ANIMAL EXPERIMENTATION

Electroejaculated Baboon (*Papio anubis*) Sperm Requires a Higher Dosage of Pentoxifylline to Enhance Motility¹

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Purpose: Sperm collected by electroejaculation often show poor motility. The objective was to determine whether the addition of the phosphodiesterase inhibitor, pentoxifylline, would stimulate electroejaculated baboon sperm motility.

Methods: Electroejaculation was performed on several occasions on a male baboon and sperm collected after familiarization. Pentoxifylline was tested at the standard concentration (1 mg/ml) and at twice the concentration. Sperm parameters were evaluated using a sperm motility analyzer, as well as acrosome and DNA integrity techniques.

Results: Sperm exposed to 2 mg/ml pentoxifylline had higher total motility when compared with the control and 1 mg/ml treatment. Rapid progression and velocities were higher after pentoxifylline. The acridine orange DNA normality test showed that over 90% of collected sperm had intact unfragmented DNA. About half the sperm population had normal morphology and intact acrosomes. A low percentage had cytoplasmic droplets.

Conclusions: Sperm collected by rectal probe electroejaculation required a higher concentration (2 mg/ml) of pentoxifylline for enhanced total motility, rapid progression, and higher velocity. This suggested differences in membrane properties or phosphodiesterase activity in electroejaculated

sperm. The electroejaculation procedure did not denature sperm DNA at the acridine orange assay level nor were the acrosomes disrupted. The present study also documented unique information on baboon kinematic parameters.

KEY WORDS: Baboon sperm; computer-aided sperm analyzer (CASA); electroejaculation; phosphodiesterase inhibitor.

INTRODUCTION

Electroejaculation for the collection of sperm is an accepted clinical technique that does not require surgery. However, information on enhancing the quality of electroejaculated sperm for assisted reproductive technology is lacking. An animal model of electroejaculation has been developed based on a nonhuman primate, the baboon. Among the nonhuman primate species, limited data are available on only two species of baboon, *Papio anubis* and *Papio ursinus* (1, 2). To collect baboon sperm, electroejaculation is employed. However, the main problem of electroejaculated sperm is the poor motility of the cells (3). Good motility is necessary for all aspects of assisted reproductive technology, including cryopreservation of sperm derived from endangered species (3).

In nonhuman primate in vitro fertilization, on the day of insemination of harvested oocytes (4) the availability and quality of sperm are important factors in determining the success of fertilization. In this study, the objective was to determine whether the addition of the phosphodiesterase inhibitor, pentoxifylline, would improve electroejaculated baboon sperm motility. The data collected will help clinicians and scientists considering strategies for improving the quality of electroejaculated sperm for assisted reproduction technology and

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for animal breeding, conservation, and gamete banking.

MATERIALS AND METHODS

Sperm Processing

Semen samples were collected from a single baboon by electroejaculation using a Standard Precision Electronics stimulator (Standard Precision Electronics, Inc., Littleton, CO) and a baboon rectal probe (2-cm diameter) with three strip electrodes (3 mm $\times$ 100 mm) that were 1 cm apart. For electroejaculation, the baboon was placed in lateral recumbency after sedation with 5 mg/kg of ketamine/HCl intramuscularly. The rectal probe was coated with K-Y jelly and inserted gently in the pelvic rectum with electrodes positioned ventrally. Rhythmic pulse stimulations were applied at a constant voltage (5). The stimulation was carried out in pulses of 3- to 5-sec duration with approximately 1-sec rest between pulses. Three series of eight to ten pulses were applied and the voltage level was increased gradually between the series. The probe was adjusted inside the rectum in order to obtain maximal erection of the penis and the penis was milked during the procedure to get maximal emission of sperm from the urethra. Ejaculates were collected with about a week of rest between collections. Once the baboon was accustomed to periodic electroejaculation (2 months), the test ejaculate was collected directly into 50-ml tube (Fisher Scientific, Los Angeles, CA) containing prewarmed 0.5 ml human tubal fluid medium (mHTF) (Irvine Scientific, Irvine, CA) supplemented with 15% synthetic serum substitute (SSS) (Irvine Scientific, Irvine, CA) and examined microscopically (light microscope 400 $\times$ magnification) within 30 min of collection. Sperm density was measured using a hemocytometer. Motility was determined by visual observation of semen. Viability of spermatozoa was assessed by utilizing dye exclusion test. The sperm was stained with 3% eosin Y (Sigma, St. Louis, MI) and dead sperm failing to exclude the dye appeared red. A total of over 100 cells were counted for each parameter each time.

Computer-Aided Sperm Analyses

Sperm kinematic parameters were measured using the Hamilton-Thorn motility (HTM-C, Hamilton Thorn Research, Danvers, MA) computer-aided sperm analyzer system. The analyses were carried out using

10- μ l aliquots of the sperm in 20- μ m deep glass slides with coverslips. Video recording of the calibrating grids and sperm in different treatments were made and the information processed through the HTM-C analyzer. A minimum of 100 sperm cells were analyzed each time. Analyses were carried out after 30 min of incubation at 37°C. The sperm kinematic parameters measured have been previously reported (6, 7) and include amplitude of lateral head displacement (ALH), total motility, beat cross-frequency (BCF), linearity, average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), and head dimensions.

Spermac Staining for Morphology and Acrosome

A drop of each sperm specimen was removed at the end of incubation and an air-dried smear was made for the Spermac acrosome procedure as previously reported (6). Briefly, each dried smear was fixed (5 min, 23°C) in formalin solution (fixative I) provided in the Spermac kit (Stain Enterprises, Onderstepoort, S. Africa, distributed by Fertility Technologies, Natick, MA). Each slide was washed through stain solutions A, B, and C (1 min, 23°C). The stained slides were air-dried and analyzed under oil immersion ($\times$ 1500) for the percentage of acrosome intact sperm. Sperm with intact acrosomes have normal oval-shaped heads with the anterior acrosomal region staining dark green and postacrosomal region staining red-pink. Sperm lacking acrosome have white or red anterior acrosomal heads. Sperm heads that showed peeled acrosomal membranes or partial green coloration were considered acrosome defective. The percentage of sperm with intact acrosome was calculated by dividing the number of sperm with dark green acrosome over the total number of sperm multiplied by 100.

Acridine Orange Test Procedure

A drop of each sperm suspension was smeared on a clean glass slide and air-dried. After 20 to 30 min, the smear was immersed in freshly made Carnoy's solution (3:1 methanol:glacial acetic acid) for 24 hr. The glass slides with smears were briefly washed in water and immersed in 0.02% acridine orange in citrate-phosphate buffer solution, pH 2.5, for 2 min (8). The stained slides were rinsed in water and covered with coverslips. The acridine orange-stained sperm were examined in an ultraviolet (UV) fluorescent microscope at 500 $\times$ magnification. A total of 100 cells were analyzed for each type of processed sperm.

Sperm undergoing apoptosis where the DNA became fragmented or denatured to single strands stained orange-red at the head, while healthy sperm with double-stranded DNA stained green (8). The percentage of sperm with intact DNA was calculated by dividing the number of green sperm over the total sperm and multiplying by 100.

The Effect of In Vitro Incubation with Pentoxifylline on Sperm Motion Characteristics

The capacitation medium used in this study was Hepes-buffered mHTF, (pH 7.4, 282 mOsmole/ml) (Irvine Scientific, Irvine, CA) supplemented with 15% SSS (Irvine Scientific, Irvine, CA). A stock solution of 4 mg/ml pentoxifylline (Sigma Chemical Co., St. Louis, MO) was prepared in mHTF medium and filter sterilized. Pentoxifylline from the stock solution was added to the experimental tubes containing sperm to achieve a final concentration of 0, 1, or 2 mg/ml. The sperm were incubated for 30 min at 37°C. The duration of incubation was based on previous reports (7, 9, 10). After incubation, the sperm kinematic parameters were measured using the HTM-C automated sperm motility digital image analyzer (see above).

Data Analyses

The results are presented as mean $\pm$ 1 SD. Differences between means were analyzed by the Student's *t* test. Categorical data such as the number of denatured sperm after acridine orange testing were evaluated using the χ^2 test statistic. A *P* < 0.05 value was considered significant.

RESULTS

Sperm cells incubated in the presence of 2 mg/ml pentoxifylline showed significantly higher total motility when compared with the untreated (0 mg/ml) and the 1 mg/ml treatment groups (Table I). There was no difference in total sperm motility between the untreated and 1 mg/ml pentoxifylline treatment. This was interesting, as the 2 mg/ml concentration showed an effect but not the 1 mg/ml concentration, the latter being the standard dose reported for stimulating sperm (9, 10). In terms of head dimensions, there was no significant difference among the treatment groups.

The number of cells showing rapid progressive motility was significantly higher in the treated groups

than in the untreated sperm. The percentage of sperm showing rapid progressive motility was lower in the presence of 1 mg/ml pentoxifylline when compared with sperm exposed to the higher concentration. Furthermore, the percentage of sperm exhibiting medium and slow forward progressive motility were higher in the 2 mg/ml pentoxifylline versus the 1 mg/ml treatment group. There were no differences in sperm velocities between the 1 mg/ml and 2 mg/ml treatment groups. There were no differences in the remaining kinematic parameters.

The concentration, motility, and viability data are presented in Table II. The volume of semen collected was approximately 200 μ l (few drops) after each electroejaculation. A second ejaculate was sometimes obtainable, but generally the quality was lower. The acridine orange DNA normality test (Table II) showed that over 90% of the electroejaculated sperm have intact unfragmented DNA, suggesting that electroejaculation was a nondestructive technique.

In terms of sperm morphology, the percentage of baboon sperm with normal morphology was about $49.5\% \pm 0.5$, well above the criteria (11) for normal human sperm morphology (30% by World Health Organization standard). Sperm in the categories of pin-head, tapered head, or duplicate were not observed. Interestingly, a low percentage of sperm with cytoplasmic droplets was observed, and this has never been reported to the best of knowledge. In terms of sperm with intact acrosomes, about half the population of sperm had intact unreacted acrosomes at the time of electroejaculation collection.

DISCUSSION

The phosphodiesterase inhibitor, pentoxifylline, enhanced the total motility, rapid progression, and velocity of electroejaculated sperm only when the concentration used was twice that of the concentration normally used (7, 9, 10) for stimulating sperm before assisted reproductive technology procedures. The 1 mg/ml standard concentration had no effect on the motility of electroejaculated sperm. The reason for the effect seen with the higher concentration remains unknown. It is possible that sperm derived from the electroejaculation technique may have different membrane properties or phosphodiesterase readiness state, hence the necessity for a higher concentration of pentoxifylline to observe an effect. The increase in intracellular cyclic adenosine 3',5'-monophosphate (cAMP) due to pentoxifylline also cause a three- to

Table I. Concentration Effect after 30 Min Incubation in the Presence of the Phosphodiesterase Inhibitor, Pentoxifylline, on Baboon (*Papio anubis*) Electroejaculated Sperm (Mean $\pm$ 1 SD)

Parameter	Units	0 mg/ml	1 mg/ml	2 mg/ml
Motility	%	39.5 $\pm$ 1.5 ^a	33.5 $\pm$ 2.5 ^b	57.0 $\pm$ 8.0 ^{a,b}
Progressive	%	3.5 $\pm$ 0.5 ^{a,b}	17.0 $\pm$ 2.0 ^a	16.0 $\pm$ 4.0 ^b
% Rapid	%	7.5 $\pm$ 3.5 ^{a,b}	23.5 $\pm$ 1.5 ^a	26.5 $\pm$ 5.5 ^b
% Medium	%	32.5 $\pm$ 1.5 ^a	10.0 $\pm$ 4.0 ^a	30.0 $\pm$ 2.0
% Slow	%	18.0 $\pm$ 1.0 ^{a,b}	4.5 $\pm$ 4.5 ^a	11.5 $\pm$ 5.5 ^b
% Static	%	42.0 $\pm$ 1.0 ^a	62.0 $\pm$ 7.0 ^{a,b}	32.5 $\pm$ 2.5 ^b
Average path velocity	μ m/sec	21.0 $\pm$ 2.0 ^{a,b}	42.0 $\pm$ 4.0 ^a	32.5 $\pm$ 1.5 ^b
Curvilinear velocity	μ m/sec	27.0 $\pm$ 1.0 ^{a,b}	62.5 $\pm$ 3.5 ^a	45.0 $\pm$ 0.0 ^b
Straight-line velocity	μ m/sec	16.5 $\pm$ 1.5 ^{a,b}	33.0 $\pm$ 0.0 ^a	24.5 $\pm$ 0.5 ^b
Linearity	%	66.0 $\pm$ 0.0	61.0 $\pm$ 0.0	56.5 $\pm$ 0.5
Lateral head disp.	μ m	1.2 $\pm$ 0.1	3.0 $\pm$ 0.4	2.0 $\pm$ 0.0
Beat cross freq.	Hertz	9.0 $\pm$ 0.4	9.6 $\pm$ 2.2	12.8 $\pm$ 0.3
Width of head	μ m	4.4 $\pm$ 0.3	3.8 $\pm$ 0.0	4.2 $\pm$ 0.0
Length of head	μ m	10.6 $\pm$ 2.6	6.3 $\pm$ 0.7	9.3 $\pm$ 0.0
Total analyzed		218	110	149

^{a,b} Values in rows with similar superscripts are different, $P < 0.05$.

fourfold increase in rapid progressive motility. Rapid progression has been linked to an increased fertilizing capacity of the sperm. Caffeine and cAMP have been reported to enhance nonhuman primate sperm motility (12–14). Caffeine and pentoxifylline are the two most widely used phosphodiesterase inhibitors in human fertility treatments (7, 9, 15, 16).

A unique feature of this study was documenting for the first time the kinematic parameters of baboon sperm. This was accomplished using the video recorder to record sperm tracks in a phase contrast microscope followed by analysis using the computer-aided sperm analyzer (CASA). The analyzer identified and tracked individual sperm cells, and thus calculated the parameters characterizing the “kinematic” of sperm motion. CASA provided improved precision of sperm motion

analysis compared to subjective visual assessments (11). At present, the kinematic values of sperm are of limited value, and the association between each parameter and fertilizing capacity or prediction for pregnancy are still being studied.

Another aspect of the study involved demonstrating that the rectal probe electroejaculation procedure itself did not fragment sperm DNA as shown by the acridine orange assay data. It has been shown that there is a good correlation between the percentage of sperm cells with unfragmented or undenatured DNA and increased reproductive potential, independent of an intact cell membrane, normal mitochondria, or motility (8). The condensed DNA in the sperm tightly bound to protamines were not denatured by the electrical current used in the electroejaculation technique here. Although subtle alterations in DNA cannot be ruled out, the study suggests that electroejaculation does not denature sperm DNA at the acridine orange binding level.

Sperm specimens collected by electroejaculation can be used, among other things, for fertility evaluation, studies on comparative sperm morphology and fertilization, phylogeny, or assisted reproductive technologies (17). Rectal probe electroejaculation has been linked to some retrograde ejaculation, and this possibility may account for variations in ejaculates. However, the focus of this study was on the sperm cells in the ejaculates and was independent of the possible retrograde condition.

The present study also showed that a high percentage of sperm had intact acrosome, which was necessary for the fertilization event. Studies using the Spermac acrosome stain showed that a fertile specimen would

Table II. Basic Semen Parameters After Electroejaculation of Baboon (*Papio anubis*)

Parameter	Results
Sperm density	68.0 $\pm$ 38.0 million/ml
Total motility	34.5 $\pm$ 14.5%
Sperm viability	69.5 $\pm$ 5.5% live
DNA normality	97.0 $\pm$ 1.0%
Acrosome intact	50.5 $\pm$ 4.5%
Morphology	
Normal forms	49.5 $\pm$ 0.5
Head defects	5.0 $\pm$ 2.0
Pinheads	0.0 $\pm$ 0.0
Tapered heads	0.0 $\pm$ 0.0
Duplicates	0.0 $\pm$ 0.0
Neck defects	3.0 $\pm$ 3.0
Tail defects	41.0 $\pm$ 2.0
Cytoplasmic droplets	2.5 $\pm$ 2.5

have over 45% of the sperm with intact acrosomes (6). The acrosome reaction is a membrane vesicle reaction that begins by intermittent fusion of the plasma membrane of the sperm and its outer acrosome membrane (18, 19). The vesiculation process releases a number of trypsinlike enzymes and includes hyaluronidase and acrosin. Hyaluronidase on the sperm surface may be responsible for the cumulus being penetrated by a sperm; acrosin helps to facilitate passage through the zona. Chan and colleagues (6) showed that there is a significant correlation between the percentage of human sperm with intact acrosome and fertilizing capacity. In the present study, the electroejaculation technique yielded specimens with a high percentage of sperm with intact acrosomes, suggesting that the fertilizing potential was not disrupted by the electroejaculation process. In summary, the electroejaculation technique was demonstrated as a safe and simple procedure for sperm collection and the technique itself did not denature sperm DNA or disrupt intact acrosomes. Enhancement of sperm motility required twice the standard concentration of pentoxifylline, suggesting that electroejaculated sperm membrane properties or phosphodiesterase activity was different from that in sperm collected by other means.

REFERENCES

1. Bornman MS, M van Vuuren, Meltzer DGA, van der Merwe CA, Rensburg SJ: Quality of semen obtained by electroejaculation from Chacma baboon (*Papio ursinus*). *J Med Primatol* 1987;17:57–61
2. Chowdury AK, Steinberger E: A study of germ cell morphology and duration of spermatogenic cycle in the baboon, *Papio anubis*. *Anat Rec* 1976;185:155–170
3. Rall WF: Cryobiology of gametes and embryos from nonhuman primates. In *In Vitro Fertilization and Embryo Transfer in Primates*. DP Wolf, RL Stouffer, M Brenner (eds). Springer-Verlag. Serono Symposium, 1993, pp 223–245
4. Hatasaka HH, Schaffer NE, Chenette PE, Kowalski W, Hecht BR, Meehan TP, Wentz AC, Valle RF, Chatterton RT, Jeyendran RS: Strategies for ovulation induction and oocyte retrieval in the lowland gorilla. *J Assist Reprod Genet* 1997;14:102–110
5. Gould KG, Warner H, Martin DE: Rectal probe electroejaculation of primates. *J Med Primatol* 1978;7:213–222
6. Chan PJ, Corselli JU, Jacobson JD, Patton WC, King A: Correlation between intact sperm acrosome assessed using the Spermac stain and sperm fertilizing capacity. *Arch Androl* 1996;36:25–27
7. Pang SC, Chan PJ, Lu A: Effects of pentoxifylline on sperm motility and hyperactivation in normozoospermic and normokinetic semen. *Fertil Steril* 1993;60:336–343
8. Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S: A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril* 1984;42:87–91
9. Rees JM, Ford WCL, Hull MGR: Effect of caffeine and of pentoxifylline on the motility and metabolism of human spermatozoa. *J Reprod Fertil* 1990;90:147–156
10. Sikka SC, Hellstrom WJ: The application of pentoxifylline in the stimulation of sperm motion in men undergoing electroejaculation. *J Androl* 1991;12:165–170
11. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction, 3rd ed. Cambridge, England, The Press Syndication of the University of Cambridge, 1992
12. Glover TD, Barratt CLR, Tyler JPP, Hennessey JF: Human Male Fertility and Semen Analysis. New York, Academic Press, 1990
13. Wolf DP, Thomson JA, Zelinski-Wooten MB, Stouffer RL: In vitro fertilization–embryo transfer in nonhuman primates: Technique and its applications. Review article. *Mol Reprod Dev* 1990;27:261–280
14. Chan PJ, Hutz RJ, Dukelow WR: Nonhuman primate in vitro fertilization: Seasonality, cumulus cells, cyclic nucleotides, ribonucleic, and viability assays. *Fertil Steril* 1982;38:609–615
15. Bavister BD, Boatman DE, Leibfried L, Loose M, Vernon MW: Fertilization and cleavage of rhesus monkey oocytes in vitro. *Biol Reprod* 1983;28:983–999
16. Harrison RF: Insemination of husband's semen with and without the addition of caffeine. *Fertil Steril* 1978;29:532–534
17. Clayton O, Kuehl TJ: Successful in vitro fertilization and embryo transfer in a nonhuman primate. *Theriogenology* 1984;21:228–
18. Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z: Presence of DNA strands breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: Analogy to apoptosis of somatic cells. *Exp Cell Res* 1993;207:202–205
19. Yanagimachi R: Mammalian fertilization. In *The Physiology of Reproduction*, 2nd ed, E Knobil, JD Neil (eds). New York, Raven Press, pp 189–317