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Frozen-thawed rhesus sperm retain normal morphology and highly progressive motility but exhibit sharply reduced efficiency in penetrating cervical mucus and hyaluronic acid gel

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Abstract

The preservation of the genetic diversity of captive populations of rhesus monkeys is critical to the future of biomedical research. Cryopreservation of rhesus macaque sperm is relatively simple to perform, yields high post-thaw motility, and theoretically, provides via artificial insemination (AI) a way to easily transfer genetics among colonies of animals. In the interest of optimizing semen cryopreservation methods for use with vaginal AI, we evaluated the ability of frozen-thawed rhesus sperm to penetrate periovulatory cervical mucus (CM). Motile sperm concentration of pre-freeze (“fresh”) and post-thawed (“thawed”) samples from 5 different males were normalized for both computer assisted sperm motion analysis and CM penetration experiments. Sperm samples were deposited into slide chambers containing CM or gel composed of hyaluronic acid (HA) as a surrogate for CM and numbers of sperm were recorded as they entered a video field a preset distance from the sperm suspension-CM (or HA) interface. Fresh and thawed sperm were dried on glass slides, “Pap”-stained, and assessed for changes in head dimensions and head and flagellar shape. While retaining better than 80% of fresh sperm progressive motility, thawed sperm from the same ejaculate retained on average only 18.6% of the CM penetration ability. Experiments using HA gel yielded similar results only with reduced experimental error and thus improved detection of treatment differences. Neither the percentage of abnormal forms nor head dimensions differed between fresh and thawed sperm. While findings suggests that sperm-CM interaction is a prominent factor in previous failures of vaginal AI with cryopreserved macaque sperm, neither sperm motility nor morphology appears to account for changes in the ability of cryopreserved sperm to penetrate CM. Our data points to a previously unidentified manifestation of cryodamage which may have implications for assessment of sperm function beyond the cervix and across mammalian species.

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Keywords

spermatozoa; cervical mucus; hyaluronic acid; artificial insemination; monkey; cryopreservation

Introduction

The rhesus monkey is a critical model for translational biomedical research. As the National Primate Research Centers (NPRCs) and other rhesus facilities expand their monkey colonies, it becomes increasingly important to both maintain genetic diversity and preserve unique rhesus lines. It follows that sharing the genetics among U.S. primate colonies is an important goal, as many genetically valuable animals are being identified and promoting their offspring could help develop models for human disease. While there are many ways to define, characterize and store genetic material from a colony of rhesus monkeys (i.e., blood samples, oral swabs and skin and/or organ biopsies), germplasm banking is now recognized as a primary strategy for maintaining the genetic diversity of captive animal populations that are used for biomedical research [1]. Cryopreservation of semen in particular is the most reliable and cost-effective way to store the genetics of a colony that will also allow those genetics to be recovered as live, breeding animals. Furthermore, sperm banking naturally lends itself to the easy transfer of genetics as the transport of only a few straws or vials of frozen semen among rhesus colonies is sufficient.

Vaginal artificial insemination is the most cost-effective approach for maintaining colony diversity with frozen-thawed semen samples. Due to the tortuous nature of the macaque cervix, intrauterine insemination (IUI) via transcervical passage of an insemination catheter would be extremely difficult, and in some females potentially impossible [2]. Moreover, transfundal IUI requires both costly equipment and considerable training [3] while embryo transfer following in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) with frozen-thawed sperm has similar limitations. By contrast, vaginal insemination is a technically straightforward and cost effective approach. Unfortunately, there is not a single report of conception in macaques with frozen-thawed sperm following vaginal AI. The failure of standard vaginal AI with current sperm cryopreservation methods may well indicate that thawed sperm are incapable of swimming up through the cervical canal to meet the oocyte. This failure may be due to barriers created by the interaction of sperm with cervical mucus and/or the length and complexity of the cervical canal that sperm must traverse.

The cervix is the most significant barrier to sperm transit in the female tract in primates. Even under normal circumstances (those leading to conception), the vast majority of sperm never advance beyond the ectocervix [4]. The small percentages of sperm that enter the cervix are likely drawn there, at least initially, by uterine contractions [5]. Subsequent movement of sperm in the cervix and progression into the upper reproductive tract requires that sperm propel themselves via vigorous flagellar movement in order to overcome resistance applied by CM [6].

It is therefore not surprising that the character and vigor of sperm motility has been shown to be a critical factor in sperm-mucus interaction. The numbers of sperm entering mucus in vitro depends on the number of sperm in semen exhibiting progressive motility [7,8]. Men whose sperm exhibit greater mean swimming velocity and amplitude of lateral head movements have greater numbers of sperm that penetrate CM [7]. Human sperm with below average velocity have difficulty penetrating cervical mucus in vitro [7,8] and are underrepresented in cervical mucus recovered post-coitus [9].

Sperm morphology, in particular, sperm head shape, also plays a role in CM penetration. Patients with semen samples that contain an elevated percentage of sperm with amorphous heads had lower numbers of sperm in post-coital CM [9] and fewer sperm that could penetrate and migrate in mucus *in vitro* [9,10] than men whose semen samples fell within the normal range of amorphous forms.

In the human, numerous studies demonstrate that cryopreserved sperm have difficulty traversing CM. *In vitro* penetration assays using human CM, bovine CM, or CM surrogate compounds show that significantly fewer frozen-thawed sperm are able to advance well beyond the CM or gel interface compared to fresh sperm from the same males [11,12,13]. Similarly *in vivo*; pregnancy rates following intracervical insemination (ICI) with frozen-thawed semen are on average only ~1/3 the rates achieved with fresh semen from the same donors [14]. Much of the inferior performance of cryopreserved human sperm has been attributed to the decrease in progressive motility following freezing and thawing [15,16], yet there are no reports in human or other primates that describe kinematics of post-thaw sperm in relationship to sperm performance in CM.

To the best of our knowledge, our study is the first in a primate species to relate objective measures of progressive motility of fresh and frozen-thawed sperm to their penetration efficiencies in CM. Ultimately, our goal is to establish a vaginal AI technique that will be effective with frozen-thawed macaque sperm, i.e. will consistently result in pregnancies. Clearly, there are a number of factors to consider and potential hurdles to overcome to achieve this end. One key factor is post-thaw sperm quality. We have recently developed methods that results in consistently high post-thaw sperm survival [17,18]. The purpose of this study was to evaluate the ability of frozen-thawed sperm cryopreserved with new methods to penetrate periovulatory CM as well as to evaluate the suitability of gels composed of hyaluronic acid as a homogeneous surrogate for CM. As part of this study, we quantitated sperm motion characteristics and assessed sperm shape in order to determine if changes in these key factors occur with cryopreservation and could account for a potential loss of performance of frozen-thawed sperm in CM.

Materials and Methods

Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise.

Animals

5 male and 3 female rhesus macaques were housed at the California National Primate Research Center (CNPRC) in compliance with American Association of Accreditation of Laboratory Animal Care Standards. The Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, approved all methods and procedures with animal subjects.

Semen Collection and Processing

Male rhesus monkeys were trained to chair restraint and semen was collected by direct penile stimulation with a Grass 6 stimulator equipped with EKG pad electrodes (30–50 V, 20 ms duration, 18 pulses s⁻¹) [19]. Samples were allowed to liquefy for 30 min before processing. Sperm suspensions were washed twice with TL-BSA at 300 g for 10 min, and resuspended to 1×10^8 cells/mL of total motile sperm (sperm density $\times$ initial motility) with TEST (43.25 g TES, 10.265 g Tris, 10g glucose in 1L distilled water, pH 7.4) yolk (20%)

solution before adding equal volume of 6% glycerol in TEST-yolk, yielding a final concentration of 3% glycerol at a sperm density of 5×10^7 cells mL⁻¹.

Freezing and Thawing Procedure

Aliquants (100 μ L) of sperm suspensions were drawn into 0.25-mL French straws (IMV International, Minneapolis) manually with a 1cc syringe and were heat-sealed (MP-4 Impulse Sealer, Midwest Pacific). Straws were placed into a 600-mL glass beaker containing 500 mL of room temperature distilled water, and equilibrated at 4 °C in a refrigerator for 2 h before initiation of the freezing process. Sperm freezing followed the methods described previously [17]. In brief, a Styrofoam box was filled to a depth of 4 cm with liquid nitrogen and a 1-cm thick Styrofoam 'boat' was floated on the surface for 10 min for temperature equilibration. Straws were placed on top of the 'boat' for 10 min before being plunged into liquid nitrogen. For post-thaw motility estimation, straws were thawed in a 37°C water bath for 30 s.

Motility Estimation and CASA

A 10- μ L drop of pre-freeze or post-thaw semen, covered with a 22 mm square coverglass, was visualized with a 20 $\times$ positive-phase objective and a condenser setting of 100 (pseudo-dark field) on an Olympus BH-series phase-contrast microscope (Scientific Instrument Co, Sunnyvale, CA) with a heated stage at 37 °C. Post-thaw motility was estimated immediately after thawing. Samples were presented in random order each time so that the operator did not know their identity.

For measurements of sperm motion characteristics with CASA, concentration of fresh and frozen-thawed samples was adjusted to 10×10^6 motile sperm/ml. Videomicrography was performed as described previously [20,21]. Briefly, 4 μ L drops of sperm suspension were loaded into two μ -cell semen analysis chambers (Fertility Technologies Inc., Natick, MA) with a 10 micron depth. In each chamber, 8–10 randomly selected microscope fields were video recorded, capturing several hundred sperm. Motion characteristics of the recorded sperm were analyzed using the HTM Ceros, version 10.9d (Hamilton Thorne Biosciences, Inc, Beverly, Mass.). Sperm tracks were digitally captured using a frame rate of 60hz and a minimum track time of 1 sec. At least 200 sperm per treatment were analyzed for curvilinear velocity (VCL), straightline velocity (VSL), and amplitude of lateral head displacement (ALH).

CM and HA gel penetration experiments

CM was collected from 2 periovulatory female rhesus macaques into polyethylene catheters as described previously [21]. Briefly, females were anesthetized with ketamine hydrochloride. A pediatric proctoscope was inserted into the vagina and a polyethylene catheter was guided to the cervical region. A stainless steel stylet fed into the catheter facilitated the insertion of the last 1 cm section of the catheter into the cervical os. Once the catheter was in position, the stylet was removed and gentle suction applied, recovering several cm of CM.

A 20 micron-deep slide chamber containing CM was prepared for CM penetration experiments as described previously [21]. The slide was warmed for 5 min on a microscope stage warmer (Motion Analysis Inc., Santa Clara, CA) set at 37°C prior to the addition of sperm. Concentration of fresh and frozen-thawed samples was adjusted to 25×10^6 motile sperm/ml. Twenty μ L of sperm samples were introduced to the open side of the CM chamber and were immediately drawn by capillary action to the CM interface. Sperm were observed in CM with an Olympus BH2 microscope and a 10X phase objective and videorecorded as described previously [21]. After 2 min from the time sperm were

introduced to the chamber, video recordings were initiated, capturing a region in the center of the microscope field that was approximately 2.75mm from the sperm-CM interface. Recordings continued for a minimum of 4 min. CM penetration was quantified from video recordings by counting the number of sperm in the video field that was paused at the very beginning of the recording ($t = 2$) and every min thereafter ($t = 3-6$) of the 4 min recording interval.

Penetration experiments were also prepared with HA gel loaded into slide chambers in place of CM. HA gel was prepared from 8mg of purified HA polymers of 1800 kDa molecular weight (Lifecore Biomedical, LLC, Chaska, MN) added to 1 ml of HEPES-buffered TL medium containing .3% BSA. TL medium was modified with reduced glucose (.15mM) and the addition of fructose (.04 mM) based on levels of these substrates described in human peri-ovulatory CM [22].

Sperm morphology and morphometry

Semen, fresh, and frozen-thawed samples from each male were smeared onto glass slides, dried, and stained using the method of Papanicolaou as described previously [23]. Two hundred sperm per male per treatment were scored for various categories of abnormal forms (head, midpiece, principal piece, midpiece and principal piece) according to the WHO '87 method used for assessing human sperm morphology (WHO laboratory manual: The examination of human semen and semen-cervical mucus interaction. 2nd edition). The length and width of sperm heads was determined using a videomicrographic system as described by Katz et al. [23] except with the following differences. While referencing a micrometer imaged on the video terminal, calipers were used to produce a measuring grid on a transparent film. The grid overlay was then applied to images of sperm on the video terminal enabling measurements of head width and length simultaneously. The heads of 50 sperm from semen smears of each of 5 males were analyzed with the grid overlay. From these "baseline" measurements, averages and standard deviations for head width and length were determined across all males and used to generate another overlay with a single standard deviation "bracket" or box for estimating length and width. The "bracket" overlay was applied to images of 100 sperm from smears of semen, fresh, and frozen-thawed samples from each male. Sperm were scored according to 5 head size categories: normal (within the length/width brackets), larger in length and/or width, smaller in length and/or width, larger in length but smaller in width ("thin"), smaller in length but larger in width ("wide").

Statistical Analysis

All data was analyzed with a 1-factor (sperm treatment) ANOVA. Response means of treatments were compared using Tukey's range test. CM and HA penetration experiments (replicates) generated values of numbers of sperm/ video field (at 1 min interval from $t = 0-4$) for fresh and frozen-thawed treatments. Data reported as mean number of sperm/ field $\pm$ standard error of the mean (SEM). All ANOVAs met assumptions of normality of data distribution. Fresh sperm exhibited greater variation in CM than did frozen-thawed sperm, these responses did not meet conditions of homogeneity of variance as determined by Levene's test. Data in experiments with CM were therefore transformed using square root function before ANOVA. Differences in treatments were considered significant at values of $p \leq 0.05$. Analyses were conducted with SAS statistical program (SAS Institute, Cary, NC), according to the principles described by Steel, Torrie, and Dickey [24].

Results

Sperm Motility

Sperm samples from 5 different males were cryopreserved for penetration experiments. One ejaculate from each of 4 males was processed for experiments with CM, a separate ejaculate from each of 4 males was processed for experiments with HA. Three of the 4 males were the same for both experiments. For CM experiments, fresh and frozen-thawed percent motility was 94.3 ± 1 and 49.0 ± 8 , respectively. For HA experiments, fresh and frozen-thawed percent motility was 87.5 ± 9 and 52.5 ± 14 , respectively. In CM penetration experiments, VCL of fresh and frozen-thawed sperm was 144.2 ± 10 and 120.8 ± 7 microns/sec. This change in VCL was shown to be significantly different ($p \leq 0.05$ Figure 1C). For HA experiments, VCL of fresh and frozen-thawed sperm was 137.8 ± 19 and 111.2 ± 13 microns/sec, respectively, a change that was not significant (Figure 2C). VSL and ALH did not differ significantly between fresh and frozen-thawed sperm from either experiment (data not shown).

CM and HA gel penetration

Frozen-thawed macaque sperm exhibited significantly reduced ability to enter and to swim in CM compared to fresh sperm (0.5 ± 0.2 vs 2.7 ± 0.8 sperm/min; $p \leq 0.05$; Figure 1B). Frozen-thawed sperm retained on average only 18.6% of the CM penetration ability of fresh sperm (Figure 1A and B) while retaining better than 83% of progressive motility (VCL; Figure 1C).

Similar results were observed for sperm penetration of HA gel. Frozen-thawed sperm had sharply reduced ability to traverse HA gel compared to fresh sperm (0.4 ± 0.2 vs 2.3 ± 0.5 sperm/min; $p < 0.01$; Figure 2B). Frozen-thawed sperm retained on average only 16.2 % of the HA gel penetration ability of fresh sperm (Figure 2A and B) while retaining over 80% of progressive motility (VCL; Figure 2C). Rates of sperm penetration into HA were very similar to rates observed with both fresh and frozen-thawed sperm in CM but with much smaller variation in response, especially across fresh sperm samples, leading to a greater power of detection of treatment differences.

Morphology/ Morphometry

The head dimensions of a total of 250 sperm (50 sperm from each of 5 different males) were measured with a grid overlay. Average sperm head width and length was 3.11 ± 0.14 and 5.18 ± 0.21 microns, respectively. Sperm heads that lay within a single standard deviation of width (2.97–3.25 microns) and a length (4.98–5.39 microns) were classified as normal. No differences were detected in the percentages of sperm with normal, smaller than normal, or larger than normal sperm heads in samples of semen, extended fresh and frozen-thawed sperm (Table 1). Fewer than 0.5% of sperm across all males and treatments had “thin” heads and no sperm had “wide” heads, therefore these categories were not included in the table.

Total normal forms (percentage of sperm with a normal shaped head and flagellum) of frozen-thawed samples compared to fresh sperm samples were not significantly different (Table 2). No differences were detected in the percentage of sperm with abnormal head, midpiece, and midpiece plus principle piece shape among sperm from semen, fresh, and frozen-thawed samples (Table 2; Figure 3). The percentage of sperm exhibiting abnormal principle piece shape, however, doubled following cryopreservation (Table 2) and corresponded with an increase in the number of sperm exhibiting a “looped” or “coiled” flagellum (Figure 3D). The increase in abnormal principle piece shape was determined by ANOVA not to be different but paired T-test analysis of fresh and thawed treatment groups indicated that increase was potentially significant ($p < 0.047$). This change in the shape of the

sperm flagellum accounted for approximately 89% of the reduction in total normal forms observed with cryopreservation.

Discussion

This study is the first to report that cryopreservation reduces the ability of sperm to penetrate cervical mucus, an essential part of sperm transport in the female reproductive tract. Furthermore, CM penetration is affected despite what is considered to be excellent post-thaw sperm motility. Sperm morphology changes associated with cryopreservation are also identified. These parameters may be important, new indications of cryodamage that have implications for the potential of cryopreserved semen from individual males to be used for artificial insemination as well as provide a more discriminatory endpoint to evaluate the success of new freezing protocols.

Our studies with rhesus sperm demonstrate that cryopreservation results in reduced sperm-CM penetration. Equal numbers of progressively motile sperm from fresh and frozen-thawed samples were loaded into CM penetration chambers. The large variation in response of fresh sperm in CM (indicated by large error bars in Figure 1A,B) was likely due in large part to differences in properties of CM collected from three different females. Previous studies of macaque sperm penetration of CM show similarly large variations in response when more than one source of CM was used for an experiment [21]. Unfortunately, the mesh structure of the hydrated mucin matrix can be damaged easily, altering CM viscoelasticity [25,26] and therefore precluding the combining and mixing of mucus samples. The lack of homogeneity of CM samples prompted the use of surrogate gels composed of HA for sperm penetration experiments.

Medium composed of hyaluronic acid has been used previously to simulate CM for *in vitro* tests of sperm function. While CM has complex biophysical properties that are derived from at least 5 distinct mucin molecules produced at the cervix [27,28], solutions prepared from HA share some of the properties of mucus, especially with respect to viscosity and charge [29]. HA gels highly resemble CM in their penetrability by human sperm [30,31,32]. We demonstrate that penetration experiments using an HA gel yield highly similar results to experiments performed with CM. Furthermore, as a defined medium, the use of HA gel results in reduced experimental error and thus improved detection of treatment differences (Figure 2A and B). Due to the varying consistency, seasonality, and cost of routine collection of rhesus macaque periovulatory CM, we propose the use of HA gel as a mucus surrogate for extending studies beyond initial observations and verifications.

While neither sperm head dimension nor overall sperm morphology changed significantly with cryopreservation, an increase in the percentage of sperm with a coiled flagellum following freezing and thawing was consistent and has recently been described in human sperm in response to cryopreservation. As with rhesus sperm, there was no significant change detected at the light level in the morphologies of the human sperm head, midpiece, or acrosome following cryopreservation [33]. By contrast, abnormal morphologies of the principal piece increased significantly with 90% of the flagellar defects characterized as looped or coiled tails [33]. This particular flagellar defect probably results from extreme osmotic stress. Rapid changes in osmolarity often occur during freezing–thawing which cause deformations of membranous structures associated with the flagellum [34]. The average increase in total flagellar defects of cryopreserved rhesus sperm in our study was 15.2%. It is tempting to speculate that the ~17% decrease in VCL of sperm following cryopreservation (Figure 1C) largely reflects the reduced function of these amorphous sperm. In future studies we will attempt to evaluate the character of motility of sperm with coiled tails.

To the best of our knowledge, we are the first to report the frequency of sperm morphological abnormalities in the rhesus monkey as well as the first to describe changes in sperm morphology with cryopreservation in a non-human primate species. A careful characterization of ejaculated cynomolgus monkey sperm morphology has been conducted previously and has been cited frequently for reference values for macaque sperm dimensions and abnormal forms [35,36]. In our and the Gago et al [35,36] studies, sperm were fixed similarly and stained with haematoxylin which provides optimal contrast and well-defined sperm head boundaries. Our findings with respect to morphology of fresh ejaculated rhesus sperm are very similar to the cynomolgus sperm reference values. As with sperm from the cynomolgus monkey [36], rhesus sperm vary only slightly in head shape with fewer than 1% of sperm exhibiting head abnormalities (characterized as enlarge, tapered, or otherwise irregularly shaped; Figure 3B). Rhesus sperm also exhibited similar defects in the flagellum (bent midpiece, coiled principal piece, coiled principle and midpiece; Figure 3C, D, and E) with 18.4% of sperm on average possessing a flagellar defect compared to 17.1% of cynomolgus sperm possessing similar abnormal forms [36]. By comparison with the cynomolgus monkey, average sperm head width and length were determined to be smaller in the rhesus by approximately 11% and 22%, respectively. While this size difference may potentially be due, at least in part to actual species differences, prior studies with human sperm suggest that the differences in both staining and measurement methods could contribute greatly to the discrepancy in sperm head size observed between these macaque studies [37,38].

In the human, decreased progressive motility of frozen-thawed sperm is one of the more obvious indications of cryodamage that results in reduced sperm fertilizing potential [15,16]. A strong positive correlation between post-thaw progressive motility and pregnancy outcome following intracervical insemination (ICI) has been demonstrated [39,40,41]. While IUI greatly improves pregnancy outcomes with cryopreserved sperm [42,43], conception rates still lag behind those achieved with fresh sperm IUI [14], presumably because sperm must still migrate through upper reproductive tract mucosal fluids and penetrate oocyte vestments. As with ICI, post-thaw progressive motility can be predictive of success with IUI, although the strength of correlation appears to be less [41,44,45,46].

On the other hand, measures of progressive motility of cryopreserved human sperm clearly don't tell the whole story. Treatments of post-thaw sperm to increase or enrich the numbers of sperm with higher VCL and LDH velocity did not improve penetration of CM [47] or pregnancy rates following IUI [48]. Furthermore, per cycle pregnancy rates with donor (cryopreserved sperm) IUI [49] have not improved appreciably since early reporting of successes [50], despite refinements in donor selection, cryopreservation technique, and post-thaw processing that have resulted in improved post-thaw sperm quality [51,52,53]. The implication here is that current assessments of sperm motility, as well as other measures of sperm function [54], don't reveal functional deficits of frozen-thawed sperm that potentially persist with current preservation methods.

As in the macaque, evaluating function of cryopreserved human sperm in high viscosity solutions may reveal aspects of cryodamage that have gone undetected previously and that may be potentially relevant to human sperm function *in vivo*. Cryopreservation results in changes in sperm membrane structure detected only by EM [33,55] as well as changes in cellular energy utilization [16]. It would not be surprising that alterations in surface properties and/or metabolism could affect efficiency of movement of cryopreserved sperm in the mucin-rich utero-tubal junction and oviductal isthmus, the highly visco-elastic matrix of the cumulus oophorus, or the semi-rigid zona pellucida. In support of this supposition, a number of studies have demonstrated that cervical mucus penetration results are positively correlated with IVF rates [56]. Reduced sperm motility in viscous medium may be

indicative of more subtle forms of cryodamage that could affect sperm performance in the upper female tract.

In order for vaginal AI with cryopreserved macaque sperm to be a viable strategy for colony management, we must first understand why cryopreservation results in reduced ability of sperm to traverse the cervix. In studies with fresh human sperm, efficient movement of sperm in CM is determined by two major factors: sperm head shape and the vigor of sperm motility. In the macaque, changes in morphology and progressive motility with cryopreservation are modest and appear to be poor predictors of sperm performance in CM. We have demonstrated previously in the macaque that negatively charged oligosaccharides associated the sperm surface coating protein DEFB126 is critical for enabling sperm to penetrate CM [21]. Preliminary studies in our lab with a limited number of males indicate that levels of DEFB126 on rhesus sperm do not change appreciably with cryopreservation (data not shown). Yet, additional studies will be required to assess if oligosaccharides are altered (which could result in a loss of surface charge) and to determine if there are male-to-male differences in loss or alteration of the sperm surface coat following cryopreservation. While progressive motility appears to be reasonably well-retained in extender with our current cryopreservation protocol, we speculate that damage to energy and force generating machinery of the flagellum could be the cause for the reduction in the ability of sperm to sustain an optimal power stroke under conditions of increased resistance as in CM. The effect of cryopreservation on sperm metabolic processes and axoneme structure is currently under investigation in our laboratory.

Conclusions

Post-thaw macaque sperm, while exhibiting only subtle differences in progressive motility and morphology with cryopreservation, display a marked decrease in the ability to penetrate CM and HA gel. CM and HA penetration assays appear to reveal a previously undescribed form of sperm cryodamage and are potentially valuable tests for evaluating the suitability of new freezing protocols for specimens to be used for AI.

Acknowledgments

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Figure 1. Sperm penetration of cervical mucus

Sperm from each ejaculate was washed free of seminal plasma, suspended into freezing diluent containing TEST-20% egg yolk-3% glycerol. Prior to freezing, extended sperm (“fresh”) were used for experiments. Sperm were then cooled, frozen and thawed. “Fresh” and frozen-thawed (“thawed”) sperm concentration was adjusted to 10×10^6 motile sperm/ml for computer assisted sperm motion analysis (CASA) and 25×10^6 motile sperm/ml for CM penetration experiments. Sperm samples were deposited into slide chambers containing peri-ovulatory CM, and after 2 minutes, sperm were recorded continuously for 4 minutes as they entered a video field 2.75mm from the sperm suspension-CM interface. Numbers of sperm in the video field were counted at one-minute intervals and reported as mean $\pm$ sem sperm/field (A). Rates of sperm penetration of CM were calculated for each male/treatment and reported as mean $\pm$ sem sperm/min (B). A minimum of 200 sperm/male/treatment were analyzed for curvilinear velocity (VCL) by CASA. VCL is reported as mean $\pm$ sem microns/sec (C). Experiments were conducted with sperm from 4 different macaques. Asterisks indicate significant differences ($p < 0.05$) in mean sperm numbers between treatments as determined by 1-way ANOVA.

Figure 2. Sperm penetration of hyaluronic acid gel

Experiments were performed and reported as described in fig. 2 with exception that HA gel replaced cervical mucus in penetration experiments. HA gel was prepared from 8mg of purified HA polymers (MW:1800 kDa) added to 1 ml of HEPES-buffered TL medium containing .3% BSA. TL medium was modified with reduced glucose, fructose, and lactate based on levels of these substrates described in periovulatory CM (Van Der Linden et al., 1992). Experiments were conducted with sperm from 4 different males. Asterisks and crosses (+) indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively in mean sperm numbers between treatments as determined by 1-way ANOVA.

Figure 3. Normal and abnormal sperm forms

Rhesus monkey sperm were dried on glass slides and stained using the method of Papanicolaou. Micrograph show a representative sperm cell with normal shape (Normal), and sperm that vary in shape from normal (Abnormal Forms) with respect to the head (HD), midpiece of flagellum (MP), principle piece of flagellum (PP), and the principle and midpiece combined (MP & PP). Scale bars equal 10 microns and 5 microns in micrographs of whole sperm (Normal) and sperm heads (HD), respectively.

Table 1

Categorization of sperm head dimensions based on “normal size” bracket overlay (N=5)

	% Normal Size	% Abnormal Size	
		Small	Large
Semen	81.8 ± 3.4	8.8 ± 2.1	9.4 ± 5.3
Fresh	87.4 ± 3	9.8 ± 2.2	2.6 ± 1.6
Thawed	85.4 ± 3.3	11 ± 4.8	3.6 ± 2.6

Table 2

Sperm morphology before and following cryopreservation (N=5)

	% Normal Forms	Head	% Abnormal Forms		MP	PP	MP & PP
Semen	80.7 ± 2.8	0.9 ± 0.4	5.7 ± 1.7	12 ± 2.9	0.7 ± 0.3		
Fresh	83.8 ± 5.2	0.9 ± 0.5	4 ± 3.9	10.8 ± 5.3	0.4 ± 0.2		
Thawed	68.1 ± 8	0.9 ± 0.7	4.9 ± 2.6	24.7 ± 7.6	1.4 ± 0.8		

MP = Midpiece PP = Principal Piece