Superoxide dismutase content in sperm correlates with motility recovery after thawing of cryopreserved human spermatozoa

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Objective: To investigate the correlation between sperm superoxide dismutase (SOD) content and motility recovery after thawing of cryopreserved human sperm, based on the rationale that this antioxidant enzyme provides protection against reactive oxygen species-induced damage during cryopreservation.
Design: Prospective study.
Setting: Private infertility institute and university-based research laboratory.
Patient(s): Forty-two consenting normozoospermic patients consulting for infertility.
Intervention(s): The SOD content was measured in sperm from unfractionated samples and in sperm recovered from the pellet fraction obtained after discontinuous density gradient centrifugation.
Main Outcome Measure(s): Sperm motility was evaluated post-thaw in the two sets of samples and motility recovery was plotted against the sperm SOD content to determine their correlation.
Result(s): There was a significant positive correlation between motility recovery after thawing and SOD content in sperm from the 90% gradient pellet containing highly purified mature sperm. There was also a significant negative correlation between motility after thawing and SOD content in the unfractionated sample.
Conclusion(s): The positive correlation between post-thaw motility recovery and SOD content in mature spermatozoa provides a good predictor of post-thaw motility recovery after cryopreservation. (Fertil Steril® 2012;97:293–8. ©2012 by American Society for Reproductive Medicine.)
Key Words: Human sperm, sperm cryopreservation, superoxide dismutase, sperm motility, reactive oxygen species

Human sperm cryopreservation has proved to be a useful procedure, particularly in infertility clinics, both for diagnosis of possible male factor and to provide samples for assisted reproductive technologies (ART). But sperm cryopreservation is not hazard-free. During cryopreservation, human spermatozoa are exposed to membrane stress (1), changes in lipid composition of the sperm plasma membrane (2), and damage induced by reactive oxygen species (ROS; 3–5). Mammalian sperm are highly susceptible to lipoperoxidative attack by ROS due to high content in the plasma membrane of polyunsaturated fatty acids, in particular docosahexaenoic acid (6–8). Lipid peroxidation reactions induced by ROS result in membrane-bound phospholipid damage leading to increased membrane permeability, loss of intracellular adenosine-5′-triphosphate (ATP), and ultimately, to sperm motility loss (4).

Sperm motility in semen is readily observed with microscope and quantified as percent of cells moving in the first instance, with vigor of movement also reported. The recent advent of computer-assisted sperm motion analysis has made possible a far more complete description of sperm movement and flagellar beat pattern. Recovery of motility post-thaw after cryopreservation of an individual sperm sample, compared with motility of the fresh prefreeze sample, offers a rapid, inexpensive, and quantitative index of cryodamage of the sperm under analysis (9, 10). This index is obtained only post-thaw; it offers no predictability as to susceptibility of the sperm in the sample to cryodamage.

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We have recently reported that thawing of cryopreserved human sperm at 40°C results in a statistically significant increase in post-thaw motility recovery compared with thawing at 37°C (11). This increase in motility recovery appears to be related to a more rapid recovery of antioxidant enzyme activity during thawing at 40°C, which suggests that the sperm cell's antioxidant enzyme defense system could provide the desired predictor of post-thaw viability. Antioxidant enzyme activities of mammalian spermatozoa comprise those provided by superoxide dismutase (SOD) and the glutathione/glutathione reductase/glutathione peroxidase system (GPX). Comparison of these two activities in rabbit, mouse, and human sperm has revealed major differences in the contribution of these two systems among species (12). Rabbit sperm, for instance, have robust SOD activity as antioxidant defense but negligible GPX system activity. However, mouse sperm have the GPX system as the major antioxidant defense enzymes. Human sperm use both SOD and GPX, the SOD activity being sensitive to cyanide inhibition, as expected for the Cu/Zn enzyme isoform (4). The GPX activity showed little variability but the SOD activity showed marked variability between individual samples (4). The human sperm samples used in those studies were optimized for high percentage of mature motile cells. The time observed for complete loss of motility due to lipoperoxidative damage for a given human sperm sample under a standard set of experimental conditions correlated strongly with the SOD activity of that sperm sample. This raised the question as to whether assay of SOD activity of the cells in a given unfractionated human sperm sample could be a valid marker for resistance to cryodamage in the context of samples being analyzed in an infertility clinic. Sperm samples in that setting have cells of varying maturity (13). We have previously reported that immature sperm subsets in a given sample display the highest content of SOD, a finding that may be related to proximal cytoplasm retention and increased hydrogen peroxide production (14). The analysis of sperm subsets in that study was accomplished by a density gradient fractionation technique that yielded a fraction, F4, of maximum density and lowest sperm SOD content, corresponding to essentially fully mature sperm. The sperm in F4 should thus be fully comparable to the sperm optimized for high motility in the studies described previously.

From these considerations, one would predict that increased resistance to cryodamage, as assayed by motility recovery post-thaw, should correlate with increased SOD activity in the F4 or equivalent sperm fraction. Conversely, one would predict that the unfractionated sample containing a substantial percentage of immature cells should show the inverse correlation, as increased SOD activity in those samples is a measure of content of less mature sperm cells with lower resistance to cryodamage. The study of Lasso et al. (15) demonstrated that SOD enzymatic activity and SOD content as measured by immunoassay were tightly correlated, allowing the use of the far more convenient immunoassay to be used as surrogate for enzymatic activity.

The objective of this study was to test this hypothesis by comparing the correlation between SOD content and motility recovery post-thaw of cryopreserved human sperm in whole samples and in the F4 or equivalent fraction from the sperm samples obtained by density gradient centrifugation. The results indicate that SOD content of the latter samples can provide a marker for prediction of post-thaw motility recovery. The SOD content of the unfractionated samples, on the other hand, provides an estimate of the immature cell content of the sample, thus adding support to our hypothesis.

MATERIALS AND METHODS

Semen Collection

Semen samples were obtained from 42 normozoospermic men who were candidates to become sperm donors. The age of the patients ranged between 20 and 30 years. The study protocol was approved by the Laboratorio de Estudios en Reproduccion (Buenos Aires, Argentina) Institutional Review Board. Semen collection was carried out by masturbation after 3–5 days of sexual abstinence, and the semen specimen was analyzed within 1 hour after ejaculation. Semen samples were allowed to liquefy at room temperature for 30 minutes. After liquefaction, sperm concentration and percent motility were measured by computer-assisted sperm motion analysis to characterize each sperm suspension used in subsequent experiments.

Motility Parameters

Aliquots of 5 μL of the sperm suspension were placed into a Makler chamber prewarmed at 37°C. Computer-assisted sperm motion analysis was performed using a Hamilton-Thorne digital image analyzer (HTR-IVOS v.10.8s; Hamilton-Thorne Research) (16). The settings used for the analysis were as follows: frames acquired, 30; frame rate, 60 Hz; minimum contrast, 85; minimum cell size, 4 pixels; straightness threshold, 80%; low path velocity cutoff, 5 mm/s; medium path velocity cutoff, 25 mm/s; head size, nonmotile, 12 pixels; head intensity, nonmotile, 130; static head size, 0.68–2.57; static head intensity, 0.31–1.25; and static elongation, 23–100. The playback function of the HTR was used to accurately identify motile and immotile sperm cells. Several motion parameters were evaluated in this study: motility percentage, track speed (in micrometers per second), progressive velocity (in micrometers per second), path velocity (in micrometers per second), straightness (percentage), beat cross frequency (in hertz), and linearity (percentage).

Density Gradient Centrifugation

Aliquots of 1 mL of the liquefied semen were loaded onto a 45% and 90% discontinuous ISolate gradient (Irvine Scientific) and centrifuged at 400 $\times$ g for 20 minutes, and the resulting interfaces between the 45% (F45) and 90% pellet (F90) were aspirated and transferred to separate test tubes (17). Sperm suspensions from the different ISolate fractions were diluted in one volume of Dulbecco's phosphate-buffered saline (PBS) and centrifuged a 400 $\times$ g for 10 minutes. The pellet was resuspended in 1 mL of PBS, and a 10-μL aliquot was used to determine the total number of spermatozoa and immature germ cells as previously described (14).

Cryopreservation Protocol

The method used for freezing and thawing was that described by Srisombut and co-workers (18). Briefly, the sperm suspension was slowly mixed with the freezing medium containing TES-TRIS citrate and 20% egg yolk with 12% glycerol at a final concentration of 6% (Irvine Scientific) in a dropwise fashion until a 1:1, vol/vol ratio was attained. Aliquots were then placed in 2-mL cryovials at a volume of 0.4 mL/vial and refrigerated at 4°C for 1 hour. Subsequently, the vials were immersed in liquid nitrogen vapors for 30 minutes (80°C) and plunged into liquid nitrogen at −196°C for a storage period that ranged between 48 and 72 hours. The cryopreserved sperm samples were thawed in a water bath at 40°C for 3 minutes (11). Thawing temperature was monitored using a high-precision thermometer (Fluke). During the thawing process the cryovials were subjected to periodic shaking to prevent the occurrence of the so-called boundary effect. In some experiments, before freezing, the seminal plasma of the patient was replaced by seminal plasma with low SOD content (<5 ng/mL) or high SOD content (>25 ng/mL). Therefore semen samples were centrifuged at 400 × *g*, their seminal plasma removed by pipette aspiration, and SOD content measured. Those semen samples with low SOD content in seminal plasma were centrifuged at 300 × *g*, their seminal plasma removed by aspiration, seminal plasma with high SOD activity added to the sperm pellet, and finally the pellet resuspended after gentle vortex mixing.

SOD Immunoreactivity Assay

An ELISA was used for the quantitative measurement of human copper-zinc superoxide dismutase (Cu/Zn-SOD ELISA, Bender Med Systems Diagnostics GmbH). The study of Lasso et al. (15) demonstrated that SOD enzymatic activity and SOD content as measured by immunoassay were tightly correlated, allowing the far more convenient immunoassay to be used as surrogate for enzymatic activity.

An aliquot of the corresponding sperm suspension (20 × 10^6 sperm/mL) was transferred to an Eppendorf tube and centrifuged at 1,000 × *g* for 10 minutes. The supernatant was discarded and the pellet was treated with 0.5 mL of 0.1% Triton X-100 in PBS, vortex-mixed three times for 10 seconds, and centrifuged at 1,000 × *g* for 3 minutes. Aliquots of the supernatant and SOD standards were added to the wells of the ELISA microplate and the assay performed according to the manufacturer's instructions. Plate wells were coated with a murine monoclonal antibody against human Cu/Zn-SOD and the secondary antibody (anti-mouse IgG) was conjugated to horseradish peroxidase. Absorbance was quantified on a spectrophotometer using 450 nm as the primary wavelength. Although this assay only measures SOD immunoreactivity, previous studies indicate that there is a strong correlation between SOD content, as measured by ELISA, and SOD activity (15, 19).

Statistical Analysis

The results are expressed as mean ± SD. Data were analyzed by Student's *t*-test, comparing groups and other selected pairs, using a computer-driven software package GraphPad Instat, version 2.02 (GraphPad Software). $P<.05$ was considered statistically significant.

TABLE 1

Standard semen parameters of samples evaluated in study.

Sperm concentration (10^6/mL)	134.6 ± 48.2
VSL (μm/s)	56.8 ± 8.8
VCL (μm/s)	82.0 ± 9.5
ALH (μm)	3.8 ± 0.8
LIN (%)	58.4 ± 5.6
Motility (%)	69.2 ± 10.3
Normal forms (strict criteria) (%)	18.5 ± 2.7
Round cells (10^6/mL)	1.0 ± 1.1

Note: Data are expressed as mean ± SD. Sperm concentration, motility, and motion parameters were assessed using a Hamilton-Thorne Digital Image Analyzer (n = 42). ALH = amplitude of lateral head displacement; LIN = linearity; VCL = track speed; VSL = progressive velocity.

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RESULTS

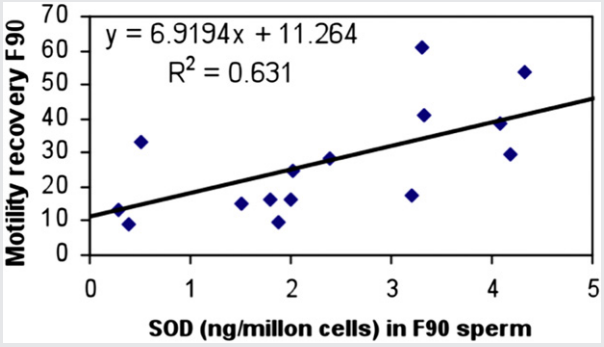
The standard semen parameters of the samples evaluated in this study are shown in Table 1. All subjects included in the study were normozoospermic men who were candidates to become sperm donors.

To evaluate the correlation between SOD content and post-thaw motility recovery of cryopreserved human sperm subsets, SOD content was measured in the F90 fraction of individual sperm samples and in the corresponding unfractionated washed sperm samples. The SOD content in sperm from the F90 fraction showed a statistically significant positive correlation with motility recovery after thawing ($P<.05$), as shown in Figure 1.

In contrast, a statistically significant negative correlation ($P<.01$) was found between SOD content in sperm from washed unfractionated semen samples and motility recovery after thawing (Fig. 2). The coefficient of correlation was high ($R^2 > 0.6$) in both cases.

Gavella et al. (20) have reported that human seminal plasma contains superoxide anion scavenging activity. This finding suggested that SOD added to seminal plasma could affect post-thaw motility recovery of cryopreserved sperm. To evaluate this potential protective effect of the addition of exogenous SOD to semen, seminal plasma with low SOD content was replaced with seminal plasma with high SOD content (see Materials and Methods section). Therefore semen samples with low SOD content in seminal plasma were centrifuged at 400 × *g*, their seminal plasma aspirated, seminal plasma with high SOD activity added to the sperm pellet, and finally the pellet resuspended after gentle vortex mixing. As shown in Table 2, increasing the SOD content of seminal plasma did not improve the motility recovery after thawing. Similar results were obtained when the seminal plasma was supplemented with commercially available SOD (data not shown). In accord with these observations, no significant correlation was found between SOD content in whole semen and motility recovery after thawing. Similarly, no significant correlation was also found between SOD content in seminal plasma and motility recovery after thawing.

FIGURE 1



Superoxide dismutase (SOD) content in sperm from the F90 fraction showed a statistically significant positive correlation with motility recovery after thawing. F90 denotes the sperm pellet from the Isolate gradient (n = 42).

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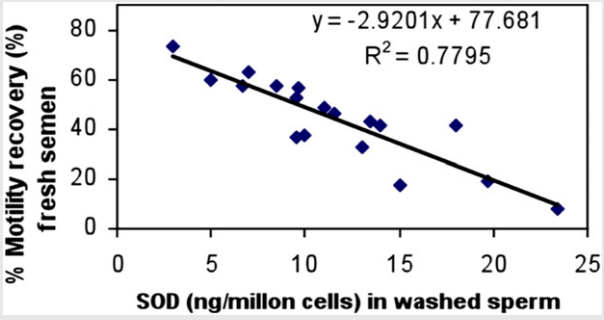
DISCUSSION

The main finding emerging from this study is the high, statistically significant positive correlation between SOD content in mature spermatozoa isolated from the 90% gradient pellet (F90 fraction) and motility recovery after thawing. The SOD has been shown to play a key role in scavenging superoxide anion intracellularly (2). The superoxide anion generated by the inner mitochondrial membrane is dismutated to hydrogen peroxide by the Mn-SOD present in the mitochondrial matrix and by the Cu/Zn-SOD present in the cytosol. In turn, hydrogen peroxide is eliminated by either GPX or catalase to avoid cell damage. Because it has been shown that sperm from most mammalian species lack catalase activity, sperm mostly relies on GPX activity to eliminate hydrogen peroxide generated intracellularly (12). Therefore, it would be expected that mature spermatozoa with a higher SOD content would be better equipped to cope with the increase in oxygen radical production reported to take place during thawing (5). However, SOD activity should not exceed that of GPX to avoid the production of toxic levels of hydrogen peroxide. Therefore, SOD and GPX activities should be coupled. In fact, it has been previously reported that both SOD and GPX play a central role in protecting mammalian sperm against oxygen radical-induced damage leading to motility loss (12).

FIGURE 2



Superoxide dismutase (SOD) content in sperm from unfractionated washed sperm samples showed a statistically significant negative correlation between SOD content and motility recovery after thawing. Fresh semen denotes sperm + seminal plasma (n = 42).

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TABLE 2

Effect of addition of seminal plasma with high SOD content to samples with low SOD content in seminal plasma on motility recovery after thawing.

	Low SOD concentration (< 5 ng/10^6 sperm)	High SOD concentration (> 25 ng/10^6 sperm)
Motility recovery	24.1 ± 4.28	23.0 ± 4.01

Note: Values are expressed as the mean ± SD (n = 6).

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How do we explain our finding that motility loss after thawing, an indicator of membrane and axoneme damage, was higher in samples with lower SOD content and lower in samples with higher SOD content? The SOD and GPX are assumed to act in concert and their activity was sufficient to cope with the oxygen radicals produced intracellularly by the sperm cell. Because an increase in the steady-state levels of superoxide anion has been reported to occur during thawing (5), sperm samples with a higher SOD content would be expected to be better equipped to prevent oxygen radical-induced membrane and axoneme damage. The reported increase in superoxide anion production observed during thawing could be either due to a slower recovery of enzymatic antioxidant activity during thawing of cryopreserved sperm, as previously suggested by Calamera et al. (11) or to partial loss of enzymatic antioxidant activity during cryopreservation. Evidence for the first hypothesis has been provided in the study by Calamera et al. (11), in which use of a thawing temperature of 40°C in sperm from the F90 fraction significantly increased motility recovery after thawing. However, despite the use of a higher temperature, possibly acting by accelerating the recovery of enzymatic antioxidant activity during thawing, percent motility after thawing is still significantly lower than prefreezing motility, suggesting that either intracellular enzymatic antioxidant activity is recovered at a lower rate than oxygen radical production or that, to some extent, there is irreversible loss of antioxidant enzyme activity during cryopreservation. This would imply that during cryopreservation structural damage of antioxidant enzymes, such as SOD, may take place. Therefore, samples with higher SOD content should better withstand either the surge in oxygen radical production or this putative partial loss in SOD activity during cryopreservation, than samples with lower SOD content. Nevertheless, for this scenario to apply, SOD and GPX activity should act in concert to avoid the generation of toxic levels of hydrogen peroxide from the dismutation of superoxide anion by SOD. However, this coupling of SOD and GPX activity in sperm needs to be confirmed experimentally.

Although there was a statistically significant correlation between SOD content and motility recovery after thawing,

as shown in Figure 1, a significant scatter in motility recovery was observed at high SOD content values. Three main hypotheses could be postulated to explain this finding. [1] The SOD content measured in the F90 fraction represents the mean value of SOD in all the spermatozoa present in this fraction. However, sperm in the F90 fraction may be comprised of sperm subsets with different SOD content. For example, sperm from a F90 fraction may have a mean SOD value of 5 ng/10^6 cells and yet 50% of the cells may have an SOD content of 2 ng/10^6 cells and the remaining 50% of 8 ng/10^6 cells. Consequently, motility recovery would be lower than that expected for a mean value of 5 ng/10^6 cells. Conversely, 75% of the cells may have an SOD content of 6 ng/10^6 cells and the remaining 25% of 2 ng/10^6 cells with a high motility recovery. Although the mean SOD content is 5 ng/10^6 cells in both cases, motility recovery is significantly different. We may, therefore, formulate the following equation to account for the cell distribution in SOD content in a given sperm sample: $\Phi SOD_T = \Phi SOD_1 + \Phi SOD_2 + \Phi SOD_3 + \Phi SOD_n$, where ΦSOD_T is the total SOD activity in a given sperm sample and ΦSOD_n is the SOD content of the corresponding sperm subset within the sample; The second hypothesis [2] postulates that GPX activity may be significantly lower than that of SOD. If GPX activity in sperm were significantly lower than that of SOD, F90 fractions with high SOD and low GPX activity would be expected to have low motility recoveries after thawing, as excess hydrogen peroxide generation from the dismutation of intracellular superoxide anion would lead to sperm damage and motility loss. The third hypothesis [3] combines both previous hypotheses. This would explain, at least in part, the observed scatter in motility recovery after thawing.

The other main finding emerging from this study is that SOD content in spermatozoa from either raw or washed semen, unlike the case of the F90 fraction, showed a negative correlation with motility recovery after thawing. Two main hypotheses can be postulated to explain this finding: [1] loss of motility in mature sperm is mediated by ROS-producing immature sperm during thawing, and [2] loss of motility in mature sperm is mediated by ROS-producing immature sperm during centrifugation of cryopreserved raw or washed semen after thawing.

With regard to the first hypothesis, it has been shown that immature sperm, particularly those with proximal cytoplasmic retention, produce high levels of superoxide anion (13, 21) and that high levels of extracellular SOD are found within the cytoplasmic droplet attached to the midpiece of these immature sperm (21). Therefore, during thawing of either raw or washed semen, the presence of immature sperm within the sample would increase oxygen radical-induced damage, mostly through the generation of high levels of hydrogen peroxide, as SOD activity in these immature sperm would most likely exceed that of GPX. Unlike the case of the F90 fraction, where most of the damage is believed to occur intracellularly, the damage that would occur in this case would be extracellular and mediated by hydrogen peroxide. This is consistent with previous reports showing that addition of catalase or reduced glutathione to the cryopreservation medium protects sperm against motility loss during thawing (22–25). Because most of this damage is mediated by hydrogen peroxide, addition of extracellular SOD would not be expected to reduce it in any significant way. And this is precisely what we have found in our study. Addition of extracellular SOD, commercial or associated to seminal plasma to semen samples with low SOD content in seminal plasma did not reduce motility loss after thawing.

Concerning the second hypothesis, it has been previously reported that centrifugation of raw or washed semen may lead to oxygen radical-induced damage and that this damage is mediated by ROS-producing immature sperm present in the sample (21, 26, 27). Therefore, loss of motility after thawing, rather than being related to SOD content, would be caused by the cocentrifugation of mature and ROS-producing immature sperm.

In conclusion, SOD content in mature sperm from the F90 fraction correlates with motility recovery after thawing. This finding could certainly find clinical applications in the selection of donors for donor sperm banking and for quality control purposes. Semen samples with higher SOD content should better withstand the cryopreservation process. In addition, this correlation could be also used for quality control purposes, as sperm from the gradient pellet with higher SOD content should always be expected to give a higher motility recovery. In those cases in which a relatively high motility recovery is not obtained in samples with a high SOD content, the adequacy of the cryopreservation protocol should be questioned. Studies are currently underway to measure SOD content and activity in sperm before and after thawing to test the hypothesis of whether the increase in oxygen radical levels observed during thawing is due to structural damage of SOD, with the subsequent loss of SOD activity. The other main conclusion that can be drawn from this study is that cryopreservation of spermatozoa isolated from the gradient pellet with a high SOD content should be highly recommended in favor of cryopreservation of whole or washed semen. This study needs to be considered as a starting point to develop diagnostic methods before cryopreserving human semen samples.

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