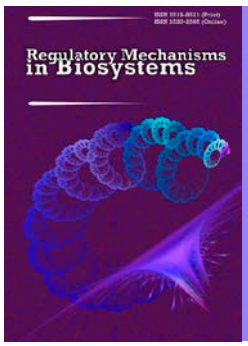

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Effect of hydrogen peroxide on Na^+,K^+-ATPase activity in spermatozoa of infertile men

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Na^+,K^+-ATPase plays an essential role in sperm motility, hyperactivation, chemotaxis, acrosome reaction etc. Na^+,K^+-ATPase is sensitive to ROS insult. Apart from production of highly reactive molecules, H_2O_2 can exert a number of direct effects on cells, their metabolism and enzymes. In the present study, exposure to exogenous H_2O_2 was used to characterize the effects of H_2O_2 on Na^+,K^+-ATPase activity in spermatozoa of infertile men with different forms of pathospermia. It was shown that Na^+,K^+-ATPase activities in spermatozoa of infertile men with different forms of pathospermia were inhibited by exposure to H_2O_2 (50−500 μM). H_2O_2, one of the most toxic oxygen species, has the ability to depress Na^+,K^+-ATPase activity in a dose-dependent manner. Severe inhibition of the hydrolytic activity was observed when higher H_2O_2 were used. The time course of incubation with 100 μM H_2O_2 showed a sharp decrease in the enzyme activity during the first 5 min of incubation for both normozoospermic and pathozoospermic men. The enzymatic activity of Na^+,K^+-ATPase in the sperm was completely destroyed at 20 min for asthenozoospermic men and 30 min for normozoospermic men. We show that an administation of H_2O_2 inhibited Na^+,K^+-ATPase activity in normozoospermic samples with IC_{50} of 106.6 ± 7.9 μM. IC_{50} for patients with asthenozoospermia was two times less than for healthy men with preserved fertility. For other studied groups, the differences in IC_{50} were not significant. These observations suggest that Na^+,K^+-ATPase in pathozoospermic samples is more vulnerable to H_2O_2-induced damage than in normozoospermic men. The Hill coefficient was significantly increased only for patients with asthenozoospermia, indicating increased positively cooperative binding. The decreases in Na^+,K^+-ATPase hydrolase activity in H_2O_2-treated sperm cells in men with normozoospermia were largely attenuated by exogenous GSH at 5 mM. This suggests that GSH partially protects the Na^+,K^+-ATPase from inhibition under experimental oxidative stress. However, treatment of oligo-, astheno- and oligoasthenozoospermic samples with 100 μM H_2O_2 and 5 mM GSH did not result in protection of Na^+,K^+-ATPase against induced oxidation, suggesting that the impaired Na^+,K^+-ATPase in pathozoospermic samples appears to be an irreversible event. In contrast, presence of GSH only after H_2O_2 treatment does not reverse Na^+,K^+-ATPase inhibition. This study has provided a deeper insight into the role Na^+,K^+-ATPase plays in sperm cells,it also could offer clues to the clinical application of antioxidant therapy in male infertility therapy.

Keywords: reactive oxygen species; enzyme inhibition; pathospermia; male infertility

Introduction

Na^+,K^+-ATPase (Na^+,K^+-activated Mg^{2+}-dependent ATP-hydrolase, EC 3.6.1.37) has been widely studied and verified in different cells, including spermatozoa (Thundathil et al., 2006). Two isoforms of the catalytic subunit of the Na^+,K^+-ATPase, α1 and α_4 which have different biochemical properties, coexist in sperm cells. Spermatozoa from knockout mice lacking α_4 were incapable of fertilizing eggs *in vitro* , indicating the essential role of Na^+,K^+-ATPase for sperm fertility (Sanchez et al., 2006).

Hyperproduction of reactive oxygen species (ROS) can damage sperm cells and is considered to be one of the mechanisms of infertility (Mahfouz et al., 2010). Spermatozoa have an unusual lipid composition of plasma membranes which is distinct from those of mammalian somatic cells. They are rich in polyunsaturated fatty acids and are very susceptible to attack by ROS (Shi et al., 2010). ROS are continuously produced in most cells and their levels are regulated by a number of enzymatic and non-enzymatic antioxidants. They have drawn attention for their potential to disrupt normal cell functions by targeting proteins for modification. ROS include hydrogen peroxide, singlet oxygen, the superoxide radical and the hydroxyl radical. Hydrogen peroxide (H_2O_2) is generated by the dismutation of the superoxide anion radical and can cross cell membranes more easily (Groeger et al., 2009). ROS can modify different biomolecules including the polyunsaturated fatty acids of sperm membranes, resulting in a lipid peroxidation and disruption of membrane proteins.

It is known that Na^+,K^+-ATPase plays a crucial role in cell signalling. Na^+,K^+-ATPase regulates cell motility, cell proliferation, glycogen synthesis, intracellular calcium and sodium homeostasis, calcium signalling, apoptosis etc. (Barwe et al., 2005; Khundmiri et al., 2006; Nguyen et al., 2007). The signaling functions of the enzyme were demonstrated in various cells including spermatozoa (Thundathil et al., 2006). Oxidative stress can activate the Na^+,K^+-ATPase signalling. H_2O_2 and exogenously added glucose oxidase (which generates a sustained low level of H_2O_2) activates Na^+,K^+-

ATPase signaling in cardiac myocytes (Liu et al., 2006). Na^+,K^+-ATPase is sensitive to ROS insult. It is known that increases in superoxide anion/hydrogen peroxide and reactive nitrogen species nitric oxide, which are mainly produced by mitochondria, NADPH oxidase, xanthine oxidase and uncoupled NO synthesis, can modify the Na^+,K^+-ATPase α and β subunit to inhibit the Na^+,K^+-ATPase enzymatic (ion exchange) activity (Petrushanko et al., 2012; Liu et al., 2012). Exogenous addition of H_2O_2 has been shown to increase intracellular ROS levels in spermatozoa (Mahfouz et al., 2008). Apart from production of highly reactive molecules, H_2O_2 can exert a number of direct effects on cells, their metabolism and enzymes. In the present study, exposure to exogenous H_2O_2 was used to characterize the effects of H_2O_2 on Na^+,K^+-ATPase activity in spermatozoa of infertile men with different forms of pathospermia.

Materials and methods

Patients. 24 infertile men with different forms of pathospermia were involved in this study. They were recruited between September 2016 and February 2017. A detailed medical history was performed in all studied cases. Exclusion criteria: subjects currently on any medication or antioxidant supplementation were not included. In addition, subjects with infertility lasting 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 reactive oxygen species levels and decreased antioxidant activity, which may affect ATPase activities.

The control group consisted of 13 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). Before participating in the study, all the men were made 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. Approval for study was taken from the Ethics Committee of Danylo Halytsky Lviv National Medical University. All patients and healthy donors gave written informed consent to participate in the research (Ethical Committee Approval, protocol No 2 from February 16, 2015).

Cell preparation. Sperm cells were washed from semen plasma by three times centrifugation at 3,000 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 the Lowry method using a kit to determine its concentration ("Simko Ltd"). The aliquots were subjected to exogenous ROS stimulation with H_2O_2 (5 min, 37 °C, 5% CO_2) at different concentrations (50, 100 and 500 μM) (if nothing else is indicated). Sperm samples without exogenous H_2O_2 served as control in the study. Also, when indicated, reduced glutathione (5.0 mM GSH) was present during the H_2O_2-treatment or was introduced for 10 min only after H_2O_2-treatment.

Assay of Na^+,K^+-ATPase activity. Determination of Na^+,K^+-ATPase activities was carried out in permeabilized spermatozoa. The detergent saponin in a final concentration of 0.5% was added to sperm suspension for permeabilization of sperm membranes. The total Na^+,K^+-ATPase activity was assayed with the following incubation medium (mM): 120 NaCl, 30 KCl, 5 $MgC1_2$, 3 ATP, 1 EGTA, 0.01 thapsigargin (specific inhibitor of SERCA), 1 NaN_3 (specific inhibitor of mitochondrial ATPase), 20 Hepes-Tris (pH 7.4; at 37 ºC). The protein concentration did not exceed 50 mg/ml. 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). Ouabain-sensitive Na^+,K^+-ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of 1 mM ouabain, which is known to completely block the Na^+,K^+-ATPase.

Statistical analysis. Experimental data were processed by methods of variation statistics using software MS Office. The results are presented as the mean ± standard error (x ± SE). 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

Na^+,K^+-ATPase is one of the targets for ROS and is directly involved in oxidative stress. Spermatozoa were subjected to oxidative stress in the form of H_2O_2. It was shown that exposure of sperm cells to various concentrations of H_2O_2 demonstrated the ability of ROS to depress Na^+,K^+-ATPase activity in a dose-dependent manner. The low concentrations of H_2O_2 noticeably decreased the Na^+,K^+-ATPase activity, but they did not lower Na^+,K^+-ATPase activity dramatically, contrary to the higher concentrations. Specifically, Na^+,K^+-ATPase activity of sperm cells decreased to 94.0 ± 7.4 and 37.6 ± 5.9% of initial activity in spermatozoa of healthy men (normozoospermia) treated with 50 μM and 100 μM H_2O_2, respectively (Fig. 1). However, the residual enzyme activities (10.6 ± 1.9% of initial activity) remain.

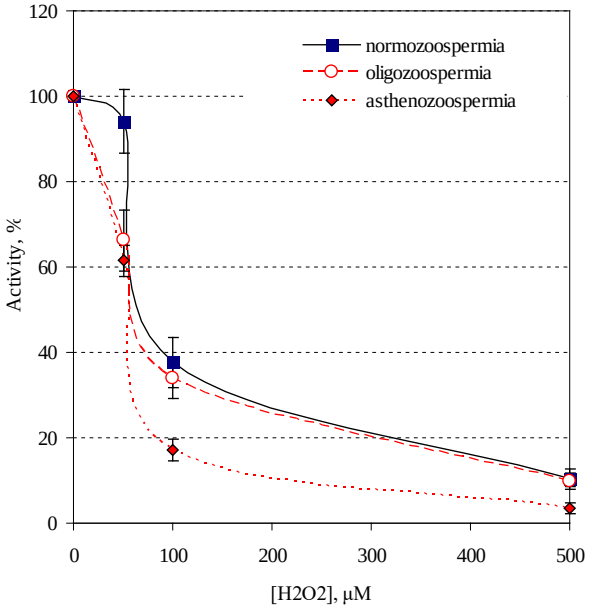

Fig. 1. Effect of hydrogen peroxide on the ouabain-sensitive Na^+,K^+-ATPase activity of spermatozoa of fertile and infertile men; data are presented as x ± SE, n = 4–6

When sperm cells of patients with pathospermia were exposed to various concentrations of H_2O_2, a dose-dependent inhibition of Na^+,K^+-ATPase enzymatic activity was also seen. For example, in patients with oligozoo- and asthenozoospermia the Na^+,K^+-ATPase activity of sperm cells decreased to 33.9 ± 4.6 and 17.3 ± 5.8% of initial activity when treated with 100 μM H_2O_2, respectively. Similar results were obtained for sperm cells of patients with oligoasthenozoospermia (data not shown). Also, sperm cells were incubated with 100 μM H_2O_2 at different times and then Na^+,K^+-ATPase activity was determined (Fig. 2). The time course of incubation with 100 μM H_2O_2 showed a sharp decrease in the enzyme activity during the first 5 min of incubation for both normozoospermic and pathozoospermic men. The enzymatic activity of the sperm Na^+,K^+-ATPase was completely destroyed at 20 min for asthenozoospermic men and 30 min for normozoospermic men.

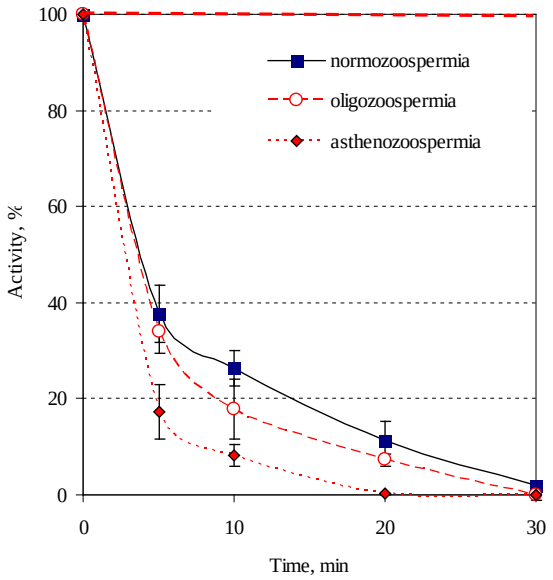

Fig. 2. Time course of Na^+,K^+-ATPase inhibition by H_2O_2; data are presented as x ± SE, n = 4–6

In order to elucidate the possible mechanism of H_2O_2 inhibitory effect the main kinetic parameters of inhibition were determined. Linearization of concentration curves in Hill's plot is represented on Figure 3. Obtained values of half maximal inhibitory concentration (IC_{50}) and Hill coefficient (nH) are represented in Table 1. The susceptibility of Na^+,K^+-ATPase to H_2O_2 in healthy men differed greatly from that of pathozoospermic patients. This is evidenced by the fact that IC_{50} for inhibition of Na^+,K^+-ATPase by H_2O_2 was greater for normozoospermic than for patients with oligo-, astheno- and oligoasthenozoospermia. IC_{50} for patients with asthenozoospermia was two times less than for healthy men with preserved fertility ($P < 0.05$). For other studied groups the differences in IC_{50} were not significant. These observations suggest that Na^+,K^+-ATPase in pathozoospermic samples is more vulnerable to H_2O_2 induced damage than in normozoospermic men. The Hill coefficient was significantly increased only for patients with asthenozoospermia indicating increased positively cooperative binding.

Table 1
Inhibition parameters of Na^+,K^+-ATPase of the ouabain-sensitive Na^+,K^+-ATPase of spermatozoa of fertile and infertile men by H_2O_2

Kinetic parameters	Normozoospermic men	Pathozoospermic men		
		oligozoospermia	asthenozoospermia	oligoasthenozoospermia
IC_{50}, μM	106.6 ± 21.9	74.6 ± 9.8	52.8 ± 3.2*	83.9 ± 8.0
n_H	1.42 ± 0.18	1.45 ± 0.16	2.00 ± 0.15*	1.52 ± 0.11

Note: data are presented as means ± standard error; n = 4–6; * – $P < 0.05$ compared to normozoospermic men (men with preserved fertility).

It is known that catalytic activity of the Na^+,K^+-ATPase protein is critically dependent on free SH groups. Therefore, we next tested whether glutathione (GSH) protected H_2O_2-induced inhibition of the enzyme. A single concentration of 100 μM H_2O_2 was chosen, as this was close to the IC_{50} value for Na^+,K^+-ATPase inhibition (Table 1). In the present study, the decreases in Na^+,K^+-ATPase hydrolase activity in H_2O_2-treated sperm cells in men with normozoospermia were largely attenuated by exogenous GSH at 5 mM (Fig. 4*a*).

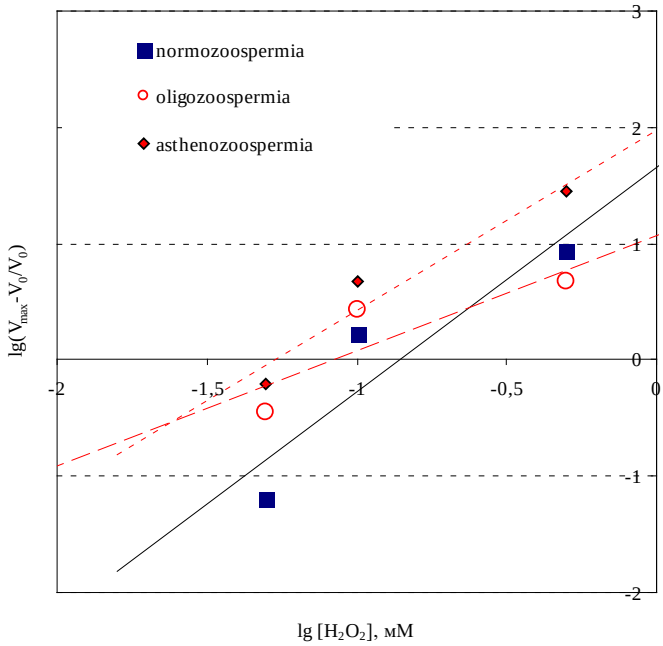

Fig. 3. Linearization of concentration curves represented on Fig. 1 in Hill's plot: x ± SE, n = 4–6; $R^2 > 0.85$

Specifically, the effect of 100 mM H_2O_2 in absence of GSH in incubation medium resulted in decrease of Na^+,K^+-ATPase activity to 37.6 ± 5.9% of initial activity, while in presence of 5 mM GSH no inhibitory effect was noted (Na^+,K^+-ATPase activity was 85.6 ± 8.8% of initial activity). This suggests that GSH partially protects the Na^+,K^+-ATPase from inhibition under experimental oxidative stress. However, treatment of oligozoospermic samples with 100 μM H_2O_2 and 5 mM GSH did not result in a protection of Na^+,K^+-ATPase against induced oxidation, suggesting that the impaired Na^+,K^+-ATPase in oligozoospermic samples appears to be an irreversible event (Fig. 4*b*). Similar results were obtained for asthenozoospermic and oligoasthenozoospermic samples (data not shown).

GSH alone significantly did not alter the Na^+,K^+-ATPase hydrolase activity in both normozoospermic and pathospermic samples (Fig. 5). Presence of GSH during H_2O_2 treatment had a protective effect on Na^+,K^+-ATPase activity and confers protection against oxidative stress. Thus it appears that GSH effectively "scavenges" H_2O_2 and, thus, prevents Na^+,K^+-ATPase inhibition.

In contrast, presence of GSH only after H_2O_2 treatment does not reverse Na^+,K^+-ATPase inhibition (data not shown). It is known that H_2O_2 can oxidize SH groups to disulfides, which leads to the enzyme disruption. However, addition of disulfide-reducing agents GSH after H_2O_2 exposure in our experiments did not result in a recovery of Na^+,K^+-ATPase activity.

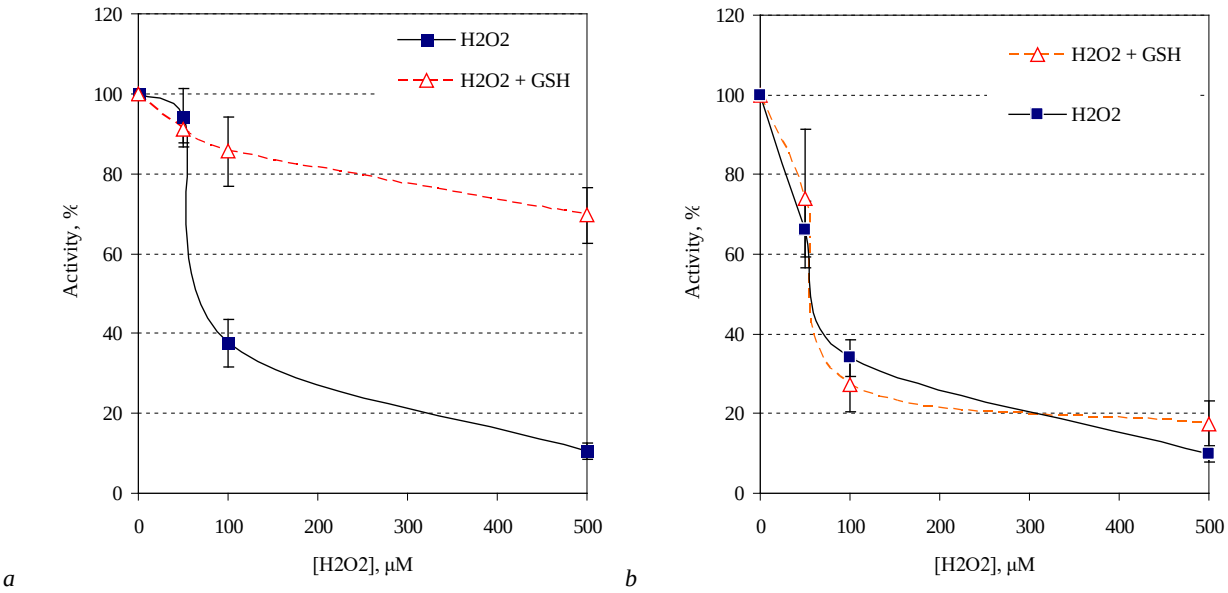

Fig. 4. Effects of 0.1 mM H_2O_2 and 5 mM GSH on the ouabain-sensitive Na^+,K^+-ATPase of spermatozoa of fertile men (*a*) and infertile men (oligozoospermia) (*b*): x ± SE, n = 4–6

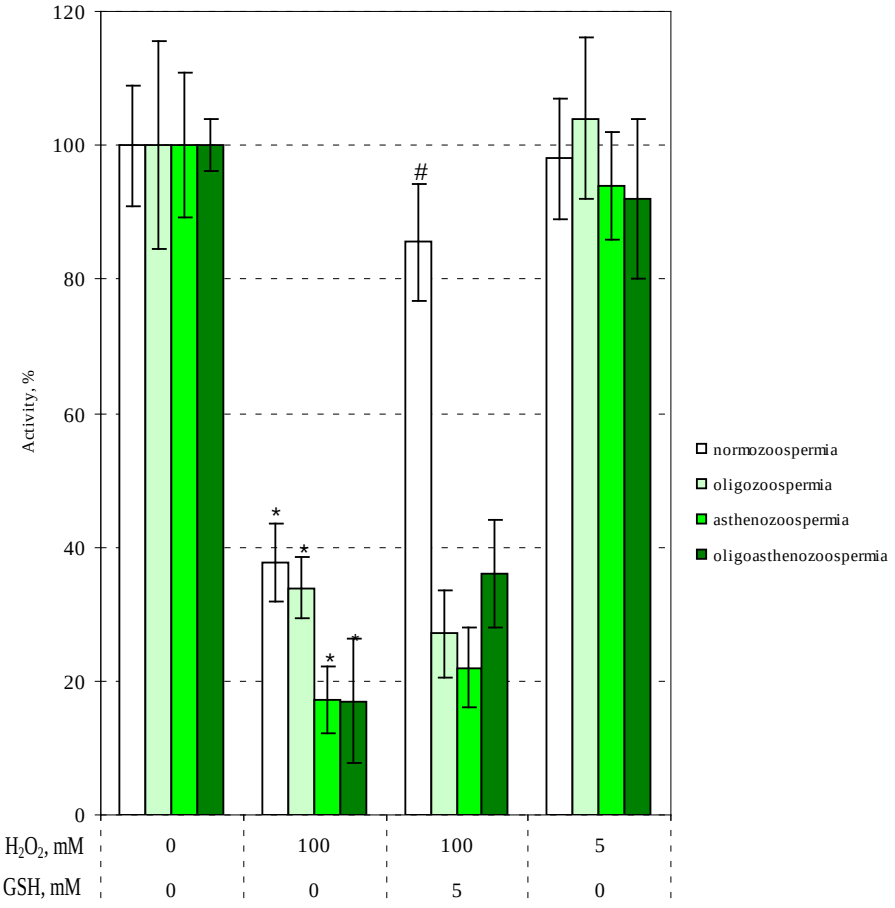

Fig. 5. Effects of 0.1 mM H_2O_2 and 5 mM GSH on the ouabain-sensitive Na^+,K^+-ATPase of spermatozoa of fertile and infertile men with different forms of pathospermia; x ± SE, n = 4–6; * – P < 0.05 compared to normozoospermic men (men with preserved fertility); # – P < 0.05 compared to data without exogenous GSH

Discussion

In our previous study (Meskalo et al., 2017), we found that spermatozoa of men with normozoospermia showed activity of ouabainsensitive Na^+,K^+-ATPase about 46.3 ± 4.2 nmol Pi/min•mg protein. Oligozoo-, asthenozoo-, oligoasthenozoo- and leucocytospermic patients have significantly impaired Na^+,K^+-ATPase compared to healthy men. In present study, H_2O_2, one of the most toxic oxygen species, has the ability to depress Na^+,K^+-ATPase activity in a dose-dependent manner.

Overall, our results are consistent with several other studies. In particular, Na^+,K^+-ATPase activity was markedly inhibited in ghost cells in the presence of 0.5 mM H_2O_2 (Derham et al., 2003). Liu et al. showed that ROS stress acted as ouabain and stimulated endocytosis of the Na^+,K^+-ATPase, which resulted in a significant inhibition of Na^+,K^+-ATPase activity (Liu et al., 2004). However, conflicting data

have been obtained in other studies. In some earlier studies researchers have shown that H_2O_2 increases Na^+,K^+-pump activity in bovine pulmonary arterial endothelial cells (Meharg et al., 1993). A sharp increase in Na^+,K^+-ATPase activity was observed in *Leishmania amazonensis* in response to low increased amounts (0.1 μM) of H_2O_2 (Rocco-Machado et al., 2015).

Oxidative stress mediated by lipid peroxidation of sperm membranes may be responsible for the inhibition of Na^+,K^+-ATPase. ROS attack polyunsaturated fatty acids of membrane phospholipids, can modify different biomolecules, resulting in a lipid peroxidation. Spermatozoa have an unusual lipid composition of plasma membranes which is distinct from those of mammalian somatic cells. They are rich in polyunsaturated fatty acids and are very susceptible to attack by ROS. Thiobarbituric acid reactive substances (TBARS) are the more important marker of lipid peroxidation, which has been used to monitor the degree of peroxidative damage in spermatozoa (Januszewski et al., 2005). In our previous study, the increased TBARS content has been noted in sperm cells of infertile men compared with men with preserved fertility (Fafula et al., 2017).

It is known that catalytic activity of the Na^+,K^+-ATPase protein is critically dependent on free SH groups. It was found that control spermatozoa (healthy men with normozoospermia) exhibited Na^+,K^+-ATPase and free -SH content significantly higher than those of as thenozoospermic patients (Vignini et al., 2009). A decrease in the number of free -SH groups suggests that the SH groups may have been oxidized to disulfides by H_2O_2. However, in another study no correlation was found between Na^+,K^+-ATPase activity and SH content (Koçak-Toker et al., 2002). It is also known that different enzyme and non-enzyme antioxidants and some inhibitors of oxidase activity can attenuate ROS mediated inhibition of Na^+,K^+-ATPase activity. Intracellular GSH depletion is capable of sensitizing many types of cells to apoptosis (Tsai-Turton and Luderer, 2006), however, little is known concerning the relationship between intracellular GSH loss and Na^+,K^+-ATPase in sperm cells. Therefore, we tested whether GSH protected H_2O_2-induced inhibition of Na^+,K^+-ATPase. We found that a decrease in Na^+,K^+-ATPase hydrolase activity in H_2O_2-treated sperm cells in men with normozoospermia was largely attenuated by exogenous GSH at 5 mM. This suggests that GSH partially protects the Na^+,K^+-ATPase from inhibition under experimental oxidative stress. Similar results which show protective effects of GSH on Na^+,K^+-ATPase against oxidation were demonstrated earlier (Schulpis et al., 2006). However, administration of GSH only after H_2O_2 treatment does not reverse Na^+,K^+-ATPase inhibition. This result may be explained by assumption that GSH led to recovery of cysteines but not enzyme activity or that inhibition is the result of modifying other aminoacids or not only -SH groups account for the Na^+,K^+-ATPase inhibition by H_2O_2.

Na^+,K^+-ATPase may serve as a molecular target for ROS and signaling via the Na^+,K^+-ATPase by ROS may generate second messengers such as intracellular Ca^{2+} ions. It was shown that ROS-induced changes in intracellular Ca^{2+} appeared to be correlated to its effect on the Na^+,K^+-ATPase. In addition, ouabain and ROS exhibited the additive effect on intracellular Ca^{2+}, suggesting a common target for both stimuli (Liu et al., 2006). It was found that Na^+,K^+-ATPase is sensitive to redox and oxygen status of cell (Bogdanova et al., 2006). Na^+,K^+-ATPase is redox-sensitive with an "optimal redox potential range," where ROS levels out of this "optimal range" are capable of inhibiting Na^+,K^+-ATPase activity (Petrushanko et al., 2006). Inhibition in the effect of ROS on Na^+,K^+-ATPase can be explained by protein modification. In purified renal Na^+,K^+-ATPase, peroxynitrite ($ONOO^-$) causes tyrosine nitration and cysteine thiol group modification of the Na^+,K^+-ATPase (Reifenberger et al., 2008).

Inhibition of sperm Na^+,K^+-ATPase activity could be explained by a direct effect of the exogenous H_2O_2, but could also result from the generation of ROS and reactive nitrogen species (RNS) by damaged spermatozoa. It was shown that mitochondria as the major site for ROS production can contribute significantly to oxidative stress in defective spermatozoa (Koppers et al., 2008). We believe that indirect effect of H_2O_2 is associated with damage to sperm cells, in particular mitochondria disruption causing the mitochondria to leak high levels of free electrons. In turn, damaged mitochondria contribute to the increase in ROS and RNS levels. The results support our assumption that exogenous H_2O_2 should adversely affect sperm motility parameters and corresponds to the results of other studies. Excessive ROS levels have been linked to lipid peroxidation of the sperm plasma membrane, resulting in a loss of membrane fluidity, structure and function (Mahfouz et al., 2009a). Undoubtedly, this study has provided a deeper insight into the role Na^+,K^+-ATPase plays in sperm cells, also could offer clues to the clinical application of antioxidant therapy in male infertility therapy.

Conclusions

H_2O_2, one of the most toxic oxygen species, has the ability to depress Na^+,K^+-ATPase activity in a dose-dependent manner. IC_{50} value for patients with asthenozoospermia was two times less than for healthy men with preserved fertility. These observations suggest that Na^+,K^+-ATPase in pathozoospermic samples is more vulnerable to H_2O_2 induced damage than in normozoospermic men. The Hill coefficient was significantly increased only for patients with asthenozoospermia, indicating increased positively cooperative binding. The decreases in Na^+,K^+-ATPase hydrolase activity in H_2O_2-treated sperm cells in men with normozoospermia were largely attenuated by exogenous GSH at 5mM.

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