An Investigation of the Effectiveness of Certain Antioxidants in Preserving the Motility of Reactivated Bull Sperm Models

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ABSTRACT

Bull sperm that had been disrupted by freezing and thawing were reactivated with 1 mM Mg-adenosine 5'-triphosphate. The antioxidants superoxide dismutase, catalase, dithiothreitol, and reduced glutathione (GSH) were tested for their ability to prolong the motility of the reactivated sperm. GSH was employed both by itself and as part of a reducing system that maintained the tripeptide in the reduced form. Three of the test agents were found to increase the duration of motility in the sperm preparations; these were reduced glutathione, dithiothreitol, and superoxide dismutase. Glutathione was the most effective protective agent, yielding reactivated preparations with a half-life for the decay of motility of 2.5 h. While dithiothreitol (DTT) is widely employed as an antioxidant, we found that DTT is measurably less effective than glutathione (half-life of 1.5 h). In spite of glutathione’s effectiveness in preserving motility, we have found, by direct assay, that mature bull sperm do not contain detectable amounts of this common biological antioxidant. Our results support the hypothesis that oxidative damage contributes to the loss of motility in reactivated sperm and suggest that oxidation could be a factor in motility loss in living sperm.

INTRODUCTION

In all living cells that utilize oxygen for aerobic respiration, the potential for damage to the cell from highly reactive oxygen radicals is present. As a consequence of this, cells that must survive for long periods in an oxygen-containing environment have internal mechanisms to protect themselves from oxidation (for review, see Meister, 1983). Sperm cells must be motile to carry out their reproductive function. Their motility is dependent on an enzyme, dynein, that interacts with tubulin and adenosine triphosphate (ATP) to generate force. Evidence that this force-producing mechanism is susceptible to destruction through oxidation was indicated by results from studies using membrane-disrupted sperm. If sperm are detergent-extracted with Triton X-100 to remove the cell membrane, motility can be restored in the cells with Mg-adenosine 5'-triphosphate (ATP) (Gibbons and Gibbons, 1972; Lindemann and Gibbons, 1975). However, it was noted in these early studies that motility lasts longer and is of better quality if an antioxidant such as dithiothreitol (DTT) is included in the reactivating mixture, and this expedient has been carried over into virtually all further studies with membrane-disrupted sperm. In this study, we examine the relative protective effects of three antioxidants in addition to DTT on the motility of membrane-disrupted sperm models. We chose to examine the effects of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase, all antioxidants of biological importance. We used sperm models produced by the freeze-thaw method reported previously from our laboratory (Lindemann et al., 1982, 1983). Frozen-thawed sperm were best suited to the present study because models prepared by this method last much longer than Triton X-100-extracted sperm models and show less evidence of proteolytic digestion, which is a separate cause of motility loss. The possible significance of our findings to intact sperm is also considered.

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MATERIALS AND METHODS

Sperm Collection and Storage

Bull sperm for these experiments were obtained through the cooperation of NOBA, Inc. (Tiffin, OH). Raw semen was diluted to $75 \times 10^6$ cells per ml with a tris(hydroxymethyl) amino (Tris)-extender containing 2% egg yolk. Diluted sperm were shipped on ice and arrived, the sperm were stored at 0–5°C until they were used for experiments. All sperm were used or frozen in liquid nitrogen within four days of receipt.

Preparation of Sperm

Bull sperm were removed from the extender before use. Two ml of the sperm-extender solution were added to 8 ml of citrate-buffered solution containing 0.1 M sodium citrate, 5 mM MgSO$_4$, 2 mM fructose, and 1 mM CaCl$_2$, pH 7.4. This mixture was centrifuged for 10 min at 1500 rpm in a cold room (0–5°C). The supernatant was discarded, and the pellet was resuspended in 10 ml of citrate buffer and centrifuged once again. The supernatant was again discarded, and the pellet was a) resuspended in 1 ml of 0.05 M ethylenediaminetetraacetic acid (EDTA) for analysis of GSH content, or b) resuspended in 1 ml of citrate buffer and used for motility experiments.

GSH Content Analysis

Washed sperm in 0.05 M EDTA were counted with the aid of a hemacytometer. A 100 volume dilution of the sperm sample was generally necessary. The diluted solution was used to fill the counting chamber, ten separate squares of the grid were counted, and the average of these was taken. The sample was shaken before dilution, and the diluted sample was shaken before the counting chamber was filled. Sperm counts were determined by the following formula: 

$$250,000 \times \text{dilution factor} \times \text{average count per square} = \text{sperm per ml}. $$

For the final determination of cell density used to set the upper limit of GSH per cell, the cell count was repeated three times to minimize the counting error. The sperm sample was then lysed with 10% trichloroacetic acid and supernatant analyzed for the presence of GSH and oxidized glutathione (GSSG) on a modified Phoenix automatic amino acid analyzer (Phoenix Precision Instrument Co., Philadelphia, PA). Detection on this instrument is reliable down to 2 nmoles/ml when 0.5 ml of sample is applied.

Preparation of the GSH-Reducing Medium

Experiments were done both with GSH alone and as part of a reducing system to keep glutathione in the reduced state and to observe the oxidation rate during motility experiments. The GSH-reducing medium was prepared in 25 ml of glutamate-Tris buffer with 1 mM MgSO$_4$. GSH, glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate (NADP) were dissolved separately in 0.4 ml aliquots of the glutamate-Tris buffer, and the pH of each was adjusted to 7.4. These aliquots were added, in turn, to the 25 ml of glutamate-Tris buffer to produce concentrations of 2 mM GSH, 2 mM G6P, and 0.1 mM NADP in the final mixture. To this mixture was added 5.2 units of glutathione reductase (GR) and 4.6 units of glucose-6-phosphate dehydrogenase (G6PD), which had been dialyzed overnight against 2 mM EDTA. The activities of GR and G6PD were determined by measuring the change in absorbance of NADP at 340 nm (Bergmeyer, 1974). The medium was assayed spectrophotometrically at this point to determine the concentrations of GSH and G6P. The concentration of GSH was determined with 5,5'-dithiobis(2-nitrobenzoic acid) (Sedlak and Lindsay, 1968). G6P content was determined by measurement of reduction of NADP to NADPH (Bergmeyer, 1974). This medium then made up the 1.5 ml of glutamate-Tris buffer into which 1.5 ml of frozen sperm sample was thawed for the motility experiments. Just before the thawed sample was added, 5 units of glutathione peroxidase were included in the reducing medium. Assays to determine GSH and G6P content were run throughout the motility experiments. This was accomplished by making duplicates of the samples containing reducing medium; one of these was carried to the spectrophotometer for assay while the other sample remained at the microscope.

Motility Experiments

Washed bull sperm were utilized in the motility assays. Sperm were kept on ice during all preparations. Sperm models were produced by the method of Lindemann et al. (1983). Briefly, 50 µl of the washed sperm in citrate buffer were suspended into 1.5 ml of glutamate-Tris buffer (0.2 M sucrose, 0.036 M potassium glutamate, 1 mM MgCl$_2$ buffered with 0.03 M Tris-HCl, pH 7.9). To approximately one-fifth of the samples, 1 mM DTT was added. Samples were placed in 1.5-ml plastic microcentrifuge tubes and frozen in liquid nitrogen for at least 10 min. All samples were
used within 3 wk of freezing. At the time of use, samples were thawed at room temperature into an additional 1.5-ml aliquot of the glutamate-Tris buffer containing a) nothing else, b) 2 mM DTT, or c) a set concentration of another reducing agent. The c) category consisted of 325 units of SOD, or 12,000 units of catalase, or 2 mM GSH with or without the constituents of the GSH-reducing system. All samples were thawed into and observed in 5-ml culture dishes with covers. Rough counting grids were scratched into the bottoms of these dishes for later ease in making observations.

The protocol used to quantify motility is essentially that developed for Triton X-100-extracted sperm in an earlier study (Lindemann, 1978) and later adapted for frozen-thawed sperm models (Lindemann et al., 1983). The protocol was adapted to the current study as follows. Each sample was observed under the microscope upon thawing for the absence of motility. If the cells were indeed still, 1 mM ATP (pH 7.9) was added to each sample. The samples were incubated for 5 min while they warmed at room temperature. Counts were taken on the percentage of motile sperm and on the number of flagellar beats per 10 s at this point. Percentage of motile sperm, or percent motility, is defined as the percentage of sperm in the sample that shows flagellar movement. Five squares of the makeshift grid were counted for percentage of motile cells each hour. Flagellar movement was also quantified by counting the frequencies or beats of the flagella per 10 s. One beat is defined as the full cycle of movement of the flagella from one side to the other and back again. Frequencies were counted by examining every motile sperm on a single line on the grid of the dish. Ten cells were counted in each sample every hour. The order in which the sperm samples were observed was rotated each hour to prevent introducing a systematic bias into the results. Each experimental condition was repeated with a number of different sperm samples; the number of separately repeated experiments is given as the n value in all of our plotted data. Samples were kept under an overturned box when not being observed under the microscope to minimize oxidation from ultraviolet light from the fluorescent lighting in the room.

A second series of experiments was run to assess the selective killing effects of hydrogen peroxide and superoxide anion. For these experiments, dishes were set up as above, but 0.1 mM H$_2$O$_2$ was added after initial motility was established. Alternatively, 1000 units of catalase followed by a dose of KO$_2$ in DMSO was added. Measurements of percentage of moving cells was then followed at 5-min intervals. Videotape recordings were made of each experiment, and counts were taken from the videotape records at a later time; this facilitated working with several samples in a 5-min timing regime. The dose of superoxide anion actually delivered was determined by measuring H$_2$O$_2$ production in the test medium with an H$_2$O$_2$ analyzer (YSI Model 27 analyzer, Yellow Springs Instrument Co., Yellow Springs, OH). The assay was done with 400 units of SOD present and without catalase. The dose of saturated KO$_2$ in DMSO needed to generate 0.1 mM H$_2$O$_2$ was 60 μl into 3 ml of reactivation solution; this was the dose selected for the motility tests. The addition of 1000 units of catalase eliminated all H$_2$O$_2$ from the preparation in less than a minute. DMSO did not have an adverse effect on motility when added alone.

Reagents

All the reagents were obtained through Sigma Chemical Co., St. Louis, MO, except for glutathione peroxidase, which was from Calbiochem-Behring Corp., La Jolla, CA. All solutions were prepared with distilled and deionized water.

RESULTS

Figure 1 shows the percentage of motile sperm as a function of time after reactivation with 1 mM Mg-ATP. The motility of sperm in the samples without any antioxidant can be used as the baseline for comparison of the other samples. It is clear from the graph that glutathione was the most effective protective agent, allowing motility to persist for over 2 h before declining to 50% of the initial value. DTT, which has long been used as the standard antioxidant in sperm model preparations, was less effective than glutathione. When the data at the 2-h measurement was subjected to a small sample test for differences between means, the difference between the DTT and GSH samples was significant to the level of p<0.001. While superoxide dismutase appeared to have some protective effect, the data were not sufficient to establish significance, and catalase had no beneficial effect by any measure. For both the DTT- and GSH-containing samples, the protective effect—when compared to time-matched controls—was highly...
significant \( p<0.001 \) for DTT and GSH vs. controls at 1 h and \( p<0.001 \) for GSH vs. controls and \( p=0.01 \) for DTT vs. controls at 2 h.

The beating frequency of the sperm flagellum is another measure of normal functioning of the flagellar apparatus. Flagellar-beating frequency was measured for the two most successful protective agents, DTT and GSH, and compared to controls without antioxidant; these results are shown in Figure 2. Using frequency as a measure of motile function, we once again observed a clear protective effect for both DTT and GSH. Furthermore, GSH out-performed DTT as a protective agent by this criterion as well.

Since GSH is readily oxidized by superoxides and peroxides, it was necessary to ascertain the degree of oxidation of GSH that occurred under the experimental conditions used for reactivation of motility. Figure 3 shows the concentrations of GSH and G6P as a function of time in sperm samples prepared with the enzymatic recycling system for GSH. The concentrations shown were determined from absorbance measurements, and the points plotted are the mean values for five trials. The graph shows that the GSH reducing system maintained a nearly constant level of GSH in the medium. G6P utilization continued throughout the experiment and provided an indication of the rate of oxidation. G6P utilization under these conditions was sufficient to account for the expected recycling of oxidized glutathione. Although further motility tests using 1 mM GSH without the enzyme system indicated that GSH alone was sufficient to protect sperm for up to 3 h, it is perhaps useful to note that the recycling system in the GSH-reducing medium we employed can be used to extend the protective effect for longer periods and at higher temperatures. It is also noteworthy that good preservation of motility was obtained in the presence of the complete enzymatic recycling system.

The impressive performance of GSH as an antioxidant in the sperm preparations prompted us to look for GSH in the intact bull sperm. GSH is a common cell constituent present in a wide variety of tissues and therefore could play a role as a physiologically important antioxidant. We found that at the highest concentrations of bull sperm we could prepare, it was not possible to detect either reduced or
oxidized glutathione. Figure 4 shows the output of the amino acid analyzer for three concentrated samples of bull sperm suspended in 0.05 M EDTA. The peaks corresponding to urea and aspartate that normally flank the glutathione peak are large and well defined, but no trace of glutathione or its oxidized product GSSG can be found. At the cell density of the preparation used, our results established an upper limit on glutathione of $1.7 \times 10^{-6}$ pmoles per cell. This translates to a cytoplasmic concentration of no more than 0.05 mM. Using the same technique, we were able to detect glutathione easily in other tissues, including testis, retina, lens, and blood (lens is shown for comparison in Fig. 4). Bull sperm appeared to be lacking in this ubiquitous and highly effective antioxidant.

In an attempt to shed light on the probable mechanism of damage, hydrogen peroxide and potassium superoxide were added to motile samples of frozen-thawed sperm and motility was recorded. The results of these experiments are displayed in Figure 5. Samples with 1 mM DTT and samples with 1 mM GSH are compared to samples with no protective agent in this experimental series. No difference was found between the two antioxidants in terms of their ability to defend against $H_2O_2$ insult; both were significantly effective ($p<0.01$) when compared to time-matched controls at 10 min. Neither agent was as effective against $KO_2$, but DTT did show a statistically significant protective effect ($p<0.05$) compared to matched controls at 10 min, whereas GSH did not. The damage done to the samples by $KO_2$ held up well to statistical examination. A one-tailed $t$-test for dependent samples was used to analyze the decline in
motility. The drop immediately upon addition of KO₂ was significant to the level of p<0.001 for the DTT-protected samples, to the level of p<0.05 for the GSH samples, and to the level of p<0.005 for both superoxide-treated samples taken together. The mechanism of damage from superoxide cannot be attributed to hydrogen peroxide production for two reasons. Firstly, all motility data displayed for KO₂-treated samples were gathered in the presence of 1000 units of catalase, an amount sufficient to degrade all the H₂O₂ generated in less than 1 min. From the H₂O₂-treated experiments, it is certain that 1-min exposure to 0.1 mM H₂O₂ is not sufficient to cause the reduction of motility seen with KO₂. Secondly, the course of motility loss is dramatically different in the KO₂-treated and H₂O₂-treated samples. The motility in the GSH and DTT samples treated with KO₂ drops in 2–3 min to a level of motility that is not reached for 30 min in the H₂O₂-treated samples. The pooled data from the GSH and DTT runs (n=12) were used to compare the H₂O₂- and KO₂-treated samples at 10 min after reactivation. The difference in motility between KO₂- and H₂O₂-treated samples is significant to the p<0.010 level.

**DISCUSSION**

GSH appears to be an effective protective agent to retard motility loss in membrane-disrupted sperm models. In this capacity, it is clearly superior to DTT and will substantially improve the longevity of sperm models for experiments that require longer periods of observation. This superiority may also extend to other isolated enzyme systems that currently employ DTT. GSH can be kept in the reduced state by using the mixed enzyme system described and adapted here for reactivated sperm models. This feature of the glutathione oxidation-reduction mechanism can be used to maintain antioxidant activity for long periods or at high temperatures. This could be a useful strategy to allow in vitro studies of enzymatic reactions to be conducted at 37°C.

The course of oxidative damage in sperm models did not appear to respond at all to catalase, which we
added at very high levels of activity. There was a small, albeit not statistically significant, response to superoxide dismutase. This raised the issue of which agent, hydrogen peroxide or superoxide anion, is the main source of oxidative insult. In an attempt to observe directly the toxicity of these two agents, we found that both can cause a deterioration of motility in the sperm models. We also found that at matched dosages superoxide is demonstrably more toxic than hydrogen peroxide and acts by a much more rapid mechanism. We can not prove that superoxide itself is the damaging agent, since other reactive species may be formed as intermediates, but our results do rule out hydrogen peroxide as the damaging agent when KO2 is added. Therefore, either superoxide anion itself, or some product formed from it, must be more toxic than hydrogen peroxide. In light of these results, it is likely that superoxide may participate in the oxidation of the motile apparatus and may be a more important factor than H2O2 in causing damage. Therefore, creation of peroxides from superoxides, as would occur in the presence of superoxide dismutase, may represent an important detoxification step. Other investigators have reported the presence of this enzyme in mammalian sperm (Abu-Erreish et al., 1978; Nissen and Kreysel, 1983).

These findings are also consistent with the reports of Alvarez and Storey (1982, 1983, 1984), who concluded that sperm are selectively sensitive to superoxides and have superoxide dismutase to protect against oxidation. Our results add a new dimension in that the effects we observe are likely to be mediated directly on the axoneme since the sperm we used were disrupted. Therefore, in addition to membrane peroxidation, dynein deactivation may also contribute to motility loss as oxidation occurs in sperm cells.

We could find no GSH in the intact mature sperm. This result extends the findings of an earlier study of GSH levels in sperm using a different assay procedure (Li, 1975), which showed that rat and boar sperm also contain little or no GSH. This fact, combined with the demonstrated efficacy of GSH as a protective agent for motility, raises the unavoidable question of why it would be lacking in sperm but present in virtually all other cell types. It is possible that some sperm may lose their glutathione as part of a scheme for planned senescence. Our present findings are consistent with that hypothesis. If this is the case, inclusion of membrane permeable antioxidants might be expected to increase the percentage of live cells in samples destined for storage in sperm banks and increase the longevity of sperm used for artificial insemination after thawing, as has been suggested previously by the work of Mounib (1978). It might also aid in understanding why GSH is found in the secretions of the male tract (Daunter et al., 1981; Pond et al., 1983) where sperm must be stored for considerable periods of time.

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REFERENCES