Validation of the Hemizona Assay (HZA) in a Monkey Model. II. Kinetics of Binding and Influence of Cryopreserved–Thawed Spermatozoa[1]

SERGIO OEHNINGER,[2,4] MAHMOOD MORSHEDI,[2] HAKAN ERTUNC,[2] CHRISTINE PHILPUT,[1,3] SILVINA M. BOCCA,[2] ANIBAL A. ACOSTA,[2] and GARY D. HODGEN[2]

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We compared fresh and frozen–thawed cynomolgus monkey spermatozoa tight binding to the zona pellucida under hemizona assay (HZA) conditions. Monkey oocytes were recovered after superovulation and stored in salt solution. Matching hemizonae were obtained by micromanipulation. Semen, obtained by electroejaculation, was used fresh or was cyropreserved, thawed, and washed by swim-up separation. At the standard initial dilution of 500,000 motile sperm/ml (or 5 × 10^4 motile sperm/hemizona), binding was significantly higher for fresh sperm (P = 0.00004). For frozen–thawed samples, there was a linear increase in the number of tightly bound sperm with increasing sperm concentration (r = 0.95). At 1.5 × 10^6 motile sperm/hemizona, binding of frozen–thawed spermatozoa was similar to that of fresh at a standard concentration. Kinetic studies showed peak binding at 1 hr of gametes coincubation. We conclude that, in this monkey model, the HZA is a valuable bioassay for evaluation of sperm binding to the zona pellucida, the initial requisite for fertilization and embryo development.

KEY WORDS: sperm; zona pellucida; binding; hemizona assay.

INTRODUCTION

The hemizona assay (HZA) was developed recently as a diagnostic test of the binding capacity of human spermatozoa to human zonae pellucidae to predict fertilization potential (1). In the HZA, the matched hemizonae created by microbisection provide two test surfaces which are functionally equal, thereby allowing a reproducible, controlled comparison of sperm–zona interactions and tight binding (2).

Previous studies on human oocytes examined the precision of zona cutting and the kinetics of human sperm binding showed maximal binding after 4 to 5 hr of coincubation (1,3). Frozen–thawed oocytes obtained from surgically removed ovaries showed binding properties equal to those of unfertilized, surplus oocytes from our in vitro fertilization (IVF) program (3). Normal sperm showed equivalent binding to dimethyl sulfoxide-stored and salt-stored human hemizonae. These observations extended the report of Yanagimachi *et al.* (4) indicating that the zonae pellucidae retained functional properties following storage in a highly concentrated salt solution. In prospective studies using this homologous bioassay, we showed that the HZA has a high predictive value for outcome in human IVF therapy (5,6).

The close similarity of the nonhuman primate menstrual cycle to that of humans has provided a useful model for ovarian response to gonadotropin stimulation, with and without gonadotropin releasing hormone (GnRH) analogues, as well as for IVF–embryo transfer (7–9). In a recent communication (10), we demonstrated the feasibility of the HZA as a test to evaluate sperm–zona pellucida interac-

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[2] The Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, 601 Colley Avenue, Norfolk, Virginia 23507.
[3] Department of Radiation Oncology (Biostatistics), Eastern Virginia Medical School, Norfolk, Virginia 23507.
[4] To whom correspondence should be addressed.

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tions as a function of oocyte maturity in the cynomolgus monkey using assay conditions previously optimized for the human system. The purposes of the present experiments were (i) to characterize the optimal kinetics of monkey sperm binding to the monkey zona pellucida and (ii) to compare the binding ability of freshly ejaculated and frozen–thawed monkey sperm under these HZA conditions.

MATERIALS AND METHODS

Primate Husbandry

Normal adult male and female cynomolgus monkeys weighing 3 to 6 kg each were caged individually in a controlled environment (temperature, 20–21°C; minimum humidity, 60%; 12/12-hr light/dark cycle) (10). The monkeys were given water ad libitum, fed commercial monkey chow twice a day, and maintained by husbandry practices previously described. Females had a history of regular menstrual cycles; daily observation of the perineum was used to detect the onset and duration of menses.

Treatment Protocols

Six females were given human menopausal gonadotropin (Pergonal; Serono Laboratories, Randolph, MA) daily, starting on day 3 of their menstrual cycles, at a dose of 37.5 IU/day intramuscularly (im) for 6 to 8 days. When serum estradiol (E_2) levels reached 800 to 1000 pg/ml, 1000 IU of human chorionic gonadotropin (Profasi; Serono) was administered im. Thirty-six hours later, laparoscopic oocyte retrieval was performed under ketamine anesthesia. These six females were subjected to 10 stimulation/retrieval cycles; a total of 60 metaphase II and 30 immature (prophase I) oocytes was recovered.

Follicular Aspiration

Follicular fluid was aspirated into Dulbecco's phosphate-buffered saline (PBS) using a 10-ml syringe incubated at 37°C. Upon collection, the follicular fluid was immediately transferred to the laboratory, where examination of the fluid in Falcon No. 3002 petri dishes (Falcon Plastics, Oxnard, NJ) was undertaken to locate and identify the oocytes under a dissecting stereomicroscope (Nikon SMZ-10) and an inverted high-resolution phase-contrast microscope (Nikon Diaphot, Garden City, NJ). Classification of oocyte maturation was performed according to the criteria of Veeck (10,11).

Oocyte Storage

After determination of their maturational stage, oocytes were stored at 4°C in capped microcentrifuge tubes containing 0.5 ml of 1.5 *M* $MgCl_2$, 0.1% polyvinylpyrrolidone, and 40 m*M* HEPES buffer (oocyte storage medium). Experiments were carried out over 15 to 40 days.

Cutting Oocytes into Hemizonae by Micromanipulation

Oocyte microbisection was performed under an inverted phase-contrast microscope equipped with a complete micromanipulation system (Model MO 102; Narishige, Tokyo). The micromanipulator was fitted with a Beaver chuck handle (No. 1312; Katena Products, Denville, NJ) with a Beaver microsharp blade (No. 7530) attached. A 100 × 20-mm plastic petri dish (Falcon No. 25832) served as a cutting chamber. The dish was loaded with 3 to 4 ml of culture medium Ham's F-10 with 3.0 mg/ml of bovine serum albumin (BSA). The blade was positioned exactly perpendicular to the dish. With micromanipulators, the blade was centered in the field of view and slowly lowered to score a groove on the bottom surface of the dish.

Approximately 0.5 ml of culture medium was added to each vial containing the oocytes. Vials were recapped and inverted 15 to 20 times. The contents were emptied into a 35 × 10-mm plastic petri dish. The oocytes were recovered and rinsed in another drop of culture medium. Each oocyte was transferred to the working area of the dish by means of a finely drawn glass pipette. The oocyte was positioned on the groove and the blade was centered over the oocyte. At 200× magnification, the blade was slowly lowered, initiating a midline cut into the zona. The blade was continually lowered until two cleanly cut hemizonae were produced. Dense ooplasm was dislodged by vigorous pipetting with a small-bore drawn glass pipette. Each hemizona pair was placed in a drop of culture medium under heavy white mineral oil (Sigma Chemical Co., St. Louis, MO) in a 35 × 10-mm petri dish and kept at room temperature until ready for use (1–3).

Sperm Preparation

Semen samples were obtained from three randomly selected male cynomolgus monkeys via electroejaculation with a rectal probe under ketamine anesthesia. For samples to be used fresh in the HZA, semen was collected into a 50-ml test tube containing PBS with 3.0 mg/ml of BSA. After mixing of the diluted sample, a 5-μl aliquot was placed in a Makler chamber (Zygoteck System, Springfield, MA) for the objective evaluation of motile characteristics by an automated, computerized sperm motion analyzer (Cellsoft Semen Analyzer, Labsoft Division of CryoResources, New York, NY) using a negative phase objective. The chamber was positioned on the stage of the microscope (Model MP-10DM; Opelco, Washington, DC); the sperm image was recorded with a videocamera and transmitted to a video monitor. Settings for the general parameters were as reported (5), except for minimum sampling velocity, which was set at 20 μm/sec, and maximum sampling velocity, which was increased to 300 μm/sec. All parameters remained constant between analyses to avoid bias. The evaluated sperm variables included percentage of motility, rectilinear velocity (μm/sec), and linearity of movement (straight:curvilinear ratio).

Following the reading, an aliquot of semen (0.3 ml) was diluted with 0.3 ml of Ham's F-10 plus 3.0 mg/ml of BSA and washed by centrifugation (8 min at 300*g*). The supernatant was discarded. Pelleted sperm was washed a second time (5 min at 300*g*). The second pellet was overlaid with 0.5 ml of Ham's F-10 plus 3% BSA and incubated at room temperature (21°C) to effect a swim-up separation. After 1 hr of incubation, the supernatant was recovered, diluted, and used for the HZA.

For samples to be cryopreserved, the ejaculate was collected into an extender solution containing the buffer medium Tes–Tris and egg yolk (see below). Evaluation of motion characteristics was performed as described, after which the samples were cryopreserved. After thawing (10 min at 37°C), a volume of Ham's F-10 plus 3% BSA equal to half the volume of the thawed semen was slowly added and kept at room temperature for 10 min. The mixture was centrifuged for 5 min at 290*g*. Approximately 0.3 ml of the supernatant was removed, mixed with an equal volume of Ham's F-10 plus 3% BSA, and recentrifuged at 290*g* for 10 min. The pellet was then overlaid with 0.25 ml of culture medium and incubated for swim-up preparation, as described for fresh samples.

For sperm cryopreservation, and extender medium was made of Tes–Tris (0.2 *M* Tes, 0.1 *M* Tris), citrate (7 m*M*), and 20% (v:v) egg yolk (12) and filtered through a 0.2-mm-pore size filter to prevent interference from egg yolk particles in the computerized readings and to provide a more uniform freezing of the semen–extender mixture. Semen was collected into this extender medium, followed by the addition of dibutyryl (db) cAMP (9) (5 m*M;* Sigma Chemical) and incubation for 30 min at room temperature; glycerol was added as cryoprotectant at a final concentration of 3.5%. Samples were aliquoted by 0.5-ml volumes into cryovials (Nunc, Baxter Scientific, Columbia, MD) and refrigerated from room temperature to 4°C for 1 hr, to allow for equilibration, in 2-ml cryotubes in a freezing tray (Handi Freeze, Taylor Wharton, Indianapolis, IN). The tray was then placed 5 cm above liquid nitrogen (LN_2) in a tank for 30 min, followed by plunging into LN_2 (−196°C) on a precooled aluminum cane. Samples were frozen for up to 3 months.

Hemizona Assay

Two 100-μl drops of the final dilution (500,000 motile sperm/ml for fresh samples and different concentrations for frozen–thawed samples, as described below) were placed in a 35 × 10-mm plastic petri dish. The drops were kept under heavy mineral oil (Sigma Chemical). Matching hemizonae were placed in parallel sperm drops of fresh and frozen–thawed samples and coincubated at 37°C (in 5° CO_2 in air) for 4 hr (10). All HZA experiments were performed under these temperature conditions. The zonae were then vigorously rinsed five times in a fresh medium drop (Ham's F-10 plus 3% BSA) using a narrow-bore glass pipette (1.5 × the diameter of the zona) to dislodge loosely attached sperm. The zonae were then transferred to another fresh medium drop and positioned with the outer surface upward. At 200× magnification, the number of tightly bound sperm on the outer (convex) zona surface was counted (1,10).

Blood Collections and Hormonal Assays

Daily serum samples (3 ml) were obtained from the female monkeys by femoral artery puncture from day 3 of the menstrual cycle until the day of

oocyte retrieval, for rapid E_2 radioimmunoassay determination (10).

Experimental Design

Oocytes used in the experiments were prophase I (PI; immature, germinal vesicle-bearing) or metaphase II (MII; extruded first polar body) at retrieval. Fresh ejaculates were tested in the HZA against frozen–thawed samples obtained from the same animal. Different oocyte classes were utilized in the three different experiments outlined below.

In experiment I, both fresh and frozen–thawed sperm were tested at the standard dilution of 500,000 motile sperm/ml (5×10^4 sperm exposed to each hemizona) and prepared at room temperature (21°C). A hemizona was placed with the fresh drop, while the matching hemizona was coincubated with the frozen–thawed sample. Sixteen PI and 14 MII oocytes were used in these experiments.

In experiment II, we evaluated the effect of increased sperm concentration in the frozen–thawed samples and sought to determine the sperm concentration required to obtain binding equivalency to that of fresh samples. Fresh sperm drops were evaluated using 5×10^4 motile sperm/hemizona. Frozen–thawed sperm drops were prepared so that 1.5×10^4, 5×10^4, 1.5×10^5, 5×10^5, 1×10^6, and 1.5×10^6 spermatozoa would be exposed to hemizonae. From 3 to 10 MII oocytes were used for each concentration of frozen–thawed sperm; sperm were prepared at 21°C.

In experiment III, kinetic studies of binding were performed for fresh and frozen–thawed samples to investigate the initial interactions during coincubation. In these studies, spermatozoa were evaluated at 5×10^4/hemizona, and fresh sperm were tested against a frozen–thawed sample. At each predetermined time of coincubation (15, 30, 45, 60, 180, and 240 min), the matching hemizonae were quickly removed from the incubating dish, rinsed, and placed in a warm drop of Ham's F-10 under oil. The sperm dish was immediately returned to the incubator. While the microscopic stage area was warmed (37°C) by a stream of air (Arenberg Safe Air Curtain Incubator, Model 279; Forma Scientific, Jamaica Plain, MA), the number of tightly bound sperm was counted. The hemizonae were then transferred to the original sperm dish for further coincubation, as needed. These kinetic studies of sperm–zona binding were performed with spermatozoa prepared as mentioned above (swim-up effected at room temperature); in addition, analogous experiments were carried out with sperm preparation at 37°C. Parallel fresh and frozen–thawed sperm drops were also kept at room temperature (21°C) and at 37°C for evaluation of motility.

Statistical Analysis

The number of tightly bound sperm in the HZA in fresh and in frozen–thawed semen for each sperm concentration were compared using a repeated-measures analysis of variance, followed by Duncan's mean comparison test. Comparisons between different maturational stages of oocytes with regard to their binding capacity to fresh and to cryopreserved semen were done using Student's t test. The correlation between the number of tightly bound sperm (Frozen–thawed semen) and the sperm concentration was carried out by regression analysis. Relationships between the number of tightly bound sperm and the time of gamete coincubation were measured with analysis of variance (two-way ANOVA) followed by Duncan's mean comparison test. Independent evaluation of these interactions (binding and time) for fresh and frozen–thawed samples was performed using Tukey's standardized range test (HSD). The effects of motility, velocity, and linearity on the relationship between tightly bound sperm and time were assessed by analysis of covariance.

All data were analyzed for normalcy of distribution. All results are expressed as the mean ± standard error of the mean (SE). P values of 0.05 or lower were considered significant.

RESULTS

Experiment I

At the initial standard dilution of 500,000 motile sperm/ml in the HZA (5×10^4 sperm/hemizona), tight binding was significantly higher for fresh than for frozen–thawed semen. This was true for PI oocytes ($n = 16$; 38.3 ± 3.3 vs 4.2 ± 2.3 in fresh and frozen–thawed semen, respectively; $P < 0.00001$), as well as for MII oocytes ($n = 14$; 113.7 ± 17.0 vs 21.0 ± 4.4; $P = 0.00002$). At this dilution, fresh sperm had significantly higher binding to MII than to PI oocytes ($P < 0.0001$). Similarly, frozen–

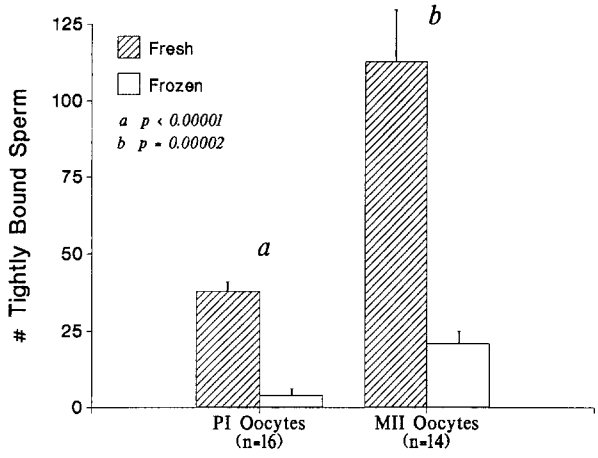

Fig. 1. Experiment I: Results of HZA comparing fresh vs frozen–thawed semen in immature and mature oocytes. Sperm prepared at 21°C (room temperature) and HZA performed at 37°C (4-hr coincubation time).

thawed semen had significantly higher binding to MII than to PI oocytes ($P = 0.01$) (Fig. 1).

Experiment II

Figure 2 shows the results of increasing sperm concentration in the frozen–thawed samples while maintaining fresh samples at 5×10^4 motile sperm/hemizona. In the range of 1.5×10^4 to 1×10^6 sperm exposed to the hemizonae, frozen spermatozoa had significantly lower binding than fresh sperm. Only at 1.5×10^6 spermatozoa/hemizona was binding not significantly different for frozen–thawed and fresh (5×10^4 motile sperm/ml) samples. Figure 3 depicts the linear relationship between tight binding and sperm concentration in the HZA after sperm concentration was increased at coincubation. The regression line for the number of tightly bound sperm obtained after increasing the sperm concentration was statistically significant at $P = 0.001$, with an intercept value of 6.49 and a slope of 0.04 ($r = 0.95$).

Experiment III

The kinetics of binding for fresh and frozen–thawed sperm are shown in Fig. 4 (5×10^4 motile sperm/hemizona for both samples). The upper panel presents the results of studies performed with sperm prepared at 37°C using MII oocytes ($n = 4$); the lower panel shows results of experiments carried out at room temperature (21°C) for sperm preparation using PI oocytes ($n = 3$). In both instances, as well as in all experiments in the present study as

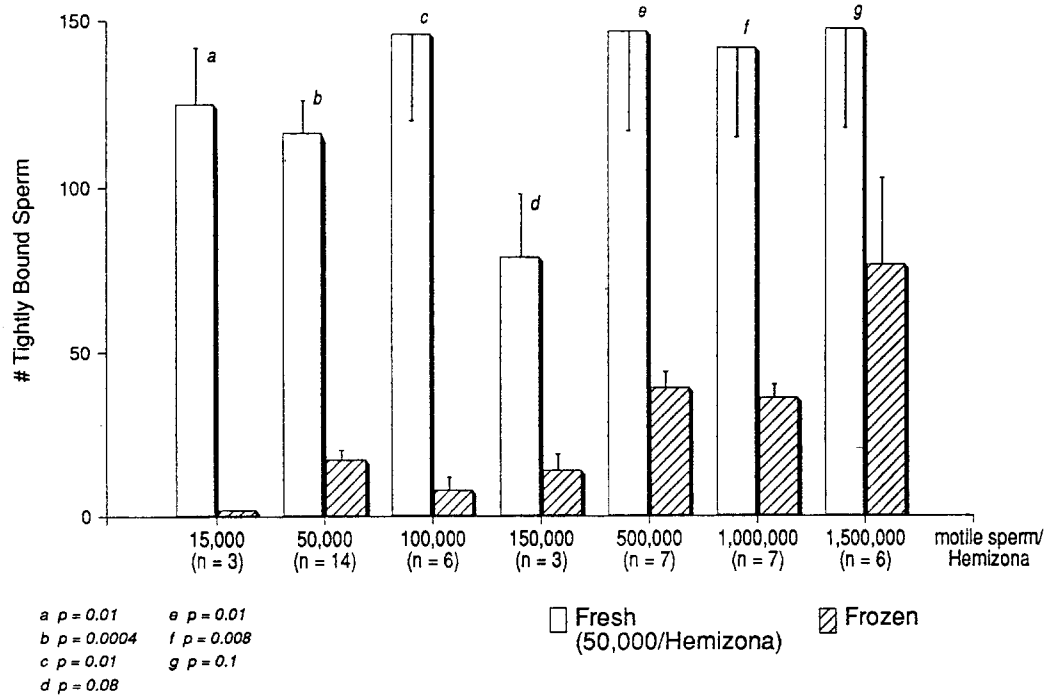

Fig. 2. Experiment II: HZA results with increasing sperm concentration in the frozen–thawed semen. Controls (fresh sperm) were kept at the standard concentration. Sperm were prepared at 21°C and HZA was performed under standard conditions (37°C).

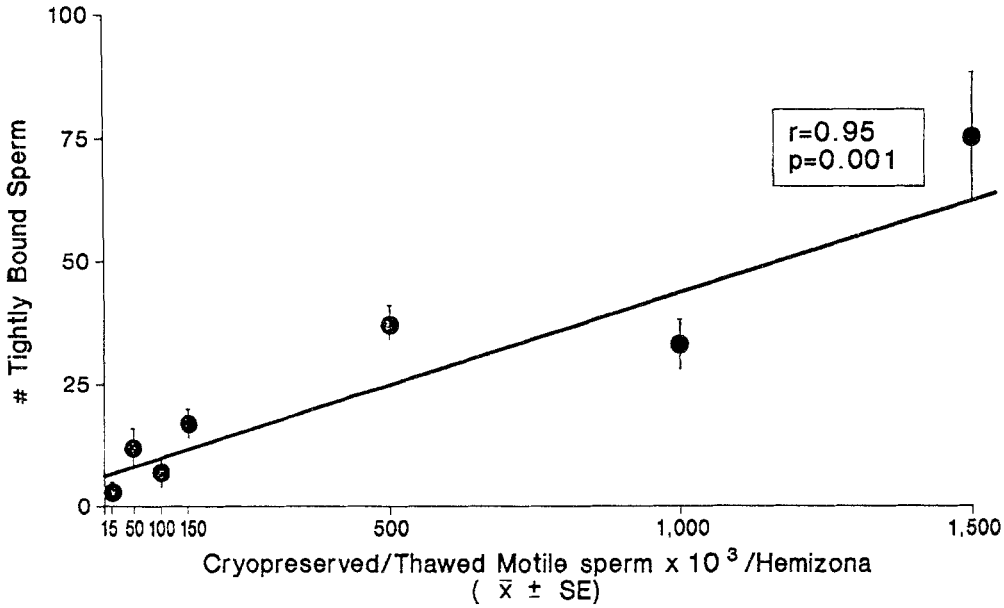

Fig. 3. Experiment II: number of tightly bound sperm after freezing–thawing and increasing sperm concentration.

described under Materials and Methods, HZA were performed under controlled-temperature conditions (37°C, 5% CO_2 in air). In both instances, tight binding was lower when frozen–thawed sperm was used. Maximum binding occurred at approximately 1 hr for both fresh and frozen–thawed sperm, with a plateau in binding during the last 3 hr of coincubation.

There was a significant relationship between the number of tightly bound sperm and the time of incubation (fresh and frozen–thawed samples) as evaluated by two-way ANOVA ($P = 0.01$). For 180 min, binding was significantly higher than at ≤45 min ($P < 0.05$), giving statistical evidence of maximum binding at 60 min, followed by a plateau. This was true for both fresh ($P < 0.05$) and frozen–thawed ($P < 0.05$) semen samples (Tukey's test).

The motile characteristics of fresh and frozen–thawed semen before and after swim-up are shown in Table 1 (mean values of seven ejaculates from three males prepared at room temperature). In fresh samples, post-swim-up motility was 58.3 ± 1.1%, velocity 91.3 ± 10.1 μm/sec, and linearity 7.1 ± 2.4. After 3 hr, there was a >50% reduction in motility, although velocity and linearity remained unchanged. For the frozen–thawed samples, post-swim-up motility was 34.0 ± 3.3%, velocity 60.5 ± 4.1 μm/sec, and linearity 4.7 ± 1.4, all lower than in fresh samples. Motility decreased at room temperature after 2 hr (23.0 ± 2.0%) and further at 3 hr (12.0 ± 5.1%). This decrease was higher at 37°C (<15% progressive motility after 2 hr of incubation).

When analysis of covariance was performed, motility and velocity accounted for the differences in binding along time for frozen–thawed samples. However, because only three measurements of motion parameters were made along time (after swim-up, 120 and 180 min), the significance of these results was not conclusive.

DISCUSSION

These results provide further evidence to support the use of the HZA as a homologous bioassay to evaluate sperm–zona interaction in this nonhuman primate model (10). Tight binding of sperm to the zona pellucida is an early, critical step leading to fertilization and embryo development. Human studies (5,6) have demonstrated that the HZA has a high predictive value for IVF outcome. Identification of the variables which influence HZA results (intraassay and interassay variation, lower limit of binding, etc.) has assisted us in optimizing this bioassay (13,14). Thus, the HZA is a practical technical advance for estimating sperm fertilizing capacity, with potential application to infertility studies and contraceptive technology (15).

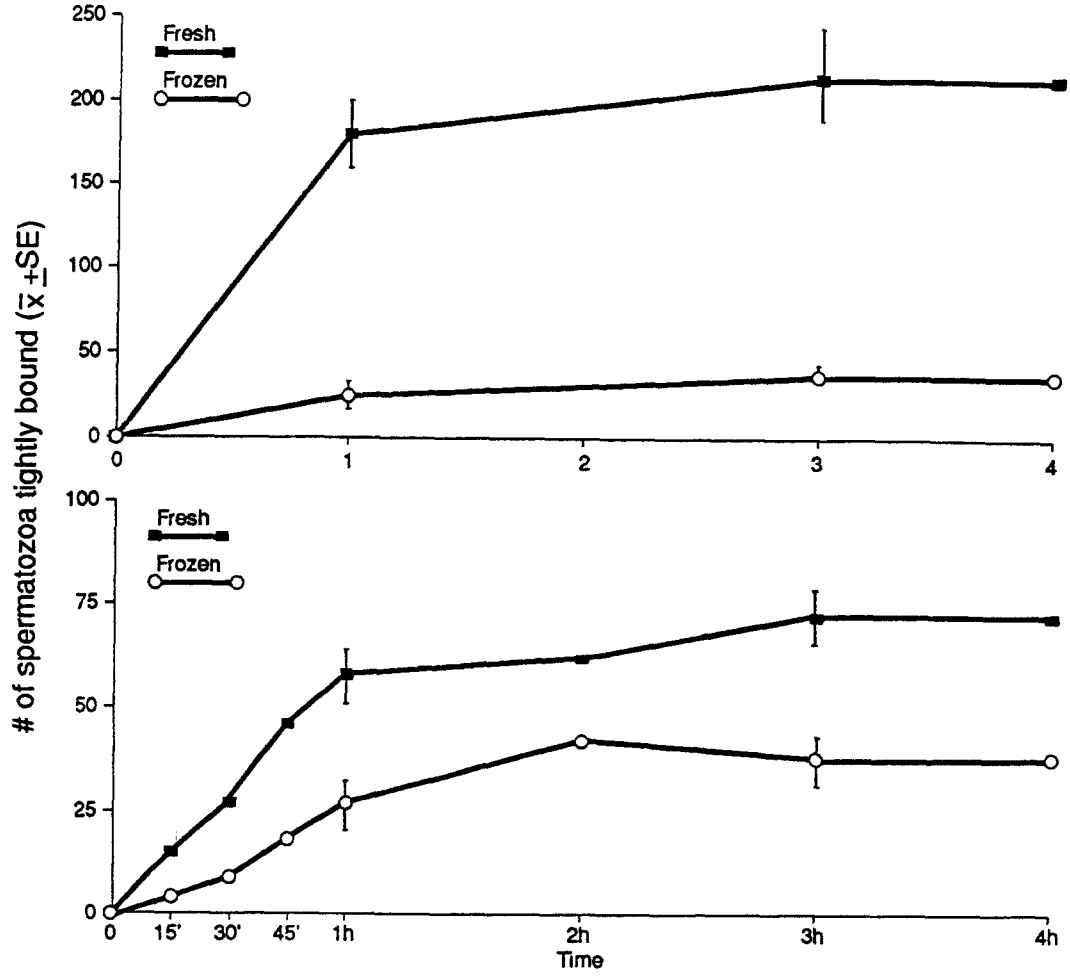

Fig. 4. Experiment III: kinetics of binding of fresh (■) and frozen–thawed (○) sperm. Upper panel: Results of sperm preparation at 37°C (MII oocytes). Lower panel: Results of sperm preparation at room temperature (PI oocytes) (5×10^4 spermatozoa were exposed to each hemizona in all experiments). All HZAs throughout the paper were performed under identical conditions (4-hr gamete coincubation at 37°C in 5% CO_2 in air).

Monkey Sperm Cryopreservation

Proven cryopreservation methods for primate sperm are scant. Roussel and Austin (16) reported successful freezing and storing of sperm in LN_2 in several monkey species, with egg yolk and glutamate as extender. Postthaw motility was 15 to 32%; the survival rate was 37 to 60%. Tollner *et al.* (17) evaluated different freezing techniques for cynomolgus monkey sperm. They concluded that 3% glycerol offers the best protection for this species and that the most effective diluent (extender) is egg yolk TEST (Tes + Tris), with 20% skin milk (compared to TEST alone and egg yolk–citrate).

Table I. Motile Characteristics of Fresh and Frozen–Thawed Samples Before and After Swim-Up (Room Temperature)

	Motility (%)	Velocity (μm/sec)	Linearity
Fresh			
Original sample	66.5 ± 2.1	54.3 ± 6.8	5.8 ± 0.5
Post-swim-up	58.3 ± 1.1	91.3 ± 10.1	7.1 ± 2.4
2 hr	31.1 ± 2.2	67.6 ± 10.1	7.0 ± 0.4
3 hr	25.0 ± 3.1	60.0 ± 8.1	8.0 ± 0.5
Frozen/thawed			
Postthaw	38.6 ± 14.1	59.0 ± 1.5	4.9 ± 0.9
Post-swim-up	34.0 ± 3.3	60.5 ± 4.1	4.7 ± 1.4
2 hr	23.0 ± 2.0	40.3 ± 3.9	3.8 ± 0.5
3 hr	12.0 ± 5.1	38.6 ± 10.1	3.7 ± 0.9

We obtained comparable results in the postthaw percentage of motility and velocity using an egg yolk–TEST–citrate buffer and 3.5% glycerol. The postthaw percentage of motility was 38.6 ± 14.1% and the velocity was 59.0 ± 1.5 μm/sec, in the same magnitude as the results of Tollner *et al.* (17) (62.0 ± 3.0% and 57.0 ± 3.0 μm/sec, respectively). After swim-up processing, we recovered a motile sperm fraction with a motility of 34.0 ± 3.3% and a veloc-

ity of 60.5 ± 4.1 μm/sec. As expected, these results were lower than those of fresh samples in which we recovered a postthaw motility of 58.3 ± 1.1% and a velocity of 91.3 ± 10.1 μm/sec.

Davis *et al.* (18) recently evaluated sperm motion in the cynomolgus monkey using a high-speed videomicrograph and a computer cell analyzer. Their results suggest that the accuracy of the system for measuring cell trajectories is higher when at least 100 frames/sec are assessed. The parameters most affected by the use of fewer frames per second were curvilinear velocity, linearity, and amplitude of lateral head displacement. However, rectilinear velocity was not significantly affected. Here, we used 30 frames/sec in the evaluation of rectilinear velocity and linearity. In future studies, we will address this issue in conjunction with the characterization of hyperactivated motility in this species.

Monkey Sperm–Oocyte Interactions

Tight binding under HZA conditions was significantly reduced for frozen–thawed sperm compared to fresh spermatozoa, irrespective of the maturational stage of the oocytes (PI or MII). The kinetics study showed peak binding at approximately 1 hr of coincubation for fresh and frozen–thawed sperm. Thereafter, a plateau in binding was observed. These results suggest that tight binding occurs more rapidly in this monkey species than in the human. In humans, tight binding in the HZA reaches a peak at about 4 to 5 hr of coincubation (1–3). However, in the mouse, maximum binding occurred within 10 to 20 min after gametes were combined in vitro, followed by the acrosome reaction (19).

We speculate that the difference between the cynomolgus monkey and the human in the kinetics of peak binding for fresh samples may be related to the high velocity of monkey sperm, which may increase the chances of collision and binding with zona receptors under HZA conditions. Whether temporal differences in capacitation in vitro or other differences in binding affinity and/or receptor density in sperm or zona also exist remains to be investigated. Cryopreserved monkey sperm also reached maximum binding at 1 hr, with a kinetic binding curve parallel to that of fresh spermatozoa. Whether the impairment in binding for frozen–thawed monkey sperm depends mainly on the loss of motility and velocity (which was more exaggerated after 3 hr of coincubation) or on other sperm defects subsequent to cryodamage remains to be established. The effects of freezing on sperm membranes, premature acrosome leakage, and loss of motility are well established (20,21). Tollner *et al.* (17) found better postthaw motility associated with a higher percentage of acrosome-intact cells in the cynomolgus monkey. In the human, it has been reported that decreased binding in the HZA occurs in some frozen–thawed semen samples, but binding kinetics are similar between fresh and cryopreserved sperm (15).

Loss of motility has been observed in freshly ejaculated monkey sperm (22). As Lopata *et al.* reported (22), we also found that slow dilution of the semen upon collection is an effective method for initial sperm preparation. However, a severe reduction in motility and velocity occurs after overnight incubation (less at room temperature than at 37°C; data not shown). For frozen–thawed samples, the loss of motility and velocity is more dramatic, with extremely poor progressive motion after 4 hr.

Influence of Oocyte Maturational Stage of Sperm–Zona Binding

The present results of experiment I also sustain our previous observation in this primate model that immature (PI) oocytes show less sperm binding ability than mature (MII) oocytes obtained after ovarian stimulation (10). Thus, final oocyte nuclear maturation may be associated with an increased zona pellucida binding capacity. Recent studies from our laboratory have demonstrated that full meiotic competence of human oocytes as evaluated in the metaphase II stage is also accompanied by a higher sperm zona pellucida binding potential compared to immature oocytes (23).

Validation of Monkey HZA

The hemizona assay evaluates tight, specific binding of sperm to the zona pellucida, as opposed to the initial reversible attachment or adhesion that initially occurs during early gamete interaction (15,24–26). Here, we have extended our initial observations that demonstrated the feasibility of the HZA as a test to evaluate monkey sperm–zona interactions at different stages of oocyte maturation (10).

In light of the present study, the monkey HZA

could be standardized to 2 hr of gamete coincubation after sperm preparation at room temperature. The standard dilution of 500,000 motile sperm/ml (or 5×10^4/hemizona) seems to be adequate in this model as it is in human studies. However, a higher concentration should be used if frozen–thawed sperm are to be evaluated, possibly exposing 1.5×10^6 sperm to each hemizona.

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