Effect of thawing temperature on the motility recovery of cryopreserved human spermatozoa

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Objective: To investigate the effects of thawing temperature on sperm function after cryopreservation. The technical aspects of sperm cryopreservation have significantly improved over the last few decades. However, a standard protocol designed to optimize sperm motility recovery after thawing has not yet been established.
Design: Prospective study.
Setting: Private infertility institute and university-based research laboratory.
Patient(s): Eighty consenting normozoospermic patients consulting for infertility.
Intervention(s): Spermatozoa from donor semen samples were thawed at different temperatures.
Main Outcome Measure(s): Sperm motility, viability, adenosine-5'-triphosphate (ATP) content, acrosomal status, and DNA integrity were evaluated as a function of thawing temperature in cryopreserved human sperm samples.
Result(s): Thawing at 40°C resulted in a statistically significant increase in sperm motility recovery compared with thawing at temperatures between 20°C and 37°C. There were no statistically significant differences in sperm viability, acrosomal status, ATP content, and DNA integrity after thawing at 40°C compared with thawing at temperatures between 20°C and 37°C.
Conclusion(s): Sperm thawing at 40°C could be safely used to improve motility recovery after sperm cryopreservation. (Fertil Steril® 2008;■:■–■. ©2008 by American Society for Reproductive Medicine.)
Key Words: Cryopreservation, human sperm, temperature, ATP, DNA damage

While the technical aspects of sperm cryopreservation have significantly improved over the last few decades (1), both the optimal rate of cooling to freeze and optimal rate of warming to thaw in sperm cryopreservation protocols vary in different systems with regard to cryodamage; these protocols (2) require further resolution for mammalian sperm (3).

During freezing, spermatozoa are exposed to physical and chemical stress (4), changes in lipid composition of the sperm plasma membrane (5), reduced head size (6), and externalization of phosphatidylserine (7). Other factors that contribute to these changes are the degree of sperm maturation and the type of cryoprotectant used (8). The optimal warming rate depends on the cooling rate in a given system (3). Rapid warming during thawing usually results in higher post-thaw sperm viability (9, 10). To date, virtually all cryopreservation proto-

cols use a thawing temperature of 37°C. Higher thawing temperatures should give even more rapid warming but are not generally used because of the perceived risk of cell damage at or above 37°C. Incubation of spermatozoa at 40°C for 4 hours constitutes the basis of the recently introduced sperm stress test, which has proved to be a useful predictor of pregnancy rates in assisted reproductive technologies (11, 12). Despite these findings, the time of exposure and the effects on sperm viability of thawing at 40°C remain to be investigated. Therefore, one of first objectives of this study was to assess the effects of thawing at temperatures between 20°C and 40°C on sperm post-thaw motility.

The plasma membrane of mammalian spermatozoa has a high polyunsaturated fatty acid content, which makes it highly susceptible to lipid peroxidation. This is a known risk factor that may contribute to sperm cryodamage. Human spermatozoa are particularly susceptible to oxygen radical–induced lipid peroxidation (13, 14). Lipid peroxidation reactions have been shown to have a steep temperature coefficient (14), and, therefore, there may be a greater risk of peroxidative damage at 40°C compared with at lower temperatures. Human sperm possess limited enzymatic antioxidant protection, which varies widely between individual sperm samples (14). Peroxidative reactions result in membrane phospholipid damage leading to increased membrane permeability and loss of adenosine-5'-triphosphate (ATP) and sperm motility (15,

16). Intracellular ATP drives the sperm flagellum and is, therefore, of paramount importance in the maintenance of sperm motility (17). Loss of ATP is a major indicator of membrane damage leading to loss of viability and sperm fertilizing ability.

However, in addition to membrane-related cryodamage, oxygen radicals have been shown to induce damage to the sperm axoneme and nuclear DNA (18). The production of a superoxide anion (O_2^-) has been shown to increase during thawing in bovine ejaculated sperm (19). This may explain, at least in part, why DNA damage increases right after thawing (16). Therefore, the second objective of this study was to assess both ATP content and DNA integrity in human sperm after thawing at 40°C as compared with thawing at temperatures between 20°C and 37°C.

MATERIALS AND METHODS
Semen Collection
Semen samples were obtained from 80 normozoospermic patients attending our clinic for infertility screening. The age of the patients ranged between 20 and 30 years. The study protocol was approved by the Laboratorio de Estudios en Reproducción 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 for 30 minutes at room temperature, and, after liquefaction, sperm concentration and percent motility were determined by computer-assisted sperm motion analysis (CASA).

Motility Parameters
Aliquots of 5 μL of the sperm suspension were placed into a Makler chamber prewarmed at 37°C. CASA analysis was performed using a Hamilton-Thorne digital image analyzer (HTR-IVOS v.10.8s; Hamilton-Thorne Research, Beverly, MA). 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 (VAP) cutoff, 5 μm/second^{-1}; medium VAP cutoff, 25 μm/second^{-1}; 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 (VCL μm/second^{-1}), progressive velocity (VSL μm/second^{-1}), path velocity (VAP μm/second^{-1}), straightness percentage, beat cross frequency (in hertz), and linearity (LIN percentage).

ISolate Density Gradient Centrifugation
Aliquots of 1 mL of the liquefied semen were loaded onto a 45% and 90% discontinuous ISolate gradient (Irvine Scien-

tific, Santa Ana, CA) and centrifuged at 400 g for 20 minutes, and the resulting interfaces between the 45% (F45) and 90% pellet (F90) were aspirated and transferred to separate test tubes. Sperm suspensions from the different ISolate fractions were diluted in one volume of Dulbecco's phosphate-buffered saline (PBS) and centrifuged a 400 g for 10 minutes. The pellet was resuspended in 1 mL of PBS, and an aliquot was used to determine the total number of spermatozoa and immature germ cells. Aliquots from each fraction were examined for sperm concentration, percent motility, DNA fragmentation, viability, acrosomal status, survival test, and ATP content.

Cryopreservation Protocol
The method used for freezing and thawing was that described by Srisombut and coworkers (20). 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 4.5% (Irvine Scientific) in a drop-wise fashion until a 1:1 volume 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 in 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 20°C, 37°C, 38°C, 39°C, or 40°C. The room temperature during the time of the year when these experiments were performed corresponded to 20 ± 1°C. Thawing temperature was monitored using a high-precision thermometer (Fluke, Everett, WA). Preliminary experiments showed that the optimal thawing time at 40°C was 3 minutes, and, therefore, this time was selected thereafter as the thawing time at 40°C. Similarly, preliminary experiments showed that there were no statistically significant differences in motility recovery between 3 and 5 minutes of thawing time at temperatures between 37°C and 39°C. Therefore, a thawing time of 3 minutes was also selected at these temperatures. It should be noted that during the thawing process the cryovials were subjected to periodic shaking to prevent the occurrence of the so-called boundary effect. Therefore, the temperature reported as the thawing temperature corresponds to the temperature of the water immediately surrounding the cryovial containing the sperm sample.

ATP Determination
An ATP bioluminescent assay kit (Sigma-Aldrich, St. Louis) was used for the quantitative determination of ATP content in the sperm samples. The ATP assay mix contained lyophilized powder containing luciferase, luciferin, $MgSO_4$, DTT, EDTA, bovine serum albumin (BSA), and tricine buffer salt. The ATP assay mix dilution buffer contained lyophilized powder including $MgSO_4$, DTT, EDTA, BSA, and tris-HCl buffer salt. The ATP standard preweighed vial contained 1 mg (2×10^{-6} moles). For the reaction, 0.1 mL of ATP assay mix solution was added to the reaction vials and allowed to

stand at room temperature for 3 minutes. During this period, any endogenous ATP that was produced would be hydrolyzed. The assay was started by the addition of 0.1 mL of ATP standard or the sperm sample and after mixing the amount of light produced was measured using a Wallac 1250 LKB luminometer.

Acrosomal Status and Sperm Viability

Aliquots of 90 μL were used to assess acrosomal status using the method of fluorescein isothiocianate (FITC)-labeled *Pisum sativum agglutinin* (PSA) (21). Viability of the cells was assessed using the hypoosmotic swelling test (HOST), which identifies living cells with intact plasma membrane (22). Viable cells were identified based on the presence of a curled tail when the spermatozoa were forced to swell by immersion in the hypoosmotic solution. We used a combination of PSA and HOST to identify acrosome-reacted viable spermatozoa (23). To study the acrosome status of the spermatozoa, 90 μL of the sperm suspension was washed twice with 100 μL of PBS-BSA (PBS solution containing 1% of BSA) and centrifuged for 5 minutes at 400 g. The pellet was then resuspended in 100 μL PBS-BSA and combined with 1 mL of hyposmotic buffer (7.35 g sodium citrate and 13.51 g fructose dissolved in 1000 mL of water, giving an osmolarity of 150 mOsm/kg) and incubated for 1 hour at 37°C. Aliquots of 20 μL of the sperm suspension were used to prepare the slides. After air-drying, the slides were dipped into methanol for 15 minutes and then allowed to dry very rapidly. Slides were washed in 100 μL of PBS-BSA and processed immediately. Methanol-fixed slides were incubated for 30 minutes at room temperature in a moisture chamber with a solution of FITC-PSA (50 μg/mL; Sigma-Aldrich, St. Louis) in PBS-BSA. The slides were then washed 3 times (10 minutes each) in PBS-BSA to remove unbound probe. After drying, the slides were mounted with 5 μL of antiquenching medium and examined by epifluorescence using a microscope L-llola GCQ 75. At least 200 cells were evaluated for each experiment.

TUNEL Assay

Oxygen radical–induced DNA damage may result in double-stranded low molecular weight DNA fragmentation as well as single-strand breaks (18). DNA strand breaks were detected by TUNEL using the Apoptosis Detection System Fluorescein Kit (Promega, Madison, WI), according to manufacturer's instructions. This kit uses fluorescein-conjugated dUTP as the fluorochrome. Briefly, the spermatozoa were washed in PBS, fixed in methanol, and permeabilized with 0.2% Triton X-100. This was followed by a 1-hour incubation in the dark at 37°C in the TUNEL reaction mixture containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme (TdT). The slides were then immersed in PBS and then in 1 μg/mL propidium iodide. Negative and positive controls (no TdT enzyme and treatment with DNAase, respectively) were performed in each experiment. At least 200 cells were analyzed per slide using epifluorescent microscopy (L-llola GPQ 75, ×1000).

TABLE 1	
Basal semen parameters of samples included in this study.	
Parameter	**Mean (95% confidence interval)**
Sperm concentration, 10^6/mL	145.3 (79.8–210.8)
VSL, μm/second	56.6 (47.1–66.1)
VCL, μm/second	99.5 (80.5–118.5)
Lateral head displacement (ALH), μm	3.9 (2.7–5.1)
LIN, %	57.9 (50.8–65.0)
Motility, %	72.0 (63.1–80.9)
Normal forms (strict criteria), %	19.0 (16.1–21.9)
Round cells, 10^6/mL	1.0 (0–2.1)

Note: Sperm concentration, motility, and motion parameters were assessed using a Hamilton-Thorne Digital Image Analyzer (n = 80).

Calamera. Thawing temperature for cryopreserved human sperm. Fertil Steril 2008.

Statistical Analysis

The results have been expressed as mean ± SD. Data were analyzed by Student's *t*-test, comparing groups and other selected pairs, and by one-way analysis of variance for time points, using a computer-driven software package GraphPad Instat, version 2.02 (GraphPad Software, Inc., La Jolla, CA). $P<.05$ was considered statistically significant.

RESULTS

The basal semen parameters obtained in the samples evaluated are shown in Table 1. Post-thaw motility recovery in fresh semen samples and in the F90 fractions was determined as a function of thawing temperature (Table 2). Although motility recovery in fresh semen and in the F90 fractions did not show significant differences when consecutive temperatures were compared, these differences were statistically significant when comparing motility recovery after thawing at 37°C versus 40°C ($P<.05$).

Post-thaw sperm kinematic parameters in F90 samples thawed at 37°C versus 40°C did not show statistically significant differences (Table 3). This beneficial effect in motility recovery after thawing did not appear to affect sperm function.

Increasing thawing temperature above 37°C could have detrimental effects on sperm viability due to an increase in the production and reactivity of oxygen radicals (11, 12). To evaluate the potential detrimental effect of thawing at

TABLE 2

Sperm motility before and after thawing the samples at different temperatures.

	Before thawing, basal	After thawing				
		Room temperature	37°C	38°C	39°C	40°C
Semen	74.4 ± 8.8	56.9 ± 17.6	48.6 ± 17.9	54.3 ± 17.7	55.1 ± 13.9	63.2 ± 12.6[a]
F90	97 ± 2.1	52.4 ± 16.6	57.0 ± 19	58.3 ± 19.3	61.7 ± 15.9	72.7 ± 17.5[a]

Note: Values are expressed as the mean ± SD (n = 80).
[a] Statistically significant differences ($P < .05$), when compared with thawing at 37°C.

Calamera. Thawing temperature for cryopreserved human sperm. Fertil Steril 2008.

temperatures higher than 37°C on oxygen radical–induced membrane damage, several sperm parameters were evaluated. These included sperm viability, acrosomal status, and sperm survival (Table 4). There were no statistically significant differences in these parameters at the different thawing temperatures used.

On the other hand, to assess non-membrane-related oxygen radical–induced sperm damage at the different thawing temperatures tested, sperm DNA integrity and ATP content were evaluated. Thawing at 40°C did not have any observable detrimental effect on DNA integrity (Fig. 1B). No significant differences in ATP content were observed after thawing at 40°C versus 37°C (Fig. 1A). However, when ATP content was compared in samples with a motility recovery after thawing >50% versus <50%, a higher ATP content was found in samples with a motility recovery >50% (data not shown; $P < .01$).

DISCUSSION

The main finding emerging from this study is the increase in motility recovery observed after thawing of cryopreserved sperm at 40°C compared with the standard thawing temperature of 37°C. Sperm thawing at 40°C resulted in a 23.1% increase in sperm motility recovery, compared with thawing at 37°C. This finding was observed after cryopreservation of either semen samples or their corresponding F90 fractions, which are known to contain spermatozoa of the highest functional quality (24).

It is well-known that cryopreservation increases the rate of lipid peroxidation and that this might be related, at least in part, to the loss of antioxidant enzymatic activity (25). Nevertheless, these authors concluded that the peroxidative contribution is relatively negligible compared with the role played by membrane stress with loss of membrane permeability barriers in sperm cryodamage (2, 25). Also, it has been previously reported that human sperm cryopreservation is associated with a loss of plasma membrane fluidity and that higher motility recovery was associated with higher membrane fluidity in the fresh samples (26).

Sperm motility is the result of a complex biomechanical process by which the flagellum projects the sperm head forward. For that reason, kinematic parameters of motility prefreeze and post-thaw were also evaluated in this study. Although the percentages of motile sperm in the fresh and thawed F90 samples were different at 37°C versus 40°C, kinematic parameters did not show any significant differences (Table 3). No significant differences in sperm viability or acrosomal status were observed when cryopreserved sperm were thawed at 37°C versus 40°C (Table 3).

Cryopreservation of human spermatozoa has been associated with increased susceptibility to sperm DNA fragmentation (27). Fertilization of oocytes by sperm with damaged DNA may lead to mutations, risk of cancer in the offspring (28), and defective embryo development (29). It was reported that thawing of human sperm at 37°C and 40°C resulted in

TABLE 3

Sperm kinematic values of samples before and after thawing at 37°C and 40°C.

	Before freezing	F90 at 37°C	F90 at 40°C
VAP, μm/second	66.8 ± 9.9	50.6 ± 8.2	51.1 ± 8.7
VSL, μm/second	56.6 ± 9.5	45.6 ± 7.2	46.5 ± 8.3
VCL, μm/second	99.5 ± 19	68.9 ± 11.1	68.1 ± 11.9
ALH, μm	3.9 ± 1.2	2.8 ± 0.3	2.8 ± 0.4
LIN, %	57.9 ± 7.1	66.3 ± 3.3	68.4 ± 4.6

Note: Values are expressed as the mean ± SD (n = 80). P = NS for all comparisons.

Calamera. Thawing temperature for cryopreserved human sperm. Fertil Steril 2008.

TABLE 4

Viability post-thawing, survival after 24 hours of incubation, and percentage of sperm with intact acrosome in samples thawed at 37°C and 40°C.

Parameter	37°C	40°C
Viability, %	82.0 ± 5.2	82.1 ± 10.1
Survival after 24 hours of incubation, %	20.6 ± 15.9	19.9 ± 12.9
Sperm with intact acrosome, %	75.6 ± 2.5	75.2 ± 7.4

Note: Values are expressed as the mean ± SD (n = 80).

Calamera. Thawing temperature for cryopreserved human sperm. Fertil Steril 2008.

a significant increase in DNA fragmentation in different sperm subsets (30). To determine whether thawing at 40°C would increase sperm DNA fragmentation compared with thawing at 37°C, sperm DNA fragmentation levels were determined by TUNEL. No significant differences in DNA fragmentation were found in the F90 fraction after thawing at 37°C versus 40°C. This observation provides further support for the notion that the physical and biological properties of those sperm that survive the freeze-thaw process and that retain their motility are similar to those found in sperm from the prefreeze sample.

One potential explanation for the observed increase in motility recovery after thawing at 40°C would be a faster rate of recovery of sperm enzymatic antioxidant activity. During thawing, there are two competing processes that determine the degree of cell damage: [1] the magnitude of oxygen radical production and [2] the rate of recovery of enzymatic antioxidant activity. The higher the temperature, the faster the recovery of enzymatic antioxidant activity, and, therefore, after thawing at 40°C, sperm will be able to neutralize more efficiently than at 37°C the increase in oxygen radical production reported to occur during thawing (19). However, the use of higher temperatures during thawing should be highly controlled in terms of magnitude and duration to avoid cell damage. Thawing at 40°C during 3 minutes was selected because it did not result in cell damage compared with thawing at 37°C and, at the same time, resulted in a significant increase in motility recovery. However, since sperm viability, acrosomal status, DNA integrity, and sperm survival were similar at 37°C versus 40°C, the type of cell damage prevented by a faster rate of recovery of antioxidant enzymatic activity might be related to ATP use in the axoneme. Rather than affecting the recovery of enzymatic antioxidant activity at the level of the membrane and/or nuclear compartments, sperm thawing at 40°C would affect antioxidant enzyme activity localized in the highly compact axonemal compartment, which is virtually devoid of cytoplasm. Although there were no statistically significant differences in ATP content in sperm from the F90 fraction after thawing at 37°C versus 40°C, a higher ATP content was found in samples with a motility recovery >50%. Since ATP steady-state levels represent the balance between biosynthesis and use of ATP, it cannot be ruled out that thawing at 40°C might have an effect in increasing the biosynthesis and/or use of ATP in the axoneme by preventing oxygen radical–induced damage to the axoneme more efficiently. This damage could be reversible in a first stage and become irreversible thereafter (31).

In summary, thawing of cryopreserved human sperm at 40°C results in a statistically significant increase in motility recovery compared with thawing at 37°C. This increase in motility recovery appears to be related to a more rapid and

FIGURE 1

ATP levels (**A**) and DNA damage (**B**) in semen samples thawed at 37°C and 40°C. Values are expressed as the mean ± SD (n = 10).
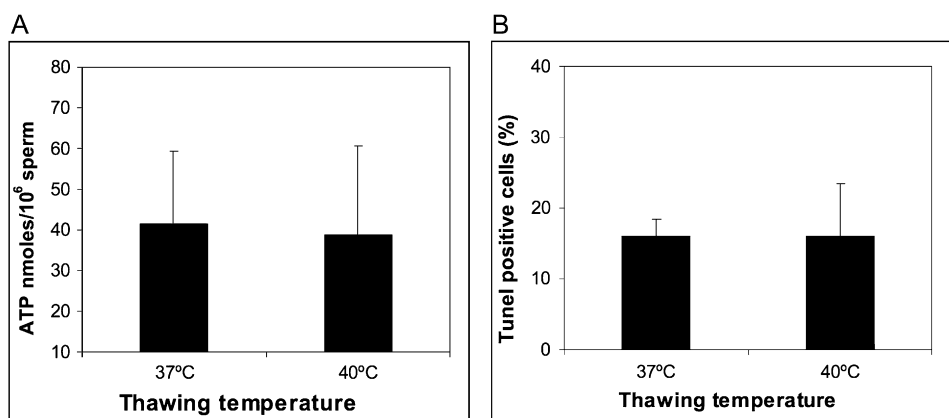
Calamera. Thawing temperature for cryopreserved human sperm. Fertil Steril 2008.

complete recovery of membrane integrity and permeability and perhaps to a more efficient preservation of the biosynthesis and use of ATP in the axoneme. The short incubation time at 40°C required for thawing does not appear to be detrimental to sperm. Thawing of human sperm at 40°C for 3 minutes may therefore prove to be advantageous for the recovery of functional human spermatozoa after cryopreservation. Additional studies are required to assess whether sperm thawed at 40°C have similar fertilization and pregnancy rates in IVF and IUI compared with those thawed at the standard temperature of 37°C.

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