diseases are known to be associated with changes in biomembrane structure and function. The membrane bond protein including integral ATP-depended transport enzyme have an important role in membrane functioning. The sperm plasma membranes contain the Na^+, K^+-ATPase which maintains the physiologic transmembrane ion gradients and resting membrane potential. Changes in ATPases activities can be used an as indicator of disturbances in structure of biological membranes at pathological conditions. To assess the functioning of the Na^+, K^+-ATPase in sperm cell the ATP

hydrolase activities were determined. Control samples showed the activity of Na^+, K^+-ATPase about 46.3±4.2 nmol P_i/min mg protein (Fig. 1). In patients with oligo-, astheno- and oligoasthenozoospermia, Na^+, K^+-ATPase activity was 22.4±3.5, 23.6±2.6 and 21.8±3.8 nmol P_i/min mg protein, correspondingly, that was two fold lower vs. control group. In the leucocytospermic patients, the Na^+, K^+-ATPase activity was 30.6±4.2 nmol P_i/min mg protein which was 1.5-fold lower than in patients with normozoospermia.

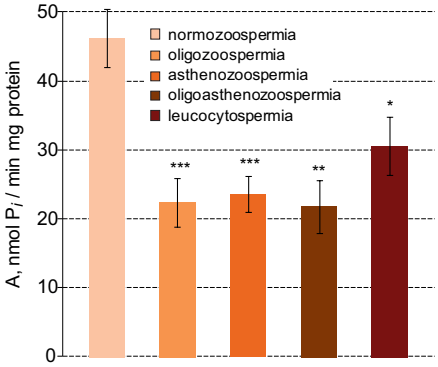

Fig. 1. Ouabain-sensitive Na^+, K^+-ATPase activity of spermatozoa of the infertile men
Hereinafter: asterisks indicate the values that significantly differ from control; *p<0.05; **p<0.01; ***p<0.001 relative to the control group (normozoospermia)

Рис. 1. Оуабаїн-чутлива Na^+, K^+-АТФ-азна активність сперматозоїдів чоловіків із неплідністю
Примітка: тут і надалі *p<0,05; **p<0,01; ***p<0,001 стосовно величин у осіб групи контролю (нормозооспермія)

The present study showed that Na^+,K^+-ATPase activity in spermatozoa of the infertile men was decreased significantly compared with that of normal control. The data presented here are consistent with that of Koçak-Toker and co-workers [9]. They found a significant decrease in Na^+, K^+-ATPase in human sperm of males with oligoasthenospermia in comparison with healthy donors. It was shown that Na^+, K^+-exchanging ATPase activity in semen is significantly lower in antisperm antibody positive infertile men compared with the controls [14]. The dynein-ATPase (Mg^{2+}-dependent, Li^+-sensitive, and insensitive to 2 mM ouabain, 1 μM oligomycin, and 1 μM thapsigargin) is of particular interest since enzyme is an intracellular motor for sperm motility. It was shown that dynein-ATPase activity was significantly lower for the asthenozoospermic donors as compared to the normozoospermic donors [24].

In present study, we have also characterized the activity of Ca^{2+}, Mg^{2+}-ATPase in sperm cells. This is of particular interest because Ca^{2+}, Mg^{2+}-ATPase is the major calcium extrusion pump in spermatozoa, and homozygous deletion of *Pmca4* results in male infertility and strikingly elevated Ca^{2+} levels [15, 20].

The thapsigargin-sensitive Ca^{2+}, Mg^{2+}-ATPase activity in sperm cells of the normozoospermic men was 4.9±0.5 nmol P_i/min mg protein (Fig. 2). The enzyme activity in spermatozoa of the oligozoospermic men did not change (5.5±0.4 nmol P_i/min mg protein) significantly. Patients with astheno-, oligoasthenozoospermia and leucocytospermia showed 2.3-, 2.6- and 3.8-fold lower activity of thapsigargin-sensitive Ca^{2+}, Mg^{2+}-ATPase (2.1±0.2, 1.9±0.2 and 1.3±0.2 nmol P_i/min mg protein, correspondingly) than did control subjects. Control samples showed thapsigargin-insensitive Ca^{2+}, Mg^{2+}-ATPase activity about 9.2±1.1 nmol P_i/min mg protein (Fig. 3). The change in enzyme activity in spermatozoa of oligozoospermic men (9.8±0.8 nmol P_i/min mg protein), was not statistically significant. In patients with astheno- and oligoasthenozoospermia, the thapsigargin-insensitive Ca^{2+}, Mg^{2+}-ATPase activity was 3.6±0.5 and 3.4±0.4 nmol P_i/min mg protein, correspondingly, that was 2.6–2.7 fold lower vs. control group. The activity of thapsiargin-

insensitive Ca^{2+}, Mg^{2+}-ATPase was 4.2-fold lower (2.2±0.4 nmol P_i/min mg protein) in leucocytospermic patients vs men with normozoospermia.

The present study showed that the Ca^{2+}, Mg^{2+}-ATPase activity in spermatozoa of patients with astheno-, oligoasthenozoospermia and leucocytospermia was decreased significantly compared with that of normal control. Similarly, a significant reduction in sperm motility and hyperactivated motility were observed to accompany elevated levels of cytosolic Ca^{2+} level and Ca^{2+} overload of mitochondria in mice where PMCA4 activity is known to be significantly decreased [1].

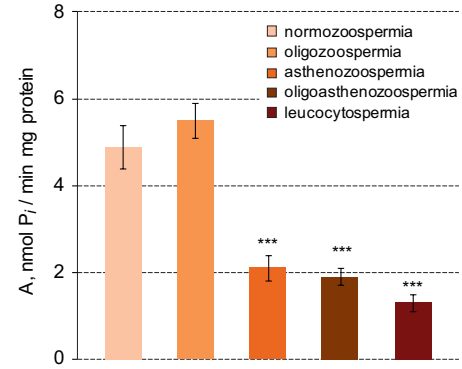

Fig. 2. Thapsigargin-sensitive Ca^{2+}, Mg^{2+}-ATPase activity of spermatozoa of the infertile men

Рис. 2. Тапсигаргін-чутлива компонента Ca^{2+}, Mg^{2+}-АТФ-азної активності сперматозоїдів чоловіків із неплідністю

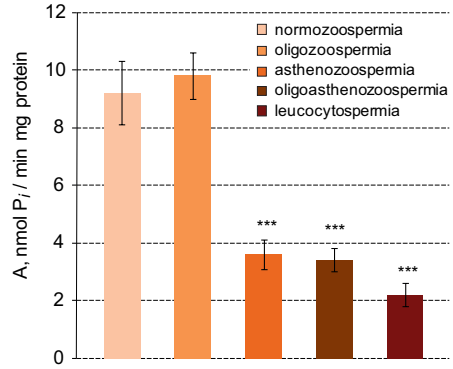

Fig. 3. Thapsigargin-insensitive Ca^{2+}, Mg^{2+}-ATPase activity of spermatozoa of the infertile men

Рис. 3. Тапсигаргін-нечутлива компонента Ca^{2+}, Mg^{2+}-АТФ-азної активності сперматозоїдів чоловіків із неплідністю

It was shown that male mice with a targeted gene deletion of isoform 4 of the plasma membrane calcium/calmodulin-dependent calcium ATPase (highly enriched in sperm tail) were infertile due to severely impaired sperm motility [20]. Genetic deletion of *pmca4* led to 100% infertility specifically in the male mutant mice due to a selective defect in sperm motility, but did not affect normal mating characteristics in these mice [3]. T. A. Kumosani et al. showed no difference in spermatozoal plasma membrane Ca^{2+}-ATPase activity in between fertile and infertile non-smokers. These authors also found significantly lower plasma membrane Ca^{2+}-ATPase activity in smokers than non-smokers either in fertile or infertile subjects [10].

The decrease in sperm motility may be attributed to the reduction in intracellular adenosine triphosphate and damage of the spermal membranes caused by lipid peroxidation products [4]. The concentration of intracellular ATP in sperm form subfertile roosters was less than in that from the fertile controls [27]. It is suggested that a decrease in Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase activity may damage sperm function and may be one of the possible causes of male infertility.

Ion exchanging ATPases could be a target for peroxynitrite $ONOO^-$ as reported by Kocak-Toker et al. [9] who described a decrease in Na^+/K^+-ATPase activity when sperm cells were incubated with increasing $ONOO^-$ concentrations. The negative strong correlations were established between Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase activities and $ONOO^-$ concentrations [23]. It was shown that $ONOO^-$ plays a central role in ion imbalance and membrane instability by the oxidation of SH groups of the enzyme [13]. In previous studies [25], we have shown that inducible NO-synthase activity was increased in spermatozoa of the infertile men. This indicates NO hyperproduction and formation of cytotoxic $ONOO^-$ which inhibits the activity of ion exchanging ATPases pumps. Association between Ca^{2+}, Mg^{2+}-ATPase and NO-synthase in sperm cells was confirmed [16]. In *pmca4*$^{-/-}$ sperm, the NO-synthase activity was elevated twofold vs. wild-type, accompanied by a twofold increase in $ONOO^-$ levels and significantly increased number of apoptotic germ cells.

In our study, the most expressed changes in Ca^{2+}, Mg^{2+}-ATPase activity were observed in the leucocytospermic patients. A significant positive correlation between leukocytospermia and sperm tail defects, acrosomal damage, and high sperm deformity index scores was found. These observations suggest that leukocytospermia is associated with compromised structural integrity of sperm [2]. It is known that leucocytospermia may indicate infection or inflammation in the male reproductive tract. Leucocytes negatively affect sperm cells, stimulate the formation of the reactive oxygen species, induction and development of oxidative stress, thus, inhibiting sperm mobility [8].

The present study has some limitations. First, our control group (normozoospermic men with proven fertility) and pathospermic patients contained a highly heterogeneous population, with large variations in spermogram parameters and infertility histories. Second, the present study investigated how sperm ion-exchanging ATPases activity was affected in the pathospermic patients only with 34 cases. Therefore, it is essential to validate the our findings with greater sample sizes and to determine the disease specificity (secretory or excretory infertility, varicocele or others) by comparing spermogram parameters.

The obtained results have both theoretical and practical importance for further elucidation of membrane mechanisms involved in maintaining of ion homeostasis in spermatozoa. They can be used as theoretical basis for improving laboratory biochemical methods for testing pathospermia.

CONCLUSIONS

1. The infertile men with oligozoo-, asthenozoo-, oligoasthenozoo- and leucocytospermia showed a significant reduction of ouabain-sensitive Na^+, K^+-ATPase activity in sperm cells compared to fertile men with normozoospermia.

2. The asthenozoo-, oligoasthenozoo- and leucocytospermic patients have significantly impaired thapsigargin-sensitive and thapsigargin-insensitive Ca^{2+}, Mg^{2+}-ATPase activity compared to healthy men.

3. Among different forms of pathospermia, the most expressed reduction in Ca^{2+}, Mg^{2+}-ATPase activity were observed in patients with leukocytospermia which could be explained by an excessive formation of the reactive oxygen species by leucocytes.

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the development of new immuno-biochemical diagnostic methods of fertility in men" (scientific supervisor – Doc.Sci. D. Vorobets) of the State Fund for Fundamental Research.

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Na^+, K^+-АТФ-азна та Ca^{2+}, Mg^{2+}-АТФ-азна АКТИВНІСТЬ СПЕРМАТОЗОЇДІВ НЕПЛІДНИХ ЧОЛОВІКІВ ІЗ РІЗНИМИ ФОРМАМИ ПАТОСПЕРМІЙ

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Транспортувальні АТФ-ази відіграють важливу роль у фізіології сперматозоїдів, зокрема, у їхній рухливості, гіперактивації, хемотаксисі, акросомній реакції та ін. Мета цього дослідження полягала у вивченні активностей Na^+, K^+-АТФ-ази та Ca^{2+}, Mg^{2+}-АТФ-ази у сперматозоїдах неплідних чоловіків із різними формами патоспермії. Встановлено значне пригнічення оуабаїнчутливої Na^+, K^+-АТФ-азної активності у сперматозоїдах неплідних чоловіків з олігозоо-, астенозоо-, олігоастенозоо- і лейкоцитоспермією. Результатами досліджень з'ясовано, що у пацієнтів із астенозоо-, олігоастенозоо- і лейкоцитоспермією значно знижена активність тапсигаргінчутливої і тапсигаргін-нечутливої компоненти Ca^{2+}, Mg^{2+}-АТФ-ази порівняно зі здоровими чоловіками. Однак Ca^{2+}, Mg^{2+}-АТФ-азна активність мала тенденцію до збільшення у пацієнтів з олігозооспермією. Інгібування АТФ-азних систем сперматозоїдів чоловіків із непліддям може бути зумовлене зниженням вмісту внутрішньоклітинного аденозинтрифосфату і пошкодженням спермальних мембран продуктами перекисного окислення ліпідів. Найбільш виражене зниження активності Ca^{2+}, Mg^{2+}-АТФ-ази спостерігали у пацієнтів із лейкоцитоспермією, коли може відбуватися надмірне утворення активних форм кисню лейкоцитами. Передбачається, що зниження активності транспортувальних АТФ-аз викликає зниження фертилізаційного потенціалу та може бути однією із ймовірних причин чоловічого неплідді.

Ключові слова: Na^+, K^+-АТФ-аза, Ca^{2+}, Mg^{2+}-АТФ-аза, чоловіче неплідді, патоспермія

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