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Postmortem recovery and cryopreservation of spermatozoa from the vas deferens of rhesus macaques (*Macaca mulatta*)

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Abstract

To determine whether sperm derived from the vas deferens could be retrieved and successfully cryopreserved, testes were collected from 20 rhesus macaques (*Macaca mulatta*). The males ranged in age from 3 to 19 years with an average of 8.5 years. No sperm was obtained from three animals which were younger than 4 years. The remaining 17 samples contained sperm with an average sperm cell number of $421.8 \pm 88.7 \times 10^6$ and an average motility of $72.8 \pm 4.4\%$.

After 24 h of culture in TALP medium at 37° C in 5% CO₂ and 95% air, the overall motility decreased significantly in all samples regardless of treatment. Freezing in TEST-yolk buffer containing 6% (vol/vol) glycerol had a significant effect on sperm reducing the immediate post-thaw motility to 42.4% in non-treated samples. Treatment with db-cAMP and caffeine further reduced sperm motility after 4 hr in fresh sperm (72.8 vs 50.4%), but increased in sperm that had been frozen (14.0 vs 23.2%). The age of the male did not influence sperm concentration or grade, but proved to be a significant factor in determining motility of frozen-thawed treated sperm with lower motility found in samples from older males. Overall the study demonstrates that motile sperm can be obtained from post-mortem males, although subsequent studies will have to determine whether the quality is sufficient to facilitate its use in assisted reproduction.

Keywords

rhesus monkey; sperm; cryopreservation; vas deferens

1. Introduction

Rhesus macaques (*Macaca mulatta*) are an important species for biomedical research because of their physiological and anatomical similarity to humans and their use is likely to be of more importance now that the rhesus genome has been sequenced [1].

The ability to obtain and store sperm from non-human primates has many significant applications for animal conservation as well as for management of captive populations that are used in research. The availability of cryopreserved sperm can accommodate the transfer of rare or valuable genomes between facilities or from wild populations into captive ones, thereby

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circumventing the costly and stressful transport of animals. Because sperm can be cryopreserved virtually indefinitely, it constitutes a safeguard against loss of genetic diversity in cases where populations are decimated by disease or other catastrophic events.

The availability of sperm has also enabled the development of methods to generate in vitro embryos in a number of non-human primate species. Such efforts have implications not only for conservation but also for the development of novel techniques that may facilitate genetic manipulations of non-inhuman primates, which in turn, will likely broaden the utility of some of these species for biomedical research [2–4].

Recovery of vas deferens-derived sperm from rhesus macaques constitutes an alternative approach to preserve genetic diversity, as it would facilitate the salvaging of genetic resources when valuable males suddenly die or are scheduled for euthanasia. Because there is little information on whether sperm can be obtained from monkeys in this way, a preliminary study was conducted to assess the viability of non-ejaculated spermatozoa retrieved from rhesus macaques during necropsy examination.

The purpose of this study, therefore, was to assess the possibility of retrieval of sperm from the vas deferens of rhesus macaques, to assess its quality, longevity after retrieval as well as the possibility of its cryopreservation with particular reference to the age of the males from which the sperm were retrieved.

2. Materials and methods

2.1. Sperm collection, cryopreservation and culture

Testes were collected from 20 male rhesus monkeys presented to the Division of Comparative Pathology at the Tulane National Primate Research Center. The animals ranged from 3 to 19 years of age and had been part of studies on SIV vaccines (15), Lyme disease (2) or were breeding animals that had developed health-related problems including neoplasm and pneumonia (3). In each case the complete male reproductive tract, from the testes to the prostate, was placed in jars containing sterile saline and transported to the laboratory within 30 to 60 minutes after death. The vasa deferentia were separated from the testis, and the upper part of each vas was grasped with forceps and the connection with the epididymis cut. The sperm was then squeezed out of the vas with a second pair of forceps. The sperm was diluted into a final volume of 3 mL of TL HEPES [5] and counted to determine the sperm cell concentration. One half of each sample was then re-suspended in TALP [5] and cultured at 37°C in a 5% CO₂-95% air incubator after initial motility and concentration had been determined [6]. The other half of each sample was frozen according to a previously described method [7]. In brief, samples were diluted in TEST-yolk buffer [8] to a concentration of motile cells of $50 \times 10^6/\text{mL}$. The sperm samples were then diluted further with TEST-yolk solution containing 6% (vol/vol) glycerol to yield a final glycerol concentration of 3% and a final sperm concentration of $25 \times 10^6/\text{mL}$. Sperm was then loaded into 0.25 cc straws (Zander Medical Supply, Vero Beach, FL) and cooled to 4°C by placing them into a beaker containing 500 mL of water at room temperature, which was then transferred into a refrigerator for 2 hours. The straws were further cooled in liquid nitrogen vapor by placing them for 10 min on a 5 cm thick Styrofoam platform that was floating in liquid nitrogen. The straws were then plunged into liquid nitrogen and stored for a minimum of 48 hours.

After removal from liquid nitrogen each straw was briefly placed in a beaker containing water at 37°C. The sperm was washed by expelling it into a 15 mL conical tube containing 5 mL of TL-HEPES. The cells were then pelleted by centrifugation and re-suspended in 200 µL of TALP, motility and grade measurements were taken and the sperm was placed into the incubator. In each case, half of the fresh and frozen-thawed samples were further divided into

two groups one of which was treated with 1mM dibutyryl-cAMP (db-cAMP) and 1mM caffeine to induce activation [9]. After placement into the incubator, motility measurements and motility grades were taken at 1, 4, 12 and 24 h for non-treated and at 4, 12 and 24 h for treated sperm. Sperm grading was performed on a modified scale of 1 to 3, with 3 denoting rapid forward movement and 1 indicating no forward movement [10].

Statistical Analysis

Random effects models were used to analyze the relationship of motility to time, age, initial motility, and grade. For the most part only random intercept models proved useful instead of random intercept-slope models because there were only three observations for most observational units (animal x procedure). All statistical analyses were carried out on R2.2.1, and the random effects analyses were performed using the contributed package nlme. [11,12]

Because of the likely strong within-animal correlations of motilities, contrasts in motility due to treatments were examined by considering paired differences of motilities for identical animals at identical times.

Because the motility variables are essentially proportions, a standard transformation (arcsine of the square root) was used to stabilize variances and bring the motility variables closer to normality. While all calculations were carried out both with the original motility variables as well as with the transformed values, for ease of interpretation, the magnitude of effects are presented only from calculations using the transformed values. Finally, to avoid prejudging the direction of any effect, all p-values are two sided.

To examine differences in motility due to treatment at each time, and to check whether these differences changed in time, paired t-tests on pairs of motility variables were conducted. Random intercept models were fit to verify the dissipation of the contrasts established with the paired t-tests. In this analysis the difference between two variables was regressed against time to determine whether the difference between observations remain consistent or declined over time. Finally, we examined the relation of certain initial motility variables and declines in motility to age.

3. Results

Sperm was successfully retrieved from both vasa deferentia of 17 males that were older than 4 years, while none of the males younger than 4 years yielded any sperm cells. The 17 samples contained on average $421.8 \pm 88.7 \times 10^6$ with a range of 24 to 1072×10^6 sperm cells, an average motility of $72.8 \pm 4.4\%$ and a mean grade of 2.6 ± 0.15 .

Initially we determined the effects of various factors on the level of motility over time. As expected, there was a significant decline in motility over time, which was seen in fresh as well as frozen-thawed samples (Figure 1). Treatment with db-cAMP and caffeine did not alter this decline and motility decreased similarly in treated samples of both fresh and frozen-thawed sperm. (Figure 2) Initial motility at the onset of culture proved to be a determinant of long-term motility only for thawed sperm, while it did not seem to be associated with long-term motility in fresh sperm. Similarly, the initial grade appeared to affect motility over time only in treated fresh sperm where a lower grade of 3 was associated with a lower level of motility (Table 1).

Freezing significantly lowered the motility in non-treated samples and modified the response to treatment, although this difference only lasted throughout the first 12 hours and had disappeared by 24 h (Table 2 and Figures 1).

Treatment with db-cAMP and caffeine also resulted in lowered motility by 4 h among fresh, but in increased motility among frozen-thawed samples. However, by 12 h these differences had again disappeared (Figure 2)

The age of male had a significant effect on motility of non-treated fresh ($p=0.016$) and of frozen-thawed ($p=0.048$) sperm (Figures 3A and B) with higher motility in sperm of older males. However, age was a significant factor in the decrease of motility (between $t = 0$ and $t = 4$) in frozen-thawed treated sperm (Figure 4).

4. Discussion

This study was designed to assess the feasibility of retrieving rhesus macaque sperm post mortem from the vas deferens and to determine whether such sperm could be successfully cryopreserved. The results show that this is, indeed, possible and this approach may contribute an important mechanism for management of captive or endangered populations. Our results confirm and expand the findings of Dong *et al.* [13] who found a similarly high initial and post-thaw motility of epididymal rhesus sperm. Motility of spermatozoa collected from the vas deferens in this study proved to be relatively high and comparable to that of sperm obtained by electroejaculation [see e.g. 7], although there was considerable variation between males with motility ranging from 35 to 90%.

Similar results have been obtained after retrieval of non-ejaculated sperm from many species including mice, rats, cats, rabbits, red deer, alpaca and monkeys [14–20] and retrieval of epididymal sperm has been an effective treatment of azoospermia in men [21–23]. The relatively high motility seen among the present samples probably reflects the fact that sperm was obtained from the vas deferens. This may be an important consideration because a number of sperm characteristics including cryosurvival can vary significantly with location of retrieval when sperm is obtained from the epididymis [24,25]. The observation that the vasa deferentia of males younger than 4 years did not yield any sperm cells is consistent with the onset of male puberty in this species [reviewed by 26].

The motility of sperm was significantly reduced after freezing, but this response differed considerably among individuals with a range from 10 to 50%. Such a highly variable response to freezing is not uncommon and has been observed by others in ejaculated rhesus sperm [7, 27]. Those studies have shown that while ejaculates from the same male seem to respond consistently to cryopreservation, samples from different animals do not. To further complicate this matter, we have recently reported that there is a significant interaction between cryopreservation protocol and sperm from individual males and testing several protocols revealed that none was consistently superior [28].

Such individual differences in the response to freezing protocols have also been reported in other species [reviewed in 29] including humans [30,31], although it has not been explained. There have been strain specific responses to cryopreservation between mouse strains, but not within individuals of inbred strains [32–34]. It is noteworthy that the cryosurvival of sperm has been linked to genetic markers in boars, where certain restriction fragment length polymorphisms seem to be predictive of cryopreservation outcome [35,36]. Identification of such markers obviously implies a genetic component which could be useful for the identification of individuals most likely to produce viable post-thaw sperm.

One factor that may have contributed to the relatively high initial motility of fresh samples in the present study was likely the fact that sperm was collected immediately after death. This may not always be possible and in some instances the testes may not be accessible until several hours after death. Most of the studies on the quality of epididymal sperm have come from human fertility clinics where such sperm has occasionally been used post-mortem to produce

pregnancies, although this procedure has significant legal and ethical implications [37,38]. Recommendations usually suggest retrieval within 24 h after death, although viable sperm has been retrieved up to 36 h postmortem [39,40]. Studies in mice and sheep have similarly shown that highly motile sperm can be obtained within 24 hours when epididymides are stored at room temperature [41,42]. Moreover, evidence from several other species including mice and deer indicates that storage of epididymal tissues at 4 °C appears to significantly extend the time during which motile sperm can be harvested [43,44].

An interesting observation of this study is that age appeared to be associated with increased initial motility of non-treated sperm, but a significantly decreased motility among treated frozen-thawed samples. Age-induced changes in female gametes are well understood, but the effects of age on sperm have not received much attention. Nevertheless, in humans, age-related changes in the testes have been well described and include a decrease in the number of Leydig cells with a concomitant reduction in the testosterone level [45,46]. It is well documented that male fertility persists much longer than female fertility and, indeed, males in most species can produce offspring well beyond the age at which this is still possible among females. However, the widely held view that sperm quality remains virtually unchanged as males age has in recent years been challenged. Several studies have shown that chromosomal abnormalities increase with age in human males [47,50], although it is not entirely clear to what degree this may affect overall fertility. Some studies have suggested that pregnancy rates in women are lower when male partners are older [51–53], while others have failed to find any such direct effects [54].

The effects of aging on sperm quality in rhesus macaques have not been investigated. Sexual activity has been observed in very old males that ranged in age up to 27 years [55]. Nevertheless, overall sexual activity tends to decrease with age but there does not appear to be a concomitant decrease in the level of gonadal hormones [55,56].

The observation that this age-induced decline was restricted to treated samples is intriguing. *In vitro* treatment of sperm with db-cAMP and caffeine to initiate activation is associated with numerous changes including altered metabolism, motility, ion concentration and membrane fluidity [reviewed by 26,57]. How age may affect any of these parameters and lower the motility of treated sperm is at present unclear.

Ideally, frozen-thawed sperm should be usable for artificial insemination although it is doubtful that the relatively short survival time of thawed samples in this study would be sufficient to accommodate sperm migration to the oviduct. However, assisted reproduction in the form of *in vitro* fertilization and subsequent embryo transfer has made significant progress during the past years and production of rhesus infants from such procedures is now common in many primate centers [58–62]. Furthermore rhesus embryos and infants have been produced by intracytoplasmic sperm injection (ICSI), a procedure that involves injection of sperm into the cytoplasm and therefore does not require motile sperm at all [10,59].

Based on the results presented here and the work of others, it is clear that retrieval of non-ejaculated sperm may be an effective method to preserve male genotypes of rhesus macaques. Further studies will be necessary to develop and refine existing freezing protocols and to determine the efficacy of this method to produce offspring.

Acknowledgments

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Figure 1. Mean motility (± SEM) of fresh and frozen-thawed sperm incubated at 37°C for 1, 4, 12 and 24 hours.

^{a,b}, Columns with different letters within the same time points differ significantly (P<0.05)

Figure 2. Mean motility ($\pm$ SEM) of fresh and frozen-thawed sperm over time following Treatment with db-cAMP and caffeine and incubation at 37°C for 4, 12 and 24 hours.
a,b, Columns with different letters within the same time points differ significantly ($P < 0.05$)

A. **B**

Figure 3.
Effects of age on initial motility of fresh (A) and frozen-thawed (B) sperm

Figure 4.
Effects of age on the decline in motility of frozen-thawed treated sperm

Table 1

The effects of various factors on sperm motility over time

Treatment group	Factors	Association	P value
Fresh sperm	Time	negative	0.00
Thawed sperm	Initial motility	positive	0.001
	Time	negative	0.00
Treated fresh sperm	Initial grade of 3	negative	0.03
	Time	negative	0.00
Treated thawed sperm	Initial motility	positive	0.039
	Age of male	negative	0.030
	Time	negative	0.00

Table 2

The effects of freezing on non-treated and treated semen

Non-treated sperm	Effect of freezing	P values, original data [*]	Mean difference in motility (%)
Initial motility	negative	0.000	28.53
1 hr	negative	0.000	39.76
At 4 hr	negative	0.000	58.76
12 hr	negative	0.009	15.71
24 hr	none	0.141	4.76
Treated sperm			
4 hr	negative	0.001	27.18
12 hr	negative	0.040	10.24
24 hr	none	0.0895	2.4

^{*} p-values given are two sided