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CHARACTERISTICS OF Ca^{2+} , Mg^{2+} -DEPENDENT ATP HYDROLYSIS IN SPERM CELLS OF INFERTILE MEN

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Disturbances of fertilizing potential of spermatozoa are closely associated with dysfunction of ion-transporting ATPases, in particular Ca^{2+} , Mg^{2+} -ATPase. Reduced activity of tapsigargin-resistant and tapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase leads to disruption of Ca^{2+} -homeostasis and is characteristic for abnormal spermatozoa (pathospermia). In order to study the peculiarities of action of Ca^{2+} , Mg^{2+} -ATPase, we determined the initial reaction rate, the maximum (plateau) amount of the reaction product and the characteristic reaction time. To determine these kinetic parameters of Ca^{2+} , Mg^{2+} -dependent hydrolysis of ATP catalyzed by Ca^{2+} , Mg^{2+} -ATPase, the dynamics of product accumulation of the ATP-hydrolases reaction was studied. The obtained curves were linearized in the coordinates $\{P/t; P\}$. Analyzing the changes in the activity of Ca^{2+} , Mg^{2+} -ATPase, the kinetics of primary-active transport of calcium ions through the plasma membrane and membranes of intracellular Ca^{2+} -stores in saponin-permeabilized spermatozoa of infertile men was studied. It was shown that in normozoospermic samples, the transport of Ca^{2+} ions through the plasma membrane is characterized by a higher capacity than through the membranes of intracellular Ca^{2+} -stores, but it occurs with practically the same initial velocity and characteristic reaction time. It was found that in pathospermic samples, transport of Ca^{2+} ions with the participation of both components of Ca^{2+} , Mg^{2+} -ATPase occurs less intensively and is characterized by a lower capacity compared to spermatozoa of men with preserved fertility. Specific changes in the kinetic parameters of Ca^{2+} , Mg^{2+} -dependent hydrolysis of ATP lead to inhibition of tapsigargin-resistant and tapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity and cause a decrease in fertilizing potential of spermatozoa.

Keywords: Ca^{2+} , Mg^{2+} -ATPase, ATP hydrolysis, spermatozoa, male infertility, pathospermia

INTRODUCTION

Infertility is one of the most important medical and biological problem. According to statistics 15 %, of married couples face this problem during the reproductive period. Nowadays, large-scale studies are being conducted all over the world to investigate the causes of impaired reproductive function and to develop methods that restore fertility [1]. Approximately 50 % of the infertility cases are related to male factor which has a tendency to increase. Defective functions of spermatozoa is the main cause of male infertility [11, 13]. The most common abnormal parameters are low motility (asthenozoospermia), low sperm count (oligozoospermia), or a combination of these abnormalities (oligoasthenozoospermia) [9, 14].

Calcium ions play a pivotal role in sperm physiology, specifically in sperm hyperactivation, chemotaxis and motility which depend on intracellular free calcium concentration [7]. For the normal functioning of sperm cells, it is necessary to change rapidly the intracellular concentration of calcium ions in response to certain stimuli. Ca^{2+} , Mg^{2+} -ATPase (EC 3.6.1.38) plays a pivotal role in Ca^{2+} extrusion from cytoplasm maintaining its concentration in low nanomolar range (10–100 nM). Total activity of Ca^{2+} , Mg^{2+} -ATPase consists of thapsigargin-resistant plasmatic membrane and thapsigargin-sensitive ATPase of internal Ca^{2+} -stores. Our previous results showed that asthenozoospermic, oligoasthenozoospermic and leucocytospermic patients have significantly impaired thapsigargin-resistant and thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity compared to healthy men [12]. Lowered activity of Ca^{2+} , Mg^{2+} -ATPase activity is likely to contribute to the disruption of Ca^{2+} homeostasis, a hallmark of abnormal sperm cells [4].

The aim of present study was to study the properties of membrane-bound Ca^{2+} -transport systems in spermatozoa of fertile (normozoospermia) and infertility men (oligozoospermia and asthenozoospermia).

MATERIALS AND METHODS

Reagents. The following reagents were used in the present study: ATP, ouabain, thapsigargin, EGTA (Sigma, USA), saponin (from Quillaja Saponaria Molina pract.; Acros organics, Belgium). Other reagents of the domestic production were of reagent grade or laboratory grade.

Donors and semen sample preparation. Human semen was obtained from 7 healthy volunteers and 12 infertile men with asthenozoospermia (AS) and oligoasthenozoospermia (OLAS) undergoing routine semen analysis for couple infertility at Lviv Regional Clinical Hospital (Ukraine). Control group consisted of healthy men with somatic fertility, normozoospermia (N) and confirmed parenthood (married for 3–10 years and having 1–3 healthy children).

Approval for study was taken from the Ethics Committee of Danylo Halytsky Lviv National Medical University (Ethical Committee Approval, protocol No 6 from March 29, 2017). Terms of sample selection meet the requirements of the principles of Convention of Europe Council on human rights, Helsinki Declaration on protection of human rights and biomedicine and the laws of Ukraine. All patients and healthy donors were matched by age and gave written informed consent to participate in research. Exclusion criteria:

subjects who are 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 serious 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 which may affect calcium level. Samples were obtained by the masturbation after 3–4 days of sexual abstinence and processed immediately upon liquefaction. The classical semen parameters of spermatozoa concentration, motility, and morphology were examined according to World Health Organization criteria (2010) [19].

Cell preparation. Biochemical studies were carried out in the Department of Medical Biology of Danylo Halytsky Lviv National Medical University. Sperm cells were washed from semen plasma by 3 times centrifugation at $3,000 \times g$ for 10 min in media which contained (mM): 120 NaCl, 30 KCl, 30 Hepes (pH 7.4). Protein concentration in the samples was determined by Lowry method using a kit to determine its concentration (“Simko Ltd”). Determination of ATPases activities was carried out in the permeabilized spermatozoa. The detergent saponin in a final concentration of 0.5% was added to sperm suspension for permeabilization of sperm membranes. Saponin interacts with membrane cholesterol, selectively removing it and leaving holes in the membrane [6].

Enzymatic studies. Ca^{2+} , Mg^{2+} -ATPase activity was assayed using the following incubation medium (mM): 150 KCl, 5 MgCl_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^{2+} -free medium (1 mM EGTA). The reaction was started by addition of aliquot of permeabilized sperm cells. After a 5 min incubation, 1 ml of a stop solution containing 1.5 M sodium acetate, 3.7 % formaldehyde, 14 % ethanol, 5 % trichloroacetic acid (pH 4.3) acid was added. P_i was determined by the Fiske-Subbarow method using assay kit “Simko Ltd” (Ukraine) [18]. Specific inhibitor of internal Ca^{2+} -stores, thapsigargin (0.01 μM), was used to evaluate thapsigargin-sensitive ATPase.

Kinetic calculations. Studies of Ca^{2+} , Mg^{2+} -ATP-dependent hydrolysis of sperm ATP were performed in a standard incubation medium, that was modified by the time of incubation. The apparent kinetic parameters of Ca^{2+} , Mg^{2+} -dependent ATP hydrolysis are the initial reaction rate V_0 , the maximum (plate) amount of the reaction product P_{max} and the characteristic reaction time was determined by linearization at $\{P/t, P\}$, where P is the amount of reaction product (P_i) and t is the incubation time [8].

Statistics analysis. Data are expressed as means \pm standard error of the numbers of determinations. Differences between paired sets of data were analysed using paired Student’s t-tests in Microsoft Excel. Differences were considered significant at $p < 0.05$ as the minimum significance level.

RESULTS AND DISCUSSION

For determination of the kinetic parameters of Ca^{2+} , Mg^{2+} -dependent ATP hydrolysis in saponin-permeabilized spermatozoa, the dynamics of inorganic phosphate (P_i) accumulation in ATP-hydrolysis reaction was studied. To do that sperm cells of fertile and infertile men were incubated in incubation medium during different time intervals (1–10 min). The experimental data showed that the curves of Ca^{2+} , Mg^{2+} -dependent hydrolysis of ATP in sperm cells tend to saturate (Fig. 1).

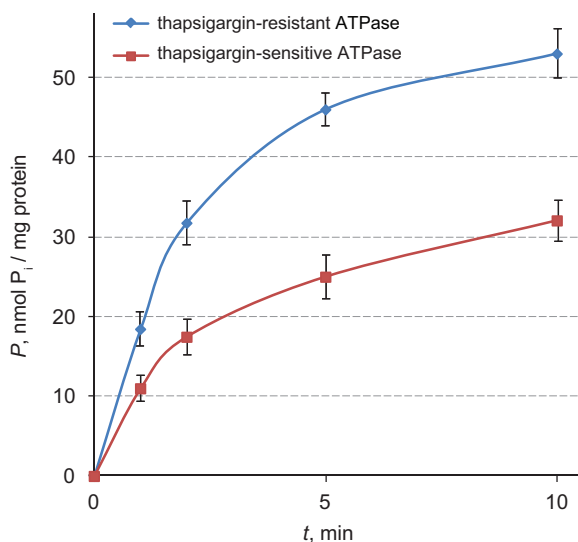


Fig. 1. Dynamics of accumulation of inorganic phosphate in Ca^{2+} , Mg^{2+} -dependent hydrolysis of ATP in normozoospermic samples, $M \pm m$, $n = 7$

Рис. 1. Динаміка накопичення неорганічного фосфату в процесі Ca^{2+} , Mg^{2+} -залежного гідролізу АТФ у сперматозоїдах нормозоспермічних чоловіків, $M \pm m$, $n = 7$

The analysis of the obtained results shows that accumulation of P_i in reaction catalyzed by thapsigargin-resistant ATPase in the normozoospermic samples in time interval 0–2 min correspond to zero order reactions. In this time interval, the graph of dependence of the amount of P_i on incubation time is almost linear. Similar dependence is observed for thapsigargin-sensitive ATPase.

By linearization of the obtained data in the coordinates $\{P/t; P\}$ (Fig. 2), the apparent kinetic parameters of the reaction of Ca^{2+} , Mg^{2+} -dependent hydrolysis of ATP were calculated (see Table).

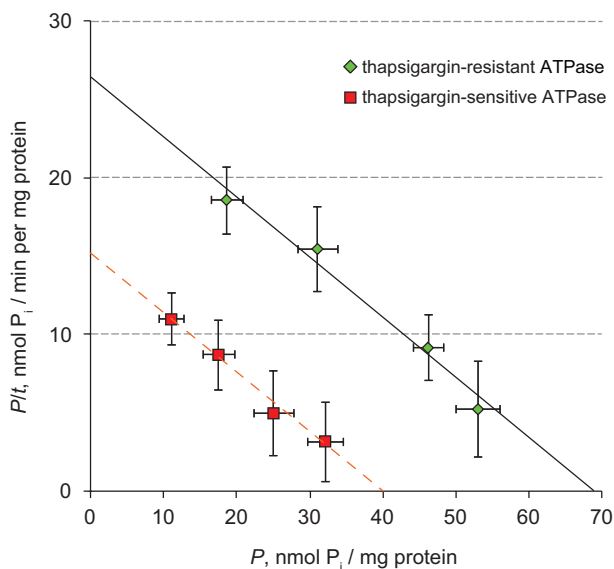


Fig. 2. Linearization of accumulation curves of inorganic phosphate P_i in reaction of Ca^{2+} , Mg^{2+} -dependent hydrolysis of ATP in normozoospermic samples in coordinates $\{P/t; P\}$; $n = 7$; $r > 0.95$

Рис. 2. Лінеаризація кривих накопичення неорганічного фосфату P_i у процесі Ca^{2+} , Mg^{2+} -залежного гідролізу АТФ у сперматозоїдах нормозоспермічних чоловіків у координатах $\{P/t; P\}$; $n = 7$; $r > 0,95$

As can be seen from the Table, the values of the apparent kinetic parameters of ATP hydrolysis catalyzed by thapsigargin-resistant and thapsigargin-sensitive ATPase

of the normozoospermic samples differ significantly among themselves. In the absence of a significant difference between values τ and V_0 of ATP hydrolysis reaction, the value P_{\max} in reaction catalyzed by thapsigargin-resistant ATPase was 1.75 times greater compared to values for thapsigargin-sensitive ATPase.

Kinetic parameters of Ca²⁺, Mg²⁺-dependent hydrolysis of ATP in sperm cells of normo- and pathospermic men, M ± m, n = 5–7

Кінетичні параметри Ca²⁺, Mg²⁺-залежного гідролізу АТФ у сперматозоїдах чоловіків із нормозоо- та патоспермією, M ± m, n = 5–7

Kinetic parameters	Groups	Pathospermia		
		Normozoospermia	asthenozoospermia	oligoasthenozoospermia
Thapsigargin-resistant Ca ²⁺ , Mg ²⁺ -ATPase				
V_0 , nmol P _i /min per mg protein		26.17 ± 4.04	7.8 ± 2.12**	6.5 ± 1.72**
P_{\max} , nmol P _i /mg protein		70.5 ± 6.8	36.6 ± 6.4**	33.7 ± 2.3***
τ , min		2.77 ± 0.68	5.02 ± 1.84	5.51 ± 1.74
Thapsigargin-sensitive Ca ²⁺ , Mg ²⁺ -ATPase				
V_0 , nmol P _i /min per mg protein		15.22 ± 2.9	8.27 ± 3.6	4.72 ± 1.9*
P_{\max} , nmol P _i /mg protein		40.1 ± 3.65 [§]	15.95 ± 1.64***	15.6 ± 2.28***
τ , min		2.7 ± 0.53	2.29 ± 0.9	3.93 ± 1.3

Comments: * P<0.05; ** P<0.01; *** P<0.001 compared to fertile men

[§] P<0.01 compared to thapsigargin-resistant Ca²⁺, Mg²⁺-ATPase

Примітки: * P<0,05; ** P<0,01; *** P<0,001 стосовно значень у здорових донорів

[§] P<0,01 стосовно значень для тапсигаргін-резистентної Ca²⁺, Mg²⁺-АТФази

Based on these results, we assume that in sperm cells of fertile men, Ca²⁺-transport by thapsigargin-resistant ATPase is characterized by a higher capacity compared to Ca²⁺-transport by thapsigargin-sensitive ATPase. These results are in disagreement with data reported in lymphocytes and cells of the submandibular salivary gland [3, 17]. It was found that transport of Ca²⁺ ions by Ca²⁺, Mg²⁺-ATPase of plasmatic membrane in healthy donors is slower and less intensive, but it is characterized by higher capacity in comparison with Ca²⁺, Mg²⁺-ATPase of endoplasmic reticulum. Our results might be explained by structural organization of spermatozoa. Since cytoplasm of spermatozoa has a restricted volume and sperm cells have highly polarized morphology, they pose a unique challenge to maintain Ca²⁺-homeostasis [15]. Standard components of Ca²⁺-signaling for somatic cell are present in spermatozoa in a slightly modified form [16]. The primary candidate for the intracellular Ca²⁺-store in spermatozoa is the acrosome, since human sperm do not contain the endoplasmic reticulum [5]. The presence of thapsigargin-sensitive Ca²⁺-ATPase SERCA 2 mainly localized to the acrosome and mid-piece was demonstrated in mammalian spermatozoa [2, 10].

The curves of Ca²⁺, Mg²⁺-dependent hydrolysis of ATP of sperm cells of the infertile men with asthenozoospermia and oligoasthenozoospermia have a similar appearance, but differ from the values for the normozoospermic samples (Fig. 3).

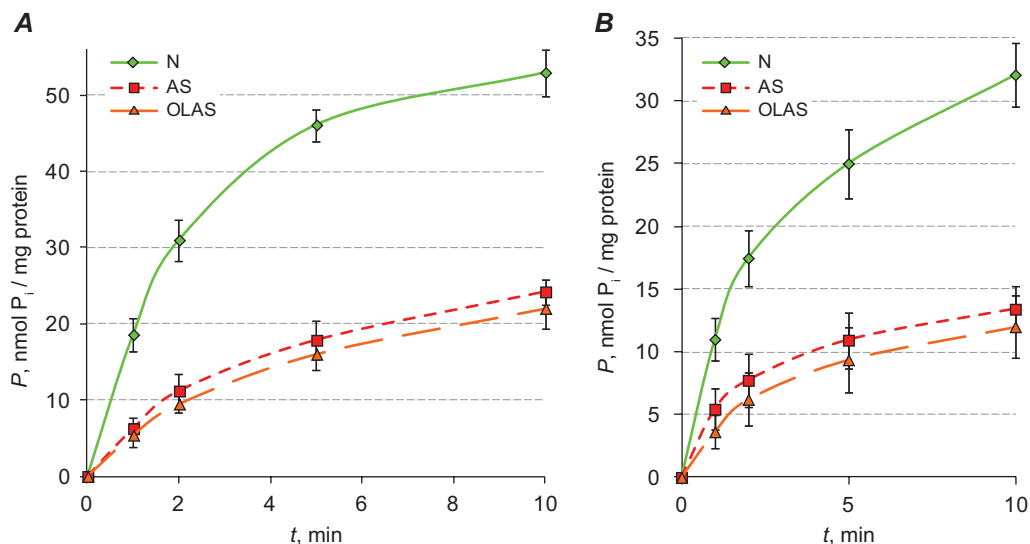


Fig. 3. Dynamics of accumulation of inorganic phosphate in the reaction catalyzed by thapsigargin-resistant (A) and thapsigargin-sensitive (B) ATPase in normozoospermic and pathospermic samples, $M \pm m$, $n = 5-7$

Рис. 3. Динаміка накопичення неорганічного фосфату у процесі Ca^{2+} , Mg^{2+} -залежного гідролізу АТФ за участю тапсигаргін-резистентної (А) і тапсигаргін-чутливої Ca^{2+} , Mg^{2+} -АТФази (В) сперматозоїдів чоловіків із нормозоо- та патозспермією, $M \pm m$, $n = 5-7$

As follows from the data in the table, the values of apparent kinetic parameters of Ca^{2+} , Mg^{2+} -dependent hydrolysis of ATP by thapsigargin-resistant ATPase in sperm cells from the infertile men differ significantly from these values in the normozoospermic samples. Specifically, the value of V_0 of ATP hydrolysis in astheno- and oligoasthenozoospermic samples is reduced 4 times compared to normozoospermic samples. The value of P_{\max} in pathospermic samples is twice lower in comparison with the normozoospermic samples. The τ values of reaction catalyzed by thapsigargin-resistant ATPase in spermatozoa from men with astheno- and oligoasthenozoospermia are 1.8–2.0 times greater than values in normozoospermic samples.

In the absence of a significant difference in the value of τ reaction catalyzed by thapsigargin-sensitive ATPase in normozoospermic and pathospermic samples, the value of V_0 in astheno- and oligoasthenozoospermic samples is reduced 2.0 times compared to normozoospermic samples. The value of P_{\max} in pathospermic samples is 2.5 times higher than normozoospermic samples. Based on these results, we assume that in sperm cells of infertile men, the transport of Ca^{2+} ions is less active and characterized by a lower capacity than in fertile men.

CONCLUSION

The analysis of the alterations in hydrolase activity of thapsigargin-resistant and thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase showed that primary active transport of Ca^{2+} ions is less intensive and characterized by lower transport capacity in sperm cells of the infertile men in comparison with fertile men.

COMPLIANCE WITH ETHICAL STANDARDS

Human Rights: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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ХАРАКТЕРИСТИКА Ca^{2+} , Mg^{2+} -ЗАЛЕЖНОГО ГІДРОЛІЗУ АТФ У СПЕРМАТОЗОЇДАХ ІНФЕРТИЛЬНИХ ЧОЛОВІКІВ

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Порушення фертилізаційної здатності сперматозоїдів тісно асоційовані з дисфункцією йон-транспортуючих АТФаз, зокрема, Ca^{2+} , Mg^{2+} -АТФази. Знижена активність тапсигаргін-резистентної і тапсигаргін-чутливої Ca^{2+} , Mg^{2+} -АТФази призводить до порушення Ca^{2+} -гомеостазу і є характерною для аномальних сперматозоїдів (патозооспермія). З метою вивчення особливостей і механізму роботи Ca^{2+} , Mg^{2+} -АТФази визначали початкову швидкість реакції, максимальну (платову) кількість утворення продукту реакції та характеристичний час реакції. Для встановлення цих кінетичних параметрів Ca^{2+} , Mg^{2+} -залежного гідролізу АТФ, який каталізується Ca^{2+} , Mg^{2+} -АТФазою, досліджували динаміку накопичення продукту АТФ-гідролізної реакції. Отримані криві лінеаризовані у координатах $\{P/t; P\}$. За оцінкою змін гідролізної активності Ca^{2+} , Mg^{2+} -АТФази досліджено кінетику первинно-активного транспортування йонів Ca^{2+} крізь плазматичну мембрану і мембрани внутрішньоклітинних Ca^{2+} -депо у сапонін-пермеабілізованих сперматозоїдах інфертильних чоловіків із астено- й олігоастенозооспермією. З'ясовано, що у сперматозоїдах нормозооспермічних чоловіків транспортування йонів Ca^{2+} крізь плазматичну мембрану характеризується вищою ємністю, ніж крізь мембрани внутрішньоклітинних Ca^{2+} -депо, однак відбувається з однаковою початковою швидкістю і характеристичним часом реакції. Встановлено, що у сперматозоїдах патоспермічних чоловіків транспортування йонів Ca^{2+} за участю обох компонентів Ca^{2+} , Mg^{2+} -АТФази відбувається менш інтенсивно і характеризується нижчою ємністю, порівняно з сперматозоїдами чоловіків зі збереженою фертильністю. Вказані зміни кінетичних параметрів Ca^{2+} , Mg^{2+} -залежного гідролізу АТФ призводять до пригнічення активності тапсигаргін-резистентної і тапсигаргін-чутливої Ca^{2+} , Mg^{2+} -АТФази та зумовлюють зниження фертилізаційного потенціалу сперматозоїдів.

Ключові слова: Ca^{2+} , Mg^{2+} -АТФаза, гідроліз АТФ, сперматозоїди, чоловіче непліддя, патоспермія

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