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Effects of Temperature on Sperm Motion characteristics and Reactive Oxygen -Species

*Navid Esfandiari, D.V.M., Ph.D. *†*
*Ramadan A. Saleh, M.D.**
Andrew P. Blaut, B.S.†
*Rakesh K. Sharma, Ph.D. *†*
David R. Nelson, M.S..‡
*Anthony J. Thomas, Jr., M.D.**
*Tommaso Falcone, M.D. *†*
*Ashok Agarwal, Ph.D. *†*

* Center for Advanced Research in Human Reproduction
Infertility, and Sexual Function
Urological Institute
† Department of Obstetrics and Gynecology, and
‡ Department of Biostatistics and Epidemiology
The Cleveland Clinic Foundation
Cleveland, Ohio

ABSTRACT: Objective-To evaluate the effect of temperature on sperm motility, sperm motion characteristics, and production of reactive oxygen species (ROS) in semen. Methods-We collected semen samples from 12 infertile patients and 12 healthy donors and divided each sample into four aliquots just after liquefaction. One aliquot was analyzed immediately after liquefaction and the remaining three were analyzed after one hour of incubation at 4°C, 25°C, and 37°C. Sperm motility and motion characteristics were measured by computer assisted sperm analysis (CASA). Levels of ROS were determined in washed sperm suspensions using a chemiluminescence assay and results were expressed as x10 counted photons per minute (cpm)/20 million sperm/mL. Results-Motility, curvilinear velocity, straight-line velocity, average path velocity, and amplitude of lateral head displacement decreased significantly from baseline values in both the patients and donors after incubation at 4°C. Percentage motility was highest and ROS levels were lowest in the samples that were incubated at 37°C. Conclusion-Based on our results, we suggest that semen samples be stored at 37°C after collection and during transportation and processing. *Int J Fertil* 47(5):227-233, 2002

KEY WORDS: male infertility, oxidative stress, reactive oxygen species, sperm motility, temperature

INTRODUCTION

Progressive sperm motility is important in evaluating the fertility potential of spermatozoa, and a prerequisite for fertilization in humans [1]. Sperm motility depends on a number of factors, including the temperature at which the semen is kept between the time of ejaculation and the time of analysis. Temperatures-lower than 4°C and higher than 37°C have been shown to affect negatively sperm functions such as motility [2], penetration into cervical mucus [3J, and hamster egg penetration [4]. The exact mechanisms that mediate decreased sperm motility at extreme temperatures have not been elucidated.

Based on the World Health Organization (WHO) guidelines [5], semen samples should be handled at temperatures ranging from 20°C to 40°C to avoid decreasing sperm motility. Some investigators have indicated that the semen should be kept at body temperature immediately after ejaculation [6], while others have found that human [7], as well as rodent [8], spermatozoa lose motility if stored at 37°C.

One mechanism that may be responsible for diminished motility and thus fertility potential of sperm is an increase in production of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion and hydroxyl radicals, in semen [9]. ROS are needed to regulate sperm function by stimulating a redox-regulated cAMP-mediated pathway that controls the induction of capacitation and the acrosome reaction [10]. However, an increase in ROS production above normal levels results in oxidative stress leading to a decline in sperm motility and a decreased capacity for sperm-oocyte fusion [9]. In addition, ROS are also known to attack DNA, causing strand breaks and damage to the nuclear content of human spermatozoa [11].

The first observation that ROS might disrupt normal sperm function came from MacLeod [12], who found that human spermatozoa rapidly lose their motility when incubated under high oxygen tensions. Studies have indicated that ROS production by sperm increases under certain circumstances, such as centrifugation [13], cryopreservation [14], and the presence of leukocytes [15]. However, the relationship between levels of seminal ROS and sperm motility after incubation of semen at different temperatures has not been elucidated.

The objective of the current study was to determine the effect of different temperatures on sperm, including ROS production, motility, and other motion characteristics.

MATERIALS AND METHODS

Semen Samples

This study was approved by the Cleveland Clinic Foundation's Institutional Review Board. Semen samples were collected from a non-selected group of infertile men (n = 12) attending our male infertility clinic with a history of infertility of more than 12 months' duration; a group of normal sperm donors (n = 12) was included as a control. Donors were selected on the basis of normal semen characteristics according to the WHO guidelines [5].

Semen Analysis

Each sample was divided into four aliquots right after liquefaction at 37°C. One aliquot was analyzed for concentration, motility, morphology, and leukocyte concentration. The remaining three aliquots were simultaneously incubated for one hour (at 4°C, 25°C, and 37°C).

Smears of fresh semen were prepared for sperm morphology assessment. The smears were fixed and stained using the Diff-Quik kit (Baxter Healthcare, McGaw Park, IL). Immediately after staining, the smears were rinsed in distilled water and air-dried. They were scored for sperm morphology using WHO criteria [5]. The presence of peroxidase-positive leukocytes in semen was assessed by a myeloperoxidase-staining test, in which a 20-µL volume of liquefied semen specimen was placed in a Corning 2.0 mL cryogenic vial (Corning Costar, Cambridge, MA) with 20 µL of phosphate-buffered saline (PBS; pH 7.0) and 40 µL of benzidine solution. The solutions were mixed and allowed to sit at room temperature for five minutes. Peroxidase-positive leukocytes staining brown were counted in a Makler chamber (Sefi Medical Instruments, Haifa, Israel). The results after correction for dilution were recorded as counts x 10^6/mL.

Assessment of Sperm Motion Kinetics

Sperm motion kinetics were analyzed in the fresh aliquot immediately after liquefaction, and after incubation in the remaining three aliquots. Measurements were made using a computer-assisted semen analyzer (CASA) (Cell-Trak, version 4.24, Motion Analysis Corporation, Palo Alto, CA). For each measurement, a 5-µL aliquot was loaded into a Microcell (Conception Technologies, La Jolla, CA) counting chamber and four to eight representative fields containing 200 or more spermatozoa were examined.

Five sperm motion characteristics were measured: curvilinear velocity (VCL, a measure of the total distance traveled by a given sperm divided by the time elapsed, µm/s); straight-line velocity (VSL, the straight-line distance from the beginning to the end of a sperm track divided by the time taken to travel that distance, µm/s); average path velocity (VAP, the average velocity of sperm movement, µm/s); linearity (LIN; %); and sperm head movements, that is, the amplitude of lateral head displacement (ALH, the mean width of sperm head oscillation, um).

The CASA calibration set-up was as follows: 2-well, 20 lam, duration of data capture (frames): 15; minimum motile speed (µm/s): 600; distance scale factor (um/pixel): 0.9457; centroid cell size minimum (pixels): 2; centroid cell size maximum (pixels): 8; number of cells to find per well: 200; minimum number of fields per sample: 3. The reproducibility of the analyzer results was determined using a calibration videotape recording.

Reactive Oxygen Species Measurement

Levels of ROS were measured in the fresh and incubated semen samples. Aliquots of semen were centrifuged at 300 g for seven minutes. The sperm pellet was washed twice with PBS and resuspended in the same medium at a concentration of 20 x 10^6 sperm/mL. The ROS production was measured by a chemiluminescence assay using luminol (5-amino-2, 3,-dihydro-1, 4-phthalazinedione; Sigma, St. Louis, MO) as a probe. Measurements were made using a Berthold luminometer Autolumat: LKB 953, Wallace Inc., Gaithersburg, MD). Eight microliters of horseradish peroxidase (HRP) 12.4U of HRP Type VI, 310U/mg: Sigma) was added to 400 µL of washed sperm suspensions to allow sensitization of the assay for measurement of extracellular hydrogen peroxide. Ten micro liters of luminol prepared as 5 mM stock in dimethyl sulfoxide (DMSO) was added to the mixture; 10 µL of 5 mM luminol added to 400 µL of PBS was used as a negative control. Levels of ROS were determined by measuring chemiluminescence in the integrated mode for 15 minutes. Results were expressed as x106 counted photons per minute (cpm) per 20 x 10^6 sperm.

Statistical Analysis

The fresh and incubated semen aliquots were compared using p-values and regression estimates from repeated measure analysis of variance (ANOVA). ANOVA included the temperature as an independent variable, and utilized the information that the same samples were included at all temperatures. Pairwise comparisons between temperatures were computed using Student's *t*-test. Correlations between variables were calculated using Spearman's rho. Statistical significance was assessed with two-tailed tests, and $P < .05$. Variables that were not normally distributed were log-transformed.

Summary statistics are presented as median and interquartile range (25th and 75th percentile) within the text. All calculations were performed with SAS version 8.1 statistical software (SAS Institute, Cary, NC).

TABLE 1
Comparison of sperm motility and motion characteristics in donors, at different temperatures.

Variable	*Fresh*	*4°C*	*25°C*	*37°C*	*A*	*B*	*C*	*D*	*E*	*F*
Motility (%)	64.5 (52.25, 81.0)	41.5 (24.5, 59.25)	59.5 (39.0, 87.25)	63 (41.0, 83.25)	<.0001	0.5	0.64	<.0001	<.0001	0.82
VCL (µm/s)	32.2 (25.1, 40.8)	17.4 (15.3, 20)	35.8 (26.8, 43.3)	34.9 (25.4, 41.2)	<.0001	0.34	0.8	<.0001	<.0001	0.48
VSL (µm/s)	13.6 (10.6, 16.7)	6.8 (5.7, 7.7)	14 (12.2, 16.5)	13.5 (11.4, 17.4)	<.0001	0.42	0.61	<.0001	<.0001	0.76
VAP (µm/s)	21.3 (16.3, 26.0)	11.3 (9.1, 14.2)	23.7 (18.6, 27.4)	23.1 (17, 26.2)	<.0001	0.28	0.54	<.0001	<.0001	0.64
LIN (%)	40.3 (38.6, 48.3)	40 (37.1, 42.2)	38.3 (36.1, 44.4)	40.2 (37.3, 47.2)	0.1	0.44	0.86	0.36	0.07	0.34
ALH (µm)	2.2 (1.7, 2.9)	1.3 (1.1, 1.5)	2.8 (1.8, 3.2)	2.5 (1.7, 3.1)	<.0001	0.22	0.65	<.0001	<.0001	0.43

Values are median and interquartile range (25th and 75th percentiles). A = p-value of fresh vs. 4°C, B = p-value of fresh vs. 25°C, C = p-value of fresh vs. 37°C, D = p-value of 4°C vs. 25°C, E = p-value of 4°C vs. 37°C, F = p-value of 25°C vs. 37°C; p-values from repeated measure analysis of variance (ANOVA).
VCL = curvilinear velocity; VSL = straight-line velocity; VAP = average path velocity; LIN = linearity; ALH = amplitude of lateral head displacement.

TABLE II
Comparison of sperm motility and motion characteristics in patients, at different temperatures.

Variable	*Fresh*	*4°C*	*25°C*	*37°C*	*A*	*B*	*C*	*D*	*E*	*F*
Motility (%)	55 (37.5, 64.75)	44 (28.25,52.75)	39 (32.75,66.25)	45.5 (27.5, 64)	0	0.42	0.13	0.04	0.17	0.48
VCL (μm/s)	35.8 (31.3, 39.6)	15.4 (13.9, 19.0)	29.6 (24.9, 37.1)	33.1 (28.9, 38.9)	<.0001	0.02	0.69	<.0001	<.0001	0.04
VSL (μm/s)	15.4 (13.8, 19.1)	7.1 (6.2, 8.4)	14.5 (11.7, 15.8)	15.0 (12.3, 17.1)	<.0001	0.06	0.51	<.0001	<.0001	0.2
VAP (μm/s)	23.9 (20.5, 27.1)	10.8 (9.9, 13.7)	20.3 (18.0, 24.9)	22.1 (20.4, 27.6)	<.0001	0.04	0.95	<.0001	<.0001	0.04
LIN (%)	44.1 (39.0, 48.9)	46.3 (44.0, 49.0)	48.3 (37.5, 51.9)	44.9 (38.1, 47.1)	0.37	0.69	0.46	0.61	0.11	0.26
ALH (μm)	2.2 (2.0, 2.3)	1.2 (1.1, 1.3)	1.8 (1.7, 2.2)	2.1 (1.8, 2.4)	<.0001	0.14	0.79	<.0001	<.0001	0.08

Values are median and interquartile range (25th and 75th percentiles). A = p-value of fresh vs. 4°C, B = p-value of fresh vs. 25°C, C = p-value of fresh vs. 37°C, D = p-value of 4°C vs. 25°C, E = p-value of 4°C vs. 37°C, F = p-value of 25°C vs. 37°C; p-values .from repeated measure analysis of variance (ANOVA). Abbreviations as in Table I.

RESULTS

Comparison of sperm characteristics between donors and infertility patients indicated no significant differences in sperm concentration (p = 0.06) and seminal leukocyte concentrations (p = 0.71). However, normal sperm morphology was significantly lower in the patients than in the donors (p = 0.04).

In both the donors and patients, the percentage motility was significantly lower for the samples incubated at 4°C compared with those incubated at 25°C and 37°C (Tables I and II); however, sperm motility was not significantly different when samples were incubated at 25°C and 37°C. Comparisons of mean and standard deviations (SD) of motility between patients and donors for the different temperatures are shown in Figure 1.

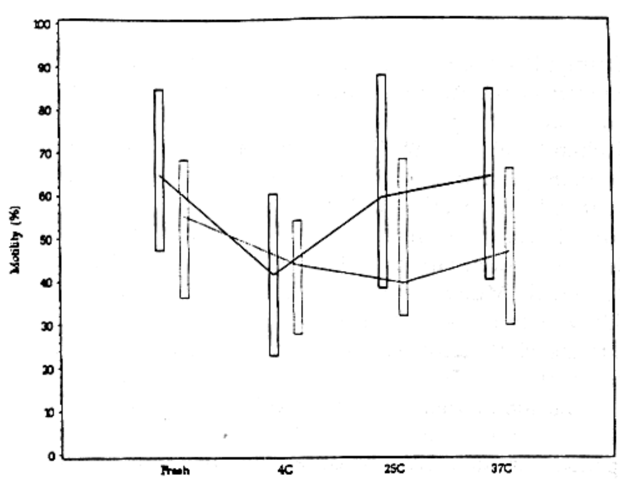

FIG. 1. Comparisons of mean (t standard deviations) of motility between patients (open boxes) and donors (closed boxes) at different temperatures.

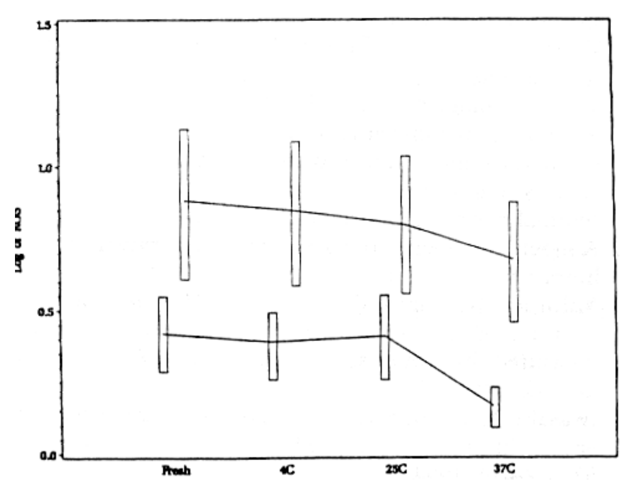

FIG. 2. Comparisons of mean (t standard deviations) of ROS (reactive oxygen species) levels between patients (open boxes/ and donors (closed boxes) at different temperatures.

Comparisons of sperm motion characteristics for donors and patients at different temperatures are shown in Tables I and II. In both the patients and donors, sperm VCL, VSL, VAP, and ALH significantly decreased from baseline values when incubated at 4°C ($P < .05$). On the other hand, baseline values of LIN were not significantly different from their values after one hour of incubation at 4°C, 25°C, and 37°C in either group. A twofold decrease was seen in VCL, VSL, VAP, and ALH in the aliquots incubated at 4°C when compared with the fresh aliquots, as well as 25°C and 37°C. The VAP was significantly lower in the patient samples incubated at 25°C than in the fresh samples ($p = 0.04$).

Comparisons of ROS levels in the patients and donors for the fresh (basal ROS) and incubated samples are shown in Figure 2. Levels of ROS in the samples incubated at 37°C were significantly lower than those in the samples incubated at 4°C and 25°C (Table III/. Levels of ROS were negatively correlated with motility ($r = -0.40$, $p = 0.02$). For every 10-fold increase in levels of ROS, the repeated-measures regression analysis indicated that motility decreased -9.1% ±3.8%.

TABLE III
Comparison of reactive oxygen species levels in donors and patients at different temperatures.

Variable	*Fresh*	*4°C*	*25°C*	*37°C*	*A*	*B*	*C*	*D*	*E*	*F*
Donors (n = 12)	1.0 (0.2, 2.6)	1.0 (0.1, 3.1)	0.4 (0, 7.2)	0.1 (0, 0.9)	0.6	0.84	0.002	0.75	0.008	0.004
Patients (n = 12)	2.2 (0.3, 67.2)	2.2 (0.4, 61.2)	1.8 (0.3, 58.5)	1.3 (0.2, 28.4)	0.6	0.18	0.001	0.4	0.004	0.04

Values are median and interquartile range /25th and 75th percentiles). A = p-value of fresh vs. 4°C, B = p-value of fresh vs. 25°C, C = p-value of fresh vs. 37°C, D = p-value of 4°C vs. 25°C, E = p-value of 4°C vs. 37°C, F = p-value of 25°C vs. 37°C; p-values from repeated measure analysis of variance (ANOVA).

DISCUSSION

Spermatozoa in warm-blooded animals, including humans, spend their entire life span inside the male and female genital tract-an environment that consistently remains at body temperature (37°C). Human sperm that is ejaculated into the female genital tract retains its fertilizing capacity for up to 85 hours [16]. Our results indicate higher sperm motility and other motion kinetics, and lower levels of ROS production, in semen samples incubated at 37°C than at 4°C and 25°C. The results obtained in this study demonstrate the close relationship between the activity of human spermatozoa and the temperature at which they are kept. The effects of different temperatures on sperm motility have been described in many studies [2,4,17].

The temperature at which semen samples should be handled is controversial. Some studies have focused solely on determining whether any direct physical damage to sperm motility occurs during incubation in vitro. It has been shown that during storage in vitro, abnormal spermatozoa produce high levels of ROS [18,19]. The generation of ROS by human spermatozoa has been implicated in the etiology of male infertility due to lipid peroxidation of the sperm plasma membrane and loss of sperm functions [9]. Altered sperm functions due to high levels of ROS include impaired motility, sperm-zona pellucida binding, and sperm-oocyte fusion, and capacity to regulate intracellular calcium levels [20,21]. The effect of high amounts of ROS on sperm motility is rapid and can cause sperm immobility within 5-30 minutes, depending on the concentration [22].

There are several mechanisms proposed to account for the decline in sperm motility associated with high amounts of ROS. The decline may be caused by a depletion of adenosine triphosphate (ATP) [22], which in turn affects sperm axonemes and negatively influences enzyme function [23]. Another possible mechanism by which ROS may adversely affect sperm motility is through an alteration in mitochondrial function [22,24]: motility of spermatozoa depends on the integrity of the mitochondrial sheath, of which phospholipids are a major component, and oxidation of these phospholipids by ROS may impair sperm motility [25]. It seems possible also that hydrogen peroxide at low concentrations inhibits sperm motility and reduces sperm ATP levels, without causing lipid peroxidation or significant declines in the mitochondria) membrane potential [26]. Thus, it is conceivable that sperm motility may be affected by ROS-mediated pathway independent of lipid peroxidation and mitochondria) membrane potential [24]. Additionally, it was reported that endogenously generated ROS can increase DNA fragmentation in human spermatozoa in a dose-dependent manner [27]. At low levels of ROS, DNA fragmentation was significantly reduced, while the rates of sperm-oocyte fusion were significantly enhanced; as the levels of ROS increased, the spermatozoa exhibited significantly elevated levels of DNA damage and yet continued to have enhanced motility and capacity for sperm-oocyte fusion [11]. At the highest levels of ROS, extreme DNA fragmentation was observed, but the spermatozoa exhibited a parallel loss in their capacities for movement and oocyte fusion [27].

Our study revealed no statistically significant differences in motility between the fresh aliquots and those incubated at 25°C and 37°C, but the levels of ROS in the samples incubated at 37°C were significantly lower. In addition, the study showed that incubating semen samples from infertility patients at 4°C or 25°C can shift the motility and other motion characteristics into the subnormal range.

In conclusion, our results indicate that 37°C is an optimum temperature at which semen samples can be collected, transported, and processed, in that handling semen samples at 37°C may help avoid deleterious effects of ROS.

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Address reprint requests to:
Ashok Agarwal, Ph.D., HCLD
Director, Center for Advanced Research
in Human Reproduction,
Infertility, and Sexual Function
The Cleveland Clinic Foundation
9500 Euclid Avenue, Desk A19.1
Cleveland, OH 44195