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ATP-INDUCED CHANGES IN $[Ca^{2+}]_i$ IN SPERMATOZOA OF INFERTILE MEN WITH OLIGO- AND ASTHENOZOOSPERMIA

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Calcium is the major second messenger which plays an important role in sperm physiology. A decreased fertility potential of spermatozoa is closely associated with the disturbances of Ca^2-homeostasis. ATP is present in the female reproductive tract and may play an important role in the fertilization process. Since intracellular calcium ($[Ca^{2+}]_i$) is the main determinant of many physiological processes occurring in sperm, we set out to describe the Ca^{2+}-signals in response to extracellular ATP (ATP_e) in spermatozoa of fertile (normozoospermia) and infertile men (oligo- and asthenozoospermia). ATP_e-induced changes in $[Ca^{2+}]_i$ in spermatozoa were studied using 2 μM fluorescent probe Fluo-4. ATP_e caused a rapid transient elevation in $[Ca^{2+}]_i$. We found that kinetics and magnitude of the $[Ca^{2+}]_i$ changes induced by the ATP_e were different in normo- and pathospermic cells. Specifically, the average value of peak amplitudes of $[Ca^{2+}]_i$ rise induced by 5 mM ATP_e in oligozoospermic samples was not significantly different from the normozoospermic samples. In the asthenozoospermic samples, the ATP_e-induced peak amplitude of $[Ca^{2+}]_i$ changes was in 1.5 fold lower ($P<0.05$) compared to that in the normozoospermic samples. ATP_e-induced increase in $[Ca^{2+}]_i$ in sperm cell has a concentration-dependent manner in both normozoospermic and pathozoospermic samples. In the oligozoospermic samples, the $[Ca^{2+}]_i$ transient response was 2.5 fold ($P<0.05$) slower than in the normozoospermic samples. Differences in ATP_e-induced $[Ca^{2+}]_i$ transients between astheno- and normozoospermic samples were also significant ($P<0.05$) although less pronounced. The obtained results clearly demonstrate ATP_e-induced increase in $[Ca^{2+}]_i$ transients are disturbed in pathozoospermic samples which may be detrimental to sperm activation and may result in fertilization failure or abnormality. Taken into account the importance reproductive techniques, specifically

for *in vitro* fertilization and intrauterine insemination present study suggest that modulation of $[Ca^{2+}]_i$ signals and sperm function by the ATP_e may be beneficial for artificial reproductive techniques used in reproductive biology and medicine.

Keywords: calcium, spermatozoa, ATP, male infertility, oligozoospermia, asthenozoospermia

INTRODUCTION

Intracellular calcium level ($[Ca^{2+}]_i$) is subjected to a strict spatiotemporal control in both somatic and germ cells [2, 4]. Calcium ions play a vital role in regulating of physiological processes in spermatozoa, in particular in motility and viability of ejaculated spermatozoa. $[Ca^{2+}]_i$ is important in the initiating process of sperm hyperactivation, capacitation, acrosome reaction and gamete interaction [7, 15]. Since cytoplasm of spermatozoa has a restricted volume and sperm cells have highly polarized morphology they pose a unique challenge to maintain Ca^{2+}-homeostasis [14].

Metabolic active form of ATP (strongly charged anion ATP^{4-}) is present in the female reproductive tract and may play an important role in the fertilization process. It is critically important for sperm cells since it is the main energy source and substrate for a second messenger cAMP in spermatozoa. Extracellular ATP (ATP_e) plays a key role as a signaling molecule. It is known that ATP_e increases fertilization rates in *in vitro* fertilization for male factor infertility [17]. The level of ATP_e depends on tissue type and presence of ecto-ATPases on the external membrane surface that hydrolyze ATP to ADP and P_i.

Since ATP is not able to cross plasma membrane freely because of its strongly charge, biological effects of ATP_e are realized via cell-surface P2 purinergic receptors [3]. Several members of the P2X family have been detected in mammalian spermatozoa [1]. Specifically, the P2X2 receptor was characterized in mouse sperm. Mice deficient in *P2rx2 gene had* normal sperm morphology and were fertile, however, their fertility potential was decreased with frequent mating [13]. ATP_e induced a rapid increase in $[Ca^{2+}]_i$ in sperm cells that was inhibited by P2 purinergic receptor inhibitors, demonstrating their ionotropic role in sperm function [16].

Defective functions of spermatozoa are the most common cause of male infertility [10]. The important determinants of male fertility are sperm count and sperm motility. Oligozoospermia (spermatozoa concentration in ejaculate $<20\times10^6$ cells/mL) and asthenozoospermia (<40% progressive motility) are the most common causes of male infertility. Up to now, data regarding ionic aspects of male infertility remain poorly understood. Since $[Ca^{2+}]_i$ is the main determinant of many physiological processes occurring in sperm, we set out to describe the Ca^{2+}-signals in response to ATP_e in spermatozoa of fertile (normozoospermia) and infertile men (oligo- and asthenozoospermia).

MATERIALS AND METHODS

Donors and preparation of semen sample. Human semen was obtained from 10 healthy volunteers (control group) and 16 pathozoospermic men undergoing routine semen analysis for couple infertility at Lviv Regional Clinical Hospital (Ukraine). Control group consisted of men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and having healthy 1–3 children). Before turning to study,

all men were aware of patient information leaflets and gave an informed consent to participate in research. Terms of sample selection meet 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 Committee of Danylo Halytsky Lviv National Medical University (Ethical Committee Approval, protocol No 6 from March 29, 2017). All patients and healthy donors were matched by age and gave written informed consent to participate in research. 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 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 masturbation after 3–4 days 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) [18]. Sperm cells were washed from semen plasma by 3 times centrifugation at 3000 ×g for 10 min in media which contained (mM): 120 NaCl, 30 KCl, 30 Hepes (pH 7.4).

Calcium measurements. Changes in ionized Ca content in spermatozoa were identified with 2 μM fluorescent probe fluo-4 acetoxymethyl ester (fluo-4 AM) (Invitrogen Inc., CA) (λ_{ex} = 495 nm, λ_{em} = 520 nm). The probe was mixed with Pluronic F-127 (0.02%) to facilitate loading [9]. All experimental procedures were conducted in physiological Henks's solution (mM): NaCl – 136.9; KCl – 5.36; KH_2PO_4 – 0.44; $NaHCO_3$ – 0.26; Na_2HPO_4 – 0.26; $CaCl_2$ – 0.03; $MgCl_2$ – 0.4; $MgSO_4$ – 0.4; glucose – 5.5; Hepes (pH 7.4, 37 °C) – 10. The sperm cells were incubated for 30 min at 37 °C. In order to compare the fluorescence intensities of different studied groups, the same number of cells was used in spectrofluorimetric measurements. The levels of Ca^{2+} in sperm cells were measured using a spectrofluorometer CM2203. ATP_e was administrated into cuvvete. The fluorescence signal was captured every second for 5 min. The following reagents were used in the study: glucose, Pluronic F-27, $CaCl_2$ (Sigma, USA), Fluo-4 AM (Invitrogen Inc., CA). Other reagents were of local manufacture and analysis-grade purity.

Statistical analysis. Data are expressed as means ± SE of the numbers of determinations. Differences between paired sets of the fluorimetric experiments were analysed using paired Student's *t*-tests. Data analyses were performed with Excel. Differences were considered significant at $p < 0.05$ as the minimum significance level.

RESULTS AND DISCUSSION

In physiological conditions, Ca^{2+} ions are intracellular second messengers which play an important role in sperm physiology. There is a strong relationship between calcium homeostasis and male infertility (sperm count and motility). A statistically significant difference between $[Ca^{2+}]_i$ in infertile versus fertile males was found, while no differences were observed for seminal plasma Ca^{2+}-concentrations [12]. Therefore, $[Ca^{2+}]_i$ seems to be a marker of sperm fertility. ATP is obligatory for sperm movement. It was found in large amounts in human seminal plasma and more significantly higher in the seminal plasma of the oligozoo- and azoospermic men [6].

To examine changes in $[Ca^{2+}]_i$ in sperm cells (expressed as $\Delta F/F_0$) observed after ATP_e treatment, we measured intracellular calcium levels using the fluorescent probe fluo-4. When spermatozoa were treated with 5 mM ATP_e, a rapid increase in $[Ca^{2+}]_i$ was observed (Fig. 1*A*). This increase in $[Ca^{2+}]_i$ was followed by a relatively slower decline. Since ATP_e was added as a disodium salt an equivalent molar amount (5 mM) of sodium ion (NaCl) was used as a control. As can be seen it had no effect on $[Ca^{2+}]_i$ in spermatozoa, which indicate a direct effect of ATP^{4-} anion on $[Ca^{2+}]_i$.

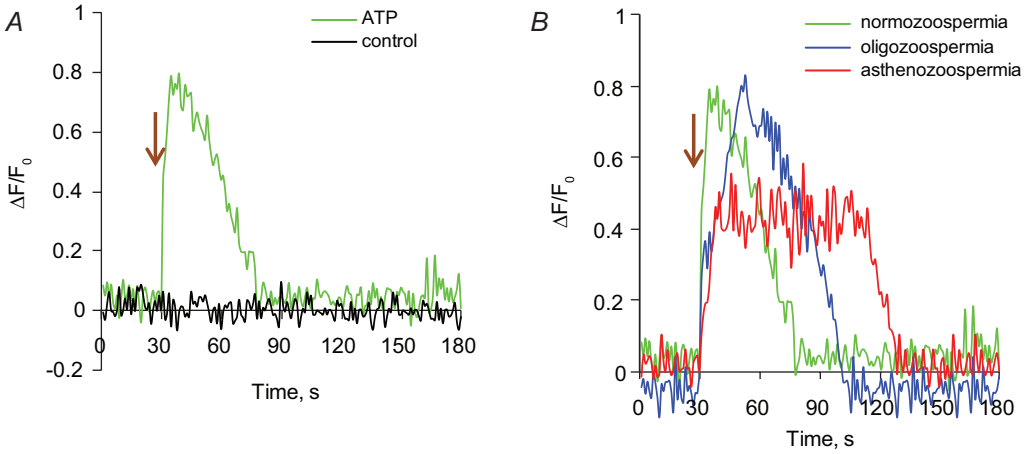


Fig. 1. Effect of ATP_e on $[Ca^{2+}]_i$ changes ($\Delta F/F_0$) in spermatozoa of normozoospermic (*A*) and pathozoospermic (*B*) men. The data show a representative experiment. Arrowheads indicate ATP or NaCl (served as a control) administration

Рис. 1. Вплив ATP_e на зміни $[Ca^{2+}]_i$ ($\Delta F/F_0$) у сперматозоїдах нормозооспермічних (*A*) і патоспермічних чоловіків (*B*). Результати типового експерименту. Стрілки вказують додавання АТР або NaCl (контроль)

ATP_e-induced $[Ca^{2+}]_i$ changes in both normo- and oligozoospermic samples were similar, but transient response was slower. However, in the asthenozoospermic samples ATP_e-induced $[Ca^{2+}]_i$ changes were characterized by lower peak levels. Specifically, the average value of peak amplitudes of $[Ca^{2+}]_i$ rise induced by 5 mM ATP_e in oligozoospermic samples was not significantly different from the normozoospermic samples (Fig. 2). In asthenozoospermic samples, the ATP_e-induced peak amplitude of $[Ca^{2+}]_i$ changes was 1.5 fold lower ($P<0.05$) compared to that in the normozoospermic samples.

ATP_e-induced increase in $[Ca^{2+}]_i$ in sperm cell has a concentration-dependent manner in both normozoospermic and pathozoospermic samples (Fig. 3). Increases in ATP_e concentration from 1 to 5 mM leads to elevation of the peak amplitudes of $[Ca^{2+}]_i$ transients. Further increase in ATP_e up to 10 mM lead to an increase in peak amplitudes of $[Ca^{2+}]_i$ transients only in the asthenozoospermic samples, but not in the oligo- and normozoospermic samples.

ATP_e-induced $[Ca^{2+}]_i$ transients were significantly slower in the pathozoospermic samples than in spermatozoa obtained from the fertile men (Fig. 4). Specifically, in oligozoospermic samples the $[Ca^{2+}]_i$ transient response was 2.5 fold ($P<0.05$) slower than in the normozoospermic samples. Differences in ATP_e-induced $[Ca^{2+}]_i$ transients between the astheno- and normozoospermic samples were also significant ($P<0.05$) although less pronounced.

Fig. 2. Peak amplitudes of $[Ca^{2+}]_i$ transients ($\Delta F/F_0$) induced by 5 mM ATP_e in spermatozoa of normozoospermic (N) and pathozoospermic (OI and Asth) men.
Hereinafter, the bars represent means ± standard error of different experiments; *p<0.05 significantly different compared to peak increase in $[Ca^{2+}]_i$ in normozoospermic samples

Рис. 2. Пікові амплітуди ATP_e-індукованих (5 мМ) $[Ca^{2+}]_i$ транзієнтів у сперматозоїдах нормозооспермічних і патоспермічних чоловіків.
Тут і надалі гістограми представляють результати середнього значення ± стандартної похибки середнього різних експериментів; *p<0,05 статистично достовірно стосовно пікової амплітуди $[Ca^{2+}]_i$ у нормозооспермічних зразках

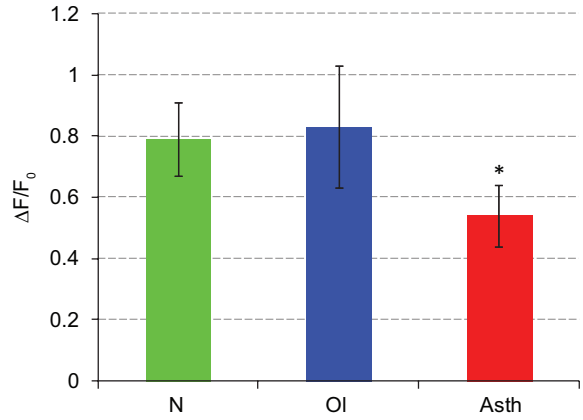


Fig. 3. Dose-responses of ATP-induced $[Ca^{2+}]_i$ rises ($\Delta F/F_0$) in spermatozoa of the normozoospermic and pathozoospermic men

Рис. 3. Дозозалежний характер ATP_e-індукованого зростання $[Ca^{2+}]_i$ у сперматозоїдах нормозооспермічних і патоспермічних чоловіків

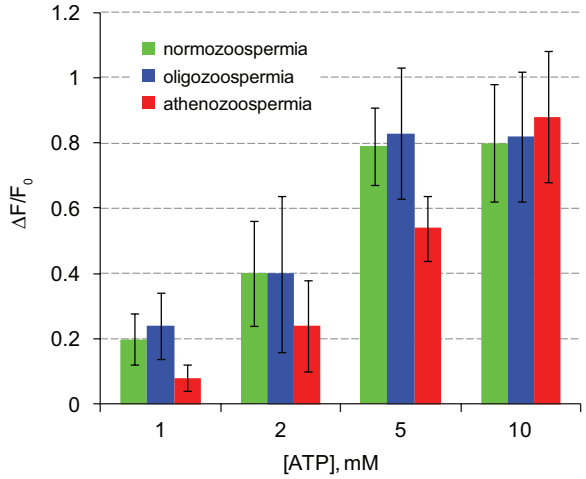
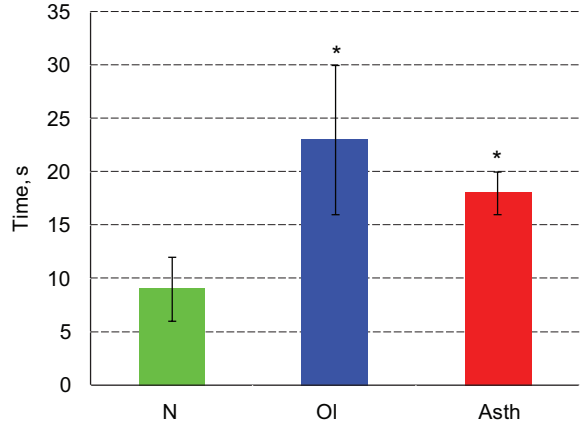


Fig. 4. The kinetics of ATP_e-induced $[Ca^{2+}]_i$ rises in the spermatozoa of the normozoospermic and pathozoospermic men; *p<0.05 significantly different compared to peak increase in $[Ca^{2+}]_i$ in the normozoospermic samples

Рис. 4. Кінетика наростання ATP_e-індукованого $[Ca^{2+}]_i$ транзієнта у сперматозоїдах нормозооспермічних і патоспермічних чоловіків; *p<0,05 статистично достовірно стосовно пікової амплітуди $[Ca^{2+}]_i$ у нормозооспермічних зразках



Earlier, the effect of ATP_e on $[Ca^{2+}]_i$ changes in sperm cells was studied. Similar results of ATP_e-induced elevation of $[Ca^{2+}]_i$ were presented in paper [11]. In contrast, in previous study, it was found that ATP_e did not increase $[Ca^{2+}]_i$ in Fura-2-loaded human sperm [8]. Present study was undertaken to evaluate differences in $[Ca^{2+}]_i$ signals that occur in human sperm in fertile and infertile men in responce to ATP_e.

There are some limitations of present study. First, a 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, in present study, Ca^{2+}-signals in response to ATP_e in the pathospermic patients only with 16 cases. Therefore it is essential to validate our findings with greater sample sizes and determine a disease specificity (secretory or excretory infertility, varicocele or others) by comparing spermogram parameters.

Since $[Ca^{2+}]_i$ is associated with sperm hyperactication, capacitation and acrosome reaction, changes in kinetics and magnitude of $[Ca^{2+}]_i$ transients induced by ATP_e in the pathozoospermic samples can be related to a reduced fertility potential. The obtained results clearly demonstrate ATP_e-induced increase in $[Ca^{2+}]_i$ transients disturbed in the pathozoospermic samples which may be detrimental to sperm activation and result in fertilization failure or abnormality. Previously, it was reported that incubation of mouse sperm with ATP_e significantly increases a success of *in vitro* fertilization, likely by altering sperm motility via purinergic receptor-mediated Ca^{2+}-fluxes [16]. Similarly ATP_e improved human sperm motility parameters [5]. These important findings have great importance as novel therapeutic strategies in treatment of male infertility.

Taken into account an importance of reproductive techniques, specifically for *in vitro* fertilization and intrauterine insemination, present study suggests that modulation of $[Ca^{2+}]_i$ signals and sperm function by ATP_e may be beneficial for artificial reproductive techniques used in the reproductive biology and medicine. The obtained results are important for further elucidation of mechanisms involved in maintaining calcium ion homeostasis in spermatozoa of fertile and infertile men.

CONCLUSIONS

Data presented reveal that ATP_e-induced increase in $[Ca^{2+}]_i$ in the asthenozoospermic samples was inhibited. In the oligozoospermic samples, ATP_e-induced increase in $[Ca^{2+}]_i$ was not significantly different from the normozoospermic samples, but kinetics of $[Ca^{2+}]_i$ transients was significantly slower compared to the normozoospermic samples.

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АТФ-ІНДУКОВАНІ ЗМІНИ $[Ca^{2+}]_i$ У СПЕРМАТОЗОЇДАХ ІНФЕРТИЛЬНИХ ЧОЛОВІКІВ З ОЛІГО- Й АСТЕНОЗООСПЕРМІЄЮ

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Йони кальцію є вторинними клітинними месенджерами, що відіграють важливу роль у фізіології сперматозоїдів. Зниження фертилізаційного потенціалу сперматозоїдів тісно пов'язане з порушеннями Ca^{2+}-гомеостазу. АТФ ідентифікований у жіночому репродуктивному тракті й може відігравати важливу роль у процесі фертилізації. Оскільки внутрішньоклітинний кальцій ($[Ca^{2+}]_i$) є основним детермінантом багатьох фізіологічних процесів у сперматозоїдах, метою роботи було описати описати Ca^{2+}-сигнали, індуковані зовнішньоклітинним АТФ ($АТФ_з$) у сперматозоїдах фертильних (нормозооспермія) та інфертильних чоловіків (оліго- й астенозооспермія). $АТФ_з$-індуковані зміни $[Ca^{2+}]_i$ у сперматозоїдах вивчали з використанням 2 мкМ флуоресцентного зонда Fluo-4. $АТФ_з$ викликав швидке транзієнтне підвищення $[Ca^{2+}]_i$. Ми виявили, що кінетика та величина $АТР_з$-індукованих змін $[Ca^{2+}]_i$ були різними у нормозоо- і патоспермічних зразках. Зокрема, середнє значення пікових амплітуд $[Ca^{2+}]_i$ підвищення індукованого 5мМ $АТФ_з$ у олігозооспермічних зразках істотно не відрізнялося від нормозооспермічних зразків. В астенозооспермічних зразках пікові амплітуди $АТФ_з$-індукованого $[Ca^{2+}]_i$ підвищення були в 1,5 разу нижчими ($P<0,05$), порівняно з показниками у нормозооспермічних зразках. $АТФ_з$-індуковане зростання $[Ca^{2+}]_i$ у сперматозоїдах мало концентраційно-залежний характер як у нормозооспермічних, так і у патоспермічних зразках. В олігозооспермічних зразках кінетика наростання $[Ca^{2+}]_i$ транзієнта була у 2,5 разу ($P<0,05$) повільніша, ніж у нормозооспермічних зразках. Відмінності в $АТФ_з$-індукованих $[Ca^{2+}]_i$ транзієнтах між астено- та нормозооспермічними зразками також були статистично достовірними ($P<0,05$), проте менш вираженими. Отримані результати чітко демонструють порушення $АТФ_з$-індукованих $[Ca^{2+}]_i$ транзієнтів у патозооспермічних зразках, які можуть негативно впливати на процеси активації сперматозоїдів і призводити до порушення процесів фертилізації. Беручи до уваги важливість методів репродукції, особливо для запліднення *in vitro* та внутрішньоматкового запліднення, ці дослідження доводять, що модуляція Ca^2-сигналів і функцій сперматозоїдів за допомогою $АТФ_з$ може бути корисною для методів штучної репродукції, що використовують у репродуктивній біології та медицині.

***Ключові слова**:* кальцій, сперматозоїди, АТФ, чоловіче неплідддя, олігозооспермія, астенозооспермія

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