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\(_2\)O\(_2\) 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\(_2\)O\(_2\). 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\(_2\)O\(_2\) 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\(_2\)O\(_2\)-induced oxidative stress, the concentration curves were linearized using Hanes–Woolf plot \([S]/V; [S]\). The apparent activation constant for Ca\(^{2+}\) (K\(_{Ca^{2+}}\)) in sperm cell obtained from men with proven fertility was not changed, whereas in the asthenozoospermic samples, it was decreased almost twice under H\(_2\)O\(_2\)-induced oxidative stress. These results indicate that in normozoospermic samples H\(_2\)O\(_2\) 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\(_2\)O\(_2\). 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$_2$O$_2$) is one of the most common ROS having potential implications in the reproductive biology. It was shown that low concentrations of H$_2$O$_2$ 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 ATPases 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$_2$O$_2$ 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$_2$O$_2$ 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 MgCl$_2$, 5 ATP, 0.05 CaCl$_2$, 1 ouabain, 1 Na$_2$S, 20 Heps-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, 14 % 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 asthenozo-, oligoastenozo- 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$_2$O$_2$ (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$_2$O$_2$ 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$_2$O$_2$ (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$_2$O$_2$ 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$_2$O$_2$ in the incubation medium.

A sensitivity to the inhibitory effect of H$_2$O$_2$ in sperm cell of the infertile men was lower than that in the control. Specifically, the effect of 25 µM H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$).

Fig. 2. The effect of increasing the concentration of H$_2$O$_2$ 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$_2$O$_2$).

The control activity at 50 µM Ca$^{2+}$, respectively). Disease related reduction in the vulnerability of PMCA to ROS in the form of H$_2$O$_2$ 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$_2$O$_2$-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$_2$O$_2$ can be a result of pathology-related chronic oxidative stress.

In order to elucidate possible mechanisms of change in PMCA activity under H$_2$O$_2$-induced oxidative stress the concentration curves were linearized using Hanes–Woolf
The concentration curves \([\frac{[S]}{V}]; [S]\) 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 \(Ca^{2+}\) \((K_{Ca^{2+}})\) of tapisgargin-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 \(Ca^{2+}\) \((K_{Ca^{2+}})\) of plasma membrane \(Ca^{2+},Mg^{2+}\)-ATPase of sperm cell of the fertile and infertile men (M±m, n = 8–10)](image)

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 \(Ca^{2+}\) \((K_{Ca^{2+}} = 0.5 \mu M\) or less) and a low affinity regulatory site \((K_{Ca^{2+}} > 10 \mu M)\) [12]. The value of \(K_{Ca^{2+}}\) 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 \(\mu M\)) inhibited PMCA activity by approximately 80 %, caused only approximately 55 % ATP depletion. Interestingly, lower \(H_2O_2\) concentration (50 \(\mu M\)) 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\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase to ATP in both normozoospermic and asthenozoospermic men under H\textsubscript{2}O\textsubscript{2}-induced oxidative stress [7].

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

H\textsubscript{2}O\textsubscript{2}-induced oxidative stress leads to a decrease in the activity of major plasma membrane Ca\textsuperscript{2+} extrusion systems resulting in a reduced Ca\textsuperscript{2+} efflux and decreased capacity to counteract potentially harmful excitotoxic Ca\textsuperscript{2+} loads [9]. Further studies are needed to elucidate the underlying mechanisms of Ca\textsuperscript{2+}, Mg\textsuperscript{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\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase activity may be useful to prevent the harmful biochemical cascades leading to Ca\textsuperscript{2+} overload and dysfunction of spermatozoa as a result of the oxidative stress.

**CONCLUSIONS**

Under oxidative stress induced by H\textsubscript{2}O\textsubscript{2} a reduction in plasma membrane Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase activity in the normozoospermic samples is associated with a decrease in maximum rate of ATP hydrolysis (V\textsubscript{max}) with no appreciable change in the affinity of the enzyme for Ca\textsuperscript{2+}. In the asthenozoospermic samples the plasma membrane Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase activity shows an increased affinity for Ca\textsuperscript{2+} under the influence of H\textsubscript{2}O\textsubscript{2} compared to age-matched normozoospermic samples. A reduction in plasma membrane Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase activity in patients with asthenozoospermia is associated with both a decrease in maximum rate of ATP hydrolysis maximum velocity (V\textsubscript{max}) and increase in the affinity of the enzyme for Ca\textsuperscript{2+}.

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