Low temperature storage of rhesus monkey spermatozoa and fertility evaluation by intracytoplasmic injection

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Abstract

The objective was to develop a sperm freezing procedure suitable for use in the propagation of valuable founder animals by assisted reproductive technologies. Here, we report a comparison of processing methods by measuring the motility of fresh and frozen–thawed rhesus monkey spermatozoa and fertility via intracytoplasmic spermatozoa injection (ICSI) of sibling oocytes. Washed spermatozoa were frozen in straws or in pellets using different cryoprotective media and processed post-thaw with or without a density gradient centrifugation step. Among the four study series, motility post-thaw was improved with density gradient centrifugation (17–24% versus 75%, \( P < 0.01 \)) achieving levels similar to fresh spermatozoa. Spermatozoa injected oocytes (total \( n = 377 \)) were co-cultured on BRL cells and observed for fertilization and development. With spermatozoa frozen in straws in liquid nitrogen vapors, the fertilization rate after ICSI was lower than with fresh spermatozoa (40–44% versus 77–86%, \( P < 0.05 \)), even with the Percoll-enriched fraction that exhibited robust motility. In contrast, somewhat slower freezing of spermatozoa in pellets on dry ice supported fertilization rates (73%) that were similar to the fresh counterpart. Developmental rates of fertilized eggs were similar in all experiments. A total of 106 embryo transfers has resulted in the first primate born after ICSI with F/T ejaculated spermatozoa plus 22 other infants to date.

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Additionally, a 3–4 h incubation after thawing improved the fertilization rate with spermatozoa from a male with poor post-thaw recovery of sperm motility. In conclusion, an acceptable fertilization rate after ICSI with motile, frozen–thawed primate spermatozoa was observed comparable to that obtained with fresh spermatozoa allowing small quantities of competent spermatozoa to be used with ICSI to facilitate propagation of desirable primate genotypes.

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1. Introduction

The non-human primate is highly relevant as a human disease model due to similar physiology. There is currently a need for populations of non-human primates with specified disease susceptible genotypes that cannot be satisfied by the importation of animals from the wild or by the identification and propagation of valuable founder animals by selective breeding. In theory, assisted reproductive technologies can be efficiently applied to the rapid propagation of select animals to satisfy the needs of the biomedical research community. Ovulation induction protocols utilizing recombinant gonadotropins that produce approximately 20 fertilizable oocytes per stimulation cycle have been developed for non-human primates [1,2]. Intracytoplasmic sperm injection is a successful fertilization procedure in this species [3,4] utilizing one spermatozoa per oocyte and thereby allowing the production of a very large number of embryos from a singleejaculate. The rapid, efficient propagation of a valuable founder male, then, might ideally involve cryopreservation of spermatozoa in small aliquots that retain fertilizing capability similar to that of freshly harvested spermatozoa.

Despite advances in cryopreservation techniques, decreases in progressive motility and in the percentage of motile spermatozoa are observable after thaw in most species [5,6] which may be reflected in decreased mucus penetration [7], decreased in vitro penetration of salt stored oocytes [8] and decreased numbers of decondensed spermatozoa heads in hamster oocytes after IVF or ICSI [9,10]. Most importantly, spermatozoa cryopreservation results in reduced function, such that conventional insemination is no longer efficient. Diminished oocyte binding and fertilization may be secondary to acrosomal and membrane damage observed after thaw [6,11]. Internal post-thaw changes in spermatozoa have also been noted, including decreased mitochondrial membrane potentials [12] and decreased chromosomal condensation [13] which may impact motility, fertilization and subsequent embryonic development. Reports are inconsistent as to the effectiveness of fresh versus frozen–thawed spermatozoa in fertilizing oocytes with ICSI. A detailed analysis of clinical cases utilizing testicular spermatozoa observed that ICSI with fresh, fully developed spermatozoa had a significantly higher fertilization rate than when frozen–thawed spermatozoa were used [14]. However, reports on this topic need to be carefully examined relative to the maturity of the spermatozoa being compared. Mature spermatozoa from fresh ejaculates yield higher fertilization rates with ICSI than immature spermatozoa of epididymal or testicular origin whether frozen–thawed or fresh [15–17]. This difference may relate to the effects of freezing and thawing, or to immaturity; as spermatozoa pass from the testis to the cauda epididymis, increases in chromosome condensation, motility, and the abilities to bind zona and activate oocytes are observed [18–20].
Cryopreservation of spermatozoa from several strains of macaques such as the rhesus [21–23] and cynomolgus [5,24,25], has been reported. However, no studies have comparatively evaluated various cryopreservation procedures by fertilization in vitro (assessed using ICSI) to evaluate cryo-damage beyond that related to motility and binding to the oocyte membranes. The present report compares four freezing–thawing protocols by evaluating fertility and embryonic development following ICSI with motile spermatozoa in an ongoing effort to identify an approach that supports retention of fertility similar to that of fresh spermatozoa and allows preservation of a large number of samples from a single ejaculate.

2. Materials and methods

2.1. Animals and collection and evaluation of semen

All animal procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Use and Care Committee of the Oregon National Primate Research Center, Oregon Health and Sciences University. Nine adult male rhesus monkeys (6–9 kg) were used in a series of four semen cryopreservation experiments while developing our current procedures. The restricted number of oocytes available for this study prevented direct comparisons of contrasting cryopreservation procedures within oocyte cohorts, as did practical considerations prevent simultaneous fertility comparisons of fresh and cryopreserved spermatozoa from the same ejaculate. However, the semen samples utilized throughout were consistently of high quality, based on sperm motion characteristics and therefore were presumed to be of similar initial fertility. Each experimental series involved three or four males, from which a total of eight to nine ejaculates were frozen. Also, a comparison of results using the same male for both fresh and frozen sperm was possible in a combined subset of freezing series data to address if reduced fertilization was a characteristic of individual males. Additionally, one founder adult male with a highly desirable genotype housed offsite was involved in the extensive in vivo application of the final cryopreservation technique. Semen was collected by penile electroejaculation [26] and allowed to liquify at 27–32 °C for 10–15 min. The liquid portion was harvested from the coagulum into a 15 mL conical centrifuge tube (05-539-12, Fisher Scientific, Tustin, CA, USA) and washed twice with 5 mL HEPES-buffered TALP medium (modified Tyrode’s solution with Albumin, Lactate, and Pyruvate medium; [27]) containing 0.3% BSA (TH3) at 130–150 × g for 5 min and the final pellet was resuspended in 0.25 mL medium to make up the washed spermatozoa pellet utilized for the different freezing procedures. Unless otherwise noted, chemicals utilized were purchased from Sigma-Aldrich, St. Louis, MO, USA. Sperm concentration and motility were evaluated microscopically with a hemocytometer (100 cells) before freezing and after thawing. Forward progression was rated on a scale of 1–4, with 1 = no forward progression (twitching) and 4 = rapid progressive movement. Only semen specimens with initial total motility of more than 70% with progression of at least grade 3 (as observed with a hemocytometer) were utilized in these experiments.
2.2. Freezing

2.2.1. Series I

In freeze–thaw series I, the washed sperm pellet was extended with medium consisting of 6% glycerol and 0.1 M sucrose in TH3 [28]. After gently mixing, the sperm suspension (in a 15 mL tube) was placed in a 500 mL beaker of 23 °C water and equilibrated to 4 °C in a refrigerator for 90 min. The suspension was then loaded into 0.25 mL straws on ice (PETS, Canton, TX, USA) that were sealed by fluid contact with the wick and powder at one end and heat-crimpled at the other end. Straws were then placed horizontally on two aluminum supports at 1 cm above the LN2 (which approximates 170 °C). The cooling of liquid within a straw to −150 °C occurred within 1 min and reached −170 °C within 2 min, as measured by a thermocouple (Tegam, Geneva, OH, USA) placed inside an identical straw filled with freezing media. Specimens were stored at least 48 h before thawing. Straws were thawed in ambient air (22 °C) for 15 s before the contents were expelled into 5 mL of TH3 rinse media that approximated a +850 °C/min warming rate. After an additional wash with 5 mL TH3, count and motility were evaluated.

2.2.2. Series II

Freeze–thaw series II involved diluting the washed pellet to 1 mL with extender medium consisting of 30% egg yolk, 20% skim milk, 0.06 M glucose in a Tes–Tris buffer adjusted to a pH 7.4 [5; Dr. C. VandeVoort, University of California, Davis, personal communication]. A separate portion of the extender medium was then modified to make a 6% glycerated solution of which an equal volume was added to the spermatozoa in thirds over 30 min at 4 °C, to give a final glycerol concentration of 3%. After equilibration with the cryoprotectant for a further 60 min at 4 °C, straws were loaded, sealed, frozen in LN2 vapors and stored as with series I. Thawing of straws frozen in experimental series II was similar to that of series I.

2.2.3. Series III

Series III involved specimens frozen in a similar procedure to series II, but subjected to an additional post-thaw purification step. The latter involved density gradient centrifugation through a column of 50, 70 and 95% Percoll (Amersham Biosciences, Piscataway, NJ, USA) [29] before recovery of the 95% fraction and washing twice in 5 mL of TH3.

Additionally, specimens prepared with this procedure were evaluated for within-ejaculate variability and cryostability of frozen spermatozoa by motility measurements of spermatozoa from straws held in LN for up to 3 month. Post-thaw retention of motility was also examined 6 h after thaw processing.

2.2.4. Series IV

Series IV involved preparation and equilibration of washed spermatozoa in 3% glycerol (as in series II), but the freezing was done by allowing 20–100 μL drops to fall directly onto solidified carbon dioxide (dry ice, −78 °C) forming pellets [18] with approximate cooling rates of −85 and −60 °C/min for 50 and 100 μL pellets, respectively. After 10 min, multiple pellets were then placed in dry-ice-cooled cryovials (#2028, Corning, Corning, NY, USA) and plunged into LN2 for storage. For thawing, a single pellet was retrieved from
the LN2 within a cryovial, placed in a dry test tube suspended in a 37 °C water bath for 40 s (which approximated a warming rate of 350 °C/min) and then washed in 5 mL of TH3. Additionally, the effect of pellet size on post-thaw motility was analyzed by comparing results from five drops frozen at 100, 50 and 20 μL each.

2.3. Post-thaw processing

With all for freeze–thaw approaches, the final washed pellet was suspended in 200 μL TH3 and post thaw motilities were determined. This suspension was then diluted, if needed, to 1–3 million motile spermatozoa/mL for ICSI, as previously described [2,4]. Note that in the ICSI experiments comparing fresh versus F/T spermatozoa, for practical purposes, the same ejaculate could not be used. However, when possible fresh and thawed spermatozoa from the same male were compared and these cases were additionally analyzed separately below.

The effect of post-thaw incubation on fertilization rate was also studied in an application of sperm from a genetically valuable male frozen with the series IV protocol. The time from thawing of the spermatozoa to initiation of ICSI was monitored and evaluated relative to fertilization rate. The interval from first observation of mature metaphase II oocytes to ICSI was also recorded, since some oocytes did not mature until several hours after retrieval.

2.4. Follicular stimulation and oocyte collection

Cumulus–oocyte-complexes (COC) were retrieved by follicular aspiration from cycling females following ovarian stimulation [1,2]. Starting 1–4 days after menses, cycling females received twice-daily injections of recombinant human FSH (rhFSH; 30 IU i.m., Ares Advanced Technology, Norwell, MA, USA) and once daily injections of Antide (a GnRH antagonist; 0.5 mg/kg s.c., Ares Advanced Technology) for eight consecutive days. On the last 2 day of rhFSH/Antide stimulation, animals also received twice daily injections of recombinant human LH (rhLH; 30 IU i.m., Ares Advanced Technology). On the last day of hormonal stimulation, ovarian morphology was evaluated by ultrasonography (ATL HDI 1000, Philips Medical Systems, Bothell WA, USA) and animals with follicles 3 mm diameter or greater received an injection of recombinant human chorionic gonadotropin (rhCG; 1000 IU i.m., Ares Advanced Technology). The COC were collected laparoscopically 30 h later and placed in TH3 after their recovery from follicular aspirates. Oocytes were freed from the COC by mechanical trituration after 1 min exposure to hyaluronidase (1 mg/mL) and held in CMRL medium (Connaught Medical Research Laboratories media; Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 10 mM L-glutamine, 5 mM sodium pyruvate, 1 mM sodium lactate, 100 units/mL of penicillin, and 100 μg/mL streptomycin for short-term culture at 37 °C in 5% CO2 for up to 4 h prior to ICSI, depending on their maturation status.

2.5. ICSI and embryo culture

The limited number of oocytes available for study necessitated evaluating spermatozoa from only one freezing procedure on each day of oocyte retrieval. Sibling oocytes were
always inseminated in parallel with fresh spermatozoa to control for oocyte quality. ICSI with frozen–thawed or fresh spermatozoa was performed on an inverted microscope (IX70, Olympus, Melville, NY, USA) equipped with Hoffman optics and micromanipulators (Narishige, Tokyo, Japan) housed in a heated room (27–30 °C) with oocytes returned to culture within 10 min. The micromanipulation chamber (Falcon 1009, Becton-Dickinson, Franklin Lakes, NJ, USA) contained oil and two drops: a sperm drop consisting of 4 μL of 10% polyvinylpyrrolidone (Irvine Scientific, Santa Ana, CA, USA) in TALP/HEPES and 1 μL of spermatozoa (3 million/mL) and an oocyte drop, 20 μL of TH3, into which mature oocytes were placed. Individual spermatozoa were immobilized by striking the tail and then injected into oocytes away from the polar body using a 7 μm outer diameter micropipette (Humagen, Charlettesville, VA, USA). Only progressively motile spermatozoa with normal morphology were selected for ICSI. Injected oocytes were cocultured on a monolayer of Buffalo Rat Liver cells (BRL; American Type Culture Collection, Manassas, VA, USA) (2.5 × 10^4 cells per well) in 4-well dishes (Nalge Nunc International Co, Naperville, IL, USA) containing 0.6 mL of CMRL culture medium with 10% FBS at 37 °C in 5% CO2. Embryos were transferred to fresh plates of BRL cells every other day. Pronuclear formation was recorded 10–16 h post-ICSI and the progression of embryo growth was recorded daily. Fertilization was defined as pronuclear formation and/or timely cleavage with nucleated blastomeres. Blastocyst formation was defined as the expansion of the compacted embryo with cavitation to include both a discernible trophoectoderm and an inner cell mass.

2.6. Embryo transfer

Embryo transfers were conducted on adult, multiparous rhesus females in which blood samples were collected daily for measurement of estradiol beginning 8 day after detection of menses. Estradiol levels were measured with an automated immunoassay (Elecsys 2010, Roche Diagnostics, Laval, Que., Canada) in order to detect the mid-cycle estradiol peak. Two to five days after the peak, animals were prepared for transcervical embryo transfers. Under ketamine anesthesia (10 mg/kg, i.m.; Fort Dodge Laboratories, Fort Dodge, IA, USA) animals were positioned in sternal recumbency, and a polyvinyl catheter aided by a stylet (Patton model, Cook OB/GYN, Spencer, IN, USA) was gently guided into the uterus. Alternative tubal transfers were via surgical or laparoscopic approaches under isoflurane gas anesthesia (Henry Schein, Sparks, NV, USA) while monitoring heart rate and peripheral oxygen saturation. Two embryos in 15 μL of TH3 medium were then slowly ejected. Pregnancy detection involved monitoring weekly hormone levels for three weeks. Ultrasonography (Philips Medical Systems) was initially performed on day 25 to detect a fetal heartbeat. Fetal status was further monitored at trimester intervals by ultrasound for the duration of pregnancy.

2.7. Data analysis

Sperm motility proportions were transformed to the arcsine of the square root before comparing by ANOVA and Fisher’s protected least significant difference test with Statview software (SAS Institute Inc., Cary, NC, USA). Sperm motility grades were converted to
proportions of the range and similarly transformed before ANOVA analysis. Fertilization rates and embryo development rates within treatment series were also transformed to the arcsine of the square root of proportions, prior to comparison between fresh and frozen results using the Student’s t-test (SAS). The regression analysis of fertilization rates versus incubation time post-thaw was evaluated using ANOVA (SAS). Values with \( P < 0.05 \) were considered different in all cases.

3. Results

3.1. Motility of fresh specimens

In series I–IV, the 26 fresh semen specimens from nine males collected for these experiments had similar motilities after washing with the percent motile and forward progression ranging from 77 to 81% and 3 to 3.5, respectively. These data were combined for presentation and provided control values for comparison to post-thaw measurements of specimens after different cryopreservation procedures (Fig. 1).

3.2. Series I

After thawing the eight specimens frozen in series I, 17 ± 6% of the spermatozoa were motile and the forward progression (1.4 ± 0.1) was significantly lower than non-frozen control samples (Fig. 1). In four ICSI trials which evaluated the fertility potential of frozen–thawed spermatozoa from three males prepared in series I, reduced fertility in sibling oocytes was observed when compared to control, non-frozen spermatozoa (40%, \( n = 40 \) versus 77%, \( n = 39 \); \( P < 0.05 \); Table 1). The blastocyst developmental rate after 8 days of culture from the embryos fertilized with thawed spermatozoa was 56% (\( n = 16 \)) and with fresh spermatozoa, 57% (\( n = 30 \)).
3.3. Series II

Eight specimens from four males cryopreserved with the procedure in experimental series II had similar motility (24 ± 5%), but higher forward progression (2.2 ± 0.1, \( P < 0.05 \); Fig. 1). However, four ICSI trials in series II using thawed spermatozoa, also yielded a reduced fertilization rate (44%, \( n = 32 \)) when compared to sibling oocytes injected with control spermatozoa (86%, \( n = 35 \); \( P < 0.05 \)) (Table 1). The blastocyst development rate from embryos fertilized with thawed spermatozoa was 57% \( (n = 7) \) compared to 50% \( (n = 30) \) with fresh spermatozoa.

3.4. Series III

In series III, an elite, motile spermatozoa population was selected after initial freezing and thawing of eight ejaculates from three males, similar to that in series II, but further processed by Percoll purification. This processing resulted in motility (75 ± 3%, \( P < 0.01 \)) and progression scores (2.8 ± 0.1, \( P < 0.05 \)) similar to those of control samples (Fig. 1). When these elite frozen–thawed spermatozoa were used in six ICSI experiments, the fertilization rate was 49% \( (n = 61) \), still less than that with sibling oocytes injected with freshly prepared spermatozoa, 77% \( (n = 52, P < 0.01) \); Table 1). The blastocyst development rate was 54% \( (n = 24) \) with frozen–thawed spermatozoa and 50% \( (n = 30) \) with fresh spermatozoa.

Using the procedure from series III, the within-ejaculate variability and cryostability of frozen spermatozoa from three males was evaluated by motility measurements of spermatozoa from nine straws held in LN for up to 3 month. The thawed motility percentages had a coefficient of variation of 4%, suggesting minimal degradation. Although ICSI was generally performed within 2 h of sperm recovery, it was also of interest to examine post-thaw retention of motility over a longer interval. In four specimens processed with the procedure from series III, high motility (81 ± 3%; 2.5 progression) was maintained through 2 h of culture; after 6 h, 72 ± 12% of the cells were still motile, though of low forward progression.

<table>
<thead>
<tr>
<th>Series</th>
<th>ICSI</th>
<th>Fertilization</th>
<th>Blastocysts(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (4 trials)</td>
<td>Frozen–thawed</td>
<td>16/40 (40%) a</td>
<td>9/16 (56%)</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
<td>30/39 (77%) b</td>
<td>17/30 (57%)</td>
</tr>
<tr>
<td>II (4 trials)</td>
<td>Frozen–thawed</td>
<td>14/32 (44%) a</td>
<td>4/7 (57%)</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
<td>30/35 (86%) b</td>
<td>15/30 (50%)</td>
</tr>
<tr>
<td>III (6 trials)</td>
<td>Frozen–thawed</td>
<td>30/61 (49%) a</td>
<td>13/24 (54%)</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
<td>40/52 (77%) b</td>
<td>15/30 (50%)</td>
</tr>
<tr>
<td>IV (6 trials)</td>
<td>Frozen–thawed</td>
<td>47/65 (73%) b</td>
<td>14/44 (32%)</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
<td>45/53 (84%) b</td>
<td>15/43 (35%)</td>
</tr>
</tbody>
</table>

Different letters within columns indicate a difference among values \( (P < 0.05) \).

\( a \) Blastocysts from embryos. In one trial from series II and two from series III and IV, embryos were utilized for other studies and unavailable for developmental analysis.
3.5. Series IV

As an alternative to LN-based, low-temperature freezing in straws, cooling of extended spermatozoa as pellets on dry ice was examined in series IV with nine specimens from four males. This approach was associated with diminished post-thaw percent motility (49 \pm 5\%) versus fresh spermatozoa (P < 0.05), similar to the outcomes from experimental series I and II. However, forward progression of spermatozoa from thawed pellets showed forward progression (3.2 \pm 0.1) compared to fresh or Percoll isolated specimens (Fig. 1). The fertility analysis in six ICSI trials using spermatozoa frozen as pellets in series IV yielded a higher fertilization rate (73\%, n = 65 oocytes) compared with frozen–thawed spermatozoa via other procedures (P < 0.01) which was not significantly different from levels obtained with control spermatozoa in sibling oocytes (84\%, n = 53 oocytes; Table 1). The blastocyst development rate was 32\% (n = 44), with embryos from spermatozoa frozen in series IV and 35\% (n = 43) with fresh spermatozoa. All six initial trials in series IV paired fresh spermatozoa with F/T spermatozoa from the same male.

The effect of pellet size on post-thaw motility was analyzed separately by comparing results from five drops frozen at 100, 50 and 20 \text{\mu L} each. The post-thaw motility ranged from 55 to 43\%, with a coefficient of variation of 8\% indicating no effect of drop volume on post-thaw motility within this range of drop sizes.

3.6. Same-male comparison

Twenty-four of the 27 trials conducted with spermatozoa processed in series I, II and III were matched with spermatozoa from the same male for both fresh and F/T ICSI albeit different specimens. An analysis of fertilization results for these 24 experiments where the same male was employed found similarly reduced fertilization when post-thaw motile spermatozoa were injected (43\%, n = 79 oocytes) compared to the rate using control spermatozoa (82\%, n = 88 oocytes; P < 0.05). This observation indicates that reduced fertilization with frozen–thawed spermatozoa from experimental procedures in series I, II and III is a characteristic of most if not all the males tested.

3.7. Post-thaw incubation

In a subsequent application of the method derived from series IV, semen was collected from a genetically valuable male at the New England National Primate Research Center and spermatozoa were cryopreserved in pellets for transportation to our laboratory. Forty-one pellets frozen from several collections were thawed with a motility of 39 \pm 1\% and when used for ICSI of 542 oocytes, an overall fertilization rate of 50.7 \pm 4.4\%. This fertility suggested that this particular male was subpar regarding the tolerance of its spermatozoa to freezing and thawing relative to our previous results with good quality spermatozoa. A study was then done evaluating the time from thawing of the spermatozoa to initiation of ICSI. There was a correlation (P < 0.01) between increased incubation time of thawed spermatozoa prior to ICSI and fertilization (Fig. 2). Incubation times <2 h (n = 18) resulted in 32.7 \pm 4.4\% fertilization, in contrast to those thaws used \geq 3 h (n = 15) which had 73.9 \pm 6.7\% fertilization. Oocyte age post-collection did not appear to
contribute to this outcome, as evidenced by a comparison between the performances of MII oocytes that were matured at collection versus those that matured late, in vitro.

3.8. Embryo transfer

An initial trial of the in vivo developmental potential of embryos produced via ICSI with frozen–thawed spermatozoa from series IV protocol involved five transcervical transfers. Two clinical pregnancies resulted in a healthy male weighing 520 grams and a healthy female weighing 385 grams delivered at 159 and 158 day of gestation, respectively, establishing the first primates born using ejaculated frozen–thawed spermatozoa and ICSI insemination. In the ongoing series utilizing frozen–thawed spermatozoa from the genetically unique male at the New England National Primate Research Center, 101 embryo transfers of 1–2 early cleavage stage embryos into recipients 2–3 day after ovulation has resulted in 21 additional pregnancies and live births.

4. Discussion

We have developed improved cryostorage procedures for rhesus monkey spermatozoa to be used for ICSI in assisted reproduction providing numerous thaws of adequate spermatozoa numbers from a few ejaculates. This is the first study comparing freezing procedures that not only assessed motility, but also evaluated the fertilizing capability of thawed ejaculated spermatozoa using ICSI (thereby circumventing motility and zona binding). We found that motility of spermatozoa frozen in straws in LN vapors did not show a relationship with fertilization, suggesting the existence of sub-lethal, cryo-damage effects on oocyte activating ability. Additional post-thaw processing to isolate spermatozoa with motility characteristics similar to freshly prepared spermatozoa, did not improve ICSI fertilization rates to levels achieved with fresh spermatozoa. An alternative procedure that involved relatively slower cooling and warming was associated with the retention of ICSI fertilizing capability, similar to that of fresh spermatozoa. Additionally, a 3–4 h incubation
after thawing improved the fertilization rate with spermatozoa from a male with poor recovery of motility post-thaw thaw.

Although current cryopreservation procedures are directly lethal to a considerable proportion of spermatozoa in a semen sample, the breadth of sub-lethal, cryo-damage, assumed to result from ice crystal formation and hypertonic intracellular environments, has not been as obvious [30]. Evaluations of fertility post-thaw of ejaculated spermatozoa have included both motility and oocyte penetration, but have not examined factors unrelated to motility, as is possible with ICSI [5,22,23]. During cryopreservation procedures, freezing rates and cryoprotective agents interact to effect cellular membrane fluidity and water channel permeability contributing to the level of cell dehydration important for post-thaw survival [32–35]. Membranes of subcellular structures throughout the cell may be compromised by the physical state changes, as shown in electron microscopy studies of frozen–thawed primate spermatozoa [21]. Moreover, reduced fertilization may be related to loss of soluble, spermatozoa-associated, oocyte activation factors, since isolation protocols for these factors utilize repeated freezing and thawing of spermatozoa [4,36]. An aggressive freeze–thaw regimen diminished the oocyte activating ability of human spermatozoa injected into hamster oocytes; this deficiency was overcome by supplementation with a sperm cytosolic factor [37,38]. Sperm-borne, oocyte activating factors have also been associated with perinuclear structures and while these structures are relatively insoluble [39–41], they still may be compromised during the freezing procedure [42]. Another possible site of freeze–thaw damage is the disulfide bond of the protamines that replace histones during DNA compaction in spermatogenesis; sperm freezing and thawing may reduce chromosomal compaction post-thaw relative to fresh spermatozoa [13]. However, intentional disruption of protamines with dithiothreitol in the mouse did not diminish pronuclear formation or blastocyst development when spermatozoa were injected into oocytes [37] suggesting that reduced chromosomal compaction may not prominently influence fertilization. In the present study, the best fertilization results were obtained from spermatozoa cryopreserved as small drops on dry ice, as compared to spermatozoa frozen in straws suspended in liquid nitrogen vapors. Inherent in the dry ice procedure is a reduced cooling rate of −60 to −85 °C/min compared to that occurring in straws in LN vapor (−150 °C/min). A reduced cooling rate has also been reported to be optimal in studies with mouse spermatozoa which incur a dramatic fall in motility above −100 °C/min [43].

A reduced ability of the spermatozoa to activate oocytes is considered the main cause of ICSI-related fertilization failures, including incomplete chromosome decondensation and premature condensation [44–47]. Oocyte activation mechanisms involve calcium oscillations, which inactivate the maturation promoting factor, trigger sperm head decondensation and, subsequently, promote pronuclear membrane formation [47]. Fertilization by ICSI bypasses the interaction of sperm and oocyte membranes, however, the micropipette penetration of the oolemma and aspiration of cytoplasm into the micropipette is thought to simulate the initial sensitization to sperm factors [48,49]. Our present ICSI procedures with fresh spermatozoa resulted in fertilization success as good as or better than can we have previously achieved with in vitro fertilization (50–75%) [50]. In vitro fertilization in the rhesus monkey is hampered by the numerous pre-activation steps required to capacitate spermatozoa and a propensity of the spermatozoa to agglutinate [51,52].
The significant improvement in ICSI fertilization rates with short-term culture for a few hours post-thaw observed with spermatozoa from a genetically valuable male, is noteworthy. Although natural mating was successful with this male, his spermatozoa were exceptionally susceptible to cryodamage (similar to other individuals of other species). Cryopreservation is known to effect sperm membrane lipids, ion channels and enzymes [53–55]. Since oocyte activating factors are sequestered in the postacrosomal perinuclear theca [40,56], they also are likely perturbed during the freeze–thaw cycle, perhaps more so in this individual. Enzyme activity such as phosphorylation of acrosomal components is known to occur during capacitation [57,58] and possibly this brief in vitro incubation allowed adequate recovery of necessary mechanisms in these markedly cryo-sensitive spermatozoa. Our incubation of thawed spermatozoa for a few hours after freezing in drops on dry ice has resulted in 21 additional live births from this genetically unique male [59].

In contrast to the range of fertilization rates observed after ICSI with frozen–thawed spermatozoa in the present experiments, in vitro embryonic development was similar between embryos produced using fresh or frozen–thawed spermatozoa. These blastocyst developmental rates were also similar to those obtained previously in the rhesus monkey with fresh spermatozoa by this and other laboratories, whether inseminations were by either ICSI or IVF [2,4,60,61]. Additionally, our successful production of 23 pregnancies from 106 embryo transfers confirms functional viability of frozen–thawed monkey sperm and is similar to the pregnancy rates we reported using embryos produced with fresh spermatozoa [2,62]. Reports in the human utilizing frozen–thawed spermatozoa have also noted no difference in embryonic development with implantation rates compared to those achieved for fresh spermatozoa [15,17,63]. The present results augmented by the above literature suggests that once fertilization is established in the primate, post fertilization embryonic development is minimally impacted by prior cryopreservation of paternal DNA with these methods. However, as shown in the mouse, suboptimal freezing procedures or, perhaps, freezing of sensitive spermatozoa from specific individuals [33] may affect subsequent fetal development, a possibility that was not intensively investigated here.

Freezing in pellets has an additional advantage relative to straws; it allows reduced volumes to be stored and retrieved separately. The retention of fertilization by ICSI with spermatozoa cryopreserved in small pellets provides the ability to extend the number of specimens 10 times that employed with straws. This advantage is important when there is limited availability of spermatozoa from a male with highly desirable genetic traits. Additionally, the use of the same ejaculate for several fertilization experiments would produce less variability from the male contribution. The room air freezing of pellets on dry ice was a concern due to the potential for contamination. However, no problems were encountered. The use of sterile dry ice and spermatozoa processing in a sterile hood could of course be considered.

In summary, the present experiments in the rhesus monkey utilized ICSI as a tool to detect sub-lethal cryo-damage and found reduced fertilization unrelated to post-thaw motility with spermatozoa frozen in straws in LN vapors. An alternative procedure, freezing specimens in drops on dry ice preserved fertility similar to that of fresh spermatozoa. The latter approach also offers the advantage of a marked increase in the number of specimens that can be obtained per ejaculate. Sperm cryopreservation, when combined with ICSI, will enable a multitude of embryos to be produced from a single
ejaculate. Proof of principle for this approach in the rhesus monkey was provided by 23 pregnancies to date. The creation of viable embryos by applying the ARTs with cryopreserved spermatozoa should allow the propagation of nonhuman primate disease models that are in great demand by the biomedical research community.

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