Journal of Andrology, Vol. 28, No. 4, July/August 2007

Damage to Chromosomes and DNA of Rhesus Monkey Sperm Following Cryopreservation

MING-WEN LI,* STUART MEYERS,† THEODORE L. TOLLNER,* AND JAMES W. OVERSTREET*

*From the *Division of Reproductive Biology, Department of Obstetrics and Gynecology, School of Medicine and †Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, Davis, California.*

ABSTRACT: Fresh and frozen-thawed rhesus monkey sperm were analyzed for DNA damage using the comet assay and for chromosome damage by cytogenetic analysis after intracytoplasmic sperm injection (ICSI) into mouse oocytes. The percentage of fresh sperm with damaged DNA in ejaculated semen was 0 to 2.7% (n = 5). Conventional cryopreservation and storage in liquid nitrogen caused DNA damage in 25.3% to 43.7% of sperm; when sperm were frozen without cryoprotectants, 52.7% to 92.0% of thawed sperm had DNA damage. However, no significant difference in chromosome damage was found between fresh sperm and frozen-thawed sperm when motile sperm were selected for ICSI. The percentage of sperm with abnormal karyotypes ranged from 0 to 8.3%. The most common structural chromosomal abnormalities in fresh motile sperm and frozen-thawed motile sperm were chromosome breaks or fragments. Our findings suggest that genetically competent frozen-thawed macaque sperm can be selected for fertilization by using only motile sperm for ICSI.

Key words: Macaque, spermatozoa, ICSI.
J Androl 2007;28:493–501

The nonhuman primate is a highly relevant model of human disease. There is currently a need for rhesus macaques (*Macaca mullata*) with well-defined genetic backgrounds for biomedical investigation in the fields of transplant biology, gene therapy, and vaccine development. As the genetic cause and predisposition for diseases are determined, the number of needed unique and valuable primate models will expand enormously. Concurrently populations of many nonhuman primate species are declining throughout the world because of habitat destruction, hunting, and genetic isolation (Dresser, 1988). It is not efficient to maintain unique nonhuman primate stocks by conventional breeding, and so additional approaches must be developed. Cryopreservation of gametes and particularly spermatozoa is an attractive approach that can be implemented immediately.

Acceptable levels of success (50% recovery of motile sperm) have been reached in cryopreservation of human sperm (Brotherton, 1990; Sherman, 1990), but much of this success can be attributed to the preselection of semen donors with sperm that are relatively resistant to cryoinjury (Thyer et al, 1999). Moreover, recent studies have determined that cryopreserved human sperm play a significant role in assisted reproductive technology in clinical practice but with decreased contribution to fertility (Kelleher et al, 2001; Wright et al, 2006). Significant effort has been expended to develop cryopreservation methods for nonhuman primate sperm (Morrell and Hodges, 1998), and offspring have been reported following artificial insemination of cryopreserved sperm in chimpanzees (Gould and Styperek, 1989), cynomolgus macaques (Tollner et al, 1990), rhesus macaques (Sanchez-Partida et al, 2000; Wolf et al, 2004; Yeoman et al, 2005), and marmoset monkeys (Morrell, 1997; Morrell et al, 1998).

The sperm cell is compartmentalized in terms of structure and function, and these compartments have different sensitivities to cryoinjury. Thus, cryoprotectants that preserve the genetic material during freezing and thawing may not preserve sperm functions required for sperm transport and survival in the female, fertilization, and preimplantation embryonic development (Rall, 1992). The combination of sperm cryopreservation and reproductive technology has the potential to obviate some of the problems that currently limit efficient production of offspring from cryopreserved nonhuman primate sperm. Already offspring have been born following in vitro fertilization (IVF) with cryopreserved sperm from marmosets (Holt et al, 1994) and gorillas (Pope et al, 1997), but the concentration of

Correspondence to: Dr Stuart Meyers, One Shields Avenue, Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616 (e-mail: smeyers@ucdavis.edu).

Received for publication June 5, 2006; accepted for publication January 17, 2007.
DOI: 10.2164/jandrol.106.000869

frozen spermatozoa required for efficient fertilization in vitro is higher than that of fresh sperm used for artificial insemination.

Intracytoplasmic sperm injection (ICSI), which requires only 1 spermatozoon per oocyte to effect fertilization, has been performed in rhesus macaques (Hewitson et al, 1996, 1998; Sutovsky et al, 1996; Meng and Wolf, 1997) and cynomolgus macaques (Ogonuki et al, 1998). Development of macaque embryos to the blastocyst stage following ICSI of nonfrozen sperm has been demonstrated (Hewitson et al, 1998), and offspring resulting from the transfer of ICSI-produced embryos have been reported (Hewitson et al, 1999; Chan et al, 2000). Cryopreserved human epididymal and testicular sperm have been used for ICSI with no recognized adverse effects on fertilization rates, embryonic development, or pregnancy rates (Friedler et al, 1998). It has been reported that chromosomal abnormalities are not increased in human sperm that survive cryopreservation, but these data are based on karyotype analysis of sperm that were capable of fusion with zona-free hamster oocytes (Martin et al, 1991). Other investigators have reported that human sperm chromatin stability is altered by cryopreservation (Hammadeh et al, 1999).

Although the use of ICSI will simplify the requirements for sperm cryopreservation protocols, the possibility of transmitting genetic damage by ICSI must be investigated because the technique bypasses the oocyte's natural ability to bind and select sperm. Evidence of increased genetic abnormalities following human ICSI has been reported (Bonduelle et al, 1995; In't Veld et al, 1995; Bonduelle et al, 1998). This concern is greater when cryopreserved sperm are used since unprotected freezing can damage sperm DNA (Rybouchkin et al, 1996). The primary objective of this study was to investigate the genetic damage caused by macaque sperm cryopreservation. Single-cell microgel electrophoresis (comet assay) was used to assess damage to sperm DNA, and sperm chromosome damage was assessed by karyotyping after ICSI of macaque sperm into mouse oocytes.

Materials and Methods

Experimental Design

Five rhesus macaques were used as semen donors. In a series of experiments, 3 ejaculates from each male were studied. Observations were made on the percentage of motile sperm (total motility and progressive motility) and the percentage of viable sperm in the fresh ejaculates. The percentage of comets was determined after single-cell microgel electrophoresis as a measure of the percentage of sperm with DNA damage in the fresh semen. Aliquots of each ejaculate were then preserved by a conventional cryopreservation method. After thawing of the cryopreserved semen, the percentages of motile sperm, viable sperm, and comets were again determined for comparison with the fresh ejaculate.

In another series of experiments, 3 ejaculates were collected from the same 5 males for sperm karyotype analysis. Motile sperm from the fresh ejaculates were injected into mouse oocytes, and the percentage of sperm with structural aberrations and aneuploidy were determined. Aliquots of these ejaculates were cryopreserved; after thawing, motile sperm were selected for ICSI and subsequent evaluation of the same cytogenetic end points.

Semen samples were obtained by electroejaculation from rhesus macaques (n = 5) as previously described (Sarason et al, 1991). Animals were housed at the California National Primate Research Center and maintained according to Institutional Animal Care and Use Committee protocols at the University of California.

Reagents

Fetal bovine serum (FBS) was purchased from Hyclone Laboratories Inc (Logan, Utah), Giemsa stain solution from Merck KGaA (Darmstadt, Germany), Dulbecco phosphate-buffered saline (DPBS) and disodium ethylenediamine-tetraacetic acid (Na_2-EDTA) solutions from Life Technologies (Rockville, Md), and all other chemicals from Sigma-Aldrich (St Louis, Mo), unless stated otherwise. Chemicals used in the comet assay, including Triton X-100, Tris base, Na_2-EDTA, DPBS, protease K, and laurylsarcosine (sodium salt), were DNase free.

Culture Media

The medium used for culturing mouse oocytes and embryos before and after ICSI was CZB medium (Chatot et al, 1990) supplemented with 5.56 mM D-glucose. The medium used for collection of oocytes from oviducts, for subsequent treatments of oocytes, and for sperm microinjection was HEPES-CZB containing 20 mM HEPES and 5 mM sodium bicarbonate and 0.1 mg/mL polyvinyl alcohol (Kimura and Yanagimachi, 1995). The medium used for slowing sperm prior to ICSI was 10% (w/v) polyvinyl pyrrolidine (PVP, 360 kd) in HEPES-CZB.

Preparation of Oocytes

Six- to 15-week-old B6D2F1 mice were obtained from Jackson Laboratory (Bar Harbor, Me) and induced to superovulate by intraperitoneal injection of 5 to 7 IU pregnant mare serum gonadotropin followed by 5 to 7 IU of human chorionic gonadotropin (hCG) 48 hours later. Oocytes were collected from oviducts 13 to 15 hours after hCG injection. Cumulus were removed by treatment with 300 U/mL bovine testis hyaluronidase in HEPES-CZB for 3 to 5 minutes. The oocytes were rinsed and kept in CZB for up to 4 hours at 37°C under 5% CO_2 in air before ICSI.

Preparation and Evaluation of Spermatozoa

All animals were housed at the California Regional Primate Research Center in compliance with the Federal Animal Welfare Act and the National Institutes of Health Guidelines for Care and Use of Laboratory Animals. The animals were maintained on a 12:12-hour light:dark cycle (lights on at 0600 hours) at 25°C to 27°C and were given a diet of Purina monkey chow and water ad libitum. The males were trained to chair restraints and were electroejaculated with a Grass 6 stimulator (Grass Medical Instruments, Quincy, Mass) equipped with electrocardiogram pad electrodes (Conmed Corp, Utica, NY) for direct penile stimulation (30–50 volts, 20-ms duration, 18 pulse/s) (Sarason et al, 1991). The semen was collected into sterile 15-mL plastic centrifuge tubes containing 5 mL of HEPES-BWW (Irvine Scientific, Santa Ana, Calif) without bovine serum albumin (BSA). The ejaculates were allowed to liquefy at room temperature for 1 hour, and the sperm suspensions were transferred to new centrifuge tubes. After the suspensions were washed twice by centrifugation at 300 × *g* for 10 minutes and resuspension in HEPES-BWW containing 0.3% BSA, sperm motility was assessed subjectively at 200× magnification using a phase-contrast microscope with warm stage (37°C). At least 200 sperm were counted in each sample to estimate the percentage of motile sperm and percentage of progressive sperm. Sperm with any evidence of flagellar activity were scored as motile; if the sperm was moving in a forward direction, it was scored as progressive. To assess sperm viability, 5 µL of sperm suspension was mixed with 5 µL of eosin Y (5 mg/mL in DPBS) on a slide. The drop was covered with a coverslip and examined after 30 seconds at 400× using a bright-field microscope with warm stage (37°C). At least 200 sperm were scored in 5 or more microscopic fields for viability. Unstained sperm were classified as live, and sperm stained red were scored as dead.

Cryopreservation of Spermatozoa

The method of Tollner et al (1990) was used for sperm cryopreservation. Macaque sperm preserved by this method have been used for artificial insemination to produce normal offspring (Tollner et al, 1990). TEST buffer (4.325% Tes, 1.027% Tris, 1.0 % dextrose [pH 7.4]) was supplemented with egg yolk obtained from freshly laid chicken eggs (30%, v/v) and commercial fluid skim milk (20%, v/v). The solution was inactivated by incubation at 56°C for 30 minutes and then centrifuged at 10 000 × *g* for 15 minutes. The supernatant was transferred to another tube and centrifuged again at 1000 × *g* for 30 minutes. The upper two thirds of the volume was collected, and 0.025% streptomycin sulfate and 0.015% penicillin G were added. The freezing medium was filtered through a 0.45-µm syringe filter and stored at −20°C before use.

Freezing medium was thawed in a 37°C water bath, and washed sperm were resuspended in the medium at a concentration of 1×10^8/mL. Then glycerol was added drop by drop to the sperm suspension and gently mixed until the final concentration of glycerol was 3%. For freezing without cryoprotectants, washed sperm were resuspended in DPBS. Aliquots (0.5 mL) of processed sperm suspension were transferred into sterile 1.2-mL cryogenic vials (Nalge Nunc International, Rochester, NY). Cryovials were sealed tightly and loaded onto canes, which were placed in the freezing chamber of a computer-controlled freezer (model 1010; Cryomed Ltd, Mansfield, United Kingdom). The sperm were cooled to 8°C at a rate of 0.2°C/min. Then the sperm sample temperature was rapidly lowered to −110°C in 6.8 minutes. After freezing, the canes were transferred immediately to a liquid nitrogen storage tank and were stored in liquid nitrogen for at least 3 days. Frozen samples were removed from liquid nitrogen and immediately thawed in a 37°C water bath. The percentages of motile sperm, progressive sperm, live sperm, and dead sperm in the extended semen were determined before and after freezing, as described above. The recoveries of motile and viable sperm were calculated as percentages of prefreeze motility and prefreeze viability.

ICSI

ICSI was carried out according to Kimura and Yanagimachi (1995) with some modifications. Holding pipettes and microinjection needles were prepared from borosilicate glass capillary tubes (Sutter Instrument Co, San Rafael, Calif) using a horizontal micropipette puller (model P-97; Sutter Instrument Co) and a Narishige microforge (Narishige Scientific Instrument Lab, Tokyo, Japan). The holding pipette had an external diameter of 50 to 60 µm and an internal diameter of 10 to 15 µm. The injection pipette was beveled at an angle of 20 degrees and had an internal diameter of 6 to 8 µm. Injection procedures were performed with an inverted Olympus IX70 microscope equipped with Hoffman modulation contrast optics, Narishige micromanipulators, and Piezo impact drive (PrimeTech, Ibaraki, Japan). The procedures were carried out at room temperature in 5-µL drops of CZB-HEPES medium covered with light mineral oil (Sigma-Aldrich) in the lid of a 100 × 15 mm tissue culture dish (BD Falcon, Franklin Lakes, NJ).

An aliquot of fresh semen or frozen-thawed extended semen was transferred to a drop of 10 % PVP in HEPES-CZB, and only progressively motile sperm were selected for ICSI. Sperm were picked up, tail first, into the injection pipette and then immobilized by applying a few piezo-pulses (intensity 2–4, speed 2–3) to the sperm tail. A mouse oocyte was fixed by the holding pipette at the 9-o'clock position, and the metaphase II spindle-chromosome complex was positioned either at the 12- or 6-o'clock position. Several piezo-pulses (intensity 2–4, speed 2–3) were applied to penetrate through the zona pellucida while a light negative pressure was applied to the zona. After the zona fragment was expelled from the injection pipette into the perivitelline space, the sperm was pushed forward and the tip of the pipette was advanced against the oolemma to the opposite side of the oocyte cortex. The oolemma was punctured by applying 1 or 2 piezo-pulses (intensity 1–2, speed 1–2), and the sperm was expelled into the ooplasm with a minimum amount of medium. After retrieving as much medium as possible, the pipette was gently withdrawn, leaving the sperm within the ooplasm. Ten to fifteen oocytes were injected with spermatozoa within 30 minutes after transfer of

the oocytes onto the operation dish. Sperm-injected oocytes were rinsed with CZB medium and incubated in this medium at 37°C under 5% CO_2 in air.

Chromosome Examination

Between 6 and 8 hours after sperm injection, oocytes in CZB medium were examined using an inverted phase-contrast microscope. Those oocytes with 2 distinct pronuclei and a second polar body were considered normally fertilized and were transferred to CZB medium containing 0.006 μg/mL vinblastine to prevent spindle formation and syngamy. The incubation was continued for an additional 12 to 15 hours when the oocytes were arrested at metaphase of the first cleavage. Oocytes with persistent pronuclei following incubation were counted and not treated further. Other oocytes were treated with 0.5 % (w/v) actinase (Kaken Pharmaceuticals, Tokyo, Japan) for 5 minutes to remove the zona pellucida. The oocytes were then treated with a hypotonic solution (1:1 mixture of 1% [w/v] sodium citrate and 30% FBS) for 10 minutes at room temperature.

Fixation and spreading of chromosomes were carried out according to the gradual fixation-air drying method of Mikamo et al (1994). Briefly, 5 to 10 oocytes with a small amount of hypotonic solution were placed gently on the bottom of a hollow glass slide filled with fixative I (methanol:acetic acid:water, 5:1:4). When the oocytes became transparent and began to move slowly, they were aspirated onto a clean glass slide. As the oocytes settled onto the slide, they were covered immediately by a gentle flow of fixative II (methanol:acetic acid, 3:1) so that the oocytes were tightly bound to the slide. The slide was placed into a jar filled with fixative II for 2 minutes. Finally, the slide was dipped into fixative III (methanol:acetic acid:water, 3:3:1) for 1 minute and then dried by warm air blown from a hair dryer. The chromosomes on the slides were stained with 2% (v/v) Giemsa solution in PBS (pH 6.8) for 10 minutes and analyzed with bright-field microscopy at 1000× . Mouse and rhesus monkey chromosome spreads were easily distinguished by their chromosome sizes (more uniform in mouse) and numbers of chromosomes (20 in mouse and 21 in rhesus monkey). Chromosome gaps and chromatid gaps were distinguished from chromosome breaks and chromatid breaks by the width of the staining discontinuity according to Brandriff et al (1984).

Comet Assay

The alkaline comet assay for DNA damage in spermatozoa was carried out according to Hughes et al (1996, 1997) with some modifications. Normal melting point agarose and low melting point agarose (Sigma-Aldrich) were dissolved in DPBS by boiling for 5 minutes. A 50-μL aliquot of 0.5% normal melting point agarose was pipetted onto a fully frosted slide (Fisher Scientific, Waltham, Mass) and spread by moving the end of another slide against it before air drying. A sperm-agarose mixture was prepared by mixing 10 μL of sperm suspension in HEPES-BWW with 90 μL of 0.5% low melting point agarose at 37°C to give a final sperm concentration of 1 million/mL. A 50-μL aliquot of sperm-agarose mixture was pipetted onto the agarose-coated slide and spread by covering with a No. 1 coverslip. The slide was maintained at 4°C for 10 minutes to allow the agarose to solidify, and then the coverslip was gently removed. Seventy-five μL of 0.5% low melting point agarose was added to the agarose gel and spread by covering with a No. 1 coverslip. After the agarose gelled, the coverslip was gently removed, and the slide was placed in freshly prepared lysing buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 1% Triton X-100, 1% laurylsarcosine sodium salt) for 1 hour at 4°C in the dark. Then the slides were treated at 37°C with protease K (200 μg/mL in lysis buffer) for 20 hours in the dark. After the protease K solution was drained from the slides, they were placed in a horizontal electrophoresis unit (Sub-Cell; Bio-Rad, Hercules, Calif) filled with freshly prepared alkaline electrophoresis buffer containing 300 mM NaOH and 10 mM EDTA for 20 minutes in the dark to allow the DNA to denature. Electrophoresis was performed at 20 V and 160 to 180 mA for 15 minutes. The slides were then neutralized in 0.4 M Tris-HCl (pH 7.5) and stained with 50 μL of 20 μg/mL ethidium bromide and mounted with a coverslip.

Two hundred sperm were scored per slide at 400× magnification using an epifluorescence microscope. Sperm nuclei with mobile fragments of DNA were classified as ''comets'' (Singh et al, 1988, 1989) (Figure 1). Overlapping sperm and sperm at the agarose edge were not scored. Fluorescence images of sperm processed in the comet assay were acquired using an AxioCam digital camera (Carl Zeiss Vision GmbH, Oberkochen, Germany) with an Olympus BX60 fluorescence microscope.

Statistical Analysis

Data were analyzed using a generalized linear mixed model (GLM analysis of variance) and Minitab statistical software (State College, Pa) on a PC computer. Due to heterogeneity of variance, sperm motility data were arcsin transformed prior to analysis; however, raw untransformed data are listed in Table 1. Results are expressed as mean ± SEM, and $P < .05$ was considered significant.

Results

Effects of Cryopreservation on Sperm Motility and Viability

The recovery of motility after freezing and thawing ranged from 50% to 72% and differed among individual males (Table 1). For all 5 males, the mean percentage of progressive sperm was lower than the total percent motility, but more than one third of the sperm were progressively motile after thawing in all cases (Table 1). The mean percentage of viable sperm was higher than the percentage of motile sperm, and the percent recovery of sperm viability after freezing and thawing was similar for all 5 males, ranging from 72% to 81% (Table 1).

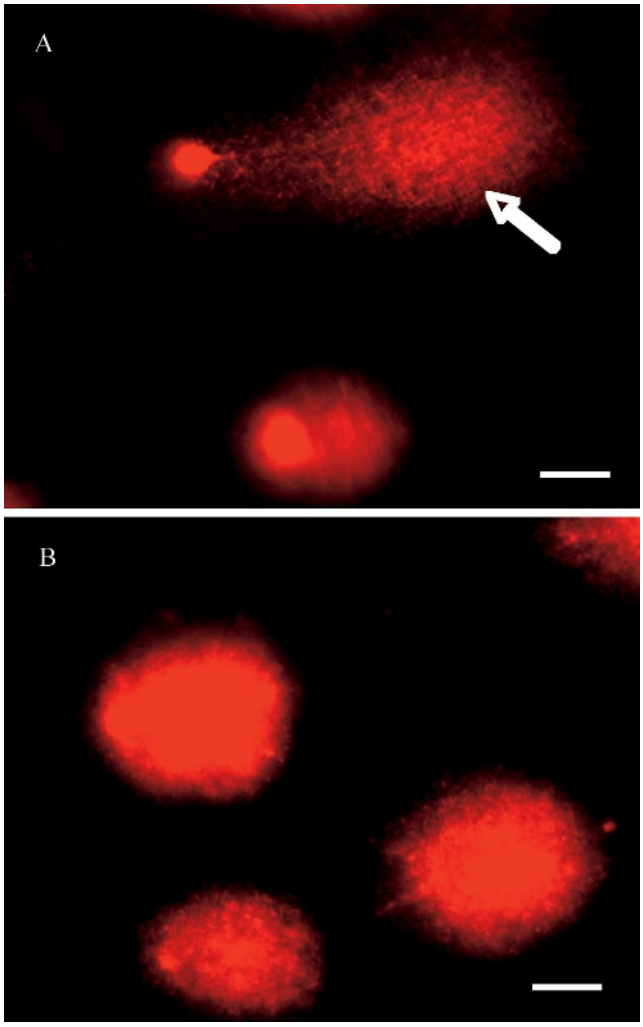

Figure 1. Evaluation of DNA damage in rhesus macaque sperm using the comet assay. **(A)** Two sperm, one of which appears as a DNA fragmented comet (arrow). **(B)** A few sperm with no evidence of comets. Scale bar = 10 μm.

Effects of Cryopreservation on Sperm DNA and Chromosome Structure

As shown in Table 2, fresh sperm from the 5 males had little evidence of DNA damage, with 0% to 3% of the sperm heads appearing as comets after electrophoresis (Table 2; Figure 1A). After cryopreservation and thawing, the percentage of comets increased greatly and ranged from a mean of 25% to 44% depending on the male (Table 2; Figure 1B). The semen samples frozen without cryoprotectants had even higher percentages of comets, with mean values ranging from 53% to 92% for individual males (Table 2).

The frequency of abnormal karyotypes was low in motile sperm from fresh ejaculates. The percentages of sperm with structural chromosomal abnormalities ranged from 0% to 6%, and the percentages of sperm with aneuploidies ranged from 0% to 8% (Table 3). The incidence of abnormal karyotypes was not significantly higher in motile frozen-thawed sperm than in fresh motile sperm (Tables 4 and 5). The structural abnormalities observed in fresh and frozen-thawed motile sperm included chromosome gaps, chromatid gaps, chromosome breaks, chromatid breaks, and chromosome exchanges (Figure 2). Chromosome breaks were the most common type structural abnormality encountered.

Discussion

Nonhuman primates, especially macaques, are studied as models of human disease, and it is important to develop strategies that can be applied for preserving unique individuals or groups of animals. The ultimate approach to preservation of some nonhuman primate models may be somatic cell cloning by nuclear transfer, as demonstrated in mice, but the efficiency of current technology is low (Wakayama et al, 1999). Storage of spermatozoa is an alternative approach that has been used extensively in numerous domestic livestock species and humans, and recently cryopreserved rhesus macaque sperm have been used for artificial insemination to produce healthy offspring (Sanchez-Partida et al, 2000; Wolf et al, 2004; Yeoman et al, 2005). Nevertheless, some males, including rhesus macaques, produce sperm samples with poor recovery of motile sperm following cryopreservation (unpublished observations),

Table 1. *Motility and viability of macaque sperm before and after cryopreservation**

Animal No.	Total Motility (%)			Progressive Motility (%)			Viability (%)		
	Fresh	Thaw	Recovery	Fresh	Thaw	Recovery	Fresh	Thaw	Recovery
21011	89.6 ± 3.9	55.7 ± 8.2	62.1	76.7 ± 3.6	44.3 ± 9.4	57.8	95.7 ± 2.0	69.0 ± 10.9	72.1
22613	91.7 ± 2.9	45.7 ± 3.6	49.8	87.3 ± 3.0	40.0 ± 2.7	45.8	94.0 ± 1.5	72.0 ± 1.5	76.6
22887	84.0 ± 5.1	59.6 ± 6.4	71.0	71.3 ± 8.3	45.3 ± 8.7	63.6	94.7 ± 3.0	76.3 ± 9.2	80.6
23022	80.7 ± 4.6	57.7 ± 2.4	71.5	65.0 ± 10.1	38.3 ± 2.0	59.0	90.0 ± 0.1	69.3 ± 3.4	77.0
26966	85.6 ± 3.2	61.6 ± 11.1	71.6	77.3 ± 4.3	52.0 ± 10.3	67.2	91.7 ± 2.0	65.0 ± 11.3	70.9
Average			65.2			58.7			75.4

* Data are mean values ± SEM. n = 3 ejaculates per male.

Table 2. *DNA strand breaks (percentage of comets) in macaque sperm before and after cryopreservation**

Animal No.	Fresh Sperm	Frozen With Cryoprotectants	Frozen Without Cryoprotectants
21011	2.67 ± 0.33	43.66 ± 7.51*	77.33 ± 2.40‡
22613	1.67 ± 0.67	25.33 ± 5.37*	52.67 ± 5.90‡
22887	0.00 ± 0.00	40.00 ± 2.31*	84.67 ± 6.39§
23022	1.00 ± 0.58	37.00 ± 9.54*	92.00 ± 3.79§
26966	1.33 ± 0.17	35.67 ± 1.78*	81.00 ± 9.50§

* Data are mean percentage of comets following microgel electrophoresis ± SEM. n = 3 ejaculates per male.
† Significantly different ($P < .01$) from percentage of comets of fresh sperm from the same male.
‡ Significantly different within columns ($P < .01$) from percentage of comets of frozen sperm with cryoprotectants from the same male.
§ Significantly different between columns ($P < .01$) from percentage of comets of frozen sperm with cryoprotectants from the same male.

Table 3. *Chromosome analysis of mouse oocytes injected with fresh motile macaque sperm*

Animal No.	No. of Ejaculates Used	No. of Oocytes Fertilized	No. of Oocytes Karyotyped	No. of Sperm With Structural Aberrations (%)	No. of Sperm With Aneuploidy (%)
21011	3	56	34	0 (0.0)	1 (2.9)
22613	3	47	25	0 (0.0)	0 (0.0)
22887	3	61	31	2 (6.5)	2 (6.5)
23022	3	65	36	2 (5.6)	3 (8.3)
26966	3	52	28	1 (3.6)	2 (7.1)

Table 4. *Chromosome analysis of mouse oocytes injected with frozen-thawed motile macaque sperm*

Animal No.	No. of Ejaculates Used	No. of Oocytes Fertilized	No. of Oocytes Karyotyped	No. of Sperm With Structural Aberrations (%)	No. of Sperm With Aneuploidy (%)
21011	3	51	31	2 (6.5)	2 (6.5)
22613	3	63	29	2 (6.9)	2 (6.9)
22887	3	56	34	1 (2.9)	2 (5.9)
23022	3	52	36	3 (8.3)	1 (2.8)
26966	3	43	24	0 (0.0)	1 (4.2)

Table 5. *Comparison of fresh motile and frozen-thawed motile sperm in chromosome aberrations*

Sperm Injected	No. of Males Used	No. of Ejaculates Used	No. of Oocytes Karyotyped	Sperm With Structural Aberrations,* % (Median ± SEM)	Sperm With Aneuploidy,* % (Median ± SEM)
Fresh motile	5	15	164	5 (3.14 ± 1.37)	8 (4.96 ± 1.53)
Frozen-thawed motile	5	15	154	8 (4.92 ± 1.52)	8 (5.26 ± 0.77)

* $P > .05$ in percentage of sperm with structural aberrations, percentage of sperm with aneuploidy, and X:Y sex ratio.

and ICSI has the potential to overcome this problem because the technique bypasses most steps in the fertilization process. However, it is unclear to what extent cryopreservation also damages the genetic material of nonhuman primate sperm and whether sperm can be selected for ICSI to minimize the risk of transmitting damaged DNA to progeny embryos.

Two methods were used in the present study for assessing cryopreservation damage to the genetic material of macaque spermatozoa. Damage to sperm DNA was assessed by single-cell microgel electrophoresis. This technique, known as the comet assay, was developed to detect DNA strand breaks induced by radiation (Ostling and Johanson, 1984). In this assay, nuclei from single cells are electrophoresed at alkaline pH and nuclei with DNA damage are recognized as ''comets,'' with mobile, fragmented DNA forming the comet tail (Singh et al, 1988, 1989). Studies of various cell types have indicated that the comet assay primarily detects single-strand breaks of DNA (Singh et al, 1988, 1989; Olive et al, 1990, 1992; Klaude et al, 1996). The comet assay has been applied previously in studies of human sperm (Singh et al, 1989; Aravindan et al, 1997), and in the present study we found that unprotected freezing

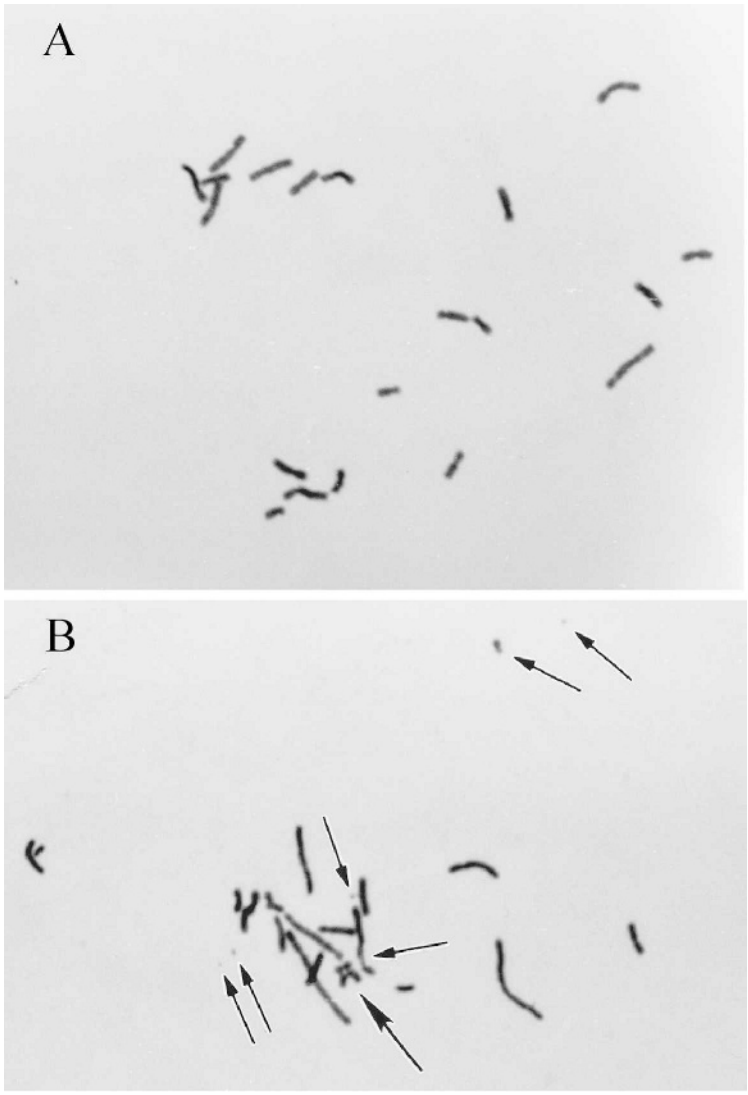

Figure 2. Chromosome spreads of rhesus macaque sperm following injection into mouse oocytes. **(A)** Normal karyotype (21-X). **(B)** Abnormal karyotype (21-Y). Large arrow shows a chromatid exchange (interchange). Small arrows show chromosome fragments.

resulted in 52% to 92% of sperm with evidence of DNA damage. Even when cryoprotectants were used, as many as 40% of sperm had DNA damage.

Damage to sperm chromosomes was evaluated by cytogenetic analysis after injection of motile macaque sperm into mouse oocytes. This method has been used previously for analysis of chromosomes in mouse (Kusakabe et al, 2001) and human sperm (Lee et al, 1996). The results of the present study showed that the percentage of sperm with chromosome damage was not increased in frozen-thawed sperm when motile sperm were selected for ICSI. It is not known whether the sperm with normal karyotypes were also free of other DNA damage because the comet assay cannot discriminate between viable and nonviable sperm.

Most studies that have evaluated frozen sperm in the nonhuman primate have used end points such as motility of sperm or fertilization rates in vitro following ICSI or IVF. With these methods, subpopulations of motile sperm within an ejaculate cannot be assessed with the exception of ICSI, and with the latter technique only very small numbers of sperm can be observed. In our study, we were able to evaluate specifically the motile sperm population for chromosomal damage using a heterologous ICSI method and cytogenetic analysis. Our results are in general agreement with those in mouse sperm reported by Kusakabe et al (2001) in that cryopreservation in the presence of cryoprotectants does not cause significant chromosomal damage to motile sperm to an extent greater than that of fresh motile

sperm, although cryopreservation results in overall increases in DNA damage, aneuploidy, and chromosome fragmentation.

Mechanisms for cryopreservation-induced DNA damage to sperm are likely to be multifactorial, but studies in other species have demonstrated that production of reactive oxygen species resulting from membrane lipid peroxidation may be a significant factor (Ball et al, 2001; Fraser and Strzezek, 2005; Jiang et al, 2005). Lipid peroxidation can also contribute directly to chromatin cross-linking, base changes, and DNA strand breaks (Hughes et al, 1996; Kodama et al, 1997; Twigg et al, 1998). Studies in equine sperm have demonstrated that cryopreservation contributes to DNA fragmentation as detected by the comet assay (Baumber et al, 2003).

In conclusion, the results of the present experiments demonstrated that protocols for cryopreservation of macaque sperm result in damage to sperm DNA. Our findings suggest that genetically competent frozen-thawed macaque sperm can be selected for fertilization by using only motile sperm for ICSI.

References

Aravindan GR, Bjordahl J, Jost LK, Evenson DP. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp Cell Res*. 1997;236:231–237.

Ball BA, Vo AT, Baumber J. Reactive oxygen species generation by equine spermatozoa. *Am J Vet Res*. 2001;62:5508–5515.

Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. *J Androl*. 2003;24:621–628.

Bonduelle M, Aytoz A, Van Assche E, Devroey P, Liebaers I, Van Steirteghem A. Incidence of chromosomal aberrations in children born after assisted reproduction through intracytoplasmic sperm injection. *Hum Reprod*. 1998;13:781–782.

Bonduelle M, Legein J, Derde MP, Buysse A, Schietecatte J, Wisanto A, Devroey P, Van Steirteghem A, Liebaers I. Comparative follow-up study of 130 children born after intracytoplasmic sperm injection and 130 children born after in-vitro fertilization. *Hum Reprod*. 1995;10:3327–3331.

Brandriff B, Gordon L, Ashworth L, Watchmaker G, Carrano A, Wyrobek A. Chromosomal abnormalities in human sperm: comparisons among four healthy men. *Hum Genet*. 1984;66:193–201.

Brotherton J. Cryopreservation of human semen. *Arch Androl*. 1990;25:181–195.

Chan AW, Luetjens CM, Dominko T, Ramalho-Santos J, Simerly CR, Hewitson L, Schatten G. Foreign DNA transmission by ICSI: injection of spermatozoa bound with exogenous DNA results in embryonic GFP expression and live rhesus monkey births. *Mol Hum Reprod*. 2000;6:26–33.

Chatot CL, Lewis JL, Torres I, Ziomek CA. Development of 1-cell embryos from different strains of mice in CZB medium. *Biol Reprod*. 1990;42:432–440.

Dresser BL. Cryobiology, embryo transfer, and artificial insemination in ex-situ animal conservation programs. In: Wilson EO, Peter FM, eds. *Biodiversity*. Washington, DC: National Academies Press; 1988;296–308.

Fraser L, Strzezek J. Effects of freezing-thawing on DNA integrity of boar spermatozoa assessed by the neutral comet assay. *Reprod Domest Anim*. 2005;40:530–536.

Friedler S, Raziel A, Soffer Y, Strassburger D, Komarovsky D, Ron-El R. The outcome of intracytoplasmic injection of fresh and cryopreserved epididymal spermatozoa from patients with obstructive azoospermia—a comparative study. *Hum Reprod*. 1998;13:1872–1877.

Gould KG, Styperek RP. Improved methods for freeze preservation of chimpanzee sperm. *Am J Primatol*. 1989;18:275–284.

Hammadeh ME, Askari AS, Georg T, Rosenbaum P, Schmidt W. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. *Int J Androl*. 1999;22:155–162.

Hewitson L, Dominko T, Takahashi D, Martinovich C, Ramalho-Santos J, Sutovsky P, Fanton J, Jacob D, Monteith D, Neuringer M, Battaglia D, Simerly C, Schatten G. Unique checkpoints during the first cell cycle of fertilization after intracytoplasmic sperm injection in rhesus monkeys. *Nat Med*. 1999;5:431–433.

Hewitson L, Takahashi D, Dominko T, Simerly C, Schatten G. Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. *Hum Reprod*. 1998;13:3449–3455.

Hewitson LC, Simerly CR, Tengowski MW, Sutovsky P, Navara CS, Haavisto AJ, Schatten G. Microtubule and chromatin configurations during rhesus intracytoplasmic sperm injection: successes and failures. *Biol Reprod*. 1996;55:271–280.

Holt WV, Wilson LJ, Marshall V. Cryopreservation of spermatozoa from the marmoset monkey (Callithrix jacchus). 1994. In: Bradley M, & Cummins J eds. *Proceedings of the 7th Symposium on Spermatology*. Cairns, Australia; 1994:17.

Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol Hum Reprod*. 1996;2:613–619.

Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. *Mut Res*. 1997;374:261–268.

In't Veld P, Brandenburg H, Verhoeff A, Dhont M, Los F. Sex chromosomal abnormalities and intracytoplasmic sperm injection. *Lancet*. 1995;346:773.

Jiang MX, Zhu Y, Zhu ZY, Sun QY, Chen DY. Effects of cooling, cryopreservation and heating on sperm proteins, nuclear DNA, and fertilization capability in mouse. *Mol Reprod Dev*. 2005;72:129–134.

Kelleher S, Wishart SM, Liu PY, Turner L, Di Pierro I, Conway AJ, Handelsman DJ. Long-term outcomes of elective human sperm cryostorage. *Hum Reprod*. 2001;16:2632–2639.

Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. *Biol Reprod*. 1995;52:709–720.

Klaude M, Eriksson S, Nygren J, Ahnstrom G. The comet assay: mechanisms and technical considerations. *Mut Res*. 1996;363:89–96.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril*. 1997;68:519–524.

Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. *Proc Natl Acad Sci U S A*. 2001;98:13501–13506.

Lee JD, Kamiguchi Y, Yanagimachi R. Analysis of chromosome constitution of human spermatozoa with normal and aberrant head morphologies after injection into mouse oocytes. *Hum Reprod*. 1996;11:1942–1946.

Martin RH, Chernos JE, Rademaker AW. Effect of cryopreservation on the frequency of chromosomal abnormalities and sex ratio in human sperm. *Mol Reprod Dev*. 1991;30:159–163.

Meng L, Wolf DP. Sperm-induced oocyte activation in the rhesus monkey: nuclear and cytoplasmic changes following intracytoplasmic sperm injection. *Hum Reprod*. 1997;12:1062–1068.

Mikamo K, Kamiguchi Y, Tateno H, Nishino T. Reliable chromosome studies of human oocytes and spermatozoa using the gradual fixation-air drying (GF-AD) method. In: Obe G, Natarajan AT eds. *Chromosome Alterations*. Berlin: Springer-Verlag; 1994: 252–261.

Morrell JM. Cryopreservation of marmoset sperm (Callithrix jacchus). *Cryoletters*. 1997;18:45–54.

Morrell JM, Hodges JK. Cryopreservation of non-human primate sperm: priorities for future research. *Anim Reprod Sci*. 1998;53: 43–63.

Morrell JM, Nubbemeyer R, Heistermann M, Rosenbusch J, Kuderling I, Holt W, Hodges JK. Artificial insemination in Callithrix jacchus using fresh or cryopreserved sperm. *Anim Reprod Sci*. 1998;52:165–174.

Ogonuki N, Sankai T, Cho F, Sato K, Yoshikawa Y. Comparison of two methods of assisted fertilization in cynomolgus monkeys (Macaca fascicularis): intracytoplasmic sperm injection and partial zona dissection followed by insemination. *Hum Reprod*. 1998;13: 2555–2560.

Olive PL, Banath JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the ''comet'' assay. *Rad Res*. 1990;122:86–94.

Olive PL, Wlodek D, Durand RE, Banath JP. Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp Cell Res*. 1992;198:259–267.

Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Comm*. 1984;123:291–298.

Pope CE, Dresser BL, Chin NW, Liu JH, Loskutoff NM, Behnke EJ, Brown C, McRae MA, Sinoway CE, Campbell MK, Cameron KN, Owens OM, Johnson CA, Evans RR, Cedars MI. Birth of a western lowland gorilla (Gorilla gorilla gorilla) following in vitro fertilization and embryo transfer. *Am J Primatol*. 1997;41:247–260.

Rall WF. Cryobiology of gametes and embryos from nonhuman primates. In: Wolf DP, Stouffer RL, Brenner RM, eds. *In Vitro Fertilization and Embryo Transfer in Primates*. New York, NY: Springer-Verlag; 1992;223–245.

Rybouchkin AV, De Sutter P, Dhont M. Unprotected freezing of human spermatozoa exerts a detrimental effect on their oocyte activating capacity and chromosome integrity. *Zygote*. 1996;4: 263–268.

Sanchez-Partida LG, Maginnis G, Dominko T, Martinovich C, McVay B, Fanton J, Schatten G. Live rhesus offspring by artificial insemination using fresh sperm and cryopreserved sperm. *Biol Reprod*. 2000;63:1092–1097.

Sarason RL, Vandevoort CA, Mader DR, Overstreet JW. The use of nonmetal electrodes in electroejaculation of restrained but unanesthetized macaques. *J Med Primatol*. 1991;20:122–125.

Sherman JK. Cryopreservation of human semen. In: Keel BA, Webster BW, eds. *CRC Handbook of the Laboratory Diagnosis and Treatment of Infertility*. Boca Raton: Fla: CRC Press; 1990;229–259.

Singh NP, Danner DB, Tice RR, McCoy MT, Collins GD, Schneider EL. Abundant alkali-sensitive sites in DNA of human and mouse sperm. *Exp Cell Res*. 1989;184:461–470.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988;175:184–191.

Sutovsky P, Hewitson L, Simerly CR, Tengowski MW, Navara CS, Haavisto A, Schatten G. Intracytoplasmic sperm injection for Rhesus monkey fertilization results in unusual chromatin, cytoskeletal, and membrane events, but eventually leads to pronuclear development and sperm aster assembly. *Hum Reprod*. 1996;11: 1703–1712.

Thyer AC, Patton PE, Burry KA, Mixon BA, Wolf DP. Fecundability trends among sperm donors as a measure of donor performance. *Fertil Steril*. 1999;71:891–895.

Tollner TL, Vandevoort CA, Overstreet JW, Drobnis EZ. Cryopreservation of spermatozoa from cynomolgus monkeys (Macaca fascicularis). *J Reprod Fertil*. 1990;90:347–352.

Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod*. 1998;13:1429–1436.

Wakayama T, Yanagimachi R. Development of normal mice from oocytes injected with freeze-dried spermatozoa. *Nat Biotechnol*. 1998b;16:639–641.

Wolf DP, Thormahlen S, Ramsey C, Yeoman RR, Fanton J, Mitalipov S. Use of assisted reproductive technologies in the propagation of rhesus macaque offspring. *Biol Reprod*. 2004;71: 486–493.

Wright VC, Chang J, Jeng G, Macaluso M. Assisted reproductive technology surveillance—United States, 2003. *MMWR Surveill Summ*. 2006;55:1–22.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*. New York, NY: Raven Press; 1994;189–317.

Yeoman RR, Mitalipov S, Gerami-Naini B, Nusser KD, Wolf DP. Low temperature storage of rhesus monkey spermatozoa and fertility evaluation by intracytoplasmic injection. *Theriogenology*. 2005;63:2356–2371.