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KINETICS OF INHIBITORY EFFECT OF HYDROGEN PEROXIDE ON ACTIVITY OF PLASMA MEMBRANE TRANSPORTING Ca^{2+}, Mg^{2+}-ATPase OF SPERM CELLS

R. V. Fafula, O. I. Meskalo, E. I. Lychkovskyy, Z. D. Vorobets

Danylo Halytsky Lviv National Medical University, 69, Pekarska St., Lviv 79010, Ukraine
e-mail: roman_fafula@ukr.net

Decreased potential of spermatozoa fertility is closely associated with the development of oxidative stress and dysfunction of ion-transporting ATPases. Oxidative stress may have negative impact on the activity of membrane-bound enzymes, such as Ca^{2+},Mg^{2+}-ATPase that is involved in maintaining calcium homeostasis in sperm cells. The aim of present work was to evaluate the exogenous H_2O_2 effect on the main kinetic parameters of ATP hydrolysis by plasma membrane Ca^{2+},Mg^{2+}-ATPase of spermatozoa of fertile (normozoospermia) and infertility (asthenozoospermia) men. Since Ca^{2+},Mg^{2+}-ATPase is one of the targets for the reactive oxygen species and is directly involved in oxidative stress, spermatozoa obtained from normo- and asthenozoospermic samples were subjected to oxidative stress in the form of exogenous H_2O_2. Then ATP hydrolysis by thapsigargin-resistant Ca^{2+},Mg^{2+}-ATPase in media with different Ca^{2+} concentrations was measured. An effective inhibitory effect of H_2O_2 on the activity of the thapsigargin-resistant component of Ca^{2+},Mg^{2+}-ATPase of sperm cells was demonstrated. In order to elucidate possible mechanisms of change in 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]}. The apparent activation constant for Ca^{2+} (K_{Ca2+}) in sperm cell obtained from men with proven fertility was not changed, whereas in the asthenozoospermic samples, it was decreased almost twice under H_2O_2-induced oxidative stress. These results indicate that in normozoospermic samples H_2O_2 implements its inhibitory action through the mechanism of uncompetitive inhibition of plasma membrane Ca^{2+},Mg^{2+}-ATPase activity. According to formal features of kinetics in the asthenozoospermic samples a mixed type of enzyme inhibition occurs under the oxidative stress induced by H_2O_2. Strategies to protect against a 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 the oxidative stress.

Keywords: Ca^{2+}, Mg^{2+}-ATPase; inhibition; hydrogen peroxide; spermatozoa; male infertility, pathospermia

INTRODUCTION

Oxidative stress is a major factor in development of male infertility. It is triggered by oxygen and oxygen-derived free radicals known as the reactive oxygen species (ROS). Low ROS concentrations have an important role in sperm physiological processes such as hyperactivation, capacitation and acrosome reaction. An excessive ROS generation appears to be related to male infertility [1]. Hydrogen peroxide (H_2O_2) is one of the most common ROS having potential implications in the reproductive biology. It was shown that low concentrations of H_2O_2 activate the antioxidant defense system in human sperm cells. Oxidative stress is the most apparent in sperm membranes. It triggers a loss of membrane integrity, changes in membrane permeability, enzyme inactivation etc. [2]. It may have a negative impact on the activity of membrane-bound enzymes, such as Ca^{2+}, Mg^{2+}-ATPase involved in maintaining calcium homeostasis in sperm cells. Ion-transporting ATPses are very sensitive to oxidation and inhibition of Ca^{2+},Mg^{2+}-ATPase may occur through a different mechanism [13, 18].

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

MATERIALS AND METHODS

Patients. 10 infertile men with asthenozoospermia were involved in this study. The exclusion criteria: subjects currently on any medication or antioxidant supplementation were not included. In addition, subjects 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.

Control group consisted of 8 healthy men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and have healthy 1–3 children). Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' 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) [17]. 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 obtained from the Ethics Committe of Danylo Halytsky National Medical University in Lviv. All patients and healthy donors gave written informed consent to participate in the research (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 ×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 as a control in the study.

Assay of plasma membrane 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 membrane. Ca^{2+}, Mg^{2+}-ATPase activity was assayed with 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). The reaction was started by addition of aliquot of permeabilized sperm cells. After a 5 min incubation, 1 ml of a 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 the Fiske-Subbarow method using assay kit "Simko Ltd" (Ukraine) [16]. Ca^{2+}, Mg^{2+}-ATPase activity was calculated as the difference between the ATPase activity in Ca^{2+} containing media and Ca^{2+}-free medium (1 mM EGTA). Plasma membrane Ca^{2+}, Mg^{2+}-ATPase (PMCA) activity was evaluated as a thapsigargin-insensitive ATPase activity (10^{-6} M thapsigargin).

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

RESULTS AND DISCUSSION

Disturbances in plasma membrane Ca^{2+}, Mg^{2+}-ATPase have been described in a large number of the pathophysiological conditions, including those linked to the oxidative stress [4]. In our previous study, it was shown that asthenozoo-, oligoastenozoo- and leucocytospermic patients have significantly impaired tapsigargin-insensitive Ca^{2+}, Mg^{2+}-ATPase activity compared to healthy men [11]. However, PMCA activity had a tendency to increase in spermatozoa of patients with oligozoospermia. Lowered plasma membrane Ca^{2+}, Mg^{2+}-ATPase activity is likely to contribute to the disruption of Ca^{2+} homeostasis that is a hallmark of abnormal sperm cells.

The depressed ATPase activity in the infertile men could thus be due to a reduction in the intracellular adenosine triphosphate and damage of spermal membranes caused by lipid peroxidation products [11]. A decrease in PMCA activity might derive from the nitration of tyrosine residues that may affect the catalytic activity of enzymes [16].

Since Ca^{2+}, Mg^{2+}-ATPase is one of the targets for ROS and is directly involved in the oxidative stress, spermatozoa were subjected to the oxidative stress in the form of exogenous H_2O_2 (25, 50, 100 and 200 μM) and then ATP hydrolysis by PMCA in media with different Ca^{2+} concentrations was measured (Fig. 1). The H_2O_2 concentrations used in these studies was in the range 25–200 μM that only slightly higher than that obtained previously half maximal inhibitory concentration of PMCA by H_2O_2 (IC_{50} = 191.7±21.9 μM).

As can be seen from Fig. 1, the monotonous increase in tapsigargin-insensitive Ca^{2+}, Mg^{2+}-ATPase activity was observed in the concentration range of Ca^{2+} from 10 to 100 μM in the incubation medium (with constant concentration of ATP). Enzymatic activity of plasma membrane Ca^{2+}, Mg^{2+}-ATPase of sperm cell was reduced in the presence of H_2O_2 in the incubation medium. Similar dependences were obtained for plasma membrane Ca^{2+}, Mg^{2+}-ATPase of sperm cell from infertile men (asthenozoospermia) (Fig. 2).

The dependences of PMCA activity on the substrate for other studied groups of the infertility men were similar (not presented). The optimal enzymes activity was observed in the presence of 50 μM Ca^{2+} in the incubation medium both normo- and asthenozoospermic samples with and without H_2O_2 in the incubation medium.

A sensitivity to the inhibitory effect of H_2O_2 in sperm cell of the infertile men was lower than that in the control. Specifically, the effect of 25 μM H_2O_2 was more expressed in normozoospermic samples than in the asthenozoospermic samples (55 % vs 98 % of

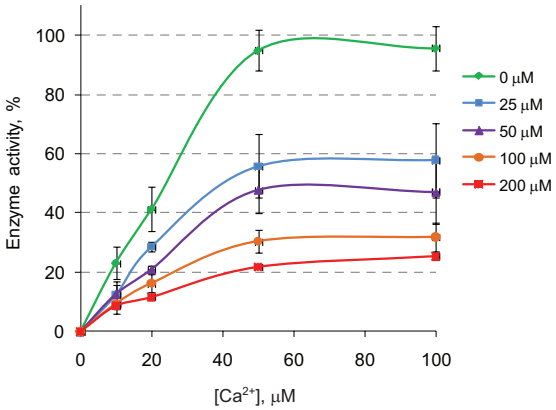

Fig. 1. The effect of increasing the concentration of H_2O_2 on the dependence of activity of plasma membrane Ca^{2+}, Mg^{2+}-ATPase of normozoospermic samples from Ca^{2+} concentration in the incubation medium (M±m, n = 8). Results are presented as a percentage of the control activity taken as 100 (enzymatic activity in the absence of H_2O_2)

Рис. 1. Вплив збільшення концентрації H_2O_2 на залежність Ca^{2+}, Mg^{2+}-АТФ-азної активності плазматичної мембрани сперматозоїдів із нормоспермічних еякулятів від концентрації Ca^{2+} в інкубаційному середовищі (M±m, n = 8). За 100 % прийнято значення питомої ензиматичної активності без внесення H_2O_2 у середовищі інкубації

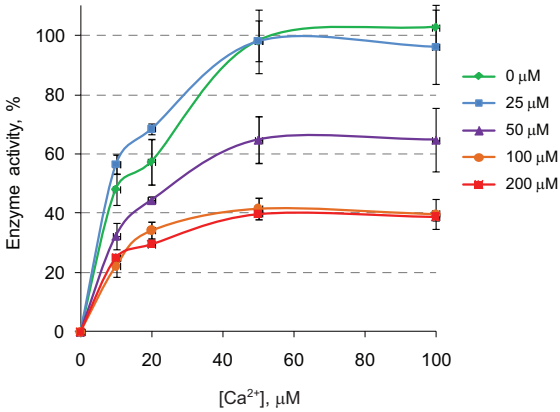

Fig. 2. The effect of increasing the concentration of H_2O_2 on the dependence of activity of plasma membrane Ca^{2+}, Mg^{2+}-ATPase of asthenozoospermic samples from Ca^{2+} concentration in the incubation medium (M±m, n = 10). Results are presented as a percentage of the control activity taken as 100 (enzymatic activity in the absence of H_2O_2)

Рис. 2. Вплив збільшення концентрації H_2O_2 на залежність Ca^{2+}, Mg^{2+}-АТФ-азної активності плазматичної мембрани сперматозоїдів чоловіків з астенозооспермією від концентрації Ca^{2+} в інкубаційному середовищі (M±m, n = 10). За 100 % прийнято значення питомої ензиматичної активності без внесення H_2O_2 у середовищі інкубації

the control activity at 50 μM Ca^{2+}, respectively). Disease related reduction in the vulnerability of PMCA to ROS in the form of H_2O_2 may be attributed to a variety of reasons such as decreased enzyme expression, its altered stability and structural alterations. Recently, a decreased PMCA4 expression was demonstrated in the asthenozoospermia group compared to normozoospermia group, however, the Ca^{2+}-ATPase activity was significantly higher in the asthenozoospermia group than in the normozoospermia group [10].

It is known that H_2O_2-induced changes in PMCA activity may be related to both direct effects on protein structure and/or due to secondary effects resulting from lipid peroxidation. Spermal membranes are rich in the polyunsaturated fatty acids and are very susceptible to attack by ROS [14, 15]. A significant increase in TBARS content as secondary products of lipid peroxidation in sperm cells of the infertile men compared with men with preserved fertility was found earlier [8]. We suggest that a decreased vulnerability of PMCA to ROS in the form of H_2O_2 can be a result of pathology-related chronic oxidative stress.

In order to elucidate possible mechanisms of change in PMCA activity under H_2O_2-induced oxidative stress the concentration curves were linearized using Hanes–Woolf

plot. The concentration curves {[S]/V; [S]} differ by the angle of inclination in the presence of H_2O_2 in the incubation medium (not presented). The obtained kinetic parameters – maximum rate of ATP hydrolysis (V_{max}) and apparent activation constant for Ca^{2+} (K_{Ca2+}) of tapsigargin-insensitive Ca^{2+},Mg^{2+}-ATPase of sperm cell of the fertile and infertile men are presented on the Fig. 3.

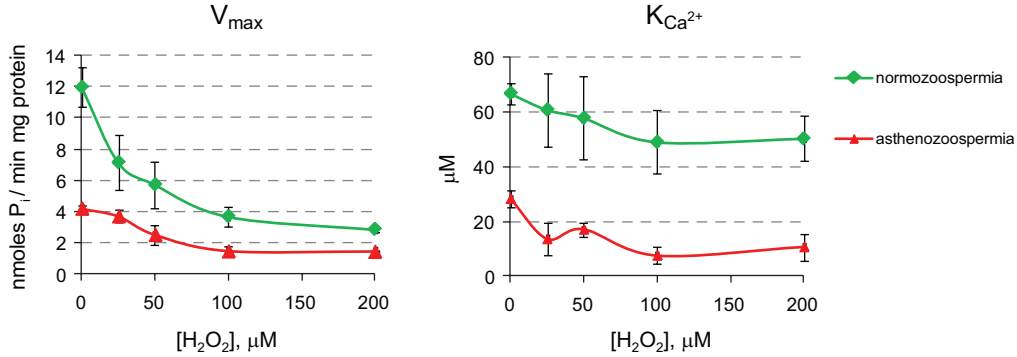

Fig. 3. The effect of different concentrations of H_2O_2 on the maximum rate of ATP hydrolysis (V_{max}) and the apparent activation constant for Ca^{2+} (K_{Ca2+}) of plasma membrane Ca^{2+}, Mg^{2+}-ATPase of sperm cell of the fertile and infertile men (M±m, n = 8–10)

Рис. 3. Вплив H_2O_2 у різних концентраціях на кінетичні параметри реакції гідролізу АТФ по Ca^{2+} (початкову максимальну швидкість реакції (V_{max}) та уявну константу активації K_{Ca2+}), що каталізується Ca^{2+}, Mg^{2+}-АТФ-азою плазматичної мембрани сперматозоїдів фертильних та інфертильних чоловіків (M±m, n = 8–10)

From the kinetics of ATP hydrolysis it is apparent that the maximum rate of ATP hydrolysis in the normozoospermic samples was dramatically decreased. In patients with asthenozoospermia, a decrease in V_{max} was not sharp but sluggish. The obtained apparent affinity constants for Ca^{2+} were in the micromolar range that corresponds to generally accepted concept about the high affinity state (site) for Ca^{2+} (K_{Ca2+} = 0.5 μM or less) and a low affinity regulatory site (K_{Ca2+} > 10 μM) [12]. The value of K_{Ca2+} in sperm cells obtained from men with proven fertility was not changed, whereas in the asthenozoospermic samples, it decreases almost twice under H_2O_2-induced oxidative stress. These results indicate that in the normozoospermic samples H_2O_2 implements its inhibitory action through a mechanism of the uncompetitive inhibition of plasma membrane Ca^{2+},Mg^{2+}-ATPase activity. According to formal features of kinetics in the asthenozoospermic samples, a mixed type of enzyme inhibition occurs.

H_2O_2 effects on plasma membrane Ca^{2+},Mg^{2+}-ATPase activity and its kinetics properties were studied earlier. It was shown that H_2O_2 can directly oxidize the plasma membrane Ca^{2+}, Mg^{2+}-ATPase and its main activator calmodulin [19]. Studies in neurons (also highly specialized cells like spermatozoa) have shown that H_2O_2 reduce the PMCA expression at the plasma membrane [9]. It was shown that cellular stress may have a profound effect on the ATP sensitivity of the PMCA [5]. Furthermore, studies have shown that high concentrations of H_2O_2 (500 μM) inhibited PMCA activity by approximately 80 %, caused only approximately 55 % ATP depletion. Interestingly, lower H_2O_2 concentration (50 μM) had no effect on ATP depletion and yet significantly inhibited plasma membrane Ca^{2+}, Mg^{2+}-ATPase activity by approximately 50 % suggesting that enzyme inhibition by H_2O_2 can occur independent by of metabolic inhibition and ATP

depletion [6]. In previous studies, we have shown a significant increase in the affinity constant of tapsigargin-insensitive Ca^{2+},Mg^{2+}-ATPase to ATP in both normozoospermic and asthenozoospermic men under H_2O_2-induced oxidative stress [7].

In present study, we studied the H_2O_2 effect on the ATP hydrolysis by Ca^{2+},Mg^{2+}-ATPase and affinity to Ca^{2+} in spermatozoa of fertile (normozoospermia) and infertility (asthenozoospermia) men. We have shown that under H_2O_2-induced oxidative stress the inhibition of PMCA activity 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+}. Similar results indicating a loss of PMCA activity due to a significant reduction in V_{max} with no change in the affinity for Ca^{2+} or K_{act} were obtained for PMCA of the synaptic membranes [20]. We have shown that a decrease in plasma membrane Ca^{2+}, Mg^{2+}-ATPase activity in sperm cell of the asthenozoospermic samples was associated with an increase in the affinity of the enzyme for Ca^{2+} ($K_{Ca^{2+}}$ decreases twice). Studies in human brain tissue from Alzheimer's disease have shown an altered PMCA affinity to Ca^{2+}, as compared to age-matched controls suggesting structural/conformational changes in the Ca^{2+} binding sites in enzyme [3].

H_2O_2-induced oxidative stress leads to a decrease in the activity of major plasma membrane Ca^{2+} extrusion systems resulting in a reduced Ca^{2+} efflux and decreased capacity to counteract potentially harmful excitotoxic Ca^{2+} loads [9]. Further studies are needed to elucidate the underlying mechanisms of 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 a 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 the oxidative stress.

CONCLUSIONS

Under oxidative stress induced by H_2O_2 a reduction in plasma membrane Ca^{2+}, Mg^{2+}-ATPase activity in the normozoospermic samples is 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+}. In the asthenozoospermic samples the plasma membrane Ca^{2+},Mg^{2+}-ATPase activity shows an increased affinity for Ca^{2+} under the influence of H_2O_2 compared to age-matched normozoospermic samples. A reduction in plasma membrane Ca^{2+}, Mg^{2+}-ATPase activity in patients with asthenozoospermia is associated with both a decrease in maximum rate of ATP hydrolysis maximum velocity (V_{max}) and increase in the affinity of the enzyme for Ca^{2+}.

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КІНЕТИКА ІНГІБІТОРНОЇ ДІЇ ГІДРОГЕН ПЕРОКСИДУ НА АКТИВНІСТЬ ТРАНСПОРТУВАЛЬНОЇ Ca^{2+}, Mg^{2+}-АТФ-ази ПЛАЗМАТИЧНОЇ МЕМБРАНИ СПЕРМАТОЗОЇДІВ ЧОЛОВІКІВ

Р. В. Фафула, О. І. Мескало, Е. І. Личковський, З. Д. Воробець

Львівський національний медичний університет імені Данила Галицького
вул. Пекарська, 69, Львів 79010, Україна
e-mail: roman_fafula@ukr.net

Порушення фертилізаційної здатності сперматозоїдів тісно асоційовані з розвитком оксидативного стресу та дисфункцією йон-транспортувальних АТФ-аз. Негативний вплив оксидативного стресу може реалізовуватися через порушення функціонування мембранозв'язаних ензимів, зокрема, Ca^{2+}, Mg^{2+}-АТФ-ази, яка відіграє провідну роль у підтриманні кальцієвого гомеостазу в сперматозоїдах. Метою роботи було оцінити вплив екзогенного H_2O_2 на основні кінетичні параметри гідролізу АТР Ca^{2+}, Mg^{2+}-АТФ-азою плазматичної мембрани сперматозоїдів фертильних (нормозооспермія) та інфертильних чоловіків (астенозооспермія). Оскільки Ca^{2+}, Mg^{2+}-АТФ-аза є однією з мішеней для активних форм Оксигену і прямо задіяна в розвитку оксидативного стресу, сперматозоїди, отримані від чоловіків з нормо- й астенозооспермією, інкубували за наявності H_2O_2 (25–200 μM) і визначали гідроліз АТФ тапсигаргін-резистентною Ca^{2+}, Mg^{2+}-АТФ-азою в інкубаційному середовищі за різних концентрацій Ca^{2+}. Продемонстровано ефективний інгібувальний вплив H_2O_2 на активність тапсигаргін-резистентної компоненти Ca^{2+}, Mg^{2+}-АТФ-ази сперматозоїдів. Методом лінеаризації отриманих концентраційних залежностей у координатах Хейнса {[S]/V; [S]} розраховано основні кінетичні параметри Ca^{2+}-активованого, Mg^{2+}-залежного гідролізу АТФ у сперматозоїдах із нормо- й астенозооспермічних еякулятів. За умов H_2O_2-індукованого оксидативного стресу уявна константа активації $K_{Ca^{2+}}$ у сперматозоїдах фертильних чоловіків не змінюється, а у сперматозоїдах чоловіків із астенозооспермією знижується удвічі. Це свідчить про неконкурентний механізм інгібування H_2O_2 Ca^{2+}, Mg^{2+}-АТФ-ази плазматичної мембрани сперматозоїдів чоловіків із нормозооспермією. За формальними ознаками у сперматозоїдах чоловіків із астенозооспермією пригнічення тапсигаргін-резистентної Ca^{2+}, Mg^{2+}-АТФ-азної активності за умов H_2O_2-індуваного оксидативного стресу відбувається за змішаним типом. Терапевтичні підходи із застосуванням протекторних речовин проти оксидативного стресу можуть бути корисними для запобігання патобіохімічним каскадам, які призводять до перенавантаження цитозолю Ca^{2+} і дисфункції сперматозоїдів.

Ключові слова: Ca^{2+}, Mg^{2+}-АТФ-аза; інгібування; гідроген пероксид; сперматозоїди; чоловіче неплідддя; патоспермія

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