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Oxidation of Glyceraldehyde-3-Phosphate Dehydrogenase Decreases Sperm Motility

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Abstract—The relation between the activity of the sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDS) and the motility of sperms was investigated. It was found that the mean value of GAPDS activity in sperm samples with low motility is 2.5-3-fold lower than that in samples with high motility. Sperm motility was shown to diminish in the presence of superoxide anion, hydroxyl radical, and hydrogen peroxide. The decrease in sperm motility in the presence of hydrogen peroxide was proportional to the concentration of the oxidant and correlated with the decrease in GAPDS activity (r = 0.96). Based on the literature data on the importance of GAPDS for the motility of sperms together with the presented observations, it was concluded that the decrease in the sperm motility in the presence of reactive oxygen species is due to the oxidation of GAPDS and inhibition of glycolysis.

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Key words: glyceraldehyde-3-phosphate dehydrogenase, motility, oxidation, reactive oxygen species, spermatozoa

At the current time, approximately 20% of couples are infertile, and in half of such cases the cause of infertility is related to the male reproductive inability. A decrease in the concentration and motility of sperms is observed all over the world. In this connection, investigation of the factors leading to the reduction of the sperm motility and searching for the ways to influence sperm motility are of special importance.

The main cause for the reduction of the sperm fertility is the toxic action of reactive oxygen species (ROS) [1-4]. In moderate amounts, ROS are produced by morphologically abnormal or immobile sperms; however, the main producers of ROS are leukocytes [1, 2, 5, 6]. Enhanced content of leukocytes in the sperm (leukospermia) significantly decreases the sperm motility [7]. Incubation of sperm with purified neutrophils reduces sperm motility in a dose-dependent manner [5, 8]. Thus, it is evident that ROS make a significant contribution into the decrease in fertility. Researchers associate the toxic action of ROS on sperms with peroxide oxidation of sperm membranes and with damage to the sperm DNA [2-4, 9], not considering the possibility of the effect of ROS on the glycolytic enzymes. However, it is known that the motility of sperms is provided mostly by glycolysis [10, 11]. One of the enzymes of glycolysis is the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPD) that catalyzes oxidative phosphorylation of glyceraldehyde-3-phosphate yielding 1,3-diphosphoglycerate. In the cells of different organs and tissues, this enzyme is localized in the cytoplasm, where the reactions of glycolysis occur. An exception is sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDS) that is tightly associated with a component of the cytoskeleton, a fibrous sheath surrounding the axoneme in the principle part of the flagellum [12-15]. The interaction of GAPDS with the fibrous sheath proteins is provided by an additional N-terminal domain that is characteristic for the sperm-specific enzyme [13, 15]. Disturbances in the expression of GAPDS affect the motility of sperms, blocking their progressive movement [16]. Glyceraldehyde-3-phosphate dehydrogenase possesses SH-groups in the active site that are readily oxidized with resulting loss of enzymatic activity. The enzyme from rabbit mus-

Abbreviations: GAPD, glyceraldehyde-3-phosphate dehydrogenase of somatic cells; GAPDS, sperm-specific glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species.

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cles is oxidized by ROS [17, 18]. GAPDS differs from the muscle enzyme by enhanced stability, but it is also easily oxidized by hydrogen peroxide (inactivation rate constant in the presence of hydrogen peroxides is 9 ± 0.3 $M^{-1}\cdot sec^{-1}$) [19]. Thus, one of the causes of the reduction of the sperm motility under the action of ROS can be decrease in GAPDS activity due to the oxidation of the SH-groups of its active site.

The goal of the present work was to investigate of the effect of ROS on sperm motility and the activity of GAPDS and to reveal a possible relationship between the reduction of sperm motility and GAPDS oxidation.

MATERIALS AND METHODS

Chemicals. We used the following chemicals in this work: mouse monoclonal antibodies against GAPDS (Abnova, Taiwan); dithiothreitol (DTT) (Fluka, Germany); diaminobenzidine hydrochloride, EDTA, glycine, glyceraldehyde-3-phosphate diethylacetal, and NAD^+ (Sigma, USA); nickel (II) chloride (Aldrich, USA). The other chemicals were of domestic production.

Samples of human sperm were provided by the Pasteur Medical Diagnostic Center (Moscow). Samples without significant morphological deviations were selected for the investigation.

Samples of frozen equine sperm were provided by the Scientific Research Institute of Horse Breeding of the Russian Academy of Agricultural Sciences (Divovo, Ryazan Region). Directly before the experiment the samples were thawed in a thermostat at 37°C.

Determination of sperm motility. A sperm sample was incubated for 30 min in a thermostat at 37°C. Then a necessary volume was taken from the original sample and diluted twice with phosphate buffered saline (PBS: 10 mM potassium-phosphate buffer, 0.2 M NaCl, pH 7.4). The sperm motility was determined using a Goryaev chamber. The number of sperms with progressive motility and the total number of sperms were counted in four different squares, and the average values of these parameters were calculated. The motility of the sample was determined as the percentage of the cells with progressive motility relative to the total number of cells.

Determination of GAPDS activity in sperm samples of different motility. Sperms were washed with PBS three times: the cells were suspended in the buffer, centrifuged at 1500*g* for 15 min, and the supernatant was removed. The pellet (50-100 μl) was suspended in an equal volume of 10 mM potassium-phosphate buffer, pH 7.4, and broken by sonication. The resulting suspension was centrifuged (14,000*g*, 10 min). The broken-cell pellet was suspended in 50-100 μl of 10 mM potassium-phosphate buffer, pH 7.4, and immediately after that the activity of GAPDS in the sample was measured. The activity was determined by the rate of accumulation of NADH that was produced during the oxidation of glyceraldehyde-3-phosphate (G-3-P) at 20°C. The reaction mixture (1 ml) contained 50 mM glycine, 50 mM potassium phosphate, 5 mM EDTA, 0.5 mM NAD^+, 0.5 mM G-3-P, pH 8.5, and 10-20 μl of the tested suspension. The reaction was started by the addition of G-3-P.

The unit of activity was determined as the amount of the enzyme catalyzing the reduction of 1 μmol NAD^+ per min. The activity was calculated per ml of the original sperm sample or per mg of total protein.

Protein concentration in the suspension of broken sperms was determined by the Bradford method [20].

Concentration of hydrogen peroxide was determined spectrophotometrically at 240 nm using molar absorption coefficient 39.4 $M^{-1}\cdot cm^{-1}$ [21].

Investigation of effect of hydrogen peroxide on sperm motility and activity of GAPDS. A sample of sperm was diluted twice with PBS and divided into two parts: one sample served as the control, and the other sample was supplemented with hydrogen peroxide. The samples were incubated in a thermostat at 37°C for 2 h, and then the sperm motility in the samples was determined. After that, the hydrogen peroxide in the samples was neutralized by the addition of equimolar concentration of DTT, and the sperms were washed free from DTT with PBS. The washed cells were broken by sonication, and the activity of GAPDS in the broken-cell pellet was determined as described above.

Immunoblotting of sperm samples. After breaking sperms by sonication, the cell pellet and the extract were diluted twice with the sample buffer for SDS-PAGE and heated at 90°C for 5 min. SDS-PAGE was run according to the standard procedure. Proteins were transferred from the gel to a nitrocellulose membrane (Sigma) using transfer buffer (25 mM Tris, 192 mM glycine, 10% ethanol, pH 8.6) (100 V, 45 min). After the transfer, the membrane was blocked with PBST (10 mM KH_2PO_4, 150 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% skim dry milk for 30 min. After washing with PBST, the membrane was incubated with a solution of mouse monoclonal anti-GAPDS antibodies (Abnova; 1 μg/ml). The secondary antibodies were peroxidase-conjugated anti-mouse antibodies (1 μg/ml). After incubation with the secondary antibodies (1 h), the membrane was washed with PBST and developed in a solution containing 3 mg of diaminobenzidine hydrochloride, 30 mg of nickel chloride, and 10 μl of 30% H_2O_2 in 10 ml of 0.1 M Tris-HCl, pH 7.6.

Generation of reactive oxygen species. Ferrous ions auto-oxidize in the presence of oxygen yielding ROS. This process is described by the Haber–Weiss reactions [22]:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^{\cdot} + OH^-.$$

EDTA significantly accelerates auto-oxidation of ferrous ions [23]. So, to produce superoxide anion and hydroxyl radical, we used solutions containing $FeSO_4$/EDTA (final concentrations, 0.1 mM/1 mM) and $FeSO_4$/EDTA/H_2O_2 (0.1 mM/1 mM/0.1 mM), respectively.

RESULTS AND DISCUSSION

To reveal the relationship between the activity of GAPDS and sperm motility, the activity of GAPDS was determined in sperm samples with various motility. Human and equine sperm was used in the investigation. Considering that GAPDS, in contrast to somatic enzyme GAPD, is bound to the cytoskeleton of the flagellum, it cannot be extracted from the cells with buffer solutions. After breaking the cells by sonication, almost all GAPDS activity remained in the insoluble fraction (broken-cell pellet) [12]. These data are supported by the results of immunoblotting (Fig. 1).

As seen from Fig. 1, in the pellet of broken sperms the major band that is stained with the antibodies against human GAPDS corresponds to ~56 kDa (Fig. 1, lane *2*). These results agree with the data on the mobility of GAPDS in a polyacrylamide gel, according to which full-length human GAPDS (molecular weight of the monomer is 44.5 kDa) yields a band of ~56 kDa during SDS-PAGE [13]. The bands of lower molecular weight are due to the proteolysis of the full-length GAPDS. In the extract, the content of GAPDS is significantly lower (Fig. 1, lane *1*).

Based on these data, the activity of GAPDS was determined in the insoluble cell fraction obtained after breaking the sperms by sonication and subsequent centrifugation. The soluble fraction was discarded, this excluding the possibility of the contamination of the samples with somatic GAPD that is contained in the leukocytes and in bacterial cells.

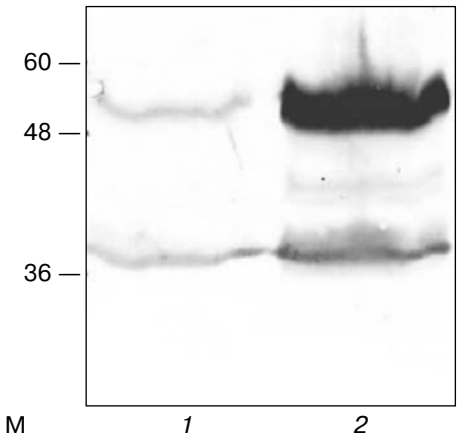

Fig. 1. Assay of different sperm fractions for GAPDS by immunoblotting. M, protein standards (kDa); *1*) extract; *2*) broken-cell pellet.

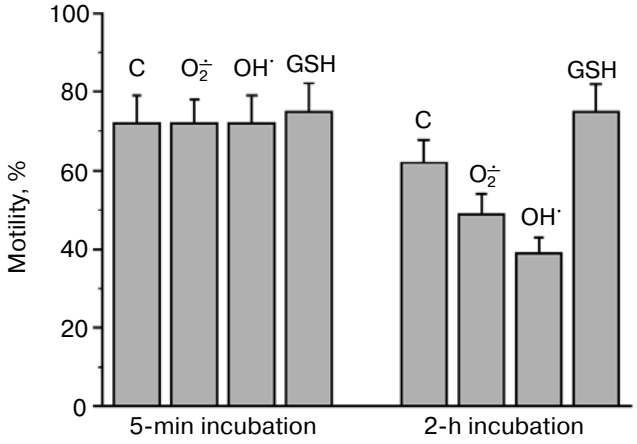

Fig. 2. Effect of reactive oxygen species and glutathione on the motility of human sperm. Sperms were washed in PBS and suspended in the same buffer. The samples were incubated with sources of ROS or 1 mM glutathione at 37°C, and after the indicated time the motility of the sperms was determined.

For the investigation, we selected sperm samples with normal morphology but differing in motility. The samples were conditionally divided into groups with high and low motility. In the case of human sperm, the group with the low motility included samples containing 6-30% of motile sperms, and the group with the high motility included samples containing 40-55% motile sperms. In the case of stud-horse sperm, the differences in the motility in the samples were less pronounced, so the first group included samples of 10-15% motility, and the second one included samples of 20-25% motility. In each group the mean value of GAPDS activity was determined. The results of this investigation are presented in the table.

The presented data indicate that the mean value of GAPDS activity in the groups with the low motility is 2.5-3 times lower than that in the groups with the high motility. In the case of the human sperm samples, the differences in the GAPDS activity in the groups with low and high motility are statistically significant according to Student's *t*-test ($p < 0.01$). The lower statistical significance ($p < 0.05$) of the differences in the GAPDS activity in the groups with low and high motility in the case of equine sperms is likely due to the less pronounced difference in the sperm motility in the investigated groups.

Thus, it was demonstrated that in the groups with low sperm motility, GAPDS activity is decreased. This observation agrees with the assumption according to which the sperm motility reduces due to decrease in GAPDS activity.

The activity of GAPDS is likely to decrease due to the oxidation of SH-groups of the enzyme by reactive oxygen species. To test this assumption, the effect of ROS

Relationship between sperm motility and activity of GAPDS

Source of sperms					
human			horse		
low motility (6-30%), μmol NADH/ml sperm		high motility (40-55%), μmol NADH/ml sperm	low motility (10-15%), μmol NADH/ml sperm		high motility (20-25%), μmol NADH/ml sperm
1	0.177	0.34	1	0.038	0.49
2	0.49	0.34	2	0.09	0.045
3	0	0.16	3	0.039	0.078
4	0.102	0.12	4	0.068	0.075
5	0.013	0.22	5	0.05	0.148
6	0.09	0.192	6		0.1
7	0	0.7	7		0.12
8	0	0.52			
9	0.024				
	0.1 ± 0.05*	0.32 ± 0.07*		0.057 ± 0.01**	0.15 ± 0.06**

Note: Data are presented as mean value of several independent experiments ± SE: *$p < 0.01$; ** $p < 0.05$ (according to Student's t-test).

and the natural antioxidant glutathione on sperm motility was studied.

As shown in Fig. 2, incubation of sperms in the presence of sources of superoxide anion and hydroxyl radical for 2 h decreased the sperm motility by 15-25% compared to the sample without additions, which illustrates the effect of ROS on the sperm motility. In the sample without additions, the motility decreased after 2 h by approximately 10%, which can be explained by the presence of leukocytes in the tested samples, as well as by the fact that the sperms produce ROS in small amounts [24]. This is confirmed by the fact that the addition of glutathione into the incubation mixture prevents decrease in the sperm motility during 2-h incubation (Fig. 2).

In contrast to the systems producing superoxide anion and hydroxyl radical, the action of H_2O_2 on the sperm motility was pronounced after 5 min of incubation (Fig. 3). Thus, hydrogen peroxide affects the sperm motility more strongly than other ROS. It should be noted that in different sperm samples the sensitivity to hydrogen peroxide can significantly differ. For example, in our experiments the concentration of H_2O_2 that was necessary for 100% loss in the motility varied from 0.5 to 7 mM. Such differences are explained by the fact that sperms and the seminal plasma possess a defensive system that controls ROS concentration. This system includes enzymes (superoxide dismutase, catalase, glutathione reductase) and a number of antioxidants (albumin, glutathione, pyruvate, vitamins E and C). Defensive abilities of the seminal plasma significantly vary [7], so at similar concentrations of oxidants the effect can be pronounced to different extent.

Thus, the following observations were made: 1) the activity of GAPDS is low in samples with low content of motile sperms; 2) the content of motile sperms decreases in the presence of ROS, and glutathione maintains the motility at a constant level. These data agree with the assumption that the reduction in sperm motility is due to the oxidation of SH-groups of GAPDS by ROS. To test this assumption, the sperms were incubated in the presence of different concentration of hydrogen peroxide, and then their residual motility was determined. After that, the hydrogen peroxide was removed, sperms were

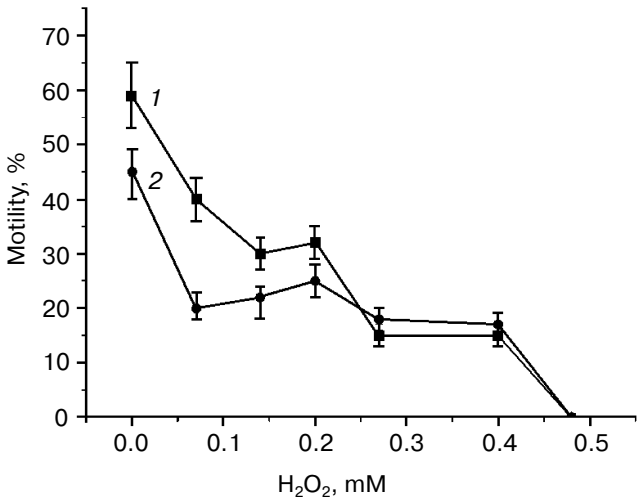

Fig. 3. Effect of hydrogen peroxide on motility of sperms. Sperm motility was determined after 5 min (*1*) and after 2 h (*2*) of incubation at 37°C.

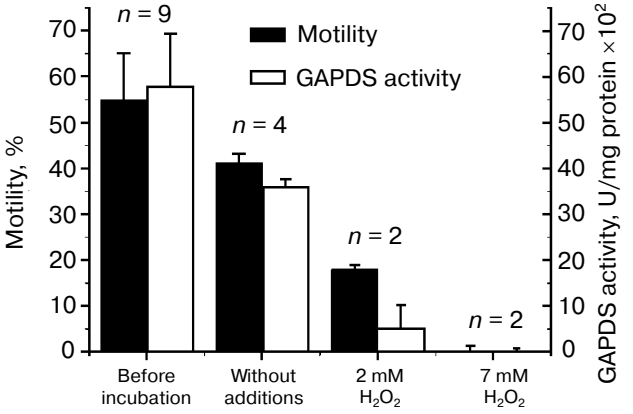

Fig. 4. Relationship of the sperm motility and GAPDS activity after incubation of sperm samples in the presence of hydrogen peroxide. Data are presented as the mean value of several independent experiments ± SE.

broken by sonication, and the activity of GAPDS was determined in the resulting suspension. Figure 4 demonstrates the relation between the sperm motility and GAPDS activity in fresh samples, after 2 h of incubation without additions, and after incubation in the presence of different concentration of hydrogen peroxide. As seen from the figure, the activity of GAPDS drops with the decrease in sperm motility. No GAPDS activity was found in the sample with zero motility. Positive correlation is observed between the activity of GAPDS and the motility of the sperms ($r = 0.96$).

Taking into consideration that the disruption of the *gapds* gene results in the loss of the sperm motility [16], our data on the inactivation of recombinant GAPDS by hydrogen peroxide [19], and the fact that hydrogen peroxide at rather high concentrations (up to 5 mM) has virtually no effect on the activity of other glycolytic enzymes [25], the presented results indicate that the reduction of the sperm motility in the presence of hydrogen peroxide is due to the oxidation of GAPDS.

The source of hydrogen peroxide in the organism is superoxide anion that is produced by neutrophils. Superoxide anion is easily transformed into hydrogen peroxide during the dismutation reaction catalyzed by superoxide dismutase. So, the conclusion can be extended as follows: sperm motility decreases in the presence of ROS due to the oxidation of GAPDS and inhibition of glycolysis that is necessary for the sperm motility. It should be noted that the pronounced oxidation of GAPDS and significant decrease in sperm motility indicates that the antioxidant defensive system of the sperms is inefficient, and consequently, the genome DNA can be damaged by ROS. Thus, the decrease in the sperm motility can be considered as a natural biological barrier preventing the fertilization of an egg cell by a sperm with a damaged genome. Consequently, efforts to increase sperm motility must be directed first to the elimination of the causes leading to enhanced ROS production (avitaminosis, intoxication of the organism, inflammatory diseases of the urogenital tract).

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