Journal of Andrology, Vol. 25, No. 4, July/August 2004

Semen Characteristics After Overnight Shipping: Preservation of Sperm Concentrations, HspA2 Ratios, CK Activity, Cytoplasmic Retention, Chromatin Maturity, DNA Integrity, and Sperm Shape

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ABSTRACT: We tested several approaches that can be used to preserve sperm attributes and the objective biochemical markers of sperm maturity and function for assessment in a remote centralized laboratory after overnight shipping of semen samples. Addition of phenyl-methyl-sulfonyl-fluoride (PMSF) to a final concentration of 20 μg/mL semen at 4°C has preserved sperm concentrations and HspA2 isoform ratios, even at room temperature, simulating a shipping delay in moderate ambient temperatures. Regarding the attributes of individual spermatozoa, the patterns of CK-immunocytochemistry (demonstrates cytoplasmic retention in diminished-maturity spermatozoa); aniline blue staining pattern (tests chromatin maturity); sperm shape assessed by both Kruger strict morphology and computer assisted morphometry; and sperm DNA integrity, as tested by DNA nick translation, all remained unchanged. Thus, the PMSF-4°C conditions preserved sperm concentrations and the cytoplasmic and nuclear biomarkers of sperm cellular maturity and function for next-day analysis. This shipping method will facilitate the early detection of subtle changes in semen quality that can affect sperm function, even when there has been no decline in sperm concentrations to signal possible toxic effects. Furthermore, sample preservation will enable investigators to evaluate semen for toxicology studies and for diagnosis of male infertility from remote locations. Home collection of semen should enhance study participation, and semen assessment in centralized laboratories will address concerns regarding interlaboratory variations and quality control.

Key words: Reproductive toxicity, male infertility, cytoplasmic and nuclear biomarkers.

J Androl 2004;25:593–604

Exposures to environmental toxicants can have detrimental effects on human male reproduction (Schrader et al, 1992; Wyrobek et al, 1997). In field studies, two levels of semen analysis are usually carried out: primary studies, which can include the conventional semen parameters of sperm concentration, motility, and morphology, as well as measurement of specific biomarkers selected by the expected health effects of the environmental toxicants causing the exposure, and secondary studies based on archived samples (ie, frozen samples, videotaped microscopic fields, or sperm smears) that are used later to further evaluate specific findings. We are developing a potential third approach, which recognizes that each semen sample is composed of sperm populations of various levels of cellular maturity. The proportions of mature and diminished-maturity sperm, which define the functional efficacy of a semen sample, can be measured by objective biochemical methods, independently from sperm concentrations and motility. This approach provides two potential advantages: 1) reliable sperm biochemical measurements, which could reflect changes in fertility and in the paternal contribution of sperm DNA to zygote development, even when a man has not reached a level of exposure that would affect his sperm concentration, and 2) detection of decline or improvement of the spermatogenetic process, and particularly of spermiogenetic maturation, during continuing exposure or removal from the adverse environment. The goal of this work is to extend the application of objective biochemical markers of sperm maturity and function to field studies by shipping semen samples with the sperm attributes preserved to a centralized laboratory.

Supported by National Institutes of Health grants OH-04061 and HD-19505. Part of this research was presented at the American Society of Andrology 2002 Annual Meeting, Seattle, WA.

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Received for publication December 9, 2003; accepted for publication February 2, 2004.

In the selection of sperm attributes for this preservation and shipping study, we focused on conventional semen parameters and on the objective biochemical markers of sperm maturity and function that we have previously identified. For the first such marker, sperm creatine kinase (CK), immunocytochemical studies indicated that high sperm CK activity was related to increased CK concentrations, as well as to abnormal and amorphous sperm head size and shape (Huszar et al, 1988a,b; Huszar and Vigue, 1993). These findings suggested to us that we had identified a sperm developmental defect in the last phase of spermiogenesis, the so-called cytoplasmic extrusion phase, when the cytoplasm (unnecessary for the mature sperm) is normally left in the adluminal area as ''residual bodies'' (Clermont, 1963). We have also found another adenosine triphosphate (ATP)–associated sperm protein that was later identified as the 70-kda testis-specific chaperone protein HspA2, the human homologue of HSP70–2 that has already been implicated in male infertility in rodents (Dix et al, 1996; Huszar et al, 2000). There was a five-fold difference in HspA2 ratios, expressed as % [HspA2/(HspA2 + CK-B)], in oligospermic (higher proportions of immature sperm) vs normospermic men (Huszar and Vigue, 1990), indicating that HspA2 is a marker of sperm biochemical maturity. Diminished sperm maturity was also related to increased frequencies of chromosomal aneuploidies (Kovanci et al, 2001).

We examined the utility of the CK activity marker in couples with oligospermic husbands whose wives were treated with intrauterine insemination. A logistic regression analysis based on 160 samples showed that increased CK activity was related to failure to cause pregnancies, whereas the sperm concentrations provided no predictive power (Huszar et al, 1990). The predictive value of low HspA2 ratios for diminished in vitro fertilization (IVF) pregnancy rates was tested in two blinded studies. First, in 84 couples undergoing IVF at Yale (New Haven, Conn) and at the Jones Institute (Norfolk, Va), no pregnancies occurred in men with HspA2 ratios below 10%. An additional important utility of the HspA2 ratio became apparent: 9 of the 22 men with HspA2 ratios below 10% were normospermic (Huszar et al, 1992). Thus, the HspA2 ratio provided, for the first time, a diagnostic tool for unexplained male infertility (infertile men with normal semen parameters but diminished sperm maturity). More recently, we demonstrated similar outcomes in 119 couples treated with conventional IVF at Yale. As in 1992, no men with HspA2 ratios below 10% caused pregnancy (13 of 25 men were normospermic). The receiver operating characteristic curve provided a cutoff value of 10.84% HspA2 ratio with a 100% positive predictive value for failure of pregnancy, whether the men were oligospermic or normospermic (Ergur et al, 2002). Thus, HspA2 ratios are reliable measures of sperm maturity and are likely to be useful in the early detection of reproductive toxicity and infertility, even if there has been no decline in sperm concentrations to signal possible toxic effects.

The goal of this study is the adaptation of conventional semen analysis and objective biochemical sperm assessment methods for portability, either in the form of kits usable at the study site or by means of preservation of semen and sperm for overnight transport. Analysis of samples collected at multiple sites in a centralized laboratory would be preferred because this method addresses concerns regarding interlaboratory and other variability and improved quality control. Also, home collection of samples is likely to enhance the participation of men with potential reproductive exposure (Schrader et al, 1992; Lahtedite, 1995; Opsahl et al, 1996; Wyrobek et al, 1997).

We report overnight storage and shipping conditions that preserve the semen attributes of sperm concentration and HspA2 ratio. The same conditions also preserve, in individual spermatozoa, the parameters of CK immunocytochemistry, aniline blue staining, DNA integrity tested with nick translation, and sperm shape, including the head and tail dimensions that are known to correlate with sperm maturity (Gergely et al, 1999; Celik-Ozenci et al, 2003). Thus, in addition to sperm concentrations, we are now able to preserve both the cytoplasmic and nuclear attributes of sperm in semen and in individual spermatozoa after overnight transportation to a remote laboratory.

Methods

Experimental Design

In these experiments, we have developed semen storage and shipping conditions that preserve semen attributes with high fidelity by addition of phenyl-methyl-sulfonide-fluoride (PMSF) to a concentration of 20 μg/mL semen, followed by storage of the semen at 2°C to 4°C for 24 hours (PMSF-4°C, simulated overnight shipping on an ice pack). First, we determined the potential effects of storage on the preservation of sperm concentrations (the number of samples studied and their respective sperm concentrations are noted at each experiment). Subsequently, we studied the effects of PMSF-4°C storage on the preservation of various sperm biochemical parameters and sperm shape. In 3 control experiments, we also tested aliquots of sperm from a subset of men for the preservation of CK activity and HspA2 ratios after 24 hours of PMSF storage at room temperature (this condition would occur in an incidental en route warming at ambient temperatures below 26°C). Furthermore, we looked at the effects of semen storage in the absence of a preservative. Finally, in another set of samples, we studied the preservation of sperm concentration, sperm CK activity, and HspA2 ratio with the use of 10 mM dithiothreitol (DTT) as an added preservative. Although the DTT results were promising in certain aspects, we abandoned this agent, because it initiated sperm

decondensation and subsequent changes in sperm head size, affecting both the strict sperm morphology and objective morphometric parameters.

In addition to testing the sperm population of semen samples, we also evaluated the potential effects of PMSF-4°C storage on the objective biochemical parameters of maturity and fertilizing potential in individual spermatozoa. These studies included 1) CK immunocytochemistry in sperm smears in order to demonstrate the conservation of cytoplasmic staining following shipping; 2) aniline blue staining to demonstrate the fidelity of preservation of chromatin maturity; 3) DNA nick translation to demonstrate retention of sperm DNA integrity during shipping; and 4) assessment of the conservation of sperm shape at the time of ejaculation and after 24 hours of PMSF-4°C incubation, using both Kruger strict morphology and objective morphometry of several sperm parameters, via the computer-based Metamorph program (Universal Imaging Co, Downington, Pa). The men investigated were those who presented for semen analysis at the Sperm Physiology Laboratory (Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Conn). All studies were approved by the Human Investigation Committee of Yale University School of Medicine.

Addition of PMSF to Semen

A PMSF stock solution of 2 mg/mL was added to semen in test tubes to a final concentration of 20 μg/mL semen, and the tubes were agitated to distribute the reagent. The PMSF concentration is not critical, providing it falls in a range above the threshold level necessary for inhibiting proteolytic enzymes. We have routinely use concentrations as low as 0.2 μg/mL for protection of proteins during chromatography or dialysis. We have also investigated whether PMSF concentrations greater than 20 μg/mL semen would affect HspA2 ratios. With concentrations of 20, 40, 60, 80, 100, and 160 μg PMSF/mL semen, the respective values were not different, with a coefficient of variation of 11% (n = 5 men).

In future shipping studies, we recommend the following procedures. 1) After ejaculation, the study subject should keep the semen in the collection vessel for 20–30 minutes to allow partial liquefaction. 2) Following the waiting period, the semen should be transferred with an eyedropper into a 5.0-mL graduated shipping tube that has been premarked to the 4.0 mL level. (The study staff will mark the tubes when placing 80 μg of PMSF in the shipping tubes.) If semen volume exceeds 4.0 mL, the leftover portion should be placed in the second tube supplied. Tubes are then sealed by their screw cups. 3) The tubes should be gently inverted 10 times and placed into the shipping container along with the prechilled ice pack. (The potential source of the transport tubes is United Lab Plastics and Equipment, St Louis, Mo).

This procedure will maintain PMSF levels at the desired concentration. (A second tube is not necessary for the semen studies but will facilitate the total semen volume determination when it exceeds 4.0 mL.) From the perspective of semen shipping, it is of interest that in our laboratory, 100 recent consecutive semen analysis samples showed a mean volume of 2.8 ± 0.1 mL (range 1.0–7.0 mL; 7 samples < 1.5 mL, 6 samples > 4.0 mL).

Determination of Sperm Concentrations

Sperm concentrations were assayed manually with a Makler chamber. After mixing the samples before each measurement, semen was applied to the Makler chamber, and 3 fields of 30–50 squares were evaluated from 2 drops in each sample. Use of a CASA instrument was not appropriate, because after the PMSF-4°C storage, sperm motility became almost negligible; thus, visual confirmation of the sperm tail during the assessment was necessary in order to maintain the accuracy of the counts.

CK Activity and HspA2 Measurements

These assays, standard procedures in our laboratory, were carried out as described previously (Huszar and Vigue, 1990; Huszar et al, 1992; Ergur et al, 2002). Aliquots of semen were washed with 10–15 volumes of ice-cold 0.15 M NaCl and 30 mM imidazole (pH 7.0) at 5000 × *g* in order to remove seminal fluid. The sperm pellets were disrupted by vortexing in 0.1% Triton, 30 mM imidazole (pH 7.0), 10% glycerol, and 5 mM DTT. The sperm extract was clarified by centrifugation at 5000 × *g*, and aliquots of the clear supernatant were subjected to CK activity determinations by a spectrophotometric CK kit (Sigma Co, St Louis, Mo).

CK-B and HspA2 were separated by electrophoresis on precast agarose gels (Helena Laboratories, Beaumont, Tex). The separated bands were developed by overlaying the gel with a fluorescent ATP substrate, and the ATP-containing CK-B and HspA2 bands were quantified under long-wave ultraviolet light with a scanning fluorometer and expressed as a ratio % [HspA2/(HspA2 + CK-B)].

CK-B Immunocytochemistry

The procedures used were described previously (Huszar and Vigue, 1993; Huszar et al, 2003). Both the initial semen and PMSF-4°C–preserved sperm fractions were fixed with 3.7% paraformaldehyde in phosphate buffer/sucrose (PB-suc) for 20 minutes at room temperature. Following the removal of the formalin, the slides were allowed to air dry. After 3 washing steps with PB-suc, the spermatozoa were exposed to a 3% bovine serum albumin blocking solution in PB-suc at room temperature. After further washing, the sperm were overlaid with a 1:1000 dilution of polyclonal anti–CK-B antiserum (Chemicon Co, Temecula, Calif). After further PB-suc washes, the slides were treated with a biotinylated second antibody at a 1:1000 dilution and were exposed to a Vector horseradish peroxidase/ABC kit (Vector Laboratories, Burlingame, Calif) according to the manufacturer's instructions. The avidin-biotin-complex (ABC)-treated slides were further processed with diaminobenzidine and hydrogen peroxide (Sigma, St. Louis, Mo). The developed brown color highlighted sperm with various degrees of cytoplasmic retention (Figure 1A). The specificity of the CK staining was established by using preimmune serum in place of the first antibody or by applying the second antibody only.

Aniline Blue Staining of Sperm Chromatin

Sperm smears were dried on glass slides and stained with a 5% aniline blue solution acidified to approximately pH 3.5 with acetic acid. The slides were washed and air dried, and a coverslip

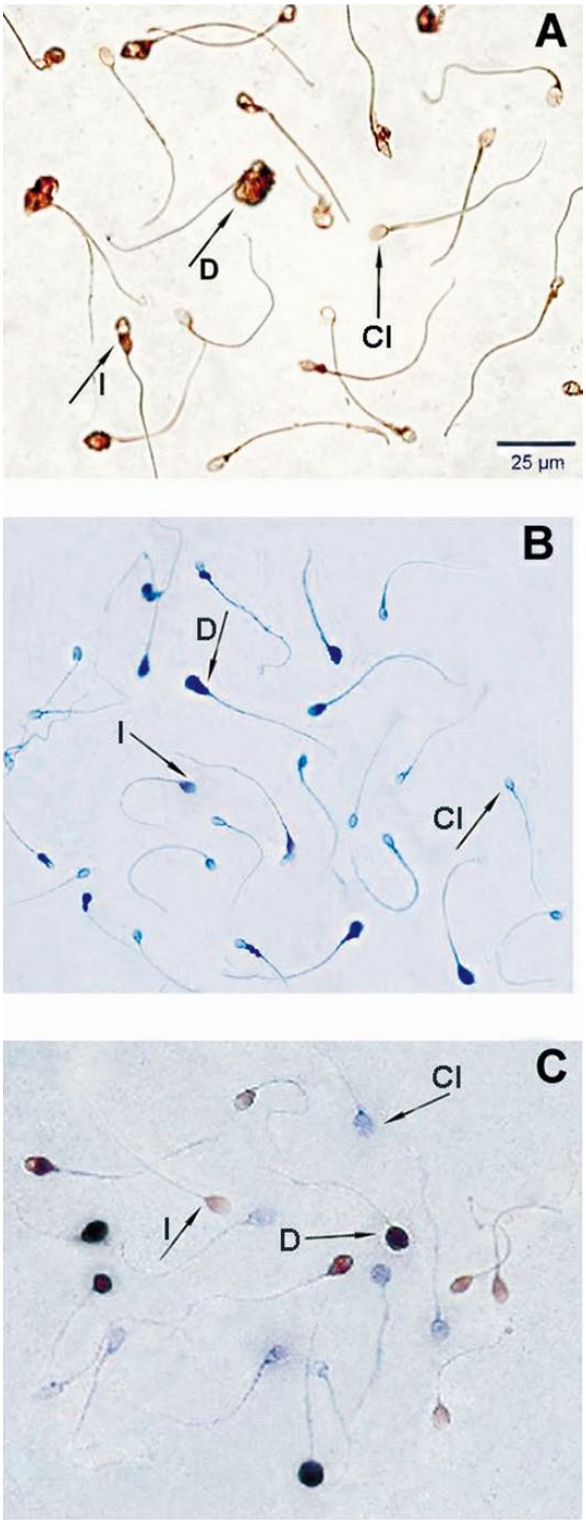

Figure 1. Composite figure of individual spermatozoa stained for the various biochemical markers. **(A)** CK-B immunocytochemistry; **(B)** aniline blue; and **(C)** DNA nick translation. The designations of CL (clear), I (intermediate), and D (dark) indicate the respective staining intensities, reflecting degree of cytoplasmic retention, persistent histones, and DNA fragmentation, respectively.

was applied before evaluation. Because of persistent histones, sperm with immature chromatin were stained to various intensities of blue (Figure 1B; Dadoune et al, 1988; Hammadeh et al, 1996; Franken et al, 1999; Huszar et al, 2003).

Assessment of DNA Integrity by Nick Translation

All steps were carried out at room temperature. The procedure was adapted from Irvine et al (2000). Briefly, slides were fixed for 15 minutes with methanol-acetic acid (3:1) and dehydrated through an ethanol series of 70%, 80%, and 100%. After air drying, the slides were treated with 10 mM DTT (Sigma) in 10 mM Tris-HCL, pH 8.0, for 30 minutes and were further exposed to 10 mM lithium diiodosalicylic acid in 10 mM Tris-HCl for 1 hour. In order to block any endogenous biotin/avidin binding sites within the sperm, the slides were subjected first to biotin and then to avidin in phosphate-buffered saline for 20 minutes. In situ nick translation was performed with the DNA polymerase I (Boehringer Mannheim Biochemica, Indianapolis, Ind). The slides were flooded with a DNA polymerase cocktail containing biotin-16-dUTP, a mixture of dGTP, dCTP, dATP, and 0.025 U/mL DNA polymerase I, for 30 minutes. The incorporation of biotin-labeled nucleotides was detected by the avidin-biotin horseradish peroxidase method. In further steps, the slides were treated with an ABC kit (Vector Laboratories). Finally, diaminobenzidine was used for the color development. Prior to counterstaining with 0.1% Coomassie Blue, the slides were air dried and mounted with Permount. All unidentified reagents were purchased from Sigma Chemical Company.

Sperm Shape Determinations

Kruger Strict Morphology—Sperm smears were dried on glass slides and stained with Diff-Quik (Dade-Behring, Newark, Del) according to the manufacturer's instructions. The slides were scored by 3 investigators in a blinded manner according to the Kruger criteria (Menkweld et al, 1990: World Health Organization, 1999). The results were averaged for each slide.

Objective Sperm Morphometry—These studies were carried out by the computer-based Metamorph program. These methods were recently described in detail (Celik-Ozenci et al, 2003). Calibration was performed by viewing an objective micrometer scale (OB-M 1/100) at 40× magnification and digitizing the image with the Metamorph program. The automated, computerized conversion of pixels to micrometers was 0.29 µm/pixel.

After digitizing the images, Metamorph overlay tools were used to delineate the head versus tail regions of individual spermatozoa in order to measure head and tail parameters separately. In the assessment of head parameters Metamorph recognizes the following elements: Area (area of head), Perimeter (distance around edge of head, measuring from midpoints of each pixel that defines its border), Long head axis (length of longest diameter through the object), Short head axis (width measured perpendicular to the longest diameter), and Shape factor (a value from 0 to 1.0 representing how closely the object approximates a circle, with 1.0 representing a perfect circle). For the sperm tail measurements, Metamorph provides the tail length.

Statistical Analysis

In order to compare the various sperm shape attributes—sperm concentrations, CK activities, HspA2 ratios, and darkness factors

Table 1. *Preserved sperm concentrations (mean ± SEM)**

Conditions†	n	Concentration, 10^6 sperm/mL (Range) Initial	24 Hours
No preservative, 4°C	20	54.2 ± 8.3 (6.7–149.2)	51.8 ± 8.3 (5.9–158.1)
PMSF-4°C	20‡	53.8 ± 8.6 (6.8–170.9)	54.03 ± 9.4 (5.9–192.7)
No preservative, RT	6	68.4 ± 24.4 (14.0–162.3)	67.7 ± 23.1 (12.1–163.5)
PMSF-RT	6	65.6 ± 23.3 (10.6–168.1)	66.7 ± 25.5 (8.5–176.5)

* All comparisons are not significant.
† RT indicates room temperature; PMSF, phenyl-methyl-sulfonyl-fluoride.
‡ Signed rank test.

before and after incubation in the shipping conditions—we used the computer-based SigmaStat program to perform paired *t* test analysis on normally distributed data and the Wilcoxon signed rank test on data that was not normally distributed (SigmaStat, version 2.0; Jandel Scientific Corporation, San Rafael, Calif). The comparisons before and after the incubations were also performed for each of the sperm concentration groups. In testing the various correlations, we used the Pearson correlation analysis of the SigmaStat program. All data are presented as mean ± SEM. Level of significance was selected as $P < .05$.

Results

Brief Overview

The purpose of this study is the development of conditions that will facilitate the preservation of sperm attributes during overnight shipment of home-collected semen samples to a central laboratory by men under investigation for reproductive toxicity exposure or infertility. The preserving agent, PMSF, is an enzyme inhibitor that has been used previously in animal semen (Hyne and Garbers, 1982; Frankel and Chapman, 1984; Imschenetzky et al, 1997). For the overnight incubations, we used conventional Falcon tubes or the Transem100 semen shipper (Fertility Solutions, Cleveland, Ohio) with no discernible differences. The semen attributes were determined at the time of ejaculation and liquefaction and after 24 hours under shipping conditions. The proposed semen shipping procedures are described in ''Methods.''

Preservation of Sperm Concentrations

In the first phase of the PMSF experiments, we focused on the preservation of sperm concentrations in semen samples from 20 men. As Table 1 indicates, there was no difference in mean sperm concentrations between the control or PMSF-4°C–treated semen samples after 1 day of storage. This was also true for the subset of 6 samples stored at the PMSF–room temperature conditions. These results indicate that addition of PMSF would preserve sperm concentrations during shipping, whether at 4°C or if an incidental warming of the samples occurred. The maintenance of sperm concentrations in the semen samples was excellent whether the samples were in the normozoospermic, 20–30 × 10^6 sperm/mL, or oligozoospermic concentration ranges with various degrees of sperm maturity. It is also of note that sperm concentrations were preserved without additives at both 4°C and room temperature conditions with variable levels of bacterial growth. However, in these no-preservative samples, the biochemical markers, such as HspA2 ratio or CK activity, did show major alterations (see later); thus, the no-preservative condition is not a viable alternative.

Preservation of Sperm CK Activity

In the second phase of the PMSF studies, we focused on the preservation of biochemical markers in 47 men (sperm concentration: 74.9 ± 11.1, range 6.0–336 × 10^6 sperm/mL). Because generally there is a relationship between sperm concentrations and the proportion of sperm with diminished maturity in samples, and because the preservation of both mature and immature sperm is very important in the assessment of fertility or reproductive toxicity, we selected samples with a diversity of concentrations. Also, we considered separately men with sperm concentrations in the range of more than 30 × 10^6 sperm/mL (normozoospermic), less than 20 × 10^6 sperm/mL (oligozoospermic), and 20–30 × 10^6 sperm/mL (Table 2). This last group is considered normozoospermic by World Health Organization (WHO) standards, but, in reality, sperm maturity of men in the 20–30 × 10^6 concentration range is inconsistent. 1) Approximately 40% of these men have diminished maturity in CK and HspA2 parameters (Huszar et al, 1988a,b). This heterogeneity is well demonstrated in the present study by the differences in CK activities (0.09 vs 0.18 IU CK/10^8 sperm) or in HspA2 ratios (52.5% vs 33.2%) in the groups with more than 30 vs 20–30 × 10^6 sperm/mL (Tables 2 and 3). 2) On repeat semen analysis, only approximately 20% of these men will present samples in the same 20–30 × 10^6 sperm/mL concentration range, whereas samples of the other 80% will be in the range of less than 20 or more than 30 × 10^6 sperm/mL. For this reason, we have previously referred to this group as ''variablespermic'' (Huszar et al, 1988b).

We studied sperm CK activity in the initial semen and after 24 hours of PMSF-4°C storage in samples from 47 men. In 13 of these men, we also examined CK activity in another semen aliquot stored at room temperature (Table 2). It is evident that the sperm CK activity shows a mean decrease of 32%. The decrease is even more pronounced, although not significantly, in the diminished maturity 20–30 × 10^6 sperm/mL and oligozoospermic sam-

Table 2. *Preservation of CK activity (mean ± SEM)*

		CK Activity, CK IU/10^8 Sperm (Range)*				
	n	**Initial**	*P*	**PMSF-4°C**	*P*	**PMSF-RT**
All Samples	47†	**0.14 ± 0.03** (0.01–0.9)	<.001	**0.10 ± 0.01** (0.01–0.41)	. . .	. . .
	13†	**0.06 ± 0.01** (0.01–0.17)	.023	**0.05 ± 0.01** (0.01–0.12)	.02	**0.04 ± 0.01** (0.01–0.11)
Normozoospermic (>30 × 10^6 sperm/mL)	29	**0.09 ± 0.02** (0.01–0.34)	.013	**0.06 ± 0.01** (0.01–0.23)	. . .	. . .
	11	**0.07 ± 0.02** (0.01–0.17)	NS	**0.05 ± 0.01** (0.01–0.12)	.04	**0.04 ± 0.01** (0.01–0.11)
Normozoospermic (20–30 × 10^6 sperm/mL)	10†	**0.18 ± 0.06** (0.03–0.41)	NS	**0.12 ± 0.03** (0.02–0.27)	. . .	. . .
	2	**0.06 ± 0.03** (0.03–0.08)	. . .	**0.05 ± 0.04** (0.02–0.09)	. . .	**0.03 ± 0.01** (0.02 ± 0.03)
Oligozoospermic (>20 × 10^6 sperm /mL)	5	**0.40 ± 0.14** (0.07–0.09)	NS	**0.24 ± 0.06** (0.04–0.41)	. . .	. . .

* RT indicates room temperature; PMSF, phenyl-methyl-sulfonyl-fluoride; and NS, not significant.
† Comparisons with signed rank test.

ples (34% and 39%, respectively; Table 2). Thus, CK activity is not a reliable parameter, particularly if the samples are accidentally warmed en route.

We also investigated the reasons underlying the decline of CK activity. The possibilities included 1) a CK activity inhibitory effect of PMSF; 2) denaturation of CK enzymatic activity during storage (CK has an SH-group on the enzymatic active site); and 3) selective attrition of the most immature sperm population, which has substantial cytoplasmic and CK retention and labile, immature sperm plasma membranes (Huszar et al, 1997). We tested these alternatives. The addition of PMSF did not affect sperm CK-activity, whether in preserved sperm or in sperm extracts. Furthermore, we stored sperm in the presence of DTT, and 10 mM DTT prevented the decline of CK activity. The storage conditions had no effect on the proportion of sperm with high levels of cytoplasmic retention. Thus, the storage- and shipping-related decline of sperm CK activity is due to the denaturation of the CK enzymatically active site.

Preservation of HspA2 Ratio

The HspA2 ratio is an established measure of sperm maturity, function, fertility, and DNA integrity and aneuploidy frequency (Huszar et al, 1992, 1997, 2000, 2003; Kovanci et al 2001; Ergur et al, 2002). Under PMSF-4°C shipping conditions, the HspA2 ratios showed only slight changes, whether we considered all 47 samples together or the normozoospermic, variablespermic, or oligozoospermic sample groups separately (Table 3). Thus, the HspA2 ratio after 24 hours of storage and shipping clearly represents the ratio at the time of ejaculation. This fidelity

Table 3. *Preservation of HspA2 ratios (mean ± SEM)*

		HspA2 Ratio (Range)*		
	n	**Initial**	4°C	**RT**
All Samples	47	**46.6 ± 3.5** (8.0–88.4)	**47.1 ± 3.4** (8.3–87.7)	. . .
	13	**55.2 ± 6.1** (18.0–88.4)	**57 ± 5.5** (23.7–87.7)	**69.4 ± 4.1** (32.7–89)
Normozoospermic (>30 × 10^6 sperm/mL)	29†	**52.5 ± 3.9** (8.0–88.4)	**54.2 ± 3.5** (8.6–87.7)	. . .
	11	**56.5 ± 7.0** (18.0–88.4)	**59.0 ± 5.6** (27.3–87.7)	**69.7 ± 4.6** (32.7–89)
Normozoospermic (20–30 × 10^6 sperm/mL)	10	**33.2 ± 8.8** (10.0–79.5)	**33.7 ± 9.1** (8.3–79)	. . .
	2	**47.6 ± 13.8** (33.9–61.4)	**45.7 ± 22.0** (23.7–67.6)	**68.0 ± 11.1** (56.9–79)
Oligozoospermic (>20 × 10^6 sperm/mL)	5	**32.8 ± 6.20** (15.1–45.5)	**29.0 ± 5.9** (15.5–49.9)	. . .

* Initial vs 4°C comparisons are not significant. Initial or 4°C vs room temperature (RT) comparisons $P < .01$.
† Comparison with signed rank test.

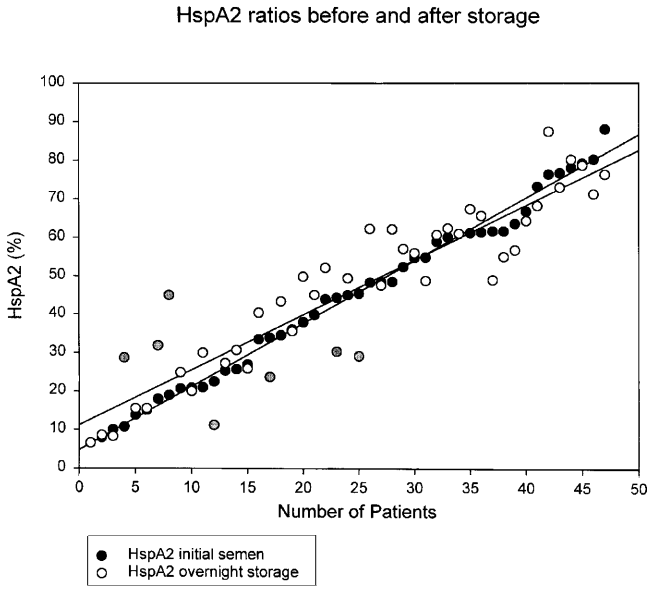

Figure 2. Distribution of HspA2 ratios before (black dots) and after (white dots) PMSF-4°C overnight storage. The sample pairs are in a vertical arrangement.

is well demonstrated by the before and after plot of Figure 2. There was a close correlation between the two sets of samples. The variation was less than 15% in 28 samples (mean 6.8% ± 0.3%), 15%–30% in 12 samples, and more than 30% in 7 samples. The last samples were in the lower HspA2 ratio range, where small absolute differences can cause more noticeable percent changes. The intra-assay variation of the HspA2 ratio determinations is approximately 8% (Huszar et al, 1992).

In the case of the 13 samples that were also stored at room temperature, the values, compared with the initial ratios, show an approximately 20%, but consistent, increase. Thus, if the sample arrived in a warm condition, the HspA2 ratios might still reflect the initial HspA2 ratios if the ambient temperature was comparable to our 20°C to 26°C room temperature range.

Relationship Between CK Activity and HspA2 Ratios

Because cytoplasmic extrusion and the second wave of HspA2 chaperone protein family expression occur simultaneously in terminal spermiogenesis, there is an inverse correlation between CK activity and HspA2 ratios (ie, the diminished maturity sperm population with high CK retention will also show lower HspA2 chaperone family expression). This has been demonstrated in several clinical studies, with a typical range of $r = -.68$ to $-.78$, $P < .001$ (Huszar and Vigue, 1990; Huszar et al, 1992; Lalwani et al, 1996; Ergur et al, 2002). In the experiments, the correlations between CK activity and HspA2 ratios were similarly maintained. The r value in the initial 47 samples was .71 ($P < .001$); in the same 47 samples after overnight storage, $r = -.78$ ($P < .001$) and in all 94 samples, $r = -.73$ ($P < .001$). This consistency provides further confirmation for the validity and fidelity of the PMSF-4°C storage and shipping conditions we have developed.

DTT and No-Preservative Conditions

We have also used 10 mM DTT as a preservation additive in the study of 32 samples. This alternative medium was also effective in preserving the various semen attributes at both cold or room temperature conditions. However, we eliminated DTT as our preservation agent of choice, because it caused decondensation of sperm DNA and a consequential alteration in sperm head size.

The maintenance of CK activity and HspA2 ratio parameters in the no-preservative conditions was studied in 14 samples (sperm concentration: $48.9 \pm 11.8 \times 10^6$ sperm/mL, range 12–146). The initial CK activity was 0.31 ± 0.09 CK IU/10^8 sperm, whereas after overnight storage, activities were 0.21 ± 0.05 at 4°C and 0.11 ± 0.02 at room temperature. The respective initial HspA2 ratio was 33.3% ± 5.0%, and after overnight incubation, the ratios were 41.6% ± 5.2% at 4°C and 52.7% ± 5.9% at room temperature. Thus, the no-preservative condition is not a satisfactory option.

Preservation of Cytoplasmic and Nuclear Markers in Individual Spermatozoa

A more specific alternative to measuring sperm CK activity or HspA2 ratio in semen samples is the assessment of cytoplasmic or nuclear maturity of individual spermatozoa (Figure 1A through C). For example, sperm cellular maturity and cytoplasmic retention can be detected by CK immunocytochemistry (Figure 1A). Observation of the stained slides can reveal proportions of clear-headed (mature), intermediate, and darkly stained (immature, with substantial cytoplasmic retention or heavily fragmented DNA) sperm in semen smears. The proportions of mature, intermediate, and immature sperm were assessed by the darkness factor. The darkness factor is the sum of the proportion of sperm unstained by the biomarker times 0, the proportion of the intermediately stained sperm times 1.5, and the proportion of the darkly stained sperm times 3. For instance, the score of a slide with 75% clear sperm, 18% intermediate sperm, and 7% dark sperm would be 48 ($1.5 \times 18 + 3.0 \times 7$). We have examined the CK immunocytochemical darkness factor (cytoplasmic retention) in sperm smears prepared from initial semen and from corresponding aliquots stored by the PMSF- 4°C protocol for 24 hours. The average sperm concentration in the samples studied was $61.6 \pm 20.4 \times 10^6$ sperm/mL (range 4–245, n = 5). The HspA2 ratios before and after storage were unchanged at 20.2% ± 6.4% (8%–29.3%) and 19.6% ± 5.9% (7.7%–28.9%), re-

Table 4. *Darkness factors before and after addition of phenyl-methyl-sulfonyl-fluoride and storage at 4°C (mean ± SEM)**

Sample	Darkness Factor (range)		
	CK-B Immuno (n = 5)	Aniline Blue (n = 10)	DNA Nick Translation (n = 10)†
Initial	43.9 ± 7.0 (28.3–61.7)	26.7 ± 3.0 (15.7–46.4)	29.9 ± 4.8 (12.3–59.6)
24 h	42.1 ± 6.9 (24.3–61.9)	28.0 ± 3.1 (15.3–47.6)	29.6 ± 4.9 (14.4–54.7)

* All comparisons are not significant.
† Comparison with signed rank test.

spectively. There were no differences between the CK-B staining patterns of the initial vs the 24-hour samples (Table 4).

Aniline Blue Staining Pattern—Chromatin Maturity

In diminished-maturity samples, there is a higher proportion of sperm with increased aniline blue staining, which highlights the presence of persistent histones in chromatin. Thus, in order to ascertain nuclear maturity of single spermatozoa, we prepared aniline blue–stained slides. (Figure 1B; Huszar et al, 2003). We tested the conservation of the aniline blue–staining darkness factor during the shipping conditions in aliquots of 10 semen samples (sperm concentration: 78.8 ± 26.0 × 10^6 sperm/mL, range 14.2–259). The HspA2 ratios were the same: 53.2% ± 7.8% (range 25.4%–88.4%) and 52.9% ± 7.5% (range 23.7%–76.7%) in the initial aliquots and those stored at PMSF-4°C.

The aniline blue–staining darkness factor also remained unchanged (Table 4). Neither the mean values nor the ranges showed changes. Thus, the PMSF-4°C shipping conditions are satisfactory with respect to the preservation of aniline blue staining intensity or the nuclear maturity of individual spermatozoa. Moreover, the sperm aniline blue–staining pattern on the next day after shipping reliably reflects the pattern of sperm in fresh semen.

Preservation of DNA Integrity—DNA Nick Translation Studies

The degree of sperm DNA strand fragmentation is a very important measure of sperm function because DNA integrity plays a key role in sperm fertilizing potential and in providing the parental contributions of the sperm to zygote development (Manicardi et al, 1995; Sakkas et al, 1999; Irvine et al, 2000; Ward et al, 2000). Sperm DNA fragmentation, which is also related to sperm maturity, is also a reflection of the level of reactive oxygen species in sperm (Aitken et al, 1994; Huszar and Vigue, 1994). In order to determine the efficacy of our storage and shipping condition with respect to preservation of DNA integrity, we used the method of nick translation. Aliquots of semen were tested after ejaculation and after 24 hours of storage. In nick translation, a modified nucleotide is inserted during the DNA polymerase–mediated repair process. In a subsequent step, horseradish peroxidase molecules are attached to the modified nucleotide residues. Thus, the extent of fragmentation and repair in DNA strands of a sperm is represented by the proportional intensity of brown color within the sperm heads, which is quantified by the darkness factor (Figure 1C).

We have studied 10 samples (sperm concentrations: 64.2 ± 20.7 × 10^6 sperm/mL, range 7.7–238). The initial and 24-hour HspA2 ratios were the same (28.6% ± 7.3%, range 8.0%–67.0%, and 28.1% ± 6.7%, range 8.5%–64.6%, respectively). A comparison of the darkness factor in the initial and 24-hour stored aliquots indicated no differences (Table 4). Thus, whether we studied oligospermic, low, or high normospermic samples within the very diverse 7.7–238 × 10^6 sperm/mL range, the DNA integrity, along with the other biochemical parameters of sperm maturity and function, remained unchanged during PMSF-4°C shipping conditions.

Preservation of Sperm Shape

An important indicator of sperm maturity and reproductive health is sperm shape, which is directly related to cytoplasmic retention and the spermiogenetic maturation process (Huszar and Vigue, 1993). In previous studies, we demonstrated a direct relationship between sperm HspA2 ratios vs midpiece area or head plus midpiece area and tail length in sperm fractions of various maturities (Gergely et al, 1999). Now we have also examined whether sperm shape is conserved during storage and shipping under the PMSF-4°C conditions by two methods: Kruger strict morphology and objective morphometry.

Kruger Strict Morphology—The strict morphology slides were prepared from semen aliquots at the time of ejaculation/liquefaction and after 24 hours of storage in the PMSF-4°C storage conditions. We studied 24 samples (sperm concentration: 64.4 ± 10.5 × 10^6 sperm/mL, range 6–155). The initial and 24-hour HspA2 ratios were 47.3% ± 4.4% (range 6.5%–88.4%) and 45.4% ± 4.3% (range 6.6%–86.8%). The strict morphology scores were essentially preserved (less than 10% variation, but $P < 0.05$ with the signed rank test). The scores for 24 men of sperm with normal morphology in the initial semen and after 24 hours of storage were 9.5% ± 1.9% (range 1.0%–46.4%, n = 4800) and 8.7% ± 1.9% (range 1.0%–47.6%, n = 4800), respectively.

Computer-Assisted Objective Morphometry—With the use of the Metamorph program, we investigated the various sperm shape components before and after the PMSF-4°C 24-hour incubation. We have assessed 8 samples with sperm concentrations of 106.7 ± 22.6 (range 24.6–259). The HspA2 ratios, before and after the 24-hour incuba-

Table 5. *Shape attributes of sperm before and after storage (mean ± SEM) of sperm attributes)**

Sample	Area, μ^2	Perimeter, μ	Long Axis, μ	Short Axis, μ	Tail Length, μ	Shape Factor
Initial	19.3 ± 1.3	16.8 ± 0.5	6.4 ± 0.2†	5.1 ± 0.1	54.0 ± 1.7	0.9 ± 0.1†
24 hours	19.5 ± 0.7	17.1 ± 0.3	6.5 ± 0.1	5.2 ± 0.1	54.0 ± 1.7	0.9 ± 0.1
Difference	1.0%	2.0%	1.0%	0	0	0

* All comparisons are not significant.
† Comparisons with signed rank test.

tions, were unchanged at 61.0% ± 6.4% (range 25.4%–88.4%) and 62.9% ± 5.4% (range 27.3%–87.7%). In each sample, we have determined the morphometric attributes of more than 50 individual spermatozoa from the initial and stored samples (976 sperm in all). As the data indicate (Table 5), the various objective morphometric sperm dimensions remained within 2% of that of the initial semen during 24 hours of incubation under storage and shipping conditions. These studies further confirmed the lack of shape changes during shipping.

On the basis of the two approaches of morphologic and morphometric assessment, we can conclude that sperm shape is faithfully preserved in the PMSF-4°C conditions. Thus, shipping of samples would allow sperm shape assessment in men investigated for reproductive toxicity or infertility in a remote, centralized laboratory the next day.

Discussion

Studies directed to male infertility or to reproductive toxicity because of occupational or environmental factors could be improved if, in addition to the conventional sperm concentration and motility parameters, validated biomarkers of sperm maturation and function were also utilized. Because men suspected to have toxic exposure or diminished fertility often reside at remote locations, the utilization of sperm biomarkers would be facilitated by analysis in a centralized laboratory rather than by special visiting teams in the field. Thus, we suggest that a better and less expensive solution is semen collection in the field or in the homes of subjects, followed by analysis at a centralized laboratory. However, this scenario assumes that semen and sperm attributes are preserved during shipping.

Within this more than 2-year research effort, we have developed and validated conditions that facilitate the overnight shipment of semen samples with the preservation of various sperm attributes, featuring both the cytoplasmic and nuclear markers of sperm maturity and function. These parameters include sperm concentrations, HspA2 ratio (a measure of sperm maturity), CK immunocytochemistry (which highlights cytoplasmic retention and thus the proportions of mature and diminished-maturity spermatozoa), aniline blue staining (which probes chromatin maturity), DNA integrity (which is important from the perspective of sperm function and paternal contribution to the zygote), and sperm shape (which we assessed by both Kruger strict morphology and objective sperm morphometry; Menkweld et al, 1990; Gergely et al, 1999; World Health Organization, 1999; Celik-Ozenci et al, 2003).

In this study, we used samples with diverse sperm concentrations of between 6 and 336 × 10^6 sperm/mL. The important finding was that preservation of semen attributes occurred in all samples, regardless of their sperm concentrations or biochemical maturities. Despite the diversity of the study subjects, the number of samples studied was limited; thus, the method requires further verification. However, the consistency of preservation in all parameters studied provides strong evidence for the efficacy of the method, the resulting data, and the conclusions. In the next phase of this study, we plan to extend the scope of the work by participation in ongoing environmental and reproductive toxicology projects.

This study also confirmed our earlier observation that sperm maturity in WHO normozoospermic men with sperm concentrations between 20 and 30 × 10^6 sperm/mL is different from that occurring in those having concentrations of more than 30 × 10^6 sperm/mL. In approximately one third of the men with 20–30 × 10^6 sperm/mL samples, there was a higher proportion of sperm with CK activity and HspA2 ratios in the diminished-maturity range (Tables 2 and 3). This group of men, whom we previously called ‘‘variablespermic,’’ because only 20% of them had sperm concentrations in the same range on repeat analysis, is likely to be important from the perspective of reproductive toxicity assessments, because their testicular function might be on the decline with respect to sperm maturation, while their sperm concentrations are still maintained at WHO normozoospermic levels. This notion is further supported by the two studies of IVF couples, in which approximately 50% of the men (from 18 of 37 couples) showed unexplained male infertility (normal sperm density and HspA2 ratios below 10%). These men failed to cause IVF pregnancies (Huszar et al, 1992; Ergur et al, 2002).

Another aspect relevant to the experimental design concerns the fact that we studied sperm attributes in the initial semen and after overnight incubation both at 4°C and at

room temperature in order to evaluate the effects of a potential shipping delay or incidental moderate warming of the samples. With the exception of sperm CK activity, which is labile because of the SH-group on the enzymatically active site, preservation of sperm concentrations, HspA2 ratios, cytoplasmic retention, and other biochemical markers was satisfactory both at the cold and the less than 26°C room temperature conditions. In other studies, we added 10 mM DTT to the semen. For most parameters, DTT was a good preservation agent at both 4°C or room temperature. However, it caused a change in sperm head size; thus, we abandoned it. It is of note in this respect that analysis of shipped samples should be viewed as a screening test. In men with abnormal test results, or after a sample warm-up, a repeat test or analysis of fresh semen in an expert laboratory is warranted.

We demonstrated earlier that both elevated sperm CK content, indicating cytoplasmic retention, and low HspA2 concentrations represent diminished sperm maturity, which is associated with diminished fertility. In addition to the increased rates of lipid peroxidation, DNA fragmentation, and high chromosomal aneuploidy frequencies demonstrated in immature spermatozoa, diminished fertility is related to another defect of terminal spermiogenesis: failure of plasma membrane remodeling and the formation of the zona pellucida binding site(s) (Huszar et al, 1994, 1997, 2000). Thus, immature sperm show diminished efficiency in conventional fertilization on the basis of sperm–zona pellucida interaction but still might serve as fertilizing sperm for intracytoplasmic sperm injection. In recent studies, we also found that formation of the sperm binding site for hyaluronic acid, which is a component of the female reproductive tract, is regulated similarly to the zona pellucida binding site(s) during spermiogenesis (Huszar et al, 2003). Sperm binding to hyaluronic acid will likely offer another test of sperm maturity and function. We are now testing sperm hyaluronic acid binding for potential inclusion with the other sperm attributes for shipping and analysis at centralized laboratories.

During spermiogenesis, in the elongated spermatid, the cytoplasm accumulates around the midpiece. This process is followed by cytoplasmic extrusion in normally maturing sperm. In immature sperm, in addition to low–HspA2 expression and a failure of plasma membrane remodeling, cytoplasmic retention causes the sperm head to be larger, rounder, or amorphous, as evidenced by abnormal sperm morphology. We also showed that sperm tail length is affected. Objective morphometry indicates that in immature spermatozoa, sperm head size increases and sperm tail length decreases in proportion to elevated CK activity and lower HspA2 ratios (Gergely et al, 1999). Thus, we suggest that objective sperm morphometric parameters, such as the tail length/long head axis ratio, be considered ''biochemical'' measures of arrested spermiogenetic development. Low levels of exposure to toxicants might cause insidious and chronic changes in testicular function and a decline in sperm maturation; thus, the fertilizing potential or paternal contribution of sperm can be diminished, without major declines in sperm concentrations or other conventional sperm measures. A long-term goal of this work stems from the recognition that detection and monitoring of reproductive toxicity or male infertility might be facilitated by more than 1 biochemical marker in the same sperm. Thus, we will have the opportunity to identify the most efficient and consistent marker to best detect toxic effects or male infertility.

Earlier studies on sperm transportation used semen extender media, primarily in animal husbandry (eg, Davis et al, 1963). However, such studies were conceptually different from this study because the main purpose was the preservation of sperm motility for artificial insemination. With respect to human semen studies, the efficacy of modified citrate–egg yolk buffer extender was tested in the preservation of sperm motility, tail viability by hypoosmotic swelling, acrosomal response to calcium ionophore, and levels of reactive oxygen species generation (Allan et al, 1997). The citrate–egg yolk buffer preserved these parameters. However, even if it preserved all parameters studied, this method is not practical because 1) the use of the citrate–egg yolk medium requires a 1:1 dilution of semen, which is undesirable with oligospermic samples, and 2) the shelf life, cold storage, and stability of the egg yolk medium is a potential problem for field studies. Recently, the effects of storage after rapid freezing of semen by liquid nitrogen were studied on sperm DNA integrity in 10 men. The data were inconclusive because chromosome breakage studied by fluorescence in situ hybridization and the TUNEL assay, both of which reflect endogenous DNA breaks, did not reveal differences. However, the neutral comet assay indicated that the frequency of double-strand DNA breaks was lower in sperm arising from freshly frozen sperm compared with frozen samples stored overnight (in Transem containers at 22°C; Young et al, 2003).

The utility of PMSF, a proteolytic enzyme inhibitor, has been explored previously for the preservation of sperm nuclear proteins in sea urchins and rodents. Addition of PMSF preserved the fertilizing ability of sperm nuclei, and inhibited the degradation of sperm histones during pronuclear remodeling (Hyne and Garbers, 1982; Imschenetzky et al, 1997). The documented stabilization of sperm nuclear proteins might be relevant to the PMSF preservation of DNA integrity and the aniline blue chromatin staining pattern of human sperm reported in this study.

Under PMSF-4°C conditions, we compared storage in test tubes and in Transem100, the only container currently available for semen shipping, with an ice pack. There was

no difference detectable in the preservation of sperm attributes. In a previous study, the majority of the Transem100 shipping container study participants, with diverse educational backgrounds, used the kit correctly with respect to collecting, packaging, and shipping the semen samples (Royster et al, 2000). Thus, it is expected that in our procedures (as described in ''Methods''), transferring semen with a device similar to an eye-dropper to volume-labeled transportation tubes containing premeasured solid PMSF will be followed easily by study subjects. It is also expected that semen collection in the home environment will increase participation compared with semen collection at an impersonal study trailer or laboratory. In addition, analysis in a centralized laboratory will reduce interlaboratory variation and will enhance quality control, which is important in the case of multicenter studies or in the monitoring of men who are exposed to or removed from the adverse environment of reproductive toxicity. In these cases, the sperm concentration or motility might remain similar, but the biochemical markers should demonstrate the adverse or beneficial effects, respectively.

We developed semen storage and shipping conditions with the use of 20 μg PMSF/mL semen at 4°C. The PMSF-4°C protocol is promising because it maintains both the conventional and the objective biochemical sperm parameters, such as HspA2 ratio, cytoplasmic retention, proportion of mature and immature sperm, chromatin maturity, DNA integrity, and sperm shape, even if a shipping delay or an accidental, moderate (less than 26°C) warm-up occurs. The fidelity of the next-day samples reflects the attributes of the initial semen well. In addition to further testing of the preservation approach described here, we are developing methods for the next-day assessment of sperm viability, motility, and hyaluronic acid binding.

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