Effect of Cryoprotective Additives and Cryopreservation Protocol on Sperm Membrane Lipid Peroxidation and Recovery of Motile Human Sperm

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ABSTRACT: Sperm membrane damage during cryopreservation reduces the recovery of motile sperm. The present study investigates changes in sperm motility and membrane lipid peroxidation (LPO) in response to two changes in the standard sperm cryopreservation/thawing methodology: 1) the addition of platelet-activating factor (PAF) and pentoxifylline (PTX) as cryoprotective additives, and 2) the alteration of sample thawing time. PAF (1 μM) and PTX (3 mM) were added to fresh sperm samples prior to cryopreservation. After 2 weeks the samples were thawed either quickly (5 minutes at 37°C) or slowly (30 minutes at 4°C) and evaluated for sperm motility and LPO. Thawing time influenced both post-thaw motility and LPO. Samples thawed quickly exhibited a 31% increase in motility recovery (35.2 ± 4.3% in quick-thaw samples; 24.3 ± 3.9% in slow-thaw

samples) and a 23% lower LPO level (23.3 ± 3.4% in quick-thaw samples; 30.09 ± 4.4% in slow-thaw samples) compared to samples thawed slowly. Results also demonstrated that PAF (49 ± 1.7%) or PTX (42.6 ± 1.5%) enhance post-thaw motility in comparison to control (35.8 ± 1.2%), whereas neither PAF nor PTX affect post-thaw LPO (19.1 ± 2.2% in controls; 20.2 ± 1.7% in PAF samples; 20.5 ± 1.4% in PTX samples). These results support observations that there is a negative correlation between sperm motility and LPO in cryopreserved samples. The results also discount the hypothesis that LPO protection is a result of the cryoprotective action of PAF or PTX.

Key words: Sperm, PAF, PTX, cryoprotection, lipid peroxidation.

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Sperm motility and normal membrane integrity are two important, interrelated factors in fertilization (Jeyendran et al, 1984; Tesarik and Testart, 1989). Sperm membranes, which must be intact and functional for ova penetration, may be damaged by the sperm cryopreservation/thawing process, thus significantly reducing sperm motility and ultimately affecting the fertilization process.

Reduced sperm motility because of cryopreservation has recently been attributed to lipid peroxidative damage to sperm membranes (Alvarez and Storey, 1992). Human sperm are highly susceptible to, and irreversibly damaged by membrane lipid peroxidation (Alvarez et al, 1987). Further evaluation of the role of membrane lipid peroxidation (LPO) in reduced post-thaw sperm motility may be helpful in the development of better cryopreservation protocols. Any improvement in the recovery of motile sperm should increase the rate of success in donor insemination programs that utilize cryopreserved semen samples (Sherman, 1990).

Currently, the potential cryoprotective abilities of several agents are being investigated (Centola et al, 1992).

Among the drugs being evaluated for use as sperm cryoprotective additives are platelet-activating factor (PAF) and pentoxifylline (PTX). In our earlier studies, both PAF and PTX improved the sperm motion of post-thaw cryopreserved samples (Hellstrom and Sikka, 1989; Sikka and Hellstrom, 1990; Hellstrom et al, 1991). The present study was designed to determine if protection of sperm membranes from LPO is involved in the cryoprotective abilities of PAF and PTX. Such information about the correlation between the recovery of motile sperm after cryopreservation and sperm membrane LPO may improve the success rate of those assisted reproductive technologies that utilize cryopreserved sperm.

Materials and Methods

Materials

Ham's F-10 nutrient mixture and TEST-Yolk freezing medium were purchased from Irvine Scientific (Santa Ana, California). All other chemical reagents were obtained from Sigma Chemical Company (St. Louis, Missouri).

Sample Collection and Preparation

Eighteen normal semen samples with a total sperm count greater than 100 × 10⁶ each and a motility grade of greater than 2 (on
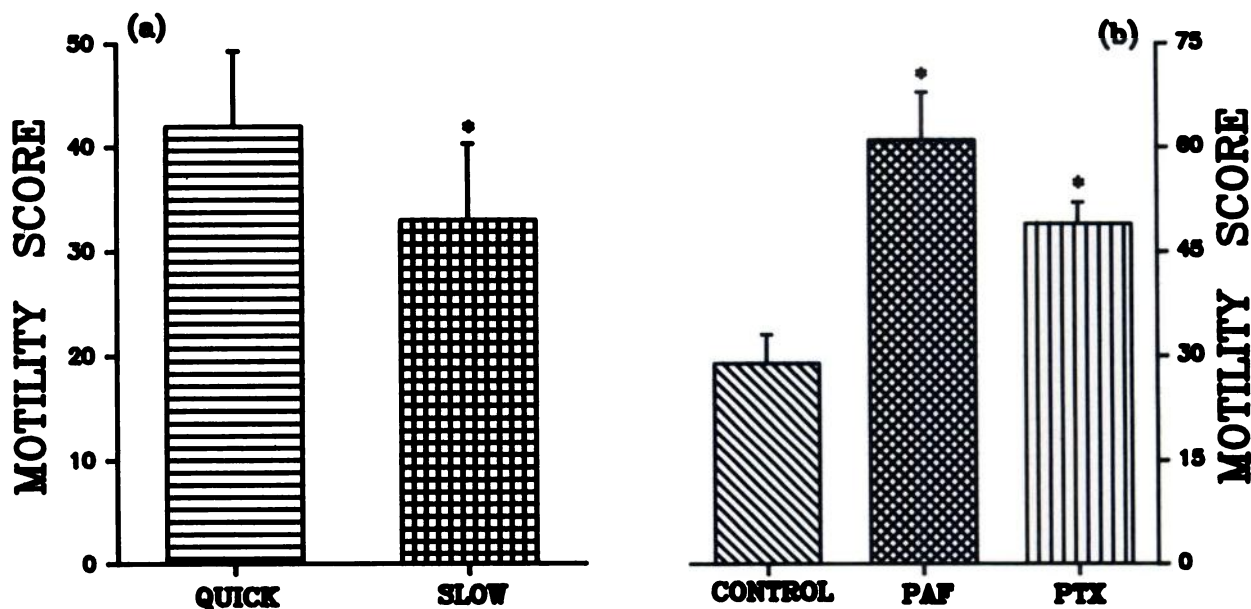
FIG. 1. Effect of experimental procedures on post-thaw sperm motility scores (percent motility × grade). (a), Motility scores (%) for quick and slow thaw samples ($n = 10$); (b), motility scores (%) for control, PAF-, and PTX-treated samples ($n = 8$). Data are shown as mean ± standard error; * = $P < 0.05$.

a scale of 1–4, as per WHO criteria) were obtained from 14 different healthy donors by masturbation.

Sperm Cryopreservation/Thawing Protocol

Ten semen samples were subjected to the following protocol: Percent motility and forward progression were measured manually by a single observer immediately upon liquefaction, and a motility score (percent motility × grade) was calculated. Each sample was subsequently divided into three equal aliquots (30–50 × 10⁶ sperm per aliquot). LPO was measured in one aliquot. The remaining two aliquots were mixed with equal volumes of Test-yolk freezing medium in a dropwise fashion with occasional gentle mixing. Once dilution was completed, they were cryopreserved by slow, gradual cooling to −79°C (1 hour total cooling time) before submersion in liquid nitrogen (Sherman, 1990). After 2 weeks of cryopreservation, one frozen aliquot was quickly thawed at 37°C for 5 minutes, while the other frozen aliquot was subjected to slow thaw at 4°C for 30 minutes. Percent motility, forward progression, and LPO were measured in each specimen immediately upon thawing.

Effect of PAF/PTX as Cryoprotectants

The remaining eight semen samples were processed for cryopreservation in the presence or absence of additives as described below: Percent motility and forward progression were measured, and motility scores were calculated in each sample immediately upon liquefaction. Each sample was then divided into four equal aliquots. The first aliquot was washed and LPO was assessed within 2 hours of liquefaction. The remaining three aliquots were cryopreserved by standard procedure as described above in the absence (control) or presence of either 1 μM PAF or 3 mM PTX.

After 2 weeks all samples were thawed at 37°C for 5 minutes before analysis.

LPO Measurement

LPO was measured within 2 hours of sample preparation by incubating washed sperm suspensions ($>20 \times 10^6$ sperm) with 0.063% ferrous sulfate and 0.223% ascorbic acid for 1 hour in a 37°C shaking water bath. Protein was precipitated by addition of ice-cold 40% trichloroacetic acid. Supernatants were collected by 25-minute cold centrifugation at 3,500 × g, reacted by 10-minute boiling with 2% thiobarbituric acid (TBA) in 0.05 N NaOH, and cooled to room temperature. Quantitation of malondialdehyde (MDA–TBA reactivity) was calculated by measuring absorbance at 534 nm and comparing it to that produced by standard MDA. LPO was expressed as nM MDA/10⁸ sperm (Bell et al, 1992).

Motility Assessment

Percent motility was determined by use of a Makler chamber (Sefi Medical Instruments, Inc., Haifa, Israel) to count the number of motile sperm in a population of 100 sperm. Forward progression was graded on a scale of 0–4 as indicated by WHO protocol.

Statistical Analysis

The Student's t-test was used to determine significance of results (P values), and the GraphPAD InStat statistics program (GraphPAD Software, San Diego, California) was used to perform linear regression analyses of the correlation between motility percentage and LPO.
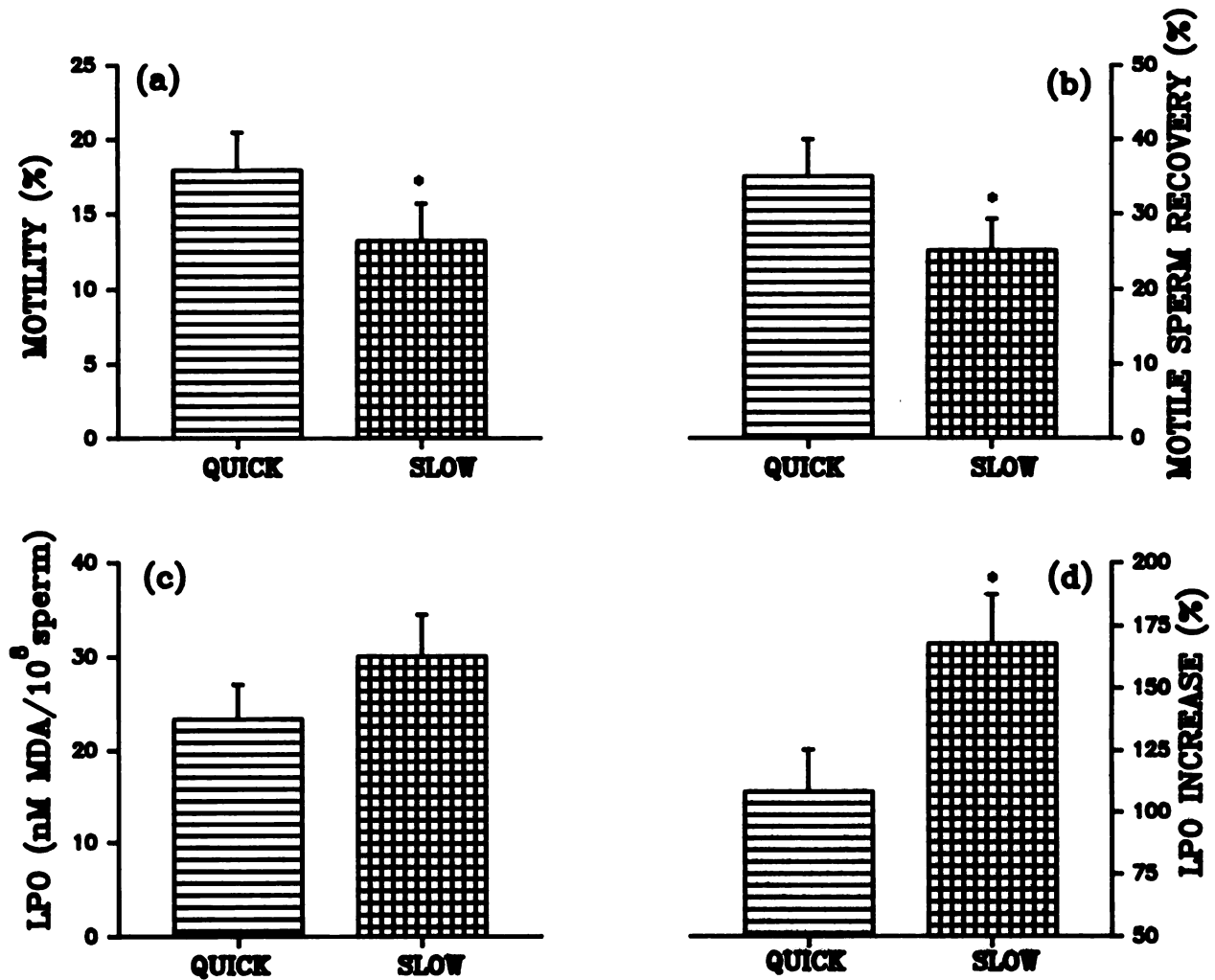
FIG. 2. Effect of quick and slow thaw on sperm parameters. (a), Percent motility; (b), percent of original motility maintained (motility recovery); (c), LPO; (d), LPO increase (compared to fresh) in quick-and slow-thawed human sperm (see Materials and Methods for details). The values for quick-thaw samples are used as controls. Data are shown as mean ± standard error; n = 10; * = P < 0.05.

Results

Effect of Quick versus Slow Thaw on Sperm Motility

Post-thaw motility scores (%) were significantly higher (Fig. 1a) in quick-thaw samples (42 ± 7) compared to those in slow-thaw samples (32 ± 7).

Recovery of motile sperm was significantly ($P < 0.05$) improved in samples thawed quickly (37°C, 5 minutes) when compared to samples thawed slowly (4°C, 30 minutes). In ten samples analyzed, post-thaw motility was 17.9 ± 2.5% in quick-thaw samples (controls) compared to 13.2 ± 2.4% in slow-thaw samples (Fig. 2a). Compared to the sperm motility in fresh samples (56.1 ± 4.0%), the percent of original motility maintained during cryopreservation (motility recovery) was 35.2 ± 4.3% in the quick-

thaw samples, but 24.3 ± 3.9% in the slow-thawed samples (Fig. 2b). The number of motile sperm recovered in quick-thaw samples (4.0 ± 0.8 × 10⁶) was 15% greater than that in slow-thaw samples (3.4 ± 0.7 × 10⁶).

Effect of Quick versus Slow Thaw on LPO

LPO was higher in samples thawed slowly than in samples thawed quickly. In the 10 samples described above, post-thaw LPO was 23.32 ± 3.7 in samples thawed quickly at 37°C and 30.09 ± 4.4 in samples thawed slowly at 4°C (Fig. 2c). When compared with fresh samples, LPO increased by 108.4 ± 12.7% in quick-thawed samples and 168.7 ± 13.1% in slow-thawed samples (Fig. 2d). This represents a significant (36%, $P < 0.05$) increase in LPO after cryopreservation and thawing in slow-thawed samples as compared to quick-thawed samples.
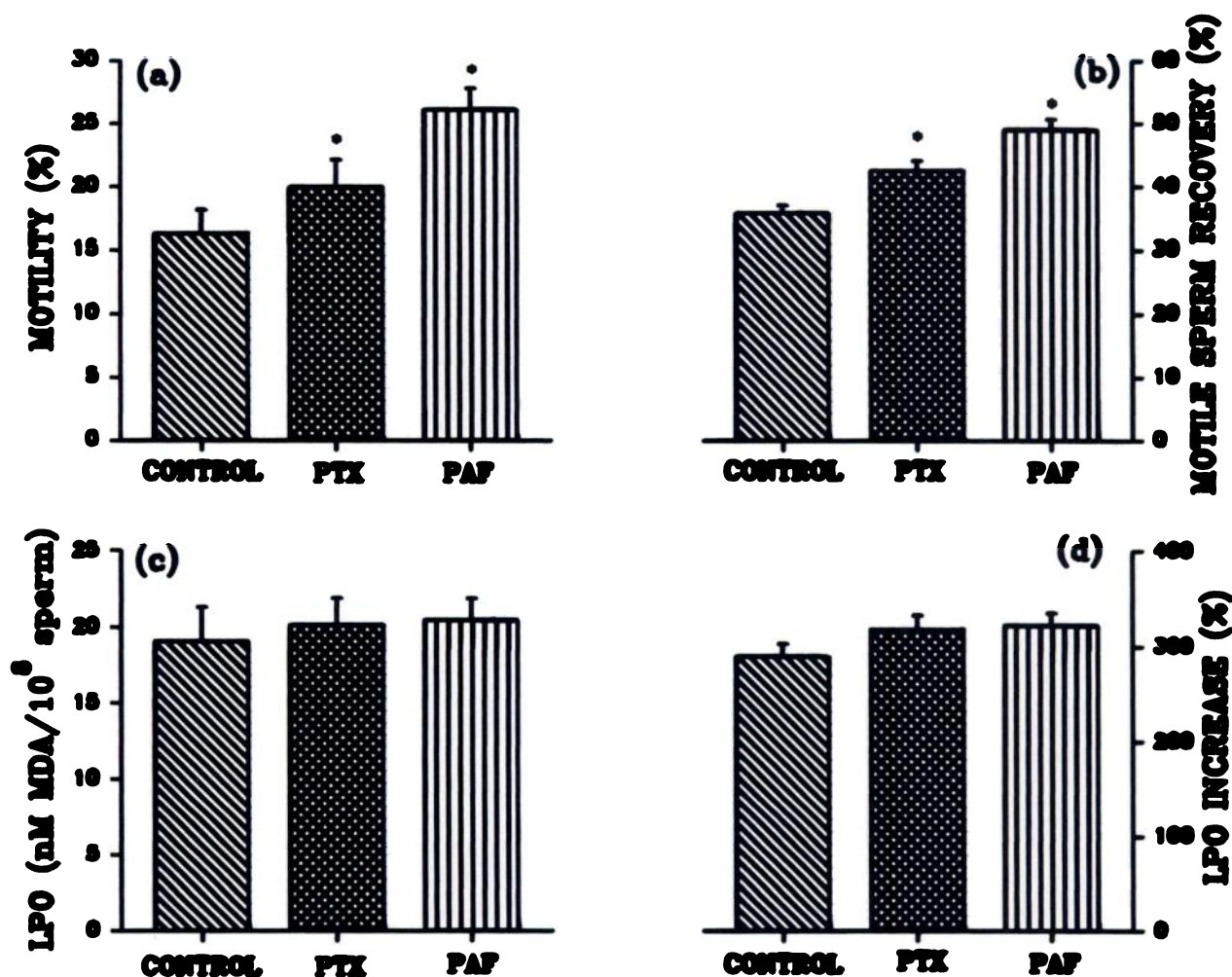
FIG. 3. Effect of cryoprotective additives (PAF and PTX) on sperm parameters. (a), Percent motility; (b), recovery of motile sperm; (c), LPO; (d), LPO increase (compared to fresh). PAF (1 μM) and PTX (3 mM) were added prior to cryopreservation (see Materials and Methods for details). Data represent mean ± standard error; $n = 8$; * = $P < 0.05$ compared to control (in absence of additives).

Effect of Cryoprotectants on Sperm Motility

Compared to controls (30 ± 3), post-thaw motility scores (%) were significantly improved by the addition of PAF (59 ± 3) or PTX (48 ± 3) prior to cryopreservation (Fig. 1b).

Samples cryopreserved in the presence of PAF (1 μM) and PTX (3 mM) exhibited a significant ($P < 0.05$) increase in the post-thaw recovery of motile sperm when compared to controls. Post-thaw motility was 16.3 ± 1.8% in controls, 20.0 ± 2.1% in PTX-treated samples, and 26.1 ± 1.7% in PAF-treated samples (Fig. 3a). When compared with the percentage of motility in fresh samples (58.0 ± 2.7%), the percentage of original motility recovered after cryopreservation/thawing was 49.0 ± 1.7% in PAF samples, 42.6 ± 1.5% in PTX samples, and 35.8 ± 1.2% in controls (Fig. 3b). The total number of motile sperm recovered was 6.5 ± 0.8 × 10⁶ in PAF samples,

4.8 ± 0.7 × 10⁶ in PTX samples, and 4.0 ± 0.6 × 10⁶ in controls.

Effect of Cryoprotectants on LPO

Sperm membrane LPO was not significantly altered by the addition of PAF or PTX during cryopreservation. Post-thaw LPO (nM MDA/10⁸ sperm) was 19.1 ± 2.2 in controls, 20.2 ± 1.7 in PTX-treated samples, and 20.5 ± 1.4 in PAF-treated samples (Fig. 3c). When compared to fresh samples (6.59 ± 1.4 nM MDA/10⁸ sperm), the percentage of increase in LPO after cryopreservation was 289.8 ± 13.2% in control samples, 318.7 ± 14.0% in PTX samples, and 322.5 ± 12.4% in PAF samples (Fig. 3d).

LPO and Sperm Motility

Figure 4 demonstrates the regression analysis results (scatter-plot and regression lines) comparing the changes in
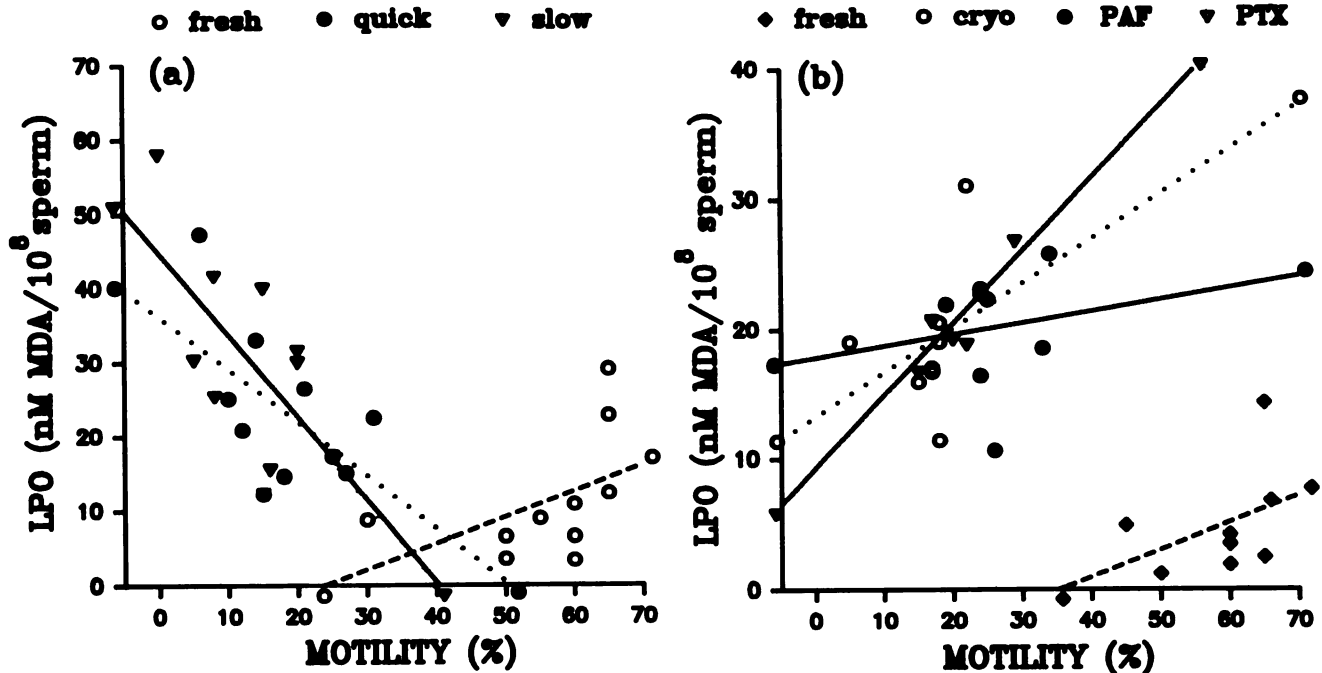
FIG. 4. Linear regression analysis of percent motility and lipid peroxidation. (a), Regression lines for quick-and slow-thaw samples compared to fresh samples. Regression line slopes for quick-and slow-thaw samples are significantly different than 0. (b), Regression lines for cryopreserved control, PAF-and PTX-treated samples compared to fresh samples.

motility percentage and LPO. A positive correlation was observed between the two parameters in fresh samples (r = 0.42), whereas a negative correlation was observed in cryopreserved samples (Fig. 4a). The degree of negativity was higher in slow-thaw samples (r = −0.62) than in quick-thaw samples (r = −0.54). A positive correlation was observed between motility percentage and LPO in fresh (r = 0.38), cryopreserved control (r = 0.30), PAF-treated (r = 0.09), and PTX-treated (r = 0.82) samples (Fig. 4b). P > 0.05 for all regression analyses.

Discussion

In sperm, as with other intact cells, damage results from the cryopreservation/thawing process. Reduced motility is one major manifestation of cell damage in cryopreserved sperm. Sperm cryopreservation has been performed for several years, and the most successful techniques involve a slow, progressive cooling of semen samples in the presence of TEST-yolk buffer before submersion into liquid nitrogen, and a rapid thawing at 37°C (Sherman, 1990).

In recent years, the AIDS crisis has contributed to the increasing necessity of successful sperm cryopreservation. World Health Organization and American Fertility Society guidelines require a 6-month quarantine of semen samples before use in any of the newly developed, assisted reproductive technologies (Ethics Committee of the American Fertility Society, 1990). The success of any

technology involving cryopreserved sperm depends upon the ability to recover large numbers of progressively motile sperm that have intact, functional membranes. As we improve our understanding of how sperm are damaged by cryopreservation, we will improve our ability to focus on mechanisms that not only reduce the damage but also improve the success of assisted reproductive technologies. The focus of our present study is cryopreservation-induced damage as reflected by membrane lipid peroxidation (LPO) and the potential benefits of two prospective cryoprotective additives, platelet-activating factor (PAF) and pentoxifylline (PTX).

Sperm membranes are damaged by the processes of cryopreservation and thawing (Hammerstedt et al, 1990). Cryopreservation and thawing, either alone or in combination, are likely to induce membrane damage and thus impair sperm motility and function. Many of the biochemical details of how cryopreservation and thawing protocols affect the recovery of motile sperm remain unknown. One measure of membrane damage is the degree of LPO of unsaturated fatty acids in cell membranes induced by the generation of free radicals (Torreilles et al, 1992). A wide range of values has been reported for human sperm LPO (Alvarez et al, 1987). Sperm membrane LPO has also been shown to increase significantly during the cryopreservation/thawing process, probably due to the release of oxygen radicals (Alvarez and Storey, 1992). The present study demonstrates one-to threefold increases in

post-thaw LPO, depending on experimental conditions. These results are in accord with recent reports by Alvarez and Storey, who demonstrated a cryopreservation-induced decrease in the activity of superoxide dismutase, an enzyme necessary for protection from lipid peroxidation (Alvarez and Storey, 1992).

A positive correlation between membrane integrity and sperm motion has been suggested (Emerit et al, 1991; Bell et al, 1992). As membrane integrity changes, ions flowing into and out of cells are changed. Proper ion balance is required for mitochondrial generation of ATP (Mishra et al, 1989). Additionally, the generation of ATP energy inside the mitochondria is a membrane-dependent process. Any damage to or alteration of cell membranes could negatively affect mitochondrial ATP generation. Without sufficient ATP energy, sperm are not progressively motile (Serres et al, 1991). How the cryopreservation and thawing process affects this ATP generation in sperm remains to be evaluated.

This study analyzed sperm motility and membrane LPO in cryopreserved samples subjected to alterations in the standard thawing protocol. The results of the thawing protocol comparison indicate a significant increase in LPO of slow-thawed samples with a simultaneous decrease in motility. This predicts a possible correlation between the two parameters as observed herein, and statistical analysis of the data yielded a higher negative correlation r-value between LPO and percentage of motility for slow-thaw samples (-0.62) than for quick-thaw samples (-0.54), as shown in Figure 4a.

The increase in LPO of control samples quickly thawed at 37°C was higher than that of control samples thawed at 37°C for the comparison of cryoprotectants (Figs. 2d, 3d). Because both control groups were cryopreserved and thawed according to the same protocol, it was expected that LPO values (percent increase compared to fresh; Figs. 2d, 3d) would be similar. The differences in these two values may be attributable to the fact that all LPO analyses of the samples used in the first set of experiments for comparison of thawing procedures were conducted within 1 hour of thawing, whereas LPO analyses of samples used for additive comparison were conducted between the first and second hours post-thaw. These results suggest that time is a critical factor in the LPO of biological membranes and should be carefully monitored. This implication is in agreement with previous reports of a time-dependent scheme of LPO increase (Emerit et al, 1991).

LPO seems to be only partially responsible for reduced post-thaw motility. The results of the cryopreservation protocol comparison indicate that recovery of sperm motility in post-thaw samples is enhanced by the addition of PAF or PTX, but lipid peroxidative damage to the sperm membranes is not reduced by their presence (Fig. 3).

The improved recovery of post-thaw motility in the presence of PAF or PTX in spite of increasing LPO during the freeze-thaw process is also evidenced by positive correlation r-values between LPO and percent motility (Fig. 4b). This suggests that protection from membrane LPO is not the mechanism for the cryoprotective effects of PAF or PTX.

Several possibilities exist as potential mechanisms of cryoprotective action for PAF and PTX. PAF is a derivative of phosphatidylcholine. It is involved in many biological activities—including platelet aggregation, anaphylaxis, and vascular permeability—and it has been shown to be a natural component of human sperm cells (Minhas et al, 1991). Maintenance of sperm membrane integrity and prevention of membrane damage during the cryopreservation/thawing processes can be attributed to PAF when used as a cryoprotective additive. Significant to this study is the known ability of PAF to increase calcium mobilization, which in turn can improve flagellar motion by increasing intracellular ATP levels (Ricker et al, 1989). It has been shown to improve the motility of post-thaw sperm samples (Hellstrom et al, 1991).

PTX is a tri-substituted methylxanthine. It functions as a phosphodiesterase inhibitor, increasing intracellular cyclic 3',5'-adenosine monophosphate (cAMP) levels, considered to be an important component of the sperm motility process (Tash and Means, 1983). The addition of PTX to post-thaw as well as to electroejaculated sperm samples with low motility has been shown to improve the recovery of motile sperm (Sikka and Hellstrom, 1990, 1991). Based on these observations, a 3 mM dose of PTX, as used in the present study, would likely stimulate sperm motility via phosphodiesterase inhibition. As expected, post-thaw motility was improved by PTX addition (Fig. 3a,b). Phosphodiesterase activity and cAMP levels were not measured in this study.

In summary, this study supports recent observations that membrane LPO is involved in freeze-thaw damage to sperm motility and suggests that peroxidative damage is increased when samples are thawed slowly. Additionally, this study has clearly demonstrated post-thaw motility improvements as a result of pre-freeze addition of PAF or PTX to normal semen samples. In patient samples with marginal motility the potential improvements that could result from PAF or PTX addition become even more critical. Although protection from LPO cannot be considered a benefit of their usage, we recommend the use of PAF or PTX as cryoprotectant additives. In order to explain the cryoprotective benefits of PAF and PTX, additional biochemical investigations are needed. Detailed studies of the changes in sperm membranes as a result of cryopreservation and thawing would be highly beneficial to our understanding of sperm membrane function.

Acknowledgments

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