Heat-Induced Hyperactivation[1]

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Purpose: *The objectives of this study were (1) to determine the sperm hyperactivation and related kinematic parameters at 40°C after using four sperm wash procedures and (2) to correlate the heat-induced hyperactivation data with cases of clinical pregnancies from either artificial insemination or standard in vitro fertilization (IVF).*
Methods: *Semen samples (n = 51) were collected by ejaculation, and semen analyses were carried out to determine the pretreatment data. Sperm kinematic measurements were performed using the Hamilton Thorn HTM-C computer-aided sperm analyzer. Hyperactivation was determined using the sort module on the HTM-C. Membrane integrity was assessed using the hypoosmotic sperm swelling procedure. Sperm morphology and acrosomal status were also determined using the Spermac stain. Each semen specimen was divided and processed through either the swim-up wash, the 1-h test-yolk buffer (TYB) wash, the 1 mg/ml pentoxifylline stimulant procedure, or the two-layer 90:47% gradient colloidal solution procedure. The washed sperm were incubated at 25 or at 40°C for 4 hr. After incubation, kinematic parameters were assessed for the posttreatment data. Semen specimens were obtained on different occasions for artificial insemination or standard IVF. Data from intracytoplasmic sperm injection cases were not included to avoid confounding factors. Live births and/or pregnancies with fetal heartbeat examined by ultrasound were considered clinical pregnancies.*
Results: *Heat-induced hyperactive motility was significantly higher in sperm of the male partner of pregnant (n = 7) patients compared with nonpregnant (n = 44) patients (mean ± SE, 10.0 ± 3.3 versus 5.5 ± 0.8%) after TYB processing followed by 4 hr of incubation at 40°C. This was also observed after colloid (Percoll) processing (11.6 ± 4.6 versus 5.8 ± 0.8%). There were no differences in hyperactivation after 4 hr at 23°C between pregnant and nonpregnant cases. Parameters such as count, volume, motility, viability, and acrosomal status were not different for the groups. However, the percentage of sperm with normal morphology (WHO classification) was twice as high in the pregnant group versus the nonpregnant group.*
Conclusions: *Heat-induced hyperactivation was associated with fertile sperm and was predictive of pregnancy obtained after artificial insemination or IVF. The association was evident only after TYB or Percoll sperm processing. The study could not confirm the finding of significant decreases in motility after heat treatment of sperm derived from infertile males. The mechanism for heat-induced hyperactivation did not involve membrane integrity or the sperm acrosome, although an involvement of heat shock proteins was postulated. Interestingly, there were no pregnancies when sperm did not exhibit heat-induced hyperactivation.*

KEY WORDS: sperm; computer-aided sperm analyzer (CASA); heat induction; hyperactivation motility.

INTRODUCTION

The motility of sperm from infertile male patients has been shown to decrease dramatically, by more than 75% of the original preincubation values, when heated at 40°C for 4 hr (1). Based on this observation, Alvarez and colleagues proposed the sperm stress test, which involves measuring the changes in sperm motility resulting from the heat treatment. They reported that the changes in motility were predictive of pregnancies in in vitro fertilization (IVF) patients in a clinical setting. However, information concerning sperm kinematic parameters such as hyperactivation after 40°C heat treatment was not considered. The sperm hyperactivation parameter is important because it serves as a marker of sperm capacitation, defined as alterations in membranes in preparation for the fertilization of the

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oocyte (2,3). Hyperactive motility is characterized by star-spin or whiplash movement with a wide amplitude of lateral head displacement, high curvilinear velocity, and low or nonlinear direction distinctly different from helical movement (3–9). Hyperactivation can be objectively analyzed on the computer-aided sperm analyzer (CASA) after defining the sort module parameters (10). The hypothesis of the present study was that 40°C heat treatment of sperm would induce hyperactivation in fertile sperm. The objectives were (i) to determine sperm hyperactivation and related kinematic parameters at 40°C after processing through one of four sperm wash procedures and (ii) to correlate the heat-induced hyperactivation of sperm with data on clinical pregnancies. The results of the study will assist clinicians in developing new diagnostic tests of sperm fertility. Furthermore, it is envisioned that new heat-induced methods may be applied to increase the fertilizing capacity of sperm from infertile males for use in assisted reproduction technologies.

MATERIALS AND METHODS

Sperm Processing

Semen samples (n = 51) were collected by masturbation, and complete semen analyses were carried out to determine the pretreatment data. Data from patients that involved intracytoplasmic sperm injection (ICSI), epididymal sperm aspiration, donor oocyte, or donor sperm were not included in the study. Each semen specimen was divided and washed using one of four sperm washing or separation procedures to determine the optimal wash procedure for observing heat-induced sperm hyperactivation. To achieve this goal, the equally divided semen specimens were processed through either the swim-up wash, the test-yolk buffer (TYB) wash, the pentoxifylline stimulant wash, or the discontinuous two-layer 90:47% colloid (Percoll; Perception, Fertility Technologies, Natick, MA) gradient centrifugation wash (11).

The swim-up from semen procedure consisted of pipetting a portion (0.5 to 1.0 ml) of each semen specimen to the bottom of the 15-ml centrifuge tube and gently overlayering the semen with 1.0 ml of HEPES buffered human tubal fluid (modified HTF; Irvine Scientific, Santa Ana, CA). The tubes were incubated in a vertical position at 37°C in room air for 30 min. After incubation, the topmost layer (1.0 ml) containing the motile sperm was pipetted into a new centrifuge tube. A portion (0.5 ml) was pipetted into a tube containing TYB (Refrigeration Medium, Irvine Scientific, Santa Ana, CA), mixed, and refrigerated for 1 hr at 4°C (unheated TYB sperm). The remaining portion (0.5 ml) was centrifuged at 300g for 10 min and the pellet resuspended in 0.4 ml of modified HTF and incubated at 23°C in room air (the unheated swim-up sperm) for 4 hr. At the start of incubation, a 0.1-ml aliquot was pipetted out from the unheated swim-up tube and incubated inside an Eppendorf microfuge tube at 40°C for 4 hr in room air atmosphere (the heat-induced swim-up sperm). The sperm were analyzed at time points described below.

The tubes of refrigerated TYB sperm were warmed back up by placing the tubes in a water bath at 37°C for 5 min (12). Each TYB tube of sperm was centrifuged at 300g for 10 min and the resultant pellet resuspended in 0.4 ml of modified HTF and incubated at 23°C in room air for 4 hr (unheated TYB sperm). At the start of incubation, a 0.1-ml aliquot was pipetted out from the TYB sperm tube and into an Eppendorf microfuge tube and incubated at 40°C for 4 hr in room air atmosphere (the heat-induced TYB sperm). At specific time points, the sperm were analyzed as detailed below.

The pentoxifylline treatment (13) consisted of mixing a portion (0.5 to 1.0 ml) of each semen specimen with an equal volume of 1 mg/ml pentoxifylline (Sigma Chemical Co., St. Louis, MO) dissolved in modified HTF medium. In this study, it was important to quality-control test each lot of pentoxifylline before use because it was our experience that some lots actually decreased sperm motility. The mixture of semen and pentoxifylline was incubated at 37°C for 30 min. It was important not to exceed the 30 min of incubation time so as to avoid deterioration of sperm motility. At the end of incubation, each mixture was layered on top of a discontinuous two layer 90:45% colloid gradient and centrifuged for 20 min at 300g. The supernatant was decanted, and the pellet was resuspended in 1 ml of modified HTF and recentrifuged for 10 min. The resultant pellet was resuspended in 0.4 ml of modified HTF and incubated at 23°C in room air atmosphere for 4 hr (unheated pentoxifylline sperm). At the start of incubation, a 0.1-ml aliquot of sperm was pipetted out from the pentoxifylline tube and into an Eppendorf microfuge tube and incubated at 40°C for 4 hr in room air atmosphere (the heated pentoxifylline sperm). Sperm analyses were performed as described below.

The two-layer colloid gradient wash (11) consisted of layering a portion (0.5 to 1.0 ml) of the semen specimen on top of a two-layer discontinuous colloid gradient inside a tube. Each colloid gradient was prepared by pipetting 1.0 ml of the 90% colloid solution

to the bottom of the 15-ml centrifuge tube and gently layering the 47% colloid solution on top of the 90% layer. For the novice, it may be helpful to add tiny drops of phenol red indicator concentrate solution (phenol red dissolved in modified HTF and sterilized by passage through a 0.2-μm syringe-filter) to the 47% colloid stock solution until the color just begins to differentiate this solution from the 90% solution. The tubes of layered semen on top of the colloid gradients were centrifuged for 20 min at 300*g*. The supernatants were decanted, and each pellet was resuspended in 1 ml of modified HTF and recentrifuged for 10 min. Each resultant pellet was resuspended in 0.4 ml of modified HTF and incubated at 23°C in room air atmosphere for 4 hr (unheated colloid washed sperm). At the start of incubation, a 0.1-ml aliquot of sperm was taken from the colloid washed sperm, placed in an Eppendorf microfuge tube, and incubated at 40°C for 4 hr in room air atmosphere (the heated colloid sperm). Sperm analyses were carried out as follows.

Computer-Aided Sperm Analyses

Sperm dimensions and kinematic parameters were measured using the Hamilton Thorn motility analyzer (HTM-C) CASA system as described previously (14). Briefly, the analyses were carried out using aliquots (10 μl each) of the processed sperm in 20-μm-deep glass slides with coverslips prewarmed at 37°C on a slide warmer. A minimum of 100 sperm cells was analyzed each time. Analyses were carried out after 0 and 4 hr of incubation at both 23 and 40°C. The percentage of hyperactive sperm was measured using the sort fraction module of the HTM-C based on published settings on this analyzer (10). For consistency, the same technician was used throughout the study. The percentage hyperactivation was based on the number of hyperactive sperm divided by the total number of motile sperm.

Spermac Staining for Acrosome

A drop from each sperm suspension was also removed at the start of incubation and an air-dried smear was made at this time for the Spermac acrosome procedure as previously reported (14). Briefly, each dried smear was fixed (5 min, 23°C) in formalin solution (Fixative I) provided in the Spermac kit (Stain Enterprises, Onderstepoort, S. Africa, distributed by Fertility Technologies, Natick, MA). Each slide was processed through stain solution A, B, and C (1 min, 23°C). The stained slides were air-dried and analyzed under oil immersion (×1000) for the percentage of sperm with intact acrosome. Sperm with intact acrosome have normal oval-shaped heads, with the anterior acrosomal region staining dark green (14) and the post-acrosomal region staining red–pink. Sperm lacking this red–pink color were not counted. Sperm lacking acrosomes have white or red anterior acrosomal heads. Sperm heads that showed peeled acrosomal membranes or partial green coloration were considered acrosome defective. The percentage of sperm with intact acrosomes was calculated by dividing the number of sperm with dark green acrosomes by the total number of sperm multiplied by 100.

Viability and Hypoosmotic Testing

Supravital staining of sperm to determine viability was performed on an aliquot of sperm as previously described using 0.5% Eosin Y stain (15). The term viability used in this study was defined as "the ability to exclude dyes which have low permeability in the membrane lipid bilayer." Sperm cells with clear-colored heads were counted as viable, whereas pink-headed sperm were counted as dead.

Another aliquot from each sperm specimen was tested for membrane integrity at the tail region as previous reported (16). Briefly, sperm (0.1 ml) were placed in 1.0 ml of a modified hypoosmotic solution (16) and incubated upright at 37°C for 30 min. After the incubation period, an aliquot (5 μl) was pipetted from the bottom of the tube onto a glass slide and a coverslip placed on top of the droplet. A total of 100 sperm cells in several random fields was analyzed for each specimen (light microscope; magnification, ×400). The percentage of sperm with intact tail membrane was calculated from the number of sperm displaying types B to G tail coiling by the total number of sperm multiplied by 100. The results were then recorded and analyzed.

Assisted Reproductive Technologies

The clinical data were based on semen specimens provided on later occasions and were either results of artificial insemination or IVF cases. As mentioned earlier, data from cases that involved ICSI, epididymal sperm aspiration, donor oocytes, and/or donor sperm were excluded for obvious reasons. The artificial insemination procedure was the same as the established clinical protocols (17). Briefly, female patients scheduled for intrauterine inseminations at the Center for Fertility and In Vitro Fertilization after routine exami-

nations were stimulated using a clomiphene citrate and/or a human menopausal gonadotropin regimen followed by a trigger injection of human chorionic gonadotropin (17). Some patients returned for more inseminations during different cycles but never more than one insemination per cycle. On the day of insemination, semen was collected from the partners after a suggested abstinence period of 2–7 days and liquefied for at least 20 min at 37°C. The semen were processed using standard sperm wash procedures and the washed sperm used for intrauterine inseminations.

The IVF procedure was the same as the established protocols (18). Briefly, female patients and partners were given routine examinations and for the female patients, ovulation induction was achieved using follicle stimulating hormone initiated on the third day of the cycle and given daily, followed by 10,000 U of human chorionic gonadotropin, generally administered on the tenth day, when the E_2 was over 500 pg/ml and the largest follicular diameter was over 18 mm. Patients on leuprolide acetate cycles followed a similar induction protocol except 0.5 mg leuprolide acetate was administered beginning on day 21 of the previous cycle and ending on the last day of stimulation (18). The culture medium used for all protocols was HTF supplemented with synthetic serum substitute. At the time of the IVF procedure, the sperm cells were washed by standard wash procedures and used in the insemination of the oocytes.

The numbers of pregnant patients mentioned in the tables are based on clinical pregnancies. The criteria for the clinical pregnancy were both an elevated serum human chorionic gonadotropin (hCG) concentration and the ultrasound detection of a uterine sac(s), (each) with a beating heart.

Data Analyses

The results (Tables I and II) are presented as the mean ± standard error of the mean (SE). Means derived from sperm sampled over a period was analyzed by repeated-measures analysis of variance (ANOVA) in which the fixed effects were temperature and exposure time (0 and 4 hr) followed by significance testing using Tukey's Q test. Student's t test was used whenever appropriate. Categorical data such as the number of false positives over the total number of cases were evaluated using the chi-square test statistic. A P value of less than 0.05 was considered significant.

Table I. Heat-Induced Hyperactivation in Human Sperm After Processing Through the Test-Yolk Buffer Wash Procedure

Parameter	Pregnant (n = 7)	Not pregnant (n = 44)
Percentage motility*		
Hr 0	74.0 ± 3.3	62.4 ± 3.2
Hr 4 (unheated)	71.4 ± 6.4	56.5 ± 3.6
Hr 4 (heated)	71.7 ± 2.9	62.7 ± 3.5
Percentage progression		
Hr 0	35.0 ± 4.7	27.9 ± 2.7
Hr 4 (unheated)	21.1 ± 2.5	19.2 ± 2.1
Hr 4 (heated)	27.0 ± 4.6	21.5 ± 2.2
Percentage hyperactivation		
Hr 0	7.0 ± 4.0	4.4 ± 0.8
Hr 4 (unheated)	2.7 ± 1.5	2.9 ± 0.6
Hr 4 (heated)	10.0 ± 3.3	5.5 ± 0.8*

* $P < 0.05$.

RESULTS

After heat treatment, the hyperactive motility (Table I) in TYB-processed sperm of the male partners of pregnant patients (n = 7) was almost twice as high ($P < 0.05$) as the hyperactive motility in sperm of partners of nonpregnant patients (mean ± SE, 10.0 ± 3.3 versus 5.5 ± 0.8%). This observed difference in sperm hyperactivation was also significant (Table II) after the two-layer colloid solution processing (11.6 ± 4.6 versus 5.8 ± 0.8%). In addition, for the two-layer colloid processed sperm, as well as the swim-up processed sperm (Table III), the difference in hyperactivation between the pregnant and the nonpregnant groups was noticeable immediately after the wash procedure (Table II). In contrast, there were no significant difference in hyperactivation for pentoxifylline-pro-

Table II. Heat-Induced Hyperactivation in Human Sperm After Processing Through the Discontinuous Two-Layer Colloid Gradient Wash Procedure

Parameter	Pregnant (n = 7)	Not pregnant (n = 44)
Percentage motility		
Hr 0	78.6 ± 5.6	75.4 ± 2.8
Hr 4 (unheated)	69.6 ± 6.7	69.5 ± 3.1
Hr 4 (heated)	76.3 ± 3.6	70.1 ± 3.5
Percentage progression		
Hr 0	31.7 ± 4.4	32.0 ± 2.1
Hr 4 (unheated)	23.4 ± 4.8	23.5 ± 2.0
Hr 4 (heated)	27.7 ± 4.4	25.7 ± 2.0
Percentage hyperactivation		
Hr 0	13.4 ± 4.4	6.6 ± 1.1*
Hr 4 (unheated)	3.7 ± 1.2	3.9 ± 0.7
Hr 4 (heated)	11.6 ± 4.6	5.8 ± 0.8*

* $P < 0.05$.

Table III. Heat-Induced Hyperactivation in Human Sperm After Processing Through the Swim-Up from Semen Wash Procedure

Parameter	Pregnant (n = 7)	Not pregnant (n = 44)
Percentage motility*		
Hr 0	77.0 ± 3.5	65.4 ± 3.7
Hr 4 (unheated)	65.4 ± 4.4	55.2 ± 3.2
Hr 4 (heated)	77.6 ± 4.7	64.9 ± 3.6
Percentage progression		
Hr 0	35.3 ± 3.1	26.2 ± 2.3
Hr 4 (unheated)	18.9 ± 2.4	16.7 ± 1.7
Hr 4 (heated)	22.4 ± 2.4	20.2 ± 2.0
Percentage hyperactivation		
Hr 0	9.0 ± 4.5	4.8 ± 0.7*
Hr 4 (unheated)	2.3 ± 0.8	3.0 ± 0.6
Hr 4 (heated)	9.7 ± 5.0	7.7 ± 1.0

* $P < 0.05$.

cessed sperm (Table IV). In the latter group however, there was a difference in the percentage progression of sperm after incubation for 4 hr at 23°C. Interestingly, the percentage of motile sperm in the pregnant group was also significantly higher than in the nonpregnant group. This difference was observed only for TYB (Table I) or swim-up-processed sperm (Table III).

For the four types of processed sperm, the final sperm motility after heat treatment at 40°C for 4 hr for both pregnant and nonpregnant groups was greater than 83% of the initial motility.

Semen analyses parameters such as count, volume, initial motility, viability, sperm velocities, kinematic parameters, and percentage acrosome intact were not different between the pregnant and the nonpregnant groups (Table V). In contrast, hypoosmotic swelling of sperm, a marker for tail membrane integrity, was paradoxically higher for the nonpregnant group. In terms of morphology, the percentage of sperm with normal forms in the pregnant group was twice as high as that for the nonpregnant group.

Table IV. Heat-Induced Hyperactivation in Human Sperm After Processing Through the 1 mg/ml Pentoxifylline Wash Procedure

Parameter	Pregnant (n = 7)	Not pregnant (n = 44)
Percentage motility		
Hr 0	77.3 ± 5.0	75.1 ± 2.9
Hr 4 (unheated)	74.4 ± 6.3	63.5 ± 3.6
Hr 4 (heated)	76.1 ± 5.6	62.7 ± 4.0
Percentage progression		
Hr 0	38.6 ± 5.5	32.7 ± 2.1
Hr 4 (unheated)	34.4 ± 4.7	24.8 ± 1.9*
Hr 4 (heated)	35.4 ± 6.4	25.7 ± 2.4
Percentage hyperactivation		
Hr 0	10.0 ± 4.2	9.9 ± 1.4
Hr 4 (unheated)	5.1 ± 2.3	5.6 ± 0.8
Hr 4 (heated)	12.7 ± 6.3	7.7 ± 1.0

* $P < 0.05$.

Table V. Basic Semen Analyses and Sperm Kinematic Parameters of Specimens Used in the Heat-Induced Hyperactivation Study

Parameter	Pregnant (n = 7)	Not pregnant (n = 44)
Semen volume (ml)	3.6 ± 1.0	3.3 ± 0.2
Sperm count (million/ml)	43.9 ± 12.7	39.4 ± 5.0
Initial motility (%)	60.7 ± 3.5	58.0 ± 2.1
Viability (%)	74.0 ± 2.4	71.2 ± 2.2
Hypoosmotic swelling (%)	58.0 ± 5.6	67.8 ± 2.2*
Curvilinear velocity (μm/sec)	54.7 ± 4.2	56.0 ± 1.9
Straight line velocity (μm/sec)	32.0 ± 1.5	33.2 ± 1.4
Linearity (%)	45.3 ± 3.2	46.5 ± 1.5
Lateral head displacement (μm)	2.2 ± 0.2	2.5 ± 0.3
Beat frequency (Hz)	7.2 ± 0.1	6.2 ± 0.3
Mean area of sperm (μm^2)	17.5 ± 1.1	20.8 ± 1.5
Normal morphology (%)	33.7 ± 2.5	16.8 ± 1.8*
Acrosome intact (%)	27.7 ± 4.3	27.6 ± 1.8

* $P < 0.05$.

DISCUSSION

The results showed that of the four types of sperm washes, only the TYB and two-layer colloid wash procedures were capable of discriminating between pregnant and nonpregnant outcomes based on heat-induced hyperactivation. The phenomenon of heat-induced hyperactivation was fascinating to observe in the laboratory, as the movement of sperm gave the appearance of worms on a hot tin roof. The temperature used for the heat treatment in this study was 40°C, or approximately 104°F, the temperature of fever in humans or after an active exercise workout. It was postulated that heat shock proteins might be involved in activating sperm hyperactivation. The presence of heat shock proteins have been documented in spermatogenic cells (19). Generally, heating at 43°C for 15 min is sufficient to induce heat shock proteins (20). So far, in the case of sperm, six types of 70-kDa heat shock proteins have been identified, HSP70 to HSP70-2. These are thought to prevent sperm apoptosis (19). In addition, these proteins activate transcription (21). The mechanism by which heat shock proteins activate hyperactivation whether through stimulated phosphorylation of essential enzymes or increased calcium channel activities remains unknown.

In the present study, the data suggest that the heat treatment either recruits new sperm displaying hyperactive motility or maintains hyperactive motility in fertile sperm. In terms of clinical application, measuring hyperactivation after heat treatment of either TYB- or two-layer colloid-processed sperm may assist in predicting the outcome of assisted reproductive technology procedures. Interestingly, there were no pregnancies when sperm did not exhibit heat-induced hyperactivation. Patients with suboptimal sperm hyperactivation may be counseled to include ICSI in their fertility treatment.

One could also observe significantly higher hyperactivation in sperm in the pregnant group as early as the start of incubation in the two-layer colloid wash- and swim-up-processed sperm. This result corroborated previous reports that documented higher hyperactivation in sperm of fertile patients (22,23). However, the hyperactivity invariably would decrease with time unless heat treatment were applied as shown by the present study.

In the case of the swim-up-processed sperm in the nonpregnant group, the low number of sperm exhibiting hyperactive motility at the beginning increased to proportions comparable to those of sperm in the pregnant group after heat treatment, a phenomenon not seen in sperm processed by the other wash procedures. This difference may be due to the minimal trauma to sperm intrinsic to the swim-up from semen procedure. However, it is clear that the mechanism did not involve acrosomal status, sperm viability, or velocities as shown by the lack of differences in these parameters.

In the sperm stress test proposed by Alvarez and colleagues (1), they observed decreases of over 75% in motility after heat treatment of two-layer colloid processed sperm in the nonpregnant group. In this study, the decreases in motility after heat treatment were minimal and were not predictive of assisted reproductive technology outcome. This difference in observations may be due to the small sample size for this study as a result of selecting a subset of patients with minimal bias or differences in sperm wash procedures. More studies are needed to evaluate the motility parameter for prognostic use.

Heat treatment has been demonstrated to activate DNA gyrase, which combines with DNA topoisomerases to catalyze DNA relaxation activity in cells (24). The temperature used in heat treatment is a critical factor and adverse effects occur at elevated temperatures. Heat applied during spermatogenesis causes spermatogenic arrest, which logically stops sperm production (25). However, information about the negative effect of a 40°C temperature on sperm that has been ejaculated is lacking. The phenomenon of heat-induced hyperactivation is certainly intriguing if the possibility exists that subfertile sperm can be rendered fertile simply by heat treatment to increase sperm hyperactivation. More studies are needed to provide answers to questions about the effects of heat treatment on sperm.

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