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Next day determination of ejaculatory sperm motility after overnight shipment of semen to remote locations

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Abstract
Purpose To develop a method for delayed assessment of sperm motility, after shipment of semen to a remote laboratory. Sperm in semen were labeled with the MitoTracker® Red CM-H_2XRos reagent, and fixed with 3.7 % formaldehyde by the laboratory technicians at the origin of the semen. This treatment reflected well sperm mitochondrial activity, and the MitoTracker® signal was related to sperm motility and velocity for 2–3 days following ejaculation.
Methods Sperm motility and velocity were evaluated manually and by computer assisted semen analysis (CASA), respectively. Fluorescence assessment of individual sperm was carried out with the computer assisted Metamorph v4.6.9 program. Emission levels of MitoTracker® spermatozoa were studied in room temperature and cooled semen, or in the respective room temperature swim-up sperm fractions following ejaculation, and on the second day ($N=103$ samples, 89 men) and third day ($N=10$ samples, 8 men).
Results Sperm with optical density (O.D.) ≥0.7 showed close correlations with ejaculatory sperm motility and velocity even after second day ($r=0.92$, $p<0.001$, $N=103$ samples). Further, the multiple of sperm motility and velocity was also related to the proportion of high MitoTracker® reagent emission sperm ($r=0.83$, $p<0.001$, $N=103$ samples). MitoTracker® dye fluorescence on the second day accurately reflected the ejaculatory sperm motility ($r=0.90$, $p<0.001$). Thus, a shipping delay would not adversely affect the results.
Conclusions The delayed assessment of sperm motility in samples treated with MitoTracker® Red CM-H_2XRos reagent and shipped to remote laboratory truly reflects the level of sperm motility at the time of the ejaculation.

Keywords Sperm mitochondrial activity · MitoTracker · Sperm velocity · Shipped semen

Capsule Next day assessment of ejaculatory sperm motility.

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Introduction

Sperm motility is an important characteristic of sperm function. In fact, sperm motility correlates well with fertilization and pregnancy rates after intrauterine insemination or in vitro fertilization (IVF) [1–5]. Exposure to environmental and occupational toxicants may adversely affect sperm motility and motion characteristics, and thus male reproductive potential. In addition to oxidative stress and mutations in the mitochondria that were implicated in infertility [6, 7], new candidate genes, such as human Polypeptide N-acetylgalactosaminyltransferase-like protein 5 (GALNTL5) were identified [8, 9]. Therefore, sperm motility is an important parameter when investigating men for potential environmental exposures or male infertility [10–12].

However, in field studies of exposure, accurate sperm motility assessment requires a computer assisted semen analyzer (CASA), instrument or personnel experienced in manual sperm motility determinations. In addition, the precise evaluation of sperm motility seems to be more difficult to assume for new personnel with limited experience [13]. Due to the complexity of these procedures, there are various technical problems, such as transporting and tuning of CASA machines [14], scheduling study subjects, finding facilities that provide

sufficient semen collection privacy. The accuracy of field motility assessments may be also affected by inter-investigator differences and variations in ambient temperature. The lack of standardization associated with the semen analysis has made it difficult for investigators to compare semen analysis results between laboratories [15]. In fact, in a recent prospective double-blinded study, semen analysis results obtained manually and through two automated approaches, were compared to evaluate the advantages and disadvantages of each method [16, 17]. Based on the data, it was concluded that detection of semen parameters of infertile couples was more consistent if testing of couples was carried out in the same clinics and Andrology laboratory facilities. Therefore, the analysis of samples in a central laboratory by an objective and repeatable method is advantageous and preferable.

There have been previous attempts to develop methods for preservation of sperm motility by various semen extenders for semen analysis or assisted reproduction [18]. For instance, citrate-egg yolk buffer for human semen, Tris-egg yolk-glutathione- citric acid for bull and ram semen, and skim milk-based extenders for horse semen [19–27]. Regarding the citrate yolk buffer, the Aitken group has demonstrated that dilution of semen to a 1:1 ratio with this buffer preserved motility and various functional attributes of sperm at ambient temperature in both normozoospermic and asthenozoospermic men. Interestingly, Amaral et al., have suggested that relevant fraction of human sperm can be maintained in simple phosphate-buffered saline for more than 1 week at room temperature, and adding glucose further enhanced sperm motility, viability, and mitochondrial membrane potential [28].

Recognizing that mitochondrial activity is a clear hallmark of human sperm function [29], we decided to use the MitoTracker® Red CM-H_2XRos reagent (Thermo Fisher Scientific, Eugene, OR, USA). Since this reagent selectively accumulates in active mitochondria in a membrane potential-dependent manner, and is well-retained after formaldehyde fixation, we decided to use it to test our hypothesis that the fluorescence of MitoTracker® reagent may provide a signal threshold that will distinguish between non-motile and motile sperm. Further, we expected that because this reagent is retained in the mitochondria after depolarization, it will facilitate the delayed assessment of the mitochondrial activity-related signal and, in turn, the proportion of motile sperm at the time of the MitoTracker® reagent loading [30].

In our ongoing studies toward developing various next day semen analysis methods, we have already demonstrated that the use of phenylmethylsulfonyl fluoride (PMSF) and 4 °C conditions during shipping can preserve various sperm attributes, including sperm concentration, heat shock chaperone protein (HspA2) ratios, chromatin maturity, DNA integrity and sperm shape [31]. The present study demonstrates that loading sperm with the MitoTracker® reagent at the laboratory where the subject to be investigated originates, and shipping of the MitoTracker® loaded sperm make possible the delayed assessment of sperm motility in remote laboratories based on the conservation of mitochondrial activity.

Materials and methods

Experimental design

All semen samples were from men presenting for semen analysis at the Sperm Physiology Laboratory, Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine after 2-3 days of abstinence. The studies were approved by the Yale Human Investigation Committee.

In developing methods see flowchart (Fig. 1) for delayed assessment of sperm motility, we studied the MitoTracker® reagent fluorescence distribution in three sperm fractions prepared from the same semen samples (N=103 samples, 89 men): as collected room temperature semen, cooled semen (diminished motility), and swim-up sperm prepared from room temperature semen (enhanced motility sperm fraction). Based on the fluorescence intensity in the three related sperm fractions, we systematically tested various MitoTracker® fluorescence emission values that might distinguish between motile and non-motile spermatozoa. In the next step, we tested the consistency of the MitoTracker® reagent staining intensity between two aliquots of the same sperm fraction, as well as in duplicate slides from the same MitoTracker® reagent-loaded sperm fraction. Further, the number of spermatozoa to be assessed in order to achieve high reliability in motility prediction was also addressed. Finally, we further validated delayed detection method in blinded studies, in which the investigator performing the analysis and predicting the ejaculatory motility was unaware of the actual motility in the samples.

Selection of study samples

In selecting semen samples for the study, we have made an effort to include collected room temperature semen samples with varied sperm concentrations and motilities. With respect to concentration, 52 out of 103 samples studied had concentrations less than 40×10^6 sperm/mL. Within this group, the distribution of sperm concentrations (x10^6/mL) was as follows: <10: N=11; 10–20: N=11; 20–30: N=15; 30–40: N=15. With respect to motility, 56 out of 103 samples had motility less than 50 %. Within this group, the distribution of motilities (%) was as follows: <10: N=12; 10–20: N=9; 20–30: N=7; 30–40: N=9; 40–50: N=19.

Initial sperm motility and velocity determination

Sperm concentration and motility in the samples were determined by computer assisted semen analysis using the

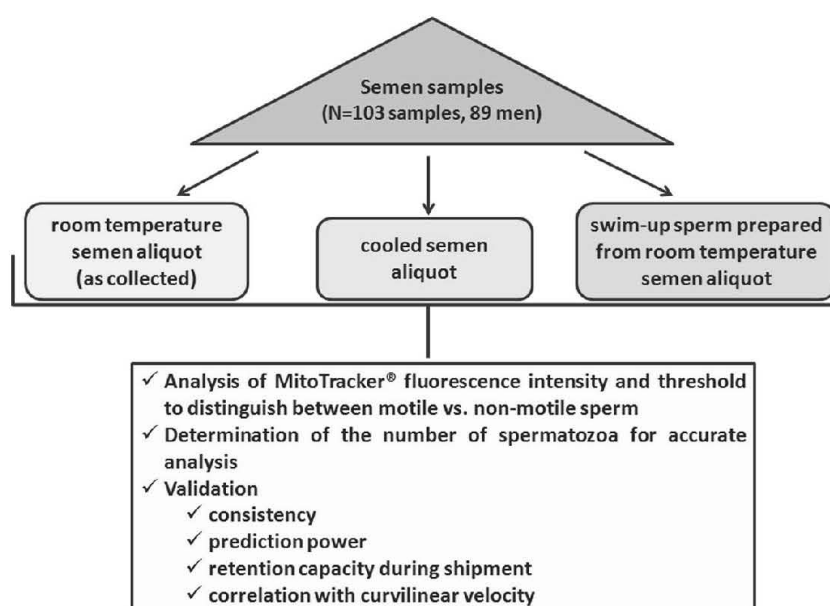

Fig. 1 Flowchart for the experimental design

Hamilton-Thorne IVOS system (Hamilton Thorne, Inc., Beverly, MA, USA). After mixing the samples before each measurement, semen was applied to the Makler Chamber and 3 fields of ten squares were evaluated from 2 drops in each sample. The accuracy of concentration measurements was checked daily with quality control Accu-beads (Hamilton Thorne, Inc., Beverly, MA, USA; 18 and 35 x10^6/ml calibration concentration). Percent motility was determined by calculating the mean value from the two drops. With respect to velocity, the parameters of straight-line velocity, path velocity, and curvilinear velocity were followed using the CASA procedure. The cutoff value for non-motile: motile sperm was 7.0 μm/s. The initial room temperature values are the initial semen measures.

Preparation of sperm fractions

We prepared three sperm fractions from each semen sample: *room temperature semen* (used as collected), *cooled semen* (prepared by placing an aliquot of semen on ice for 2–3 minutes to reduce the motility to <10 %, and *swim-up sperm fractions of the room temperature semen*. Swim-up sperm was prepared by layering semen, diluted with 2-3 volumes of human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA, USA)-0.5 % bovine serum albumin (BSA; fraction V; Sigma-Aldrich, St. Louis, MO USA), facilitated by a flat platform created by pouring electron microscopy embedding medium, subsequently solidified by heat treatment, into the bottom of 15 mL Falcon® centrifuge tubes. The diluted semen was then centrifuged for 20 min at 300xg. Following centrifugation, all but 1.0 mL of the supernatant was removed using a Pasteur pipette. The tube was then incubated at 36 °C for 30 min. The top 0.5 mL of medium, enriched in the upward-swimming motile sperm, was recovered. The sperm concentration and motility in each of the three fractions were determined, and the sperm was then loaded with MitoTracker® reagent, as described below.

MitoTracker® red CM-H_2XRos dye loading of sperm and preparation of smears

The MitoTracker® reagent for sperm labeling was prepared by diluting a 1 mM stock solution (prepared in dimethyl sulfoxide) in saline imidazole (pH 7.0) (Sigma-Aldrich, St. Louis, MO USA), to a concentration of 1 μM. The MitoTracker® probe solution was then added to the sperm fraction to a final concentration of 250 nM, and the tubes were gently agitated. The tubes were then wrapped in aluminum foil to exclude light, and the sperm was incubated in the MitoTracker® probe solution for 35 min at 36 °C. Following incubation, the excess reagent was removed by diluting the sperm suspension with 10 volumes saline imidazole-0.3 % BSA (pH 7.0), and centrifuging at 800xg for 8 min. The supernatant was removed and the pellet was gently re-suspended in saline imidazole-0.3 % BSA (pH 7.0). Sperm smears were prepared on glass slides, mounted with Cytoseal™ 60 (Richard-Alan) and cover slips were applied. The MitoTracker® dye fluorescence was evaluated on the first and second days, and in some experiments, also on the third day. The sperm smears were stored at 4 °C.

Fluorescence detection and analysis

The sperm smears were observed at 40X magnification by epifluorescence microscopy using a rhodamine filter cube (excitation: 520–550 nm; emission: 580 nm, long pass) and

the Olympus® BX-51 microscope. Fluorescence and phase contrast images were captured and the fluorescence emission strength of individual sperm was assessed using the Metamorph® v4.6.9 software (Universal Imaging, Downingtown, PA, USA). For each sample, 200–500 fluorescing sperm cells were individually delineated with Metamorph®, and fluorescence intensity measurements were performed.

Statistical analysis

All data were exported directly to Microsoft® Excel® and statistical analysis was performed by Microsoft® Excel® and SigmaStat® 3.5 program (Jandel Corporation, San Rafael, California). The coefficient of variation between the fluorescence values in neat room temperature semen, cooled semen and swim-up sperm fractions of the room temperature semen were calculated and then a respective plot was created. The data are presented as mean ± SEM.

Results

MitoTracker® red CM-H_2XRos dye fluorescence properties of sperm fractions from semen samples

In the first phase of the study, we determined: a) whether or not there were differences in fluorescence intensity among individual spermatozoa stained with the MitoTracker® dye, and b) whether there was a greater proportion of high emission sperm in samples with higher percentages of motile sperm. To address these questions, we examined the distribution of sperm with various degrees of fluorescence intensity in fractions of seven semen samples, including room temperature semen (sperm concentration: $39.7 \pm 3.5 \times 10^6$/mL, motility: 54.4±7.9 %), cooled semen (sperm concentration: $39.7 \pm 3.5 \times 10^6$/mL, motility: 2.0±1.4 %), and swim-up sperm prepared from room temperature semen (sperm concentration: $13.8 \pm 3.8 \times 10^6$/mL, motility: 87.1±2.1 %). There were well-distinguishable variations in fluorescence intensity among individual spermatozoa. We also found differences in the distribution of high and low emission sperm among the three fractions. These observations were true for all samples, regardless of their sperm concentrations or motility characteristics.

Relationship between motility and proportion of sperm with high fluorescence intensity

In the next phase, we focused on establishing a fluorescence intensity threshold that would distinguish between motile vs. non-motile sperm and would facilitate an accurate assessment of sperm motility. In line with the O.D. distributions in sperm fractions of various motility we analyzed, in 0.1 O.D. increments, the potential relationship between the % motile sperm and the proportion of sperm staining brighter than various O.D. threshold values between 0.6 and 1.0 O.D. units. We established, that the best fit was provided by the proportion of sperm below and above the threshold value of O.D. 0.7 ($r=0.93$, $p<0.001$, $N=32$ samples).

For instance, in samples with a mean motility of 83.9± 3.1 % ($N=8$), 94.5±2.8 % of sperm cells stained brighter than 0.7, whereas in samples with a mean motility of 7.2±2.3 % ($N=12$) only 3.7±0.9 % of cells stained brighter than 0.7 O.D. Based on all 103 samples studied (including the samples studied in the blinded experiments, see below), the correlation between measured motility and the proportion of sperm staining brighter than O.D. 0.7 was $r=0.92$ ($p<0.001$, Fig. 2). The coefficient of variation between the fluorescence values in these samples was a low 7.5±0.5 %.

Number of sperm to evaluate for an accurate prediction

Further, we examined the number of sperm cells that needed to be evaluated for consistent and accurate prediction of motility based on the MitoTracker® dye fluorescence intensity. In 5 fresh samples, we compared the manually assayed motility to the proportion of high emission sperm after 50, 150, 200, 300 and 400+ sperm were analyzed (Fig. 3). The results suggest that the evaluation of at least 400 sperm in a sample gave the most reliable sperm motility assessment. Within each of the five samples, as the number of sperm recorded reached 400, the predicted motility became accurate within ±5 %.

Validation of staining intensity consistency

In an effort to validate whether MitoTracker® dye staining was consistent, we explored the fluorescence values between two slides of the same sperm aliquot, and also between two aliquots of the same MitoTracker® dye-loaded semen samples. First, we divided each of 10 samples (concentration: $30.4 \pm 7.4 \times 10^6$ sperm/mL; motility: 55.7±7.1 %) into two aliquots and loaded them independently with the MitoTracker® dye. Further, we prepared two sample slides from each aliquot. Thus, from each of the 10 samples, we studied four slides in order to evaluate inter-aliquot and inter-slide differences. In both comparisons, the variation was <5 %: the inter-aliquot coefficient of variation was 3.0±1.0 % while the inter-slide variation was 4.0±1.0 %. Indeed, these differences were in line with the variations found in fluorescence intensity between days 1 and 2.

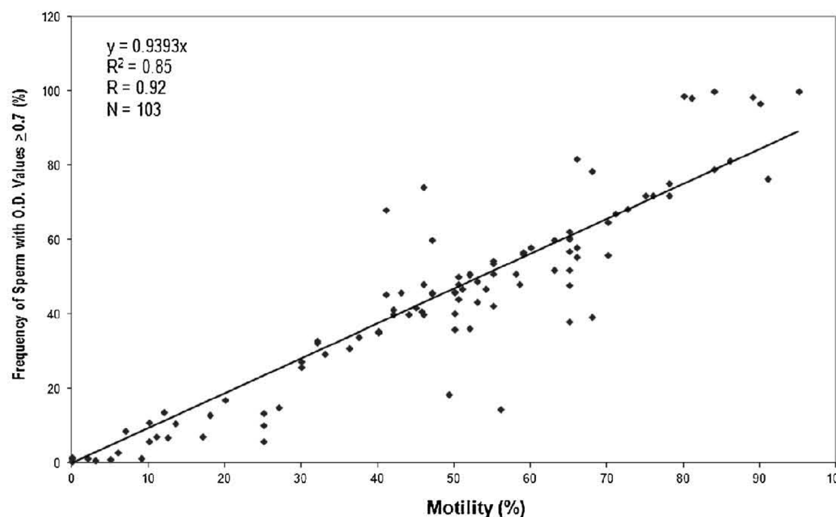

Fig. 2 Correlation between % motile sperm and the proportion of sperm with high MitoTracker® fluorescence emission (≥OD 0.7)

Blinded studies

In the last phase of the MitoTracker® dye studies, we conducted 50 blinded experiments in order to validate our methods in the prediction of ejaculatory sperm motility from the delayed analysis of MitoTracker® dye fluorescence. Sperm motility and velocity were assayed by one investigator while another team member performed the MitoTracker® dye analysis on 400+ sperm per sample (mean=404.8±2.9). In the 50 samples, the ejaculatory motility was correctly predicted by the MitoTracker® dye pattern (±3 %) in 27 samples (54 %), while at the ±5 % tolerance range, the correct prediction occurred in 36 samples (72 %). In the fourteen samples where variation exceeded ±5 % level, six were within the ±5–10 % range, five were in the ±10–15 % range, one was in the 15–20 % range, and only two were in the ±20–30 % range. No prediction deviated in the>±30 % range. The overall correlation between measured and blindly predicted motility was $r=0.91$ ($p<0.001$, $N=50$, Fig. 4) and the coefficient of variation between measured and predicted motility was 9.5±0.9 %. The low level of variation is indicated, independently from sperm concentrations and motility, is well substantiated by the overall consist pattern of Fig. 4.

Retention of MitoTracker® red CM-H_2XRos dye fluorescence during a shipping delay

In evaluating the potential impact of possible shipping delays, we performed repeated daily measurements of MitoTracker® dye-loaded sperm fluorescence for 5 days to test the delay during which we could still accurately predict the initial motility at the time of ejaculation, based on the MitoTracker® dye signal. The fluorescence intensity of the sperm slides remained steady for 3 days, with a mean coefficient of variation of 5.0±2.0 % ($N=10$ samples). After the third day, the motility prediction was less reliable due to a greater variability in the MitoTracker® dye fluorescence.

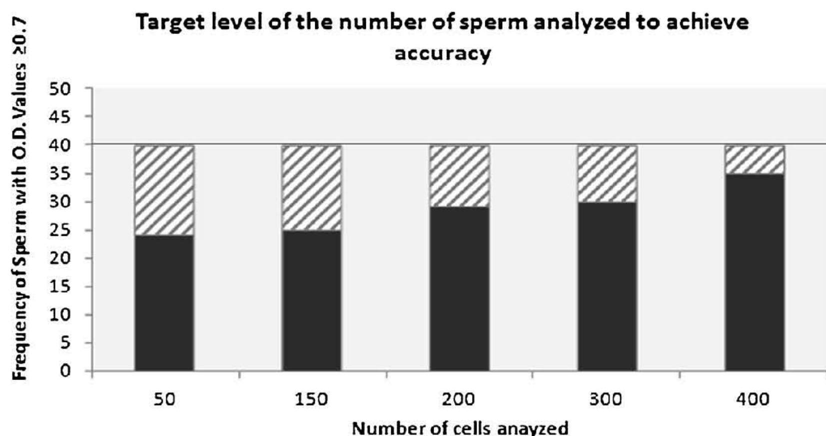

Fig. 3 Target level of the number of sperm analyzed to achieve accuracy in the prediction of sperm motility in a sample with 40 % sperm motility. As the number of sperm evaluated approaches 400, predicted motility becomes accurate within ±5 % (The solid staining pattern is rising)

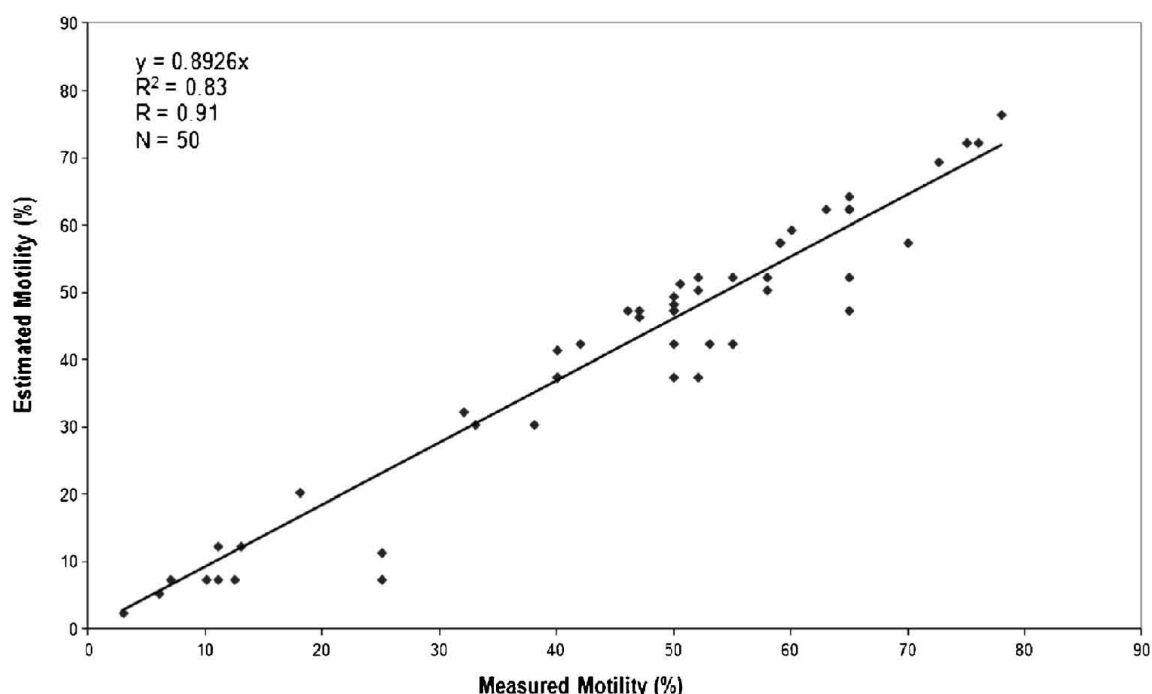

Fig. 4 Correlation between estimated and manually measured motility in a blinded study

Curvilinear sperm velocity and the proportion of high emission sperm

The potential relationship between the mean sperm curvilinear velocity (µm/sec) and the proportion of sperm with fluorescence emission of≥O.D. 0.7 was also studied. The correlation was $r=0.69$ ($p<0.001$, $N=103$ samples).

As the MitoTracker® dye fluorescence reflects both sperm motility and curvilinear velocity, it was of interest to examine a third, derived, factor: the multiple of motility and velocity. This value was calculated by multiplying the percent motility expressed in real numbers (i.e. 30 % motility as 0.3) by the mean curvilinear velocity. When this motion factor multiple was plotted against the proportion of sperm with fluorescence of≥O.D. 0.7., the correlation was very impressive, $r=0.83$ ($p<0.001$, $N=103$ samples, Fig. 5).

Discussion

An increasing number of recent human studies focus upon the evaluation of potential effects of environmental or occupational toxic exposure that would affect sperm quality and thus fertility potential of men [32]. The monitoring of semen quality in subjects who reside and work in remote locations, often necessitate a visit by medical teams, in order to evaluate the exposure and perform semen analysis. Such studies could be greatly improved if semen analysis in laboratories at remote locations was possible. This concept requires that semen attributes reflecting sperm function, are preserved during shipment. We have already developed and validated conditions that would facilitate the overnight shipment of semen samples while preserving various sperm attributes including cytoplasmic and nuclear markers of sperm development and maturity [31]. However, reliable methods for preserving sperm motility for overnight shipment and delayed analysis were not yet available.

In the present study, we are reporting on a method and shipping conditions that would facilitate an accurate delayed determination of sperm motility at ejaculation after shipment of MitoTracker® probe-treated sperm to remote laboratories. Using the MitoTracker® probe, we have shown that the proportion of sperm with fluorescence emission of≥O.D. 0.7 and percent of motile sperm in semen at ejaculation are closely related. It was also confirmed that the objective O.D. value of 0.7 is indeed an accurate threshold for indication of motile sperm in independent aliquots of semen, as well as in duplicate slides of samples. The hypothesis that mitochondrial activity would reflect sperm motility and velocity was well supported by the data as the blinded next day MitoTracker® probe intensity measurements very accurately predicted ejaculatory sperm motility ($r=0.91$, $p<0.001$). The proportion of high emission sperm was also related to the mean curvilinear velocity, and particularly to the multiple of sperm motility and curvilinear velocity.

The sperm motility values predicted by the MitoTracker® dye deviated>±20 % in only 2 of the 50 samples. No estimates deviated by >30 %. This accuracy is expected to be enhanced by the use of a cell sorter which facilitates the quick

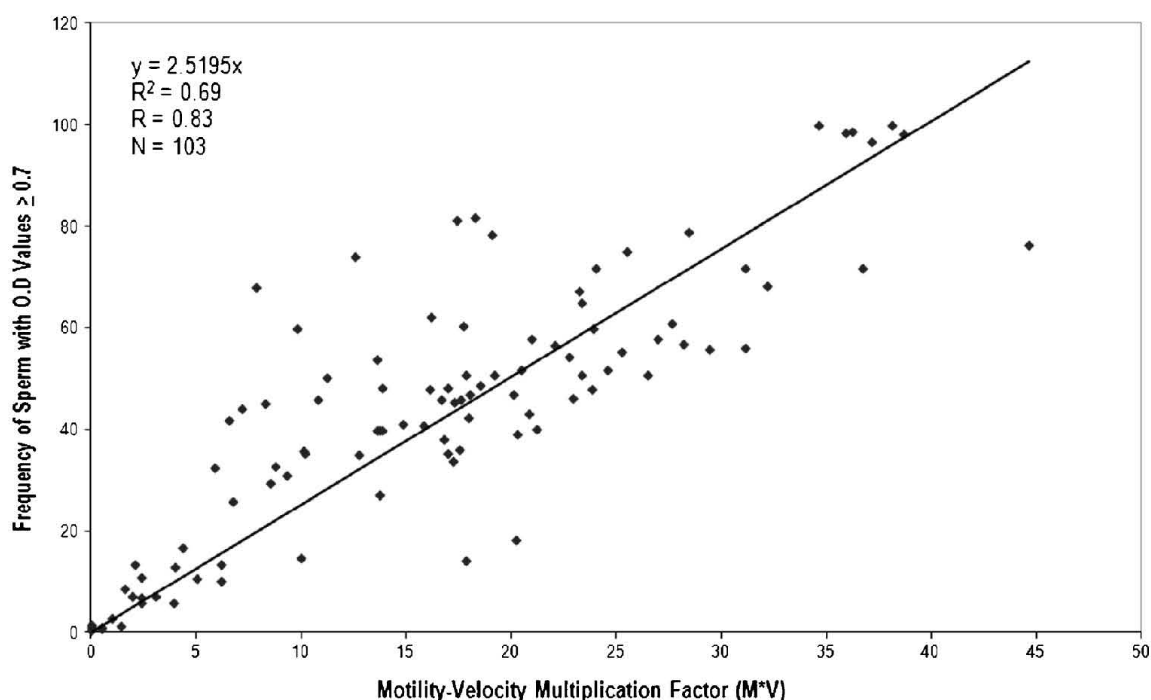

Fig. 5 Correlation between the multiple of motility and velocity vs. the proportion of sperm with high MitoTracker® fluorescence emission (≥OD 0.7)

and efficient evaluation of more sperm cells compared the assessment of individual sperm by epifluorescence microscopy. Considering potential shipping delays, it is of note that the level of MitoTracker® fluorescence remained steady and virtually unchanged for 3 days. As a result, the MitoTracker® probe method allows the evaluation of sperm motility in remote laboratories following shipping, even if a delay is involved.

The methods of loading sperm with MitoTracker® and the preparation of slides are quick (approximately 40 min), and can be easily performed by a skilled technician at the location where the sample originates. Further, we consider it an advantage that the technician may prepare 6 to 8 samples at the same time, depending on the capacity of the centrifuge available, while still observing the 35 min MitoTracker® dye loading period which is optimal and should not be exceeded in order to prevent sperm from becoming overloaded. Conversely, on the receiving remote laboratories only a fluorescence microscope is needed to complete the collaboration.

Experimental evidence suggesting an association between mitochondrial functionality and sperm quality was presented in previous papers [7, 33, 34]. Indeed, the structural and functional defects in sperm mitochondria, and the presence of mutant mitochondrial DNAs are associated with decreased sperm motility in men [35, 36]. Thus, our data confirmed these previous studies regarding the relationship between sperm mitochondrial activity and motility [5, 37, 38].

Other investigators have studied the mitochondrial membrane potential (MMP) in human sperm in association with other aspects of sperm function [5, 39]. Indeed, it has been suggested that measurement of MMP may provide useful information about the fertilizing potential of individual sperm, and thus MMP might have clinical value [40, 41]. Furthermore, sperm with high levels of mitochondrial membrane activity had increased levels of forward motility, and the related IVF results indicated higher fertilization rates [5, 37]. It is of interest that human spermatozoa can also be stored in an electrolyte free media without freezing while maintaining motility, viability, and sperm DNA integrity without cryopreservation [42] .

In a more recent study, Marchetti et al., measured 91 random couples undergoing IVF to determine whether the outcome of in vitro fertilization (IVF) is influenced by the percentage of spermatozoa with functional mitochondria [43]. This approach directed to the inner mitochondrial membrane potential would facilitate the identification of couples who would or would not benefit from assisted reproduction following ICSI. However, the reproductive outcomes following IVF have not yet been evaluated in this respect [43, 44].

In summary, our effort to develop methods for delayed semen analysis after shipment to remote laboratories, we have already reported approaches for preservation of sperm concentrations, the maturity marker HspA2 chaperone ratio, sperm cytoplasmic retention, the presence of persistent histones, DNA chain integrity, and preservation of the sperm shape [31, 45, 46]. We have now furthered sperm motility assessment in adding the MitoTracker®-mediated next day determination of ejaculatory sperm motility.

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Conflict of interest MJ is an employee of Thermo Fisher Scientific, the manufacturer of MitoTracker Red. The other listed authors have no financial, commercial or corporate conflict of interest.

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