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Live Rhesus Offspring by Artificial Insemination Using Fresh Sperm and Cryopreserved Sperm[1]

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ABSTRACT

Artificial insemination (AI) and the cryopreservation of sperm with full reproductive capabilities are vital in the armamentarium of infertility clinics and reproductive laboratories. Notwithstanding the fantastic successes with AI and sperm cryopreservation in numerous species, including humans and cattle, these assisted reproductive technologies are less well developed in other species of importance for biomedical research, such as genetically modified mice and nonhuman primates. To that end, AI at high efficiency in the rhesus macaque (*Macaca mullata*) and the successful cryopreservation of rhesus sperm is presented here, as are the complexities of this primate model due to differences in reproductive tract anatomy and gamete physiology. Cryopreservation had no effect on the ability of sperm to fertilize oocytes in vitro or in vivo. Post-thaw progressive motility was not affected by cryopreservation; however, acrosome integrity was lower for cryopreserved (74.1%) than for fresh sperm (92.7%). Fertilization rates did not differ when fresh (58.1%; n = 32/55) or cryopreserved sperm (63.8%; n = 23/36) were used for in vitro fertilization. Similarly, pregnancy rates did not differ significantly after AI with fresh (57.1%; n = 8/14) or cryopreserved sperm (62.5%; n = 5/8). Seven live rhesus macaques were born following AI with fresh sperm, and three live offspring and two ongoing pregnancies were obtained when cryopreserved sperm were used. Cryopreservation of rhesus sperm as presented here would allow for the cost-effective storage of lineages of nonhuman primates with known genotypes. These results suggest that either national or international centers could be established as repositories to fill the global needs of sperm for nonhuman primate research and to provide the experimental foundation on which to explore and perfect the preservation of sperm from endangered nonhuman primates.

sperm capacitation/acrosome reaction, sperm motility and transport

INTRODUCTION

The genetic and physiological similarities between the rhesus macaque (*Macaca mullata*) and humans make the former an excellent model for clinically relevant biomedical research. Nonetheless, use of this nonhuman primate for the study of human diseases, such as retinitis pigmentosa and hemophilia, and for age-related or reproductive studies is limited by the low availability of specimens. The use of assisted reproductive technologies (ART), such as in vitro fertilization (IVF) [1] and intracytoplasmic sperm injection (ICSI) with ejaculated [2] or plasmid-bound sperm [3], has resulted in a number of live rhesus monkey births. Notwithstanding the relevance of these ART procedures, a less-demanding method is needed to propagate these macaques. Artificial insemination (AI), which has been successful in the propagation of other mammalian species, including humans, could be most useful to increase the number of rhesus macaques available for biomedical research. Nevertheless, AI in the rhesus macaque is somewhat difficult, with pregnancy rates using fresh sperm ranging between 4% to 40% [4–6] and only one pregnancy reported after AI with cryopreserved sperm, in which the embryo was aborted at an early stage [7]. In the rhesus macaque, AI is limited by features of the female reproductive tract and by the viability of cryopreserved sperm. In contrast to other mammalian species, the cervix in the female rhesus macaque has five to six folds that do not allow easy passage of an insemination pipette for the deposition of semen into the uterus. Furthermore, rhesus macaque sperm are susceptible to cryopreservation and lose their motility and viability shortly after freeze-thawing [7, 8].

Development of sperm storage and AI protocols for nonhuman primates would also be important to assist in the preservation of endangered nonhuman primate species from captive or fresh postmortem males when available. These protocols could also be used to aid in solving difficulties with mating performance, to increase the population of individuals with desirable genetic traits, or to preserve genetic diversity. To this end, the present study describes a noninvasive method for AI in the rhesus macaque and a method for the cryopreservation of rhesus sperm.

MATERIALS AND METHODS

Animals

Mature, specific pathogen-free rhesus males and females housed in individual cages were used in the present study. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Oregon Regional Primate Research Center/Oregon Health Sciences University.

Semen Collection

Semen was collected by electric penile stimulation from six male rhesus macaques of proven fertility. Ejaculates were allowed to liquefy for 15–20 min at 37°C [9], and samples were then assessed for motility and processed as described later.

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Fresh Sperm

Samples used for IVF were washed twice by centrifugation (400 × *g* for 8 min) in Tyrode albumin lactate pyruvate-HEPES (TALP-HEPES) and diluted to 20×10^6 sperm/ml with TALP [9]. The sperm suspension was then incubated at 37°C for 4–5 h in a humidified atmosphere containing 5% CO_2 in air before IVF. In contrast, semen for AI was diluted fivefold at 30°C with TALP-HEPES medium [9] and stored at 30–32°C for 2–3 h.

Cryopreserved Sperm

Semen samples were diluted fivefold at 30°C with a Tris-based medium consisting of 300 mM Tris (Sigma, St. Louis, MO), 28 mM glucose (Sigma), 95 mM citric acid (Sigma), 5% (v/v) glycerol (Sigma), 1000 IU/ml gentamicin (Sigma), 1 mg/ml streptomycin (Sigma), and 20% (v/v) chicken egg yolk. Diluted semen was cooled slowly to 5°C over 90–120 min in a 4°C room. Aliquots of diluted semen (200 μl) were pellet frozen in solid dry-ice wells for 60–90 sec and then transferred and stored at −196°C in liquid nitrogen for at least 1 wk [10]. Before IVF or AI, pellets were thawed individually in dry test tubes, which were hand shaken in a water bath at 37°C for 45–60 sec. For IVF, frozen-thawed sperm were diluted to 20×10^6 sperm/ml with TALP [9]. Sperm for AI were not diluted and were used immediately after thawing for insemination.

Sperm Evaluation Assays

Progressive motility. An 8-μl aliquot of diluted sperm was placed on a clean, warm (37°C) slide and covered with a coverslip. Progressive motility (sperm moving in a forward direction) of the sperm samples was assessed subjectively by light microscopy at 100× before AI or IVF. Before motility assessment, sperm samples were thoroughly mixed and were not subjected to any separation method, such as swim-up or Percoll wash. Samples were presented in random order each time so that the operator did not know their identity.

Acrosome integrity. Sperm were attached to poly-L-lysine-coated microscopy coverslips by a 5-min incubation in a 1-ml drop of warm PBS and then fixed for 1 h in 2% formaldehyde in PBS. Following a rinse in PBS, sperm were incubated with 100 μl of PBS containing 1 μg of fluorescein isothiocyanate-conjugated peanut lectin (FITC-PNA; Sigma) in the dark for 1 h at room temperature [11]. Next, 2 μl of PBS containing 10 μg of 4′,6′-diamindino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) were added to the FITC-PNA solution 5 min before the end of incubation. Coverslips were then rinsed in PBS, mounted in Vectashield (Vector Labs, Burlingame, CA), and examined by conventional epifluorescent microscopy using a Zeiss Axiphot microscope (Princeton Instruments, Trenton, NJ). Slides were presented in random order to the operator, who then assessed 250 sperm cells per slide for acrosome integrity. A cell labeled by DAPI but not by FITC-PNA was considered as having an intact acrosome.

Follicle Stimulation and Oocyte Collection

Females exhibiting normal menstrual cycle were hyperstimulated by a regimen of exogenous gonadotropic hormones [12–14]. At the beginning of menses, females were down-regulated by daily s.c. injections of a GnRH antagonist (Antide; Ares Serono, Aubonne, Switzerland) at 0.5 mg/kg body weight for 6 days, during which recombinant human (rh) FSH (Organon, West Orange, NJ) was administered twice daily (30 IU). This was followed by 1–3 days of rhFSH plus rhLH (30 IU each, twice daily; Serono). Ultrasonography was performed on Day 7 to confirm adequate follicular development. Recombinant hCG (1000 IU; Ares Serono, Randolph, MA) was administered when follicles were 3–4 mm. Follicles were aspirated 27 h after rhCG, and the collected oocytes were immediately assessed for nuclear maturity [14].

In Vitro Fertilization

Sperm from two males were used for IVF. Samples ($n = 6$) from each male were assessed for progressive motility and acrosome integrity as described earlier. Before IVF, hyperstimulation was induced by addition of 1 mM caffeine (Sigma) and 1 mM dibutyryl cyclic adenosine monophosphate (Sigma) to sperm suspensions, and the suspension was then further cultured for 15 min at 37°C in a humidified atmosphere containing 5% CO_2 in air. Fertilization was performed in 100-μl drops of TALP medium overlaid with oil by the addition of 1×10^6 sperm/ml [9]. Oocytes containing two pronuclei and two polar bodies 16 h after IVF were considered to be fertilized and were maintained and cultured in TALP until the two-cell stage [9].

Artificial Insemination

Females showing normal menstrual cycle, with at least two births by means of vaginal delivery, and at least 7 yr old were screened for serum estrogen and progesterone. Before AI, females were anesthetized with 10 mg/kg of ketamine hydrochloride (Ketaset; Ford Dodge Animal Health, IA). Artificial insemination was performed twice, 1 day before and 1 day after estrogen surge during a single menstrual cycle for each female. After aseptic preparation of the perivaginal area, a stylet was introduced into the vagina and placed into the cervical os. Rectal palpation was used to manipulate the cervix and to direct the stylet through the cervical canal and into the uterine body. A cannula was then placed onto the stylet. The stylet was removed, and a catheter with 100 μl of sperm suspension (containing $26.7 \pm 3.3 \times 10^6$ fresh or $20.1 \pm 1.2 \times 10^6$ cryopreserved sperm) was placed into the cannula. Once the catheter reached the uterine body, the cannula was withdrawn up to the cervical os and the suspension was slowly injected (Fig.

TABLE 1. Progressive motility, intact acrosome, and in vitro fertility of fresh or cryopreserved sperm.

Sperm	Progressive motility (%)	Intact acrosome (%)	Pronuclear formation after in vitro fertilization	
			(No.)	(%)
Fresh	87.1 ± 0.1	92.7 ± 1.0†	32/55	58.1
Cryopreserved	85.0 ± 0.1	74.1 ± 0.9	23/36	63.8

* The percentages of motile sperm and intact acrosome are the means of 12 samples.
† Significantly higher than cryopreserved sperm ($P < 0.01$). $\chi^2 = 27.9$ value for pronuclear formation.

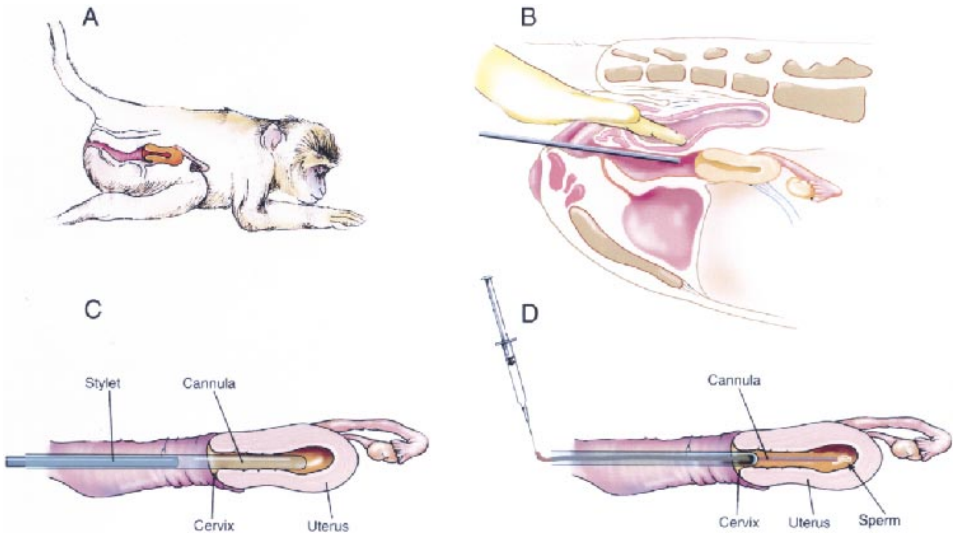

FIG. 1. Noninvasive artificial insemination in the rhesus macaque. **A**) Female rhesus macaques were positioned in ventral recumbency, with the tail elevated from the perineum. **B**) A stylet was introduced into the vagina and placed into the cervical os. Rectal palpation was used to manipulate the cervix and to direct the stylet through the cervical canal and into the uterine body. A cannula was then placed onto the stylet. **C**) The stylet was then removed, and a catheter with 100 μl of sperm suspension containing fresh or cryopreserved sperm was placed into the cannula. **D**) Once the catheter reached the uterine body, the cannula was withdrawn up to the cervical os and the suspension was slowly injected therein.

1). After each insemination, sperm samples (n = 3) were assessed for progressive motility and acrosome integrity as described earlier.

Diagnosis of Pregnancy

Pregnancies were detected by radioimmunoassay of plasma estrogen and progesterone 30 days after initiation of the menstrual cycle [15] and were confirmed by real-time ultrasonography between Days 50 and 60 after insemination. Ultrasound was performed once more during the second trimester to determine developmental normality. Pregnant females gave birth approximately 170 days after insemination.

Statistical Analyses

Data reported as percentages were subjected to angular transformation and to one-way ANOVA using GENSTAT 5 (Release 3.1, 1994; Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Significant differences between means were determined by Student's *t*-test, and $P < 0.05$ was considered to be significant. Where appropriate, pregnancy data were analyzed by χ^2 analysis of contingency tables.

RESULTS

Progressive Motility

Cryopreservation had no effect on the post-thaw progressive motility of rhesus sperm (Tables 1 and 2). Furthermore, no differences between males on the post-thaw progressive motility were observed.

Acrosome Integrity

Localization of FITC-PNA was observed only on the acrosomal cap of both fresh and cryopreserved sperm (Fig. 2). However, the proportion of sperm labeled with FITC-PNA was higher for cryopreserved rather than for fresh sperm ($P < 0.01$; Table 1).

IVF Rates with Fresh or Cryopreserved Sperm

The ability of cryopreserved sperm to fertilize oocytes in vitro was not affected by the freeze-thawing process, and IVF rates were similar for cryopreserved or fresh sperm (Table 1).

Artificial Insemination

Deposition of sperm using the noninvasive technique was possible in all but three females; in those three, sperm were deposited into the cervix in the first or second insemination. Regardless of the place of insemination in these females, one became pregnant.

Blood was observed in the catheter of two females after AI; only one of these became pregnant. Pregnancy rates after AI with fresh or cryopreserved sperm are presented in Table 2. Intrauterine insemination of 14 females with fresh sperm resulted in eight pregnancies at 30 days and 60 days as determined by progesterone serum levels and

TABLE 2. Percentage of progressive motility, intact acrosome, and pregnancy rates from plasma progesterone assay and ultrasound scanning of female rhesus macaques after intrauterine insemination in a single menstrual cycle with fresh or cryopreserved sperm.*

Sperm	Progressive motility (%)	Intact acrosome (%)	Pregnancy rates 30 days (No.)	Pregnancy rates 30 days (%)	Pregnancy rates 60 days (No.)	Pregnancy rates 60 days (%)	Live offspring (No.)	Live offspring (%)
Fresh	84.8 ± 0.1	87.5 ± 2.0	8/14	57.1	8/14	57.1	7/8	87.5
Cryopreserved	83.3 ± 1.9	71.4 ± 1.1	5/8	62.5	5/8	62.5	3/5	†

* The percentages of motile sperm and intact acrosome are the means of 84 and 48 samples for fresh and cryopreserved sperm, respectively.
† Two pregnancies are still ongoing.

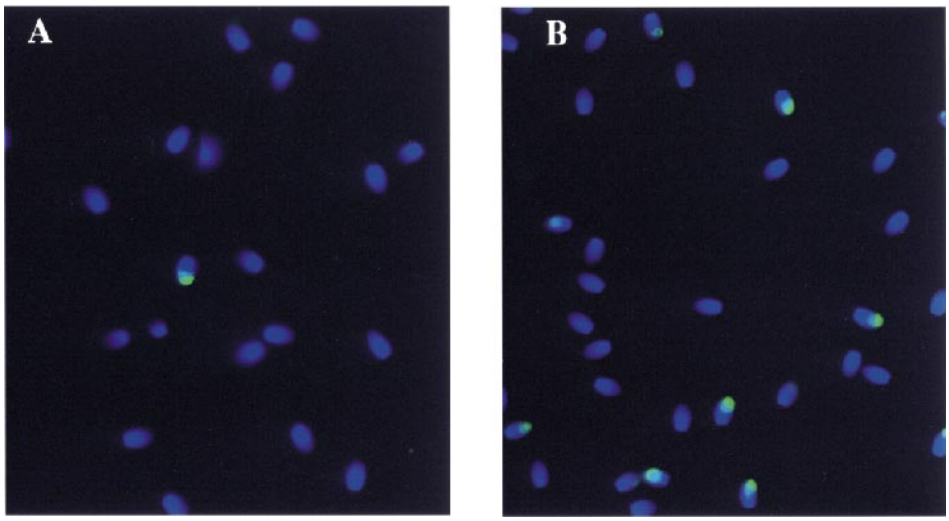

FIG. 2. Acrosome integrity of fresh (**A**) and cryopreserved (**B**) sperm as determined FITC-PNA binding assay. A cell labeled by DAPI but not by FITC-PNA was considered as having an intact acrosome.

ultrasound, respectively, and seven live and one stillborn rhesus macaques were born. In addition, insemination of eight females with cryopreserved sperm resulted in the birth of two males (Fig. 3), two females, and one stillborn.

DISCUSSION

Artificial insemination and sperm cryopreservation have been successfully used for the propagation of both humans and farm animals. Although rhesus macaques are extensively used in biomedical research, their availability for such studies is limited, as also is the case for research on AI and cryopreservation of the gametes of this nonhuman primate. The tortuous anatomy of the female reproductive tract together with the reported low survival of cryopreserved sperm have restricted the use of these technologies in the rhesus macaque. Here, we report the use of a noninvasive AI technique for propagation of this nonhuman primate and the successful cryopreservation of rhesus sperm.

FIG. 3. Fraiser, the first rhesus macaque born following AI with cryopreserved sperm.

Processing and storage inevitably cause changes to the sperm membranes. These changes are generally assumed to be detrimental and have been associated with a loss of motility and fertility [16, 17]. In the case of rhesus sperm, motility and viability were reported to decrease immediately after storage [7, 8]. In the present study, we describe a protocol for the successful cryopreservation of rhesus sperm as assessed by post-thaw progressive motility, acrosome integrity, and fertilizing ability both in vivo and in vitro.

Post-thaw progressive motility and acrosome integrity may not be the only relevant parameters with which to assess the potential fertilizing ability of sperm; nevertheless, motile cells with an intact acrosome are required to achieve fertilization in standard IVF and AI programs. Progressive motility at levels similar to those of fresh sperm was maintained after storage at −196°C (Tables 1 and 2) and was higher (85 vs. 2%–47%) compared with those reported elsewhere [7, 8]. Furthermore, progressive motility was maintained (50%) after incubation for 7 h at 37°C in 5% CO_2 (unpublished observations). No difference was observed in the post-thaw progressive motility of sperm between the males used in this study, and this could be accounted for by the selection of these males based on their ability to produce high-quality sperm samples.

Freezing and thawing rates play an important role in the preservation of cell viability. Lower but consistent freezing rates are obtained when samples are frozen on dry ice (100°C min^{-1}) than in liquid nitrogen vapor (170°C min^{-1}). This difference in freezing rates could account for the successful cryopreservation of rhesus sperm reported here. Furthermore, freezing sperm as pellets and not in ampules or straws is also better for the cryopreservation of sperm from other species, such as the squirrel monkey [18], the African elephant [19], and the ram [20]. Although no direct comparison between freezing methods was made in the present study, our findings suggest that freezing rates of 100°C min^{-1} rather than 170°C min^{-1}, as reported elsewhere [7, 8], are more suitable for the cryopreservation of rhesus sperm.

Spermatozoa must undergo the acrosome reaction to penetrate the zona pellucida and to fertilize the oocyte. However, if the acrosome has reacted before sperm contact with the zona pellucida, fertilization is not likely to occur.

The freeze-thawing process significantly affects the acrosome integrity [16, 17]; thus, assessment of this parameter is relevant to determine the efficiency of protocols used for sperm cryopreservation. The high affinity of FITC-PNA for the acrosomal region makes this lectin a useful probe with which to determine acrosome integrity [11, 21, 22]. In the present study, FITC-PNA was localized on the acrosomal cap of sperm only, and this finding is similar to that reported for ejaculated rhesus sperm by Navaneetham et al. [11]. These authors also reported that the majority (>87%) of live ejaculated sperm did not label with FITC-PNA. Similarly, we observed a high proportion of fresh sperm that were not labeled by FITC-PNA. On the other hand, cryopreservation significantly increased the proportion of sperm cells that were labeled by FITC-PNA (Fig. 2).

Indeed, post-thaw motility and acrosome integrity are relevant parameters with which to assess the success of the storage protocol; however, the ultimate test for cryopreserved sperm is their ability to penetrate and to fertilize oocytes after storage. In the present study, progressive motility and the acrosome integrity of cryopreserved sperm were not only maintained, the IVF rates and pregnancy rates with cryopreserved sperm were similar to those obtained with fresh sperm (Tables 1 and 2). The use of a noninvasive insemination technique resulted in pregnancy rates of 57% and 62.5% for fresh and cryopreserved sperm, respectively. Pregnancy rates of as much as 40% after AI with fresh semen during one menstrual cycle have been previously reported in the rhesus macaque [6]. The difference between our findings and those reported elsewhere could be attributed to the deposition of sperm directly into the uterus and not into the vagina or cervix of the female rhesus [4–6, 23] and to the efficiency of the cryopreservation protocol at preserving the fertilizing ability of the sperm. Furthermore, the results presented here could also be attributed to the use of sperm suspensions containing a known cell concentration for AI rather than portions of the coagulum from the ejaculate [4], in which the sperm concentration was not determined. To our knowledge, little information is available on the optimal time for insemination in the rhesus macaque, and the pregnancy rates presented here could likely be improved if AI can be performed closer to the time of ovulation.

Given the genetic similarity between humans and the rhesus macaque, this nonhuman primate is extensively used around the world for biomedical research. As demand for the development of treatments to cure diseases that afflict mankind increases, so does the pressure on captive-breeding programs to provide specimens to researchers. Low pregnancy rates are obtained after natural mating in the rhesus macaque [4, 5, 23]. The ability to use cryopreserved sperm would assist in the successful implementation of AI programs, in the propagation of rhesus macaques for biomedical research, and in the preservation of primate endangered species. Furthermore, as technologies for the production of genetically modified nonhuman primates emerge [3], successful cryopreservation of rhesus sperm is likely to affect the cryobanking of nonhuman primate lineages.

The cryopreservation of rhesus sperm could also improve the well-being of captive males, because the number of semen collections per macaque required for reproductive studies will be significantly reduced. Furthermore, ejaculates will be efficiently used. Depending on the volume and sperm concentration, a diluted ejaculate could be cryopreserved in small aliquots and used for different experiments within a laboratory or around the world instead of using one ejaculate per experiment, as is the case now.

Notwithstanding the relevance of AI and sperm cryopreservation for the propagation of rhesus macaques in research centers, the latter is likely to assist other ART technologies, such as IVF [1] and ICSI [2]. Finally, development of protocols to maintain the viability of cryopreserved rhesus sperm together with the ability of express courier services to deliver packages worldwide provide an excellent opportunity for the shipment of sperm to other research centers or zoos for the propagation of nonhuman primates.

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