“ЕКСПЕРИМЕНТАЛЬНА ТА КЛІНІЧНА ФІЗІОЛОГІЯ І БІОХІМІЯ”
“EXPERIMENTAL AND CLINICAL PHYSIOLOGY AND BIOCHEMISTRY”
Науково-практичний журнал/Scientific-practical journal

Наукові статті / Research article
ECPB 2018, 1(81): 33–38. <https://doi.org/>

УДК 615.25:612.616.2

Hydrogen Peroxide Effect on the Thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase Activity of Sperm Cells

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Introduction. It is known that mammalian and human spermatozoa are able to generate reactive oxygen species (ROS) which are important regulators of sperm functions (hyperactivation, capacitation and acrosome reaction). However, an excessive ROS generation appears to be related to male infertility. An uncontrolled production of ROS or impaired antioxidant defense enzyme and non-enzyme mechanisms leads to oxidative stress which is harmful to sperm cells. It has been demonstrated recently that oxidative stress is implicated in male infertility. ROS-mediated damage to sperm is a considerable pathology contribution in 30% to 80% of unselected infertile patients [1, 2].

One of the most common ROS having potential implications in the reproductive biology is hydrogen peroxide (H_2O_2). It was shown that human spermatozoa incubated and exposed to artificial H_2O_2 has detrimental effects on sperm motility and give rise to a significant increase in the overall ROS [4].

Oxidative stress may have negative impact on the activity of membrane-bound enzymes such as Ca^{2+} , Mg^{2+} -ATPase which is involved in maintaining calcium homeostasis in spermatozoa. Ion-transporting ATPases are very sensitive to oxidation. The inhibition of Ca^{2+} , Mg^{2+} -ATPase may occur through a different mechanism [9, 12].

The aim of the present work was to evaluate the H_2O_2 effect on the main kinetic parameters of ATP hydrolysis by thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase of spermatozoa of fertile (normozoospermia) and infertile men (asthenozoospermia).

Material and Methods. Patients. 10 infertile men with asthenozoospermia were involved in this study. The main exclusion criteria: men who were currently on any medication or antioxidant supplementation were not included. In addition, men with infertility over 10 years, azoospermia, genital infection, chronic illness and serious systemic diseases, smokers and alcoholic men were excluded from the study because of their well-known high seminal ROS levels and decreased antioxidant activity which may affect ATPase activities. Infertile men were age-matched to fertile control cases. The control group consisted of 8 healthy men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and have 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 (WHO, 2010) [11]. Before turning to study, all men were aware of patient information leaflets and gave informed consent to participate in the 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. All patients and healthy donors gave a written consent to participate in the research. The approval for the study was taken from the ethics committee of Danylo Halytsky Lviv National Medical University (Ethical Committee Approval, protocol No 6 from March 29, 2017).

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 to determine its concentration ('Simko Ltd'). The aliquots were subjected to exogenous ROS stimulation with H_2O_2 as an oxidizing agent (5 min, 37 °C, 5 % CO_2) at different concentrations (25, 50, 100 and 200 μM). Untreated cells were used for controlling in the study.

Assay of Ca^{2+} , Mg^{2+} -ATPase activity. The detergent saponin in a final concentration of 0.5 % was added to sperm suspension for permeabilization of sperm membranes. Ca^{2+} , Mg^{2+} -ATPase activity was assayed with 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). The reaction was started by adding an aliquot of permeabilized sperm cells. After a 5 min incubation, 1 ml of a final solution containing (mM) 1.5 M sodium acetate, 3.7 % formaldehyde, 14 % ethanol, 5 % trichloroacetic acid (pH 4.3) was added. Pi was determined by the Fiske-Subbarow method using assay kit 'Simko Ltd' (Ukraine) [10]. Ca^{2+} , Mg^{2+} -ATPase activity was calculated as the difference between the ATPase activity in Ca^{2+} containing media and Ca-free medium (1 mM EGTA). Thapsigargin-sensitive ATPase activity was evaluated in presence of 10^{-6} M thapsigargin.

Statistical analysis. Experimental data were processed by methods of variation statistics using software MS Office. The results are presented as the mean $\pm$ standard error ($M \pm m$). The analysis of variance (ANOVA) was used to compare the difference in the means between infertile and healthy men. Differences were considered statistically significant at $p < 0.05$ for all analyses.

Results and Discussion. Disturbances in Ca^{2+} , Mg^{2+} -ATPase have been described in a large number of pathophysiological conditions, among them those connected with the oxidative stress. Significant correlations were found among pro-oxidative reactions with Ca^{2+} ATPases [3]. Our previous results showed that asthenozoospermic, oligoasthenozoospermic and leucocytospermic patients have significantly impaired thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity compared to healthy men [7]. However, Ca^{2+} , Mg^{2+} -ATPase activity has a tendency to increase in patients with oligozoospermia. The lowered activity of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity is likely to contribute to the disruption of Ca^{2+} homeostasis, a hallmark of abnormal sperm cells. The depressed ATPase activity in pathozoospermic samples could be due to the reduction in intracellular ATP and the damage of the spermal membranes caused by lipid peroxidation products [7].

Since Ca^{2+} , Mg^{2+} -ATPase is one of the targets for ROS and is directly involved in oxidative stress, spermatozoa obtained from normo- and pathozoospermic samples were subjected to oxidative stress in the form of exogenous H_2O_2 (25, 50, 100 and 200 μM) and then ATP hydrolysis by thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase in media with different Ca^{2+} concentrations was measured (Fig. 1). The H_2O_2 concentrations used in these studies were in the range 25–200 μM which is only slightly higher than the previously obtained half maximal inhibitory concentration of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase by H_2O_2 ($IC_{50} = 123.8 \pm 22.6 \mu M$) (unpublished/forthcoming).

Fig. 1. The effect of increasing the concentration of H₂O₂ on the dependence of thapsigargin-sensitive Ca²⁺, Mg²⁺-ATPase activity of normozoospermic samples on the Ca²⁺ concentration in incubation medium ($M \pm m$, $n = 8$). Results are given as percentage of the control activity taken as 100 (enzyme activity in the absence of H₂O₂)

As can be seen from Fig. 1 the monotonous increase in thapsigargin-sensitive Ca²⁺, Mg²⁺-ATPase activity was observed in the concentration range of Ca²⁺ from 10 to 50 μM in incubation medium (with constant concentration of ATP). The enzyme activity of thapsigargin-sensitive Ca²⁺, Mg²⁺-ATPase was reduced in the presence of H₂O₂ in the incubation medium. Similar dependences were obtained for thapsigargin-sensitive Ca²⁺, Mg²⁺-ATPase of sperm cell obtained from infertile (not presented in this article). The optimal enzyme activity was observed in the presence of 50 μM Ca²⁺ in the incubation medium in both normo- and asthenozoospermic samples with and without H₂O₂ in the incubation medium.

In order to elucidate the possible mechanisms of change in thapsigargin-sensitive Ca²⁺, Mg²⁺-ATPase activity under H₂O₂-induced oxidative stress the concentration curves were linearized using Hanes-Woolf plot $\{[S]/V; [S]\}$. The concentration curves differ by angle of inclination in the presence of H₂O₂ in the incubation medium (not presented in this article). The obtained kinetic parameters – the maximum rate of ATP hydrolysis (V_{max}) and the apparent activation constant for Ca²⁺ (KCa^{2+}) of thapsigargin-sensitive Ca²⁺, Mg²⁺-ATPase of sperm cell of fertile and infertile men are presented in Fig. 2 and Fig. 3.

From the kinetics of hydrolysis of ATP it is apparent that the maximum rate of ATP hydrolysis in normozoospermic samples decreased dramatically (more than in 2.5 fold). In patients with asthenozoospermia the decrease in V_{max} was not sharp, but gradual. The apparent activation constant for Ca²⁺ (KCa^{2+}) has not changed under H₂O₂-induced oxidative stress in sperm cells obtained from both normo- and pathozoospermic samples. However, the apparent activation constant for Ca²⁺ in pathozoospermic samples had a tendency to be lower than in normozoospermic samples, but these changes were not significant. All these results indicate that in both normo- and pathozoospermic samples H₂O₂ implements its inhibitory action through mechanism of uncompetitive inhibition of thapsigargin-sensitive Ca²⁺, Mg²⁺-ATPase activity (V_{max} decreases with no appreciable change in KCa^{2+}).

Fig. 2. The effect of different H_2O_2 concentrations on the maximum rate of ATP hydrolysis ($V_{\max}$) of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase of sperm cell of fertile and infertile men ($M \pm m$, $n = 8^{10}$)

Fig. 3. The effect of different concentrations of H_2O_2 on the apparent activation constant for Ca^{2+} ($K_{\text{Ca}^{2+}}$) of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase of sperm cell of fertile and infertile men ($M \pm m$, $n = 8^{10}$)

The obtained apparent affinity constants for Ca were in a micromolar range which corresponds to the generally accepted concept about a high affinity state (site) for Ca^{2+} ($K_{\text{Ca}^{2+}} = 0.5 \mu\text{M}$ or less) and a low affinity regulatory site ($K_{\text{Ca}^{2+}} > 10 \mu\text{M}$) [8]. Results concerning changes in kinetics properties of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase are limited. The affinity constant of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase to ATP shown in our previous studies has increased significantly in spermatozoa of men with asthenozoospermia in comparison with men with proven fertility [5].

H_2O_2 -induced oxidative stress leads to a decrease in activity of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase resulting in the reduced Ca^{2+} efflux and the decreased capacity to counteract potentially harmful excitotoxic Ca^{2+} loads [6]. Further studies are needed to elucidate the underlying mechanisms of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase inhibition and determine whether the observed changes in its activity and kinetic properties are a cause or consequence of the dysfunction of spermatozoa. Strategies to protect against loss in Ca^{2+} , Mg^{2+} -ATPase activity may be useful to prevent the harmful biochemical cascades leading to Ca^{2+} overload and dysfunction of spermatozoa as a result of oxidative stress.

Conclusion. Under H_2O_2 -induced oxidative stress the reduction in thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity in both normo- and pathozoospermic

samples was associated with a decrease in maximum rate of ATP hydrolysis maximum velocity (V_{max}) with no appreciable change in the affinity of the enzyme for Ca^{2+} .

Acknowledgement. The publication contains the results of studies conducted on the President's of Ukraine grant for competitive projects (project No Ф63/97-2016 from 10.08.2016 'Molecular biological regulatory mechanisms of disturbance of fertilizing ability spermatozoa and the development of new immuno-biochemical diagnostic methods of fertility in men' of the State Fund for Fundamental Research.

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Стаття надійшла до редколегії 06.02.2018

RESEARCH ARTICLES

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The objective of the present work was to evaluate the H_2O_2 effect on the main kinetic parameters of ATP hydrolysis by thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase of spermatozoa of fertile (normozoospermia) and infertile men (asthenozoospermia).

Material and Methods. 10 infertile men with asthenozoospermia and 8 healthy men with somatic fertility and confirmed parenthood (normozoospermia) were involved in this study.

Ca^{2+} , Mg^{2+} -ATPase activity was determined spectrophotometrically by production of Pi. Thapsigargin-sensitive ATPase activity was evaluated in presence of 10^{-6} M thapsigargin. The aliquots of sperm cells were subjected to exogenous reactive oxygen species stimulation with H_2O_2 as an oxidizing agent (5 min, 37 °C, 5 % CO_2) at different concentrations (25, 50, 100 and 200 μM). Untreated cells were used for controlling the study.

Results. Since Ca^{2+} , Mg^{2+} -ATPase is one of the targets for reactive oxygen species and is directly involved in oxidative stress, spermatozoa obtained from normo- and pathozoospermic samples were subjected to oxidative stress in the form of exogenous H_2O_2 and then ATP hydrolysis by thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase in media with different Ca^{2+} concentrations was measured. Enzyme activity of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase was reduced in the presence of H_2O_2 in the incubation medium. In order to elucidate the possible mechanisms of change in thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity under H_2O_2 -induced oxidative stress the concentration curves were linearized using Hanes–Woolf plot $\{[S]/V; [S]\}$. It is apparent from the kinetics of hydrolysis of ATP that the maximum rate of ATP hydrolysis in normozoospermic samples was decreased dramatically (more than in 2.5 fold). In patients with asthenozoospermia the decrease in V_{max} was not sharp, but gradual. The apparent activation constant for Ca^{2+} ($K_{\text{Ca}^{2+}}$) has not changed under H_2O_2 -induced oxidative stress in the sperm cell obtained from both normo- and pathozoospermic samples. All these results indicate that in both normo- and pathozoospermic samples H_2O_2 implements its inhibitory action through the mechanism of uncompetitive inhibition of thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity (V_{max} decreases with no appreciable change in $K_{\text{Ca}^{2+}}$). Strategies for protecting against loss in Ca^{2+} , Mg^{2+} -ATPase activity may be useful to prevent the harmful biochemical cascades leading to Ca^{2+} overload and dysfunction of spermatozoa as a result of oxidative stress.

Conclusion. Under H_2O_2 -induced oxidative stress, the reduction in thapsigargin-sensitive Ca^{2+} , Mg^{2+} -ATPase activity in both normo- and pathozoospermic samples was associated with a decrease in maximum rate of ATP hydrolysis (V_{max}) with no appreciable change in the affinity of the enzyme for Ca^{2+} .

Key words: Ca^{2+} , Mg^{2+} -ATPase, hydrogen peroxide, thapsigargin, inhibition, spermatozoa, male infertility, pathospermia.