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Role of Mitochondria in Progesterone-evoked Intracellular Calcium Signals in Ejaculated Spermatozoa from Infertile Men

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Calcium ions plays a pivotal role in sperm physiology [1]. Specifically, sperm hyperactivation, chemotaxis and motility depend on intracellular free calcium concentration ($[Ca^{2+}]_i$) [2]. $[Ca^{2+}]_i$ has intracellular and extracellular sources. Regulation of $[Ca^{2+}]_i$ is achieved by a combination of channels, pumps and exchanges on both the plasma membrane and intracellular stores. Sperm mitochondria may serve as intracellular Ca^{2+} stores, although their contribution to $[Ca^{2+}]_i$ homeostasis and signalling cascades in spermatozoa is still unclear [3,4]. Mitochondria facilitate Ca^{2+} uptake via the mitochondrial uniporter, which is dually mediated by the electrochemical membrane potential gradient. Subsequently, Ca^{2+} can be released from mitochondria either by Na^+/Ca^{2+} or H^+/Ca^{2+} exchangers [5]. Also at elevated mitochondrial calcium level and upon mitochondrial depolarization it can be released thorough permeability transition pore [6].

The stored Ca^{2+} can be liberated from intracellular Ca^{2+} stores as a result of adequate stimuli [7]. Progesterone plays a crucial role in human sperm physiology, since it affects sperm motility, acrosomal reaction and chemotaxis [8]. It is a Ca^{2+} -mobilizing classical agonist which is able to deplete the intracellular Ca^{2+} stores. Also, progesterone induces Ca^{2+} influx into the spermatozoa and intracellular alkalization potentiates this effect [7].

Since mitochondria are important in the regulation of the $[Ca^{2+}]_i$ in sperm, we set out to describe the progesterone-evoked intracellular Ca^{2+} -signals in response to uncoupling agent CCCP in spermatozoa of fertile (normozoospermia) and infertile men (oligo- and asthenozoospermia).

Materials and methods. *Donors and semen sample preparation.* 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 have healthy 1–3 children). Before turning to study, all men were aware of patient information leaflets and gave 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 Halytskyi 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 Ca^{2+} 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) [9]. Sperm cells were washed from semen plasma by 3 times centrifugation at 3000 $\times g$ for 10 min in media which contained (mM): 120 NaCl, 30 KCl, 30 Hepes (pH 7.4).

Calcium measurements. Changes of ionized Ca content in spermatozoa were identified with 2 μM fluorescent probe fluo-4 acetoxymethyl ester (fluo-4 AM) (Invitrogen Inc., CA) ($\lambda_{\text{ex}} = 495 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$). The probe was mixed with Pluronic F-127 (0.02%) to facilitate loading. 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. 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 $\pm$ standard errors of the numbers of determinations. Differences between paired sets of 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. As a physiological agent we used progesterone, a Ca^{2+} -mobilizing classical agonist which is able to deplete the intracellular Ca^{2+} stores. It was shown that evaluation of human sperm to respond to progesterone by increasing $[\text{Ca}^{2+}]_i$ may be used as a fast and objective tool for the diagnosis of the human sperm quality, especially in cases of unexplained sterility [10]. Treatment of spermatozoa with progesterone caused a typical transient increase $[\text{Ca}^{2+}]_i$.

To investigate the role of mitochondria in Ca^{2+} signaling, we used an uncoupling agents carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP) at a concentration of 10 μM which leads to dissipation of the mitochondrial membrane potential and blocking of Ca^{2+} -uptake. Preapplication of CCCP induced an increase in the amplitude of $[\text{Ca}^{2+}]_i$ rise caused by progesterone (20 μM), which indicate that mitochondria regulate rapid Ca^{2+} uptake. However, in oligo- and asthenozoospermic samples the preapplication with CCCP induces Ca^{2+} signal much smaller compared to Ca^{2+} signal obtained in spermatozoa from normozoospermic men.

CCCP application after $[\text{Ca}^{2+}]_i$ transient induced an additional $[\text{Ca}^{2+}]_i$ rise in normozoospermic samples, which indicates significant Ca^{2+} release that was previously accumulated by mitochondria (Fig. 1 a). However, in oligozoospermic samples the amplitude of CCCP-induced $[\text{Ca}^{2+}]_i$ transient was much smaller compared to Ca^{2+} signal obtained in spermatozoa from normozoospermic men (Fig. 1 b, c). Contrary to normo- and oligozoospermic samples, there was a sustained (not transient) $[\text{Ca}^{2+}]_i$ rise induced by CCCP application after $[\text{Ca}^{2+}]_i$ transient induced by progesterone in sperm cells obtained from men with asthenozoospermia.

Fig. 1. Effect of CCCP on progesterone-induced $[Ca^{2+}]_i$ changes ($\Delta F/F_0$) in spermatozoa of normozoospermic (a) oligo- (b) and asthenozoospermic (c) men. The data show a representative experiment. Arrowheads indicate progesteron and CCCP administration

Peak amplitudes of $[Ca^{2+}]_i$ rise ($\Delta F/F_0$) induced by CCCP in spermatozoa of oligozoospermic and asthenozoospermic men were in 1.3 and 2.0 fold < 0.05) lower than normozoospermic samples (Fig. 2 a). In asthenozoospermic samples CCCP-induced $[Ca^{2+}]_i$ rise was twice slower ($P < 0.05$) than in spermatozoa obtained from fertile men (Fig. 2 b). Differences in CCCP-induced $[Ca^{2+}]_i$ transients between oligo- and normozoospermic samples were not significant ($P > 0.05$).

Fig. 2. Peak amplitudes (a) and kinetics (b) of CCCP-induced $[Ca^{2+}]_i$ transients ($\Delta F/F_0$) after progesteron-induced $[Ca^{2+}]_i$ transient in spermatozoa of normozoospermic (N) and oligo (Ol and asthenozoospermic (Asth) men.

Under physiological conditions mitochondria play an important role in Ca^{2+} buffering and signalling, shaping (and often extending) the kinetics of Ca^{2+} signals [11]. However, previously it was found that major role in Ca^{2+} clearance in sperm cell belong to Ca^{2+} -ATPase pump of plasma membrane [6]. In mouse spermatozoa it was demonstrated that under resting conditions the contribution of mitochondrial Ca^{2+} buffering was marginal, but became more significant when plasma membrane Ca^{2+} pumps were inhibited [12]. The mitochondrial Ca^{2+} uniporter brings Ca^{2+} into mitochondria when $[\text{Ca}^{2+}]_i$ is elevated above ~ 500 nM [13].

In sperm of the sea urchin *Strongylocentrotus purpuratus* it was demonstrated that CCCP and other mitochondrial inhibitors cause mobilization of stored Ca^{2+} followed by sustained Ca^{2+} influx, apparently due to mobilization of mitochondrial Ca^{2+} and consequent activation of store-operated Ca^{2+} channels [14]. Our results indicate that pathospermia is associated with a significant decrease mitochondria accumulation of Ca^{2+} . The obtained results are important for further elucidation of mechanisms involved in maintaining Ca^{2+} homeostasis in spermatozoa of fertile and infertile men.

Conclusion. We presume that disrupted Ca^{2+} mobilization in spermatozoa from pathozoospermic men might be associated with decrease in the Ca^{2+} -accumulating properties of mitochondria.

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RESEARCH ARTICLE

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Calcium is the major second messenger which has an important role in sperm physiology. Decreased fertility potential of spermatozoa is closely associated with the disturbances of Ca^{2+} -homeostasis. Sperm mitochondria may serve as intracellular Ca^{2+} stores, although their contribution to $[\text{Ca}^{2+}]_i$ homeostasis and signalling cascades in spermatozoa is still unclear. Since mitochondria are important in the regulation of the $[\text{Ca}^{2+}]_i$ in sperm, we set out to describe the progesterone-evoked intracellular Ca^{2+} -signals in response to uncoupling agent CCCP in spermatozoa of fertile (normozoospermia) and infertile men (oligo- and asthenozoospermia).

Human semen was obtained from 10 healthy volunteers (control group) and 16 pathozoospermic men undergoing routine semen analysis for couple infertility. Human ejaculates were obtained from healthy volunteers and pathozoospermic men by masturbation after 3–4 days sexual abstinence and processed immediately upon liquefaction. For determination of cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$), spermatozoa were loaded with 2 μM fluorescent probe Fluo-4.

In this study we have investigated the progesterone-evoked intracellular Ca^{2+} signal in response to uncoupling agent CCCP in ejaculated spermatozoa from infertile men with different form of pathospermia. To investigate the role of mitochondria in Ca^{2+} signaling, we used an uncoupling agents carbonyl cyanide m-chloro phenyl hydrazone (CCCP) at a concentration of 10 μM which leads to dissipation of the mitochondrial membrane potential and blocking of Ca^{2+} -uptake. Preapplication of CCCP induced an increase in the amplitude of $[\text{Ca}^{2+}]_i$ rise caused by progesterone (20 μM), which indicate that mitochondria regulate rapid Ca^{2+} uptake. CCCP application after $[\text{Ca}^{2+}]_i$ transient induced an additional $[\text{Ca}^{2+}]_i$ rise in normozoospermic samples, which indicates significant Ca^{2+} release that was previously accumulated by mitochondria. Our results show that amplitude of CCCP-induced $[\text{Ca}^{2+}]_i$ transient after progesterone-induced $[\text{Ca}^{2+}]_i$ transient in oligo- and asthenozoospermic samples was much smaller compared to calcium signal obtained in spermatozoa from normozoospermic men. In asthenozoospermic samples CCCP application after progesterone-induced $[\text{Ca}^{2+}]_i$ caused sustained (not transient) $[\text{Ca}^{2+}]_i$ rise.

We presume that disrupted calcium mobilization in spermatozoa from pathozoospermic men might be associated with decrease in the Ca^{2+} -accumulating properties of mitochondria.

Key words: calcium, spermatozoa, mitochondria, male infertility, oligozoospermia, asthenozoospermia.