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Concentration of Glutathione and Expression of Glutathione Peroxidases 1 and 4 in Fresh Sperm Provide a Forecast of the Outcome of Cryopreservation of Human Spermatozoa

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ABSTRACT: Oxidative stress imbalance potentially leads to damage of the structure of the cell and macromolecules such as plasma membrane components, proteins, and DNA. The plasma membrane of the sperm cell, which has high levels of polyunsaturated fatty acids, renders it particularly sensitive to free radical–mediated attacks. The freezing and subsequent thawing of sperm is a physically stressful process carried out during routine procedures in assisted reproduction techniques, which results in a highly variable and unpredictable reduction in the number of motile sperm cells. Subsequently, oxidative status can positively or negatively affect the motility, viability, and fertilizing capacity of thawed sperm. These effects are counteracted by various oxidative defense enzymes and antioxidants such as glutathione peroxidase isoforms GPx1 and GPx4, glutathione reductase (GR), and cellular glutathione (reduced) (GSH). In this way, oxidative status could represent a predictive marker of sperm quality following the freeze-thaw process. This study was based on 56 human sperm samples. We observed direct positive and negative relationships between the postthaw motile sperm recovery rate and GPx1 and GPx4 expression and activity, on the one hand, and GSH concentrations, on the other. No correlation was found between this recovery rate and GR or basic semen parameters. Predictive values clearly demonstrate that, among the molecules analyzed, the most accurate diagnoses result when analyses are conducted for GPx1 and GPx1 messenger RNA expression, GPx1 and GPx4 enzymatic activity, and GSH concentration. In conclusion, a reserve of glutathione, together with GPx expression, is necessary to eliminate free radicals using GSH or a like structural protein and seems to be essential for a good postthaw recovery. These molecules can be employed as indicators of postthaw sperm quality.

Key words: Oxidative stress, freezing, thawing, sperm motility, assisted reproduction.

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Oxidative stress is defined as the imbalance between pro-oxidative and antioxidative molecules in a biological system, in which the former is favored, thus resulting in the presence of free radicals. This imbalance can lead to damage to the structure of cells and macromolecules such as plasma membrane components, proteins, and DNA (Aitken et al, 1999). It has long been known that spermatozoa produce reactive oxygen species (ROS), mitochondria being the main source of the ROS production. Furthermore, leukocyte infiltration of semen is associated with decreased fertility due to leukocyte-produced ROS. Because of this dual effect—sperm's extreme sensitivity to ROS and the production of ROS by the sperm and their environment (testicular and epididymal fluids)—intricate defense systems have evolved to counter the effects of free radical–mediated attacks; these systems are based on the expression of various antioxidant enzymes and molecules (Saleh and Agarwal, 2002).

The plasma membrane of sperm, which contains high levels of polyunsaturated fatty acids, renders it particularly sensitive to free radical–mediated attacks. One of the most common types of damage induced by free radicals is membrane lipid peroxidation (LPO). Spontaneous LPO has been well characterized in human semen samples (Alvarez et al, 1987; Mazzilli et al, 1995). This LPO considerably impairs sperm function by adversely affecting its viability and motility and, ultimately, its fertilizing potential (Critser et al, 1987; Centola et al, 1992; Mc Laughlin et al, 1992). Increased susceptibility to ROS and cell death has been reported in cryopreserved sperm. The freezing and subsequent thawing of sperm is a physically stressful process carried out during routine procedures in assisted reproduction techniques (ARTs), which results in a highly variable and unpredictable reduction in the number of motile sperm cells. Various oxidative defense enzymes, such as superoxide dismutase, glutathione peroxidase (GPx), and glutathione reductase (GR), are crucial

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for the prevention of LPO (reviewed by Storey, 1997). GPx and GR have provoked special interest, since LPO increases 20-fold following the inhibition of GPx activity with mercaptosuccinate (Alvarez et al, 1987). Additionally, the GPx/GR system has been shown to play a major role in maintaining membrane integrity in human sperm, since inhibition of GPX with mercaptosuccinate increases membrane damage (Alvarez and Storey, 1989).

The complete glutathione system consists of a complex series of interactions. In short, GPx isoforms catalyze the reduction of inorganic and organic hydroperoxides. During catalysis, glutathione acts as a reducing equivalent, inactivating free radicals through the action of GPx isoforms and thereby converting the reduced form (glutathione [GSH]) to the oxidized form (GSSG). Conversely, GR restores glutathione to its reduced form.

GPx isoforms are a family of enzymes whose properties vary slightly, depending on the tissues. The classic intracellular GPx1 is expressed in sperm and in the genital tract, and a direct relationship has been demonstrated with sperm motility (Dandekar et al, 2002). However, knockout mice lacking GPx1 have been found to be fertile (de Haan et al, 1998).

More significantly, a direct relationship has been reported between male fertility and phospholipid hydroperoxide glutathione peroxidase (PHGPx, or GPx4), a selenoprotein that is highly expressed in testicular tissue (Foresta et al, 2002). Furthermore, genetic variations of GPx4 have been detected in humans. Eleven variant sites have been discovered in 25% of all infertile males, yet only 25% of these affect activity or transcription, which rules out a relationship with peroxidase content and fertility-related parameters. However, GPx4 does deserve further attention as a potential cause of infertility in certain cases (Maiorino et al, 2003). Its importance is reflected by a selenium deficiency–related suppression of spermatogenesis in rats (Behne et al, 1996). GPx4 has a dual function as an active peroxidase that may protect rapidly dividing cells against oxidative injury and as a structural protein that cross-links with itself and other proteins and subsequently builds up the mitochondrial capsule (Foresta et al, 2002).

To date, there is a lack of markers for prognosticating the quality of semen samples following the freeze-thaw cycle. Moreover, there are no sperm parameters or biochemical features that provide a reliable predictive value for the success of postthaw recovery.

The goal of the current study was to correlate oxidative stress status with the postthaw recovery of progressive motile cells, both in terms of GSH levels and GPx and GR expression and activity, as a first step in improving sperm cryostorage protocols. Evidently, these measurements could serve as indicators of the postthaw quality of sperm samples.

To do this, we determined the expression of GPx1, GPx4, and GR by quantitative fluorescent polymerase chain reaction (qfPCR) and, by using spectrophotometry in controlled reactions, we measured the activity of these enzymes as well as cellular GSH.

Materials and Methods

The study was approved by the Institutional Review Board of the Instituto Valenciano de Infertilidad. Semen samples were acquired from 56 men who visited our center for infertility screening or were semen donors. All samples were obtained via masturbation following 3–5 days' abstinence from sexual activity. All participants gave their informed consent for a part of their samples to be used for research purposes.

After 10 minutes of liquefaction at 37°C with 5% CO_2, semen samples were examined for concentration and motility in a Mackler chamber (Sefi Laboratories, Tel Aviv, Israel) according to World Health Organization (1999) guidelines. Sperm vitality was analyzed using the dye exclusion method with eosin (Sigma Chemical Co, St Louis, Mo). The number of sperm used in the experiments described was standardized to 50 million to provide enough spermatozoa for all the experiments, and the sperm were processed by a 10-minute centrifugation at 400 × *g*. The supernatant was then discarded, and the pellet was resuspended in an isotonic buffer. Aliquots of these suspensions were used to determine protein concentration, enzymatic activity, and messenger RNA (mRNA) expression. No ejaculate showed significant (>0.1 million/mL) levels of leukocytes, immature germ cells, or other somatic cell contaminants, as demonstrated by Quick Panoptic (QCA, Barcelona, Spain) staining of semen smears.

Semen samples were frozen in pills on the surface of dry ice using a glycerol-based cryoprotectant (Sperm Freezing Medium, MediCult, Jyllinsge, Denmark) as described by Meseguer et al (2004). Aliquots of the samples were transferred to new tubes that were immersed in room temperature water and thawed for 10 minutes, after which they were treated for 10 minutes at 37°C.

Samples were then analyzed for concentration, motility, and vitality as explained above, and determinations were recorded independently by 2 different observers (M.M. and N.G.). Final results are presented as a mean of the 2 observers' recordings and only when there was less than 5% discordance. Samples showing greater differences were disregarded.

RNA Extraction and Reverse Transcription-PCR

Sperm RNA extraction was performed with pure, unadulterated, undiluted semen (n = 27) using the Trizol reagent (TelTest, Friendswood, Tex). The total amount of RNA was quantified by spectrophotometry on a BioRad (Durviz, Valencia, Spain) spectrophotometer. Then, RNA was reverse transcribed using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, Calif) following the manufacturer's instructions. The product was diluted to a final volume of 100 μL with diethylpyrocarbonate-treated water and stored at −20°C until PCR analysis.

Primer sequences that targeted the mRNAs of GPx1 and GR in sperm cells were obtained from previously published articles (Dubrovskaya and Wetterhahn, 1998; El Mouatassim et al,

1999). Sequences for human GPx4 were obtained from GenBank, and primers were acquired from Genotek (Barcelona, Spain) and designed using GeneFisher software.

Prior to the qfPCR experiments, all primers were tested via nonfluorescent PCR in an Eppendorf (Hamburg, Germany) Personal Thermocycler, and their products were run in a 2% agarose gel and stained with ethidium bromide to verify that the amplification targeted the correct sequence and that the product size was adequate.

All of the results of qfPCR were normalized by the analysis of β-globin expression, and the primers used were those provided by the qfPCR kit, as described in the following paragraphs.

Each nonfluorescent PCR was programmed for 36 cycles, and the complete series of PCR reactions is documented in other publications by our group (Garrido et al, 2001; Meseguer et al, 2001, 2002).

qfPCR was performed with 5 μL of complementary DNA (cDNA) using the LightCycler—FastStart DNA Master SYBR Green I Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions.

A calibration curve was included for each experiment from 5 serial dilutions (1/5) of a sample of endometrial cDNA to interpolate the results.

Green fluorescence was measured during each cycle, and the results were analyzed with the aid of an amplification curve, taking into account the starting point, using Roche Molecular Biochemical Lightcycler Software. The units used were the ratios between the expressions of each gene vs that of β-globin.

Enzymatic Activity Measurements

Sperm samples destined for total protein experiments (n = 29) were lysed, and protein fractions were extracted using 350 μL of lysis buffer (20% perchloric acid in 1 mM EDTA). The suspensions were centrifuged once more, and the supernatants were stored at −20°C until protein and enzymatic determinations were carried out.

The readings of enzymatic activity were normalized with the total amount of protein. These determinations were performed according to the Bradford method (BioRad).

One unit of GR activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol of GSSG per minute at pH 7.6, 25°C. This activity was determined indirectly, by measuring the consumption of NADPH and observing the decrease in absorbency at 340 nm as a function of time (25°C), with a Bioxytech GR-340 Spectrophotometric Assay (OxisResearch, Portland, Ore).

Selenium-dependent GPx (GPx1) and PHGPx activities (units) are defined as the amount of enzyme catalyzing the oxidation of 1 μmol of GSH per minute at pH 7, 37°C.

GPx1 and GPx4 activities were again measured indirectly by spectrophotometry, based on the consumption of NADPH as the result of added GR, by the decrease of absorbency at 340 nm as a function of time, with GPx first converting GSH to GSSG through the reduction of cumene hydroxide, which acts as an electron donor for GPx1 (Romero et al, 1999) and PHGPx. Phosphatidylcholine hydroperoxide was used as a specific substrate for PHGPx, mainly GPx4 (Imai et al, 2001).

To summarize, 10 μL of thawed sample was added to 590 μL of solution 1 (1 mM EDTA, 1 mM sodium azide, and 0.1 M potassium phosphate buffer, pH 7; all from Sigma). Later, 100 μL of GR with an activity of 2.4 U/mL (using the previous buffer as a diluent) plus 100 μL of 10 mM GSH were added, and the solution was incubated for 5 minutes at 37°C. Afterward, a 100-μL solution of 1.5 mM NADPH was dissolved in 0.1% $NaHCO_3$, and only the oxidation of NADPH that was not dependent on hydroperoxides was monitored for 3 minutes. Finally, 100 μL of either 1.5 mM cumene hydroperoxide (Sigma) or 40 μM phosphatidylcholine hydroperoxide (kindly provided by Dr Matilde Maiorino, University of Padova, Italy) was added to the reaction, and the decrease in absorbency at 340 nm (37°C) was recorded.

GSH Concentration

To study glutathione concentrations, a similar experiment was carried out. The acidic media in which samples were suspended as basified by the addition of known volumes of 40% K_2HPO_4, and the samples were centrifuged at 12 000 × *g* for 5 minutes to precipitate sodium perchlorate.

Aliquots of 930 μL of solution 1 (1 mM EDTA, 1 mM sodium azide, and 0.1 M potassium phosphate buffer, pH 7; all from Sigma) were first added to the cuvette, followed by 10 μL of solution 2 (0.02 g of 1-chloro-2,4-dinitrobenzene, from Sigma, in 10 mL of ethanol) and 50 μL of the study sample. The cuvette was then introduced into the spectrophotometer.

The reaction was initiated with the addition of 20 μL of solution 3 (500 IU of GSH-*S*-transferase per milliliter from Sigma in 0.1 M phosphate buffer, pH 7.0), and we monitored the increase in absorbency at 340 nm (37°C) until it reached a plateau, when all available GSH had reacted.

Calculations were performed on the basis of the volume of the sample, light path length, corresponding dilution factors, absorbency decrease, and molar extinction coefficient.

Statistical Analysis

GR, GPx1, and GPx4 mRNA expression and activity and glutathione concentrations were correlated with postthaw seminal parameters using linear regression analysis followed by analysis of variance. Significance was defined as $P < .05$.

Thawing was considered successful when the percentage of postthaw total progressive motility was 40% higher than fresh total motility. Our goal was to use the determinations from these experiments as a diagnostic test for predicting the state of semen samples after thawing.

The diagnostic capacity of a test, or its ability to discriminate between situations, is evaluated using receiver operating characteristic (ROC) curve analysis (Zweig and Campbell, 1993). ROC curves can also be used to compare the diagnostic performance of 2 or more diagnostic tests. In this way, we compared ROC analyses of successful and unsuccessful thawing for all the parameters. By analyzing the area under the ROC curve (AUC), we can be advised about the accuracy of the parameters (their ability to discriminate between the 2 conditions). Such analyses also permit us to determine the optimum discriminating point of each parameter as well as its sensitivity and specificity.

We determined the positive and negative predictive values of GPx1, GPx4, and GR expression and activity and GSH concen-

Table 1. *Mean basic semen parameters (after liquefaction), presented as mean ± SEM with the range between gap, of the 56 semen samples studied before and after freezing and thawing*

Semen Parameters	Fresh	Thawed
Sperm concentration (million/mL)	96.5 ± 6.8 (12–255)	. . .
% progressive motile sperm	54.6 ± 1.4 (20–79)	. . .
Volume (mL)	3.4 (1–9)	. . .
Total progressive motile sperm (million)	164.2 ± 11.6 (11–445)	54.6 ± 5.9 (2–262)
Sperm vitality (%)	77.8 ± 1.9 (56–93)	25.8 ± 1.7 (14–45)

tration. These values illustrate the probability of samples thawing successfully when the test for GPx1 and GPx4, GR, and GSH is positive and the probability of samples thawing poorly when the same test is negative.

Also, statistical significance was compared between the ROC curves for GPx1, GPx4, GR, and GSH to determine which parameter should be used as the most effective diagnostic tool.

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc, Chicago, Ill) and MedCalc (Ghent, Belgium) statistical software.

Results

The mean basic semen parameters (postliquefaction) of the 56 semen samples before and after freezing and thawing are presented in Table 1. The mean progressive postthaw recovery rate (PTRR), expressed as the percentage of total progressive motile sperm cells in the ejaculate recovered after thawing, was 33.8 ± 2.5% (range, 5%–96%). When linear regression analysis was performed, no direct relationship between basic semen parameters and PTRR was observed, as illustrated in Table 2.

Relationship Between GPx1 and GPx4 and PTRR

We identified a statistically significant positive correlation between GPx1 and GPx4 activity and mRNA expression and PTRR: for GPx1 activity, $P = .013$; for PHGPx activity, $P = .022$; for GPx1 gene expression, $P = .019$; and for GPx4 gene expression, $P = .020$.

The correlation coefficient (r) was .493 for the GPx1 assay, .431 for the PHGPx assays (enzyme activity), .471 for the qfPCR GPx1 experiments, and .585 for the qfPCR GPx4 experiments (mRNA) (Figure 1).

Table 2. *Linear regression analysis of the relationship between basic semen parameters and PTRR of motile sperm; note that none of the parameters considered was statistically related to PTRR**

Semen Parameter	Correlation Coefficient	*P*
Sperm concentration	.03	.80
% progressive motile sperm	.06	.64
Volume	.21	.10
Total progressive motile sperm	.15	.22
Sperm vitality	.02	.92

* PTRR indicates postthaw motile sperm recovery rate.

Relationship Between GSH and Postthaw Motile Sperm Recovery

A contrary pattern was discovered when GSH concentrations were analyzed in sperm cell lysates. There was a statistically significant negative correlation between GSH and PTRR, with a significance of $P = .011$ and an r value of $-.472$ (Figure 2).

Relationship Between GR and Postthaw Motile Sperm Recovery

When GR activity and gene expression were studied, no correlation of statistical significance was initially found. Indeed, GR activity was considerably low when compared to that of GPx. Thus, GR is unlikely to be an enzyme implicated in cellular metabolism. Significance was $P = .104$ when we performed mRNA analysis by quantitative PCR, and significance was $P = .062$ when we carried out spectrometry enzymatic activity assays. Nevertheless, as in the GSH study, there seems to be a negative correlation between GR expression and activity and PTRR ($r = -.320$ vs $r = -.351$) (Figure 3).

Predictive Value of Oxidative Stress Status and Postthaw Sperm Quality

To further investigate the possibility of forecasting successful or unsuccessful sperm thawing, we determined AUC^{ROC} curves, using ROC analysis, for sperm GPx and GR mRNA, GPx and GR activity, and GSH, and these are presented in Table 3. In this way, we clearly demonstrate that the most accurate diagnoses provided by the molecules analyzed are those for GPx4 and GPx1 mRNA expression, followed by GPx1 and PHGPx enzymatic activity and GSH concentrations. The higher AUCs for these parameters indicate the greater likelihood of a good postthaw recovery of semen samples (with a total motile sperm recovery of >40%).

The highest AUC values that discriminate between successful and unsuccessful thawing of semen samples were more than 7.2 (sensitivity, 80.0%; specificity, 100%) for GPx4 mRNA and more than 445 U (sensitivity, 45.5%; specificity, 94.1%) for GPx1 activity (Table 3).

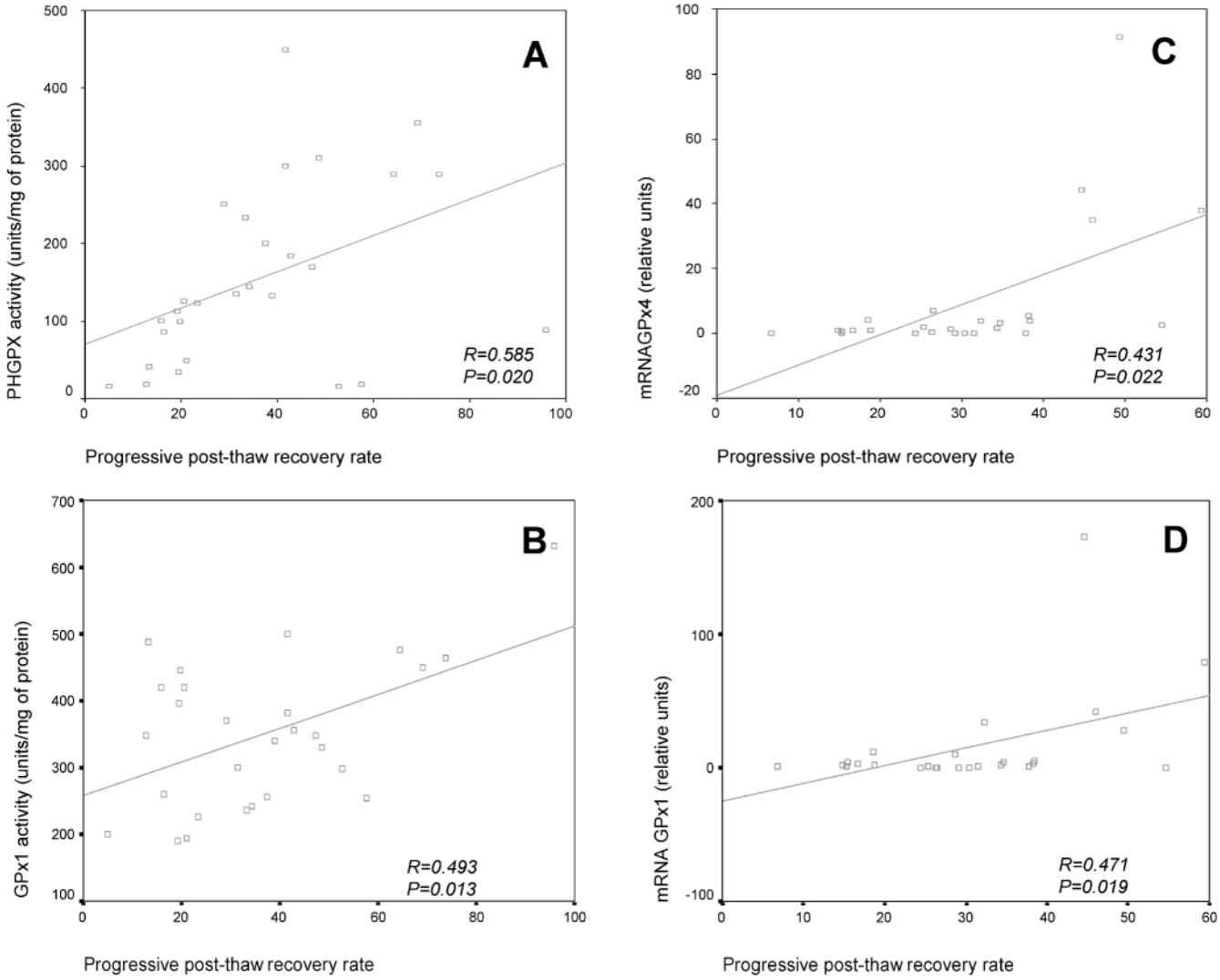

Figure 1. Linear regression analysis of the relationship between enzymatic activity (using spectrophotometry) of phospholipid hydroperoxide glutathione peroxidase (PHGPx) **(A)** and glutathione peroxidase isoform 1 (GPx1) **(B)** and messenger RNA (mRNA) expression (using quantitative fluorescent polymerase chain reaction [qfPCR]) of GPx4 **(C)** and GPx1 **(D)** (Y-axis) in raw sperm and postthaw motile sperm recovery rate (PTRR) of motile sperm (X-axis). Each panel represents a scattergram and the regression line. Correlation coefficients and *P* values are plotted in every graphic.

Discussion

In the present study, we confirmed the presence of a complete intracellular glutathione system (including the tripeptide enzyme and the enzymes implicated in its metabolism) and a correlation between this system and the protection of sperm cells against the oxidative processes induced by free radicals in human sperm. Nevertheless, our data show that sperm GR activity is rather low when compared to that of GPx and PHGPx, which indicates that GR is not an important supplier of reduced glutathione, which in turn suggests that GSH is provided by seminal plasma.

The glutathione system has a determinant role in the protection of sperm cells because it inactivates free radicals. The pivotal component is the tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine), whose free thiol group in the cysteine residue is extremely reactive (Meister and Anderson, 1983). This molecule exists in a thiol-reduced form (GSH) and in an oxidized form (GSSG).

Further components of this system are the molecules implicated in glutathione metabolism, which are responsible for their redox state in physiological conditions.

GPx isoforms are a family of selenium-containing enzymes that are present in all mammals and are particularly active in the liver, where there is a relatively high exposure to reactive agents. Their importance to sperm function has been shown in elaborate experiments by Alvarez and Storey (1989), in which enzyme inhibition with mercaptosuccinate (permeate to plasma membrane) increased

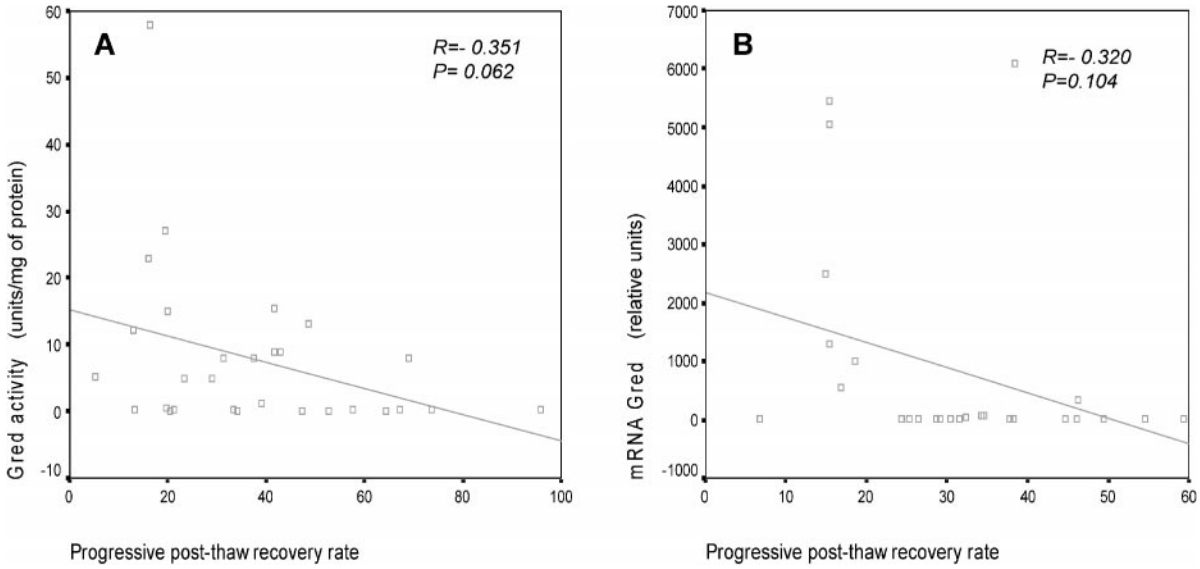

Figure 2. Linear regression analysis of the relationship between enzymatic activity (using spectrophotometry) **(A)** and messenger RNA (mRNA) expression (using quantitative fluorescent polymerase chain reaction [qfPCR]) **(B)** of glutathione reductase (GR) (Y-axis) in raw sperm and postthaw motile sperm recovery rate (PTRR) of motile sperm (X-axis). Each panel represents a scattergram and the regression line. Correlation coefficients and *P* values are plotted in every graphic.

the LPO process 20-fold and in which glutathione was present in its GSH form, thus indicating that functioning GPx, together with a reductive substrate, is necessary for preventing LPO. Depleting GSH or inhibiting GPx dramatically increases LPO.

GR renews the GSH, which is required by GPx; thus, they are all of equal importance. The reductive substrate employed by this enzyme in human sperm is NADPH (Storey, 1997).

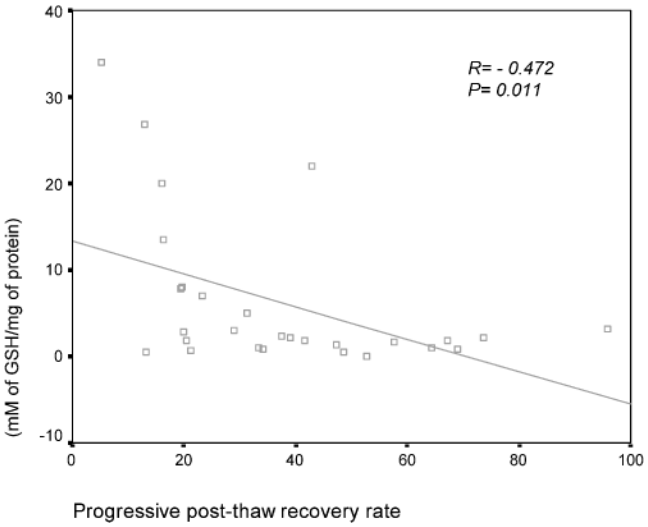

Figure 3. Linear regression analysis of the relationship between glutathione (X-axis) in sperm cells from raw semen and postthaw motile sperm recovery rate (PTRR) (Y-axis) of motile sperm. The panel represents a scattergram and the regression line. The correlation coefficient and *P* value are plotted in the graphic.

Oxidative stress–induced LPO is undoubtedly related to sperm function in physiological conditions, such as high leukocyte levels and immature sperm, as well as in nonphysiological conditions, such as semen freezing (Gomez et al, 1998; Aitken et al, 1999; Schuffner et al, 2001).

Protection against the harsh process of cryopreservation is necessary to preserve sperm viability and fertilization potential. To this end, GPx isoforms must be relatively abundant and functional in sperm.

The interest in developing a satisfactory system for recovering as many healthy sperm as possible after thawing is, without doubt, a primary objective. Optimal sperm recovery following cryopreservation is especially relevant when samples are subject to elevated oxidative stress levels, as in the case of testicular biopsies and prechemotherapy, prevasectomy, and sperm donor samples (Meseguer et al, 2003).

Through our results, we can conclude that GR mRNA expression and activity are not related to sperm susceptibility to cryodamage, as shown by the lack of correlation between the quality of the sperm sample after thawing and both the gene expression and activity inside sperm cells. GR activity was considerably low in all the samples tested. Therefore, its role in sperm function does not seem to be significant.

Conversely, the activity of GPx and its ability to eliminate free radicals via GSH seems to be essential for postthaw recovery. A stressful oxidative environment seems to sensitize sperm to the traumatic procedure of freezing and thawing. The impact of PHGPx on sperm survival is less easily explained. In early spermatogenesis, as an ac-

Table 3. *Diagnostic accuracy of GPx and GR expression and activity and GSH concentrations in fresh semen with respect to forecasting successful and unsuccessful thawing results**

Parameters	AUC^{ROC} (95% CI)	Significance	Threshold	Sensitivity (%)	Specificity (%)	PPV	NPV
GPx1 mRNA	0.82 (0.62–0.94)	0.03[a]†	>12.1	80.0	95.2	80.0	95.2
GPx1 activity	0.74 (0.53–0.88)	0.04[a]	>445	45.5	94.1	83.3	72.7
GPx4 mRNA	0.94 (0.78–0.99)	0.02[a]	>7.2	80.0	100.0	100.0	95.5
GPx4 activity	0.74 (0.54–0.89)	0.02[b]	>145	72.7	82.4	72.7	82.4
GR mRNA	0.65 (0.44–0.82)	0.27[b]	≤12.3	83.3	57.1	35.7	92.3
GR activity	0.60 (0.41–0.78)	0.35[b]	≤0.4	58.3	70.6	58.3	70.6
GSH	0.73 (0.52–0.87)	0.04[a]	≤2.1	81.8	70.6	64.3	85.7

* GPx indicates glutathione peroxidase; GR, glutathione reductase; GSH, glutathione (reduced); AUC^{ROC}, area under the curve in a receiver operating characteristic analysis; PPV, postive predictive value; NPV, negative predictive value; and mRNA, messenger RNA.
† Values with different letters were significantly different ($P < .01$).

tive peroxidase, it may protect the rapidly dividing cells against oxidative injury, but it is difficult to attribute this antioxidant function, widely assumed to be mandatory in the protection of sperm against abundant oxidants, to PHGPx in mature spermatozoa. When overwhelming proportions of sperm in the capsule are inactive, the nuclear variant is cross-linked to protamine, and the residual active enzyme is unable to function as an antioxidant system. This is because it lacks the major reducing substrate, GSH, the loss of which has been attributed to processes that occur during final sperm maturation (Foresta et al, 2002). Subsequently, PHPGx adopts the peculiar function of a structural protein. In this way, the lower postfreeze-thaw survival rate could be associated with sperm alterations, such as impaired motility—a fuzzy appearance to the midpiece, which is most likely a result of impaired PHGPx biosynthesis and which affects the sperm's survival potential.

GSH concentrations are inversely correlated with PTRR, which could be related to GPx function; that is to say, lower GPx activity increases GSH reserves, and higher GPx activity reduces GSH levels. Thus, it would seem that, during sperm metabolism, GSH does not inactivate free radicals alone (as occurs during cellular metabolism), but requires the mediation of GPx in order to reduce oxidative components present in the sperm cells.

It also has been demonstrated that GPx and PHGPx activity, gene expression of GPx1 and GPx4, and concentrations of GSH are reliable predictors of postthaw semen quality, as observed in the recovery of progressive motile sperm cells after thawing when compared to the initial seminal analysis.

Both of the GPx isoforms analyzed in the present study showed similar levels of expression and activity and similar predictive values.

Although further experiments are required, given the implications that oxidative stress has on the quality of sperm cells after thawing, oxidative-protective substances (ie, enzyme enhancers) could be added to the medium used to improve postthaw recovery. It seems that antioxidants alone do not inactivate free radicals, as observed with GSH. Subsequently, it is unlikely that the addition of these molecules to the medium would improve the sperm quality for the purpose of ART procedures. Indeed, we suspect that a direct effect on antioxidative enzymes would be more effective.

Although intracytoplasmic sperm injection can overcome severe male factor infertility and combat poor reproductive results after thawing, there are specific cases in which an improved freezing method is essential, such as in sperm donor management (Garrido et al, 2002).

Redox status determination could also be employed for predicting the total motile cells expected after thawing. This would be especially useful in the selection and management of sperm donors.

Furthermore, our data clearly demonstrate that no predictive values can be derived from the basic parameters of fresh semen samples.

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