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Sperm Mitochondrial Integrity Is Not Required for Hyperactivated Motility, Zona Binding, or Acrosome Reaction in the Rhesus Macaque[1]

Pei-hsuan Hung,[3,6] Marion G. Miller,[4] Stuart A. Meyers,[5] and Catherine A. VandeVoort[2,3,6]

Molecular, Cellular, and Integrative Physiology,[3] Pharmacology and Toxicology,[4] Department of Anatomy, Physiology, and Cell Biology,[5] and California National Primate Research Center,[6] University of California, Davis, Davis, California 95616

ABSTRACT

Whether the main energy source for sperm motility is from oxidative phosphorylation or glycolysis has been long-debated in the field of reproductive biology. Using the rhesus monkey as a model, we examined the role of glycolysis and oxidative phosphorylation in sperm function by using alpha-chlorohydrin (ACH), a glycolysis inhibitor, and pentachlorophenol (PCP), an oxidative phosphorylation uncoupler. Sperm treated with ACH showed no change in percentage of motile sperm, although sperm motion was impaired. The ACH-treated sperm did not display either hyperactivity- or hyperactivation-associated changes in protein tyrosine phosphorylation. When treated with PCP, sperm motion parameters were affected by the highest level of PCP (200 μM); however, PCP did not cause motility impairments even after chemical activation. Sperm treated with PCP were able to display hyperactivity and tyrosine phosphorylation after chemical activation. In contrast with motility measurements, treatment with either the glycolytic inhibitor or the oxidative phosphorylation inhibitor did not affect sperm-zona binding and zona-induced acrosome reaction. The results suggest glycolysis is essential to support sperm motility, hyperactivity, and protein tyrosine phosphorylation, while energy from oxidative phosphorylation is not necessary for hyperactivated sperm motility, tyrosine phosphorylation, sperm-zona binding, and acrosome reaction in the rhesus macaque.

acrosome reaction, sperm, sperm capacitation

INTRODUCTION

Mammalian spermatozoa are highly specialized cells. One of the distinct features of sperm is motility generated by the elongated flagellum. The motility of sperm is essential to transport the genetic materials to the site of fertilization. The percentage of motile sperm is used as one of the major indices in fertility clinics to determine whether an individual man is fertile. The source of ATP in support of sperm motility has been long debated in the field of gamete research. The two major mechanisms that have been considered for energy production in spermatozoa are oxidative phosphorylation from the mitochondria and glycolysis in the principal piece of the flagellum. Both of these possibilities have been investigated many times, but the results remain inconclusive.

The mitochondria are located in the midpiece of the flagellum and have been thought to supply the energy to drive the flagellum. In support of mitochondrial involvement in sperm motility, studies have shown that bull sperm motility is correlated with oxygen consumption [1]. Moreover, men with mitochondrial DNA mutations related to oxidative phosphorylation have reduced sperm motility [2]. Bull sperm were able to sustain motility under aerobic condition when glycolysis was inhibited [3], and inhibition of respiratory chain of ATP synthesis in the mitochondria by an uncoupling agent decreased sperm motility in bovine samples [4, 5]. An early study has also shown that human sperm can obtain energy from oxidative phosphorylation, and because of its high efficiency, the net yield of ATP might play an important role in sperm physiology [6]. However, whether the ATP produced by the mitochondria can be delivered in sufficient quantities to supply the entire flagellum is still under debate [7–9].

Glycolytic enzymes are located mainly in the principal piece and in the fibrous sheath of the flagellum in a variety of mammalian species including rabbits, mice, and foxes [10–12]. A recent study used a proteomic approach to identify a sperm flagellar energy carrier protein along with seven glycolytic enzymes in the fibrous sheath in the principal piece of human sperm [13]. These findings support the theory that the ATP produced adjacent to the site of energy requirement can effectively support the demand. Glycolysis plays a significant role in the energy production in boar sperm [14]. In stable isotope and radiotracer studies with glucose, boar sperm readily converted glucose to glucose-6-phosphate and lactate via glycolysis with no involvement of pentose phosphate metabolism [14]. Another study showed that mice with a gene knockout for the sperm specific isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have immotile sperm, which supports the importance of glycolysis in sperm motility [15]. In addition to its role in sperm motility, in some studies glucose was found to be involved in sperm capacitation in mice since ATP produced by glycolytic pathways was sufficient to support capacitation associated sperm tyrosine phosphorylation. [16, 17]. However, other studies showed that ram and mouse sperm treated with the GAPDH inhibitor, α-chlorohydrin (ACH), can maintain motility and ATP concentration for at least 1 h without external glucose, indicating that glycolysis is not required to support normal motility [18, 19]. Another study also showed that when mouse sperm were incubated in a medium without glucose for 90 min, the percentage of sperm showing capacitation and progressive motility as well as values for curvilinear velocity and linearity were not different from the sperm in medium with glucose [20]. Such species differences suggest that mechanisms underlying sperm ATP production and sources may differ between species.

Previous studies have been mostly focused on whether oxidative phosphorylation or glycolysis supports sperm motility by measuring the consumption of glycolysis substrates or oxygen for oxidative phosphorylation. In the present study,

[1]Supported by Philip Morris External Research Program, NIH grant RR00169, and by R01 RR016581.
[2]Correspondence: Catherine A. VandeVoort, California National Primate Research Center, Roads 98 and Hutchison, University of California, Davis, CA 95616. FAX: 530 752 2880;
e-mail: cavandevoort@ucdavis.edu

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we used specific inhibitors to these processes to examine whether glycolysis or oxidative phosphorylation is the major energy source supporting sperm motility in rhesus monkey sperm. ACH, which inhibits the glycolytic enzymes GAPDHs and triosephosphate isomerase (TPI) in sperm to prevent ATP production from glycolysis, was a potential male contraceptive until its epididymal toxicity as well as neurotoxic effect and bone marrow damage were found [21–25]. Pentachlorophenol (PCP) is an oxidative phosphorylation inhibitor and has been used to study sperm motility in other species [26, 27]. With these chemicals, the importance of glycolysis or oxidative phosphorylation can be more easily assessed. In addition to sperm motility, capacitation, sperm-zona binding, and zona-induced acrosome reaction were also examined to provide more information about the role of glycolysis and oxidative phosphorylation in rhesus monkey sperm physiology.

MATERIALS AND METHODS

Sperm Collection and Washing

Three fertile adult male rhesus macaques were caged individually at the California National Primate Research Center in compliance with the Federal Animal Welfare Act and the NIH Guidelines for Care and Use of Laboratory Animals. The animals were maintained on a 12L:12D light cycle (lights on at 0600 h) at 25~27°C and were given a diet of Purina monkey chow and water ad libitum. The males were trained to chair restraint and were electroejaculated with a Grass 6 stimulator (Grass Medical Instruments, Quincy, MA) equipped with EKG pad electrodes (Conmed Corp., Utica, NY) for direct penile stimulation (30~50 V, 20-msec duration, 18 pulse/sec) [28]. Semen samples, collected in 50-ml centrifuge tubes, were allowed to stand for 30 min at 22°C before the coagulum was removed. Each semen sample was processed separately. Semen was transferred to a 15-ml tube and washed three times for 10 min at $300 \times g$ with 15 ml Tyrode medium (TL-BSA) that was modified and supplemented with 3 mg/ml bovine serum albumin and lactate [29] and resuspended in media described later. For some experiments, semen was split into two 15-ml tubes. One was washed with TL-BSA containing glucose, and the other was washed with TL-BSA without glucose (TL-BSA-NG).

Experimental Design

Sperm were suspended in TL-BSA (with or without glucose), with ACH (0.1~5 mM with or without glucose), or pentochlorophenol (PCP, 50~200 μM) at a concentration of 10×10^6 sperm per milliliter. Sperm concentration was determined with a hemocytometer. Sperm from each male were treated with different chemicals (randomized complete block design) and were incubated for 1 h at 37°C in 5% CO_2 before assessment. Sperm were evaluated immediately after incubation for motility, CASA analysis, and mitochondrial integrity. Sperm were then activated by incubating with 1 mM dbcAMP (Alexis Chemicals, San Diego, CA) and 1mM caffeine (Sigma Chemical Co., St. Louis, MO) for an additional 30 min [30]. Further sperm motility, CASA analysis, and sperm-zona binding assay were performed.

Assessment of Percentage of Motile Sperm

A sperm was considered motile when it displayed any movement in this study. The percentage of motile sperm (% motility) was determined by visual observation at 200× magnification of 100 sperm using an Olympus BH2 microscope (Scientific Instruments Co., Sunnyvale, CA). Prior to treatment, semen samples used in these experiments had greater than 90% motile sperm, of which 90% had forward progression. After 1 h of treatment and after 30 min of activation, a 10-μl aliquot of sperm suspension was placed onto a slide and overlaid with a 22-mm^2 cover slip, and the slide was maintained at 37°C during analysis by a heated stage, and the percentage of motile sperm was evaluated [30].

CASA Assessment of Sperm Motion Parameters

After either 1 h of treatment or 1 h of treatment followed by 30 min of activation, a 10-μl aliquot of sperm suspension was placed onto a microscope slide and overlaid with a 22-mm^2 cover slip, and the slide was maintained at 37°C during analysis by a heated stage. Sperm motility of at least 10 fields at 37°C was recorded onto videotape using a VHS recorder (Panasonic Co., Secaucus, NJ) utilizing a CCD video camera (MTI Technology Co., Tustin, CA) mounted on an Olympus BH2 microscopy. A dark-field 4× objective was used, and the total magnification for videotaping was 40. The VHS tape video was transformed to a digital videocassette for analysis. At least 200 cells per sample in a minimum of four fields were analyzed by computer-automated sperm analysis (CASA) utilizing HTM Ceros (Version 12.2 g; Hamilton-Thorne Research, Beverly, MA). Instrument settings for the CASA analysis were as follows: frame rate 60 Hz, frames acquired 30, minimum contrast 80, minimum cell size four pixels, static VAP cutoff 20 μ/sec, static VSL cutoff 10 μ/sec, progressive VAP threshold 25 μ/sec, progressive STR threshold 80%, static intensity limits 0.6–1.4, static size limits 0.6–2.31, and static elongation limits 0–80. Average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), and percentage of hyperactivated sperm (HYP) were determined. The threshold to determine the hyperactivated sperm is for VCL $\geq$ 130 μm/sec, ALH $\geq$ 7.5 Hz, and LIN $\leq$ 69% [31, 32].

Evaluation of Mitochondrial Integrity and Changes of Membrane Potential

Live mitochondria were labeled with 200 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) for 30 min after 1 h of ACH or PCP treatment in glucose-containing media. A 10-μl sample of each sperm suspension was placed onto a glass slide and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Sperm images were taken with Provis Production Microscopy at California National Primate Research Center. With the same field, DAPI and MitoTracker Red images were taken by using a 40× objective lens. The resolution of each image was set to 300 dpi. The DAPI and MitoTracker Red images were combined to create one fluorescent image. Sperm mitochondria showing red fluorescent labeling were intact. Sperm that underwent several freeze–thaw cycles served as negative control.

To evaluate the changes of mitochondrial membrane potential, sperm were labeled with potential-sensitive 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes) for 10 min after 1 h of ACH or PCP treatment, and after 30 min of chemical activation. Sperm then were fixed with 50 μl 4% formaldehyde, and the supernatant was removed and the sperm were resuspended in DPBS. The percentage of sperm with green (inactive mitochondria, no membrane potential), bright orange (active mitochondria, high membrane potential), or faint orange staining was evaluated visually by using Olympus BH2 fluorescence microscopy, and a total of 100 sperm were scored in each treatment.

Measurement of Intracellular ATP

After 1 h incubation with ACH or PCP, sperm protein was precipitated by addition of ice-cold 100% ethanol (1:3.3 dilution, final 70%). The samples were vortexed, ultrasonically homogenized, and dried by liquid nitrogen. The samples were resuspended in dH_2O, and ATP was measured by the luminometric methods as previously described [33] by using Adenosine 5′-triphosphate (ATP) Bioluminescent Assay Kit (Sigma) on a Turner Luminometer (TE20).

Sperm-Zona Binding and Zona Pellucida-Induced Acrosome Reaction

Sperm-zona binding experiments were performed as previously described [30]. Briefly, sperm were activated by incubating with 1 mM dbcAMP (Alexis Chemicals) and 1 mM caffeine (Sigma) for 30 min. Then, two to four oocytes were placed in 100-μl drops of sperm suspension under silicone oil (Aldrich Chemical Co., Milwaukee, WI). After 30 sec of coincubation, the oocytes were quickly rinsed in DPBS and immediately fixed in cold absolute ethanol for 1–2 min. After fixation, oocytes were placed on glass slides, and sperm bound to the zona pellucida were labeled by indirect immunofluorescence using a polyclonal antisperm antiserum produced and employed as described [34]. The number of sperm on the zona pellucida and percentage of acrosomal reacted sperm were evaluated by using Olympus BH2 fluorescence microscopy.

Immunofluorescence Labeling for Protein Tyrosine Phosphorylation

The percentage of sperm with tyrosine phosphorylation was evaluated as described previously [32]. Briefly, sperm were washed three times in DPBS containing 1 mg/ml polyvinyl alcohol (Sigma) after activation, fixed 10 min in 2% paraformadehyde, permeabilized with cold 95% ethanol for 10 min, blocked for 10 min in 5% BSA in DPBS, incubated in 4G10 monoclonal

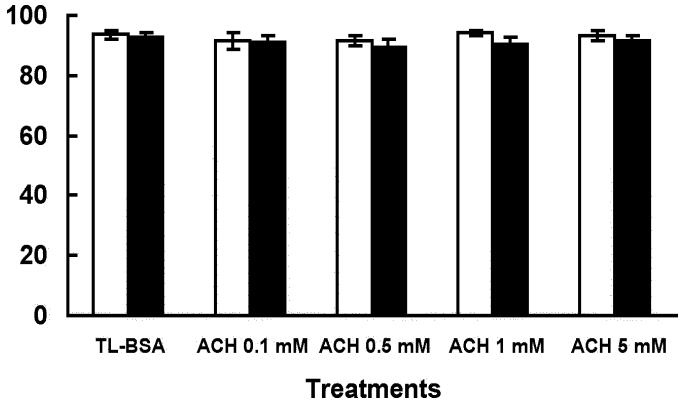

FIG. 1. Percentage of motile sperm after 1 h of ACH treatment in glucose (open bars) or nonglucose media (closed bars). The percentage of motile sperm after 1 h of incubation showed no difference among groups in both treatments. Data are from four males and presented as mean ± SEM.

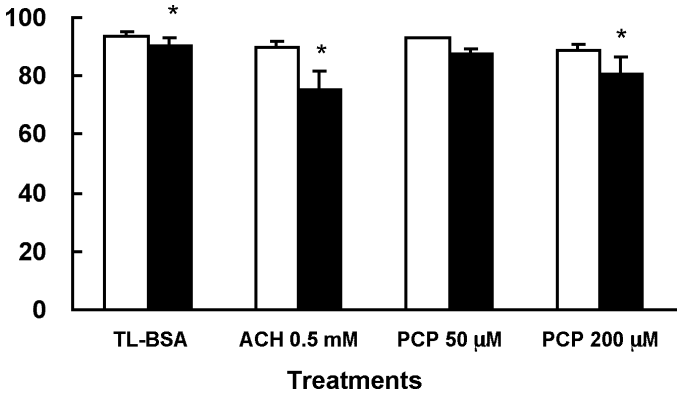

FIG. 2. Percentage of motile sperm after 1 h of ACH or PCP treatment (open bars) and after 30 min of chemical activation (closed bars) in glucose-containing media. The percentage of motile sperm after 1 h of incubation showed no difference among groups. After chemical activation, the ACH 0.5-mM treatment group showed a significant decrease of percentage of motile sperm compared to other treatment groups. Data are from three males and presented as mean ± SEM. *, $P < 0.05$ when comparing mean differences between preactivation and postactivation.

antibody (dilution, 1:500) (Upstate USA, Inc., Charlottesville, VA) at 4°C overnight, and then incubated with fluorescein-conjugated goat anti-mouse (Fab) IgG (Molecular Probes) (dilution, 1:32) for 1 h at room temperature in the dark. One hundred sperm were scored with Olympus BH-2 fluorescence microscopy for the percentage of sperm with tyrosine phosphorylation.

Statistical Analysis

All data are expressed as mean ± SEM. For all data, *N* represents the number of male monkeys used for each experiment. The data were examined by two-way analysis of variance when comparing the mean differences among treatment groups. Paired *t*-test was used when comparing the mean differences between pre- and postactivation. A two-sample *t*-test was used when comparing the mean differences between glucose and no-glucose treatments.

RESULTS

Percentage of Motile Sperm

After 1 h of treatment, sperm treated with ACH in glucose or nonglucose media showed no significant difference in the percentage of motile sperm when compared to the TL-BSA control groups (Fig. 1). It should be noted that this manual measurement of percentage motility does not necessarily indicate changes in motion characteristics. However, visually it could be seen that sperm treated with 0.5 mM and 5 mM ACH in glucose-containing media showed slower forward progression when compared to other groups. The 0.5 mM ACH was used treat sperm to compare to the PCP-treated sperm for the rest of the experiments in glucose-containing media. When sperm were treated with ACH or different levels of PCP in glucose-containing media, the percentage of motile sperm did not show any significant change when compared to the TL-BSA control group (Fig. 2). There was no change in percentage motility observed in both PCP treatment groups compared to the control group. Sperm were then chemically activated by dbcAMP and caffeine for 30 min, and the percentage of motile sperm was evaluated. After activation, the percentage of motile sperm decreased in TL-BSA control, ACH 0.5-mM, and PCP 200-μM treatment groups. Although there were changes compared to preactivation in some groups, none of the treatment groups showed significant differences compared to the TL-BSA control group after activation (Fig. 2).

Sperm Motion Parameters by CASA

Detailed CASA analysis of sperm motion parameters was carried out after 1 h of treatment and after 1 h of treatment followed by 30 min of incubation with the activators (dbcAMP and caffeine) The CASA data of ACH treated sperm in glucose or nonglucose media are shown in Table 1. Sperm treated with different level of ACH in glucose-containing media for 1 h had dramatically decreased ($P < 0.05$) VAP, VSL, VCL, LIN, and HYP compared to the TL-BSA control group, while in sperm treated with a different level of ACH in nonglucose media for 1 h, only in the highest ACH level (0.5 mM) was the sperm motion affected (VCL, $P < 0.05$). After activation, sperm treated with a different level of ACH in glucose-containing media did not show hyperactive motion, while sperm in nonglucose media showed changes only in the 5-mM ACH treatment group (VAP, ALH, and HYP) when compared to the TL-BSA control group.

Table 2 depicts a comparison between the effect of ACH and PCP on CASA values in glucose-containing media. Similarly to the data reported in Table 1, sperm treated with 0.5 mM ACH in glucose-containing medium for 1 h had dramatically decreased ($P < 0.05$) VAP, VSL, VCL, ALH, and BCF compared to the TL-BSA control group (Table 2). The PCP 200-μM treatment group also showed a significant decrease ($P < 0.05$) of VAP, VSL, VCL, and ALH. The sperm motion parameters of the PCP 50-μM treatment group were similar to those of the TL-BSA control. After activation, the sperm in the TL-BSA control group demonstrated changes consistent with hyperactivity, such as an increase in VCL, ALH, and HYP and a decrease in VSL ($P < 0.05$), BCF, STR ($P < 0.05$), and LIN ($P < 0.05$). The ACH treatment group showed no signs of hyperactivated motility at all, and the sperm motion parameters did not display any changes compared to before activation. The PCP 50-μM treatment group displayed hyperactivity after activation, and the motion parameters and HYP were similar to those of the TL-BSA control. The PCP 200-μM treatment group displayed some impairments of hyperactivity after activation, such as low VCL and ALH as well as high STR, and the HYP was significantly lower when compared to the TL-BSA control group.

Mitochondrial Integrity and Membrane Potential

The MtoTracker Red accumulates and fluoresces in active sperm mitochondria after 30 min of incubation. Sperm treated with TL-BSA and 50 μM PCP in glucose-containing media

TABLE 1. Computer assisted semen analysis of sperm treated with ACH in glucose or non-glucose media (data are presented as mean ± SEM; N = 3).

Treatment	VAP (μm/sec)	VSL (μm/sec)	VCL (μm/sec)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)	HYP (%)
CASA analysis after 1 h treatment								
With glucose								
TL-BSA	134.20 ± 7.77^{a}	126.73 ± 8.31^{a}	179.30 ± 5.25^{a}	5.00 ± 0.38^{a}	39.40 ± 0.55^{a}	91.75 ± 1.31^{a}	68.25 ± 3.77^{a}	11.00 ± 2.12^{a}
ACH 0.5 mM	67.53 ± 3.69^{b}	59.18 ± 4.50^{b}	117.50 ± 6.39^{b}	4.50 ± 0.31^{a}	32.38 ± 1.37^{a,b}	85.25 ± 2.56^{a,b}	51.00 ± 5.12^{b}	4.75 ± 1.11^{b}
ACH 5 mM	55.98 ± 4.46^{b}	47.20 ± 6.06^{b}	101.68 ± 1.86^{b}	4.03 ± 0.17^{a}	31.43 ± 2.40^{b}	82.25 ± 4.03^{b}	46.75 ± 4.78^{b}	2.75 ± 0.48^{b}
Without glucose								
TL-BSA	146.20 ± 7.60^{a}	137.65 ± 8.95^{a}	193.75 ± 5.65^{a}	5.08 ± 0.33^{a}	42.90 ± 0.25^{a†}	91.50 ± 2.10^{a}	69.25 ± 4.03^{a}	9.25 ± 3.79^{a}
ACH 0.5 mM	125.45 ± 9.53^{a†}	117.35 ± 9.45^{a†}	167.20 ± 8.33^{a,b†}	4.63 ± 0.24^{a}	39.35 ± 1.55^{a}	91.00 ± 1.47^{a}	68.25 ± 3.01^{a†}	8.75 ± 2.10^{a}
ACH 5 mM	114.28 ± 6.76^{a†}	108.33 ± 6.92^{a†}	149.50 ± 6.14^{b†}	4.28 ± 0.05^{a}	38.83 ± 1.62^{a†}	92.25 ± 0.48^{a†}	69.50 ± 1.44^{a†}	6.50 ± 1.04^{a†}
CASA analysis after activation								
With glucose								
TL-BSA	119.45 ± 4.66^{a}	93.50 ± 6.91^{a*}	225.08 ± 8.07^{a*}	9.43 ± 0.33^{a*}	30.33 ± 1.14^{a*}	76.75 ± 3.82^{a*}	42.00 ± 2.61^{a*}	61.25 ± 5.44^{a*}
ACH 0.5 mM	62.98 ± 4.02^{b*}	56.20 ± 4.76^{b*}	111.68 ± 5.68^{b}	4.15 ± 0.25^{b}	35.95 ± 2.47^{a}	86.50 ± 2.47^{a}	51.00 ± 3.85^{a}	3.00 ± 0.71^{b}
ACH 5 mM	50.90 ± 3.66^{b}	44.35 ± 4.50^{b}	93.28 ± 4.59^{b}	3.58 ± 0.15^{b}	34.95 ± 1.84^{a*}	85.25 ± 2.95^{a}	48.25 ± 3.82^{a}	1.00 ± 0.71^{b}
Without glucose								
TL-BSA	109.95 ± 4.91^{a*}	83.85 ± 4.58^{a*}	204.75 ± 11.39^{a}	8.28 ± 0.24^{a*†}	28.98 ± 2.03^{a*}	76.00 ± 3.81^{a*}	42.50 ± 1.85^{a*}	60.75 ± 3.79^{a*}
ACH 0.5 mM	96.28 ± 4.86^{a,b†}	74.40 ± 3.80^{a*†}	184.95 ± 10.24^{a†}	8.50 ± 0.48^{a*†}	26.33 ± 0.90^{a*†}	76.00 ± 1.47^{a*†}	41.25 ± 0.95^{a*†}	59.50 ± 6.65^{a*†}
ACH 5 mM	83.88 ± 5.09^{b†}	69.88 ± 4.54^{a*†}	154.43 ± 7.67^{a†}	6.53 ± 0.17^{b*†}	29.70 ± 0.83^{a*†}	80.50 ± 1.71^{a*}	45.25 ± 2.10^{a*}	26.75 ± 3.09^{b*†}

^{a,b} $P < 0.05$ when comparing the mean differences between the treatment groups.
* $P < 0.05$ when comparing mean differences between pre- and post-treatment.
† $P < 0.05$ when comparing mean differences between glucose and non-glucose treatment.

showed similar mitochondrial labeling indicated by the brightly labeled midpieces (Fig. 3). The sperm in the ACH 0.5-mM treatment group consistently showed more intense mitochondrial labeling compared to those of the TL-BSA control group, suggesting that the mitochondria were more active. In contrast, sperm treated with 200 μM PCP showed no MitoTracker Red labeling (Fig. 3), indicating that the mitochondria were damaged and had lost membrane potential. To further investigate the mitochondrial membrane potential after the treatments, the sperm mitochondria were labeled with JC-1, and the percentage of sperm with active (bright orange), partial active (faint orange) and inactive (green) mitochondria was scored (Table 3). After 1 h of treatment, sperm treated with 200 μM PCP showed significantly lower numbers of sperm with active mitochondria ($P < 0.05$) and significantly higher numbers of sperm with inactive mitochondria ($P < 0.05$). After 30 min of chemical activation, the majority of sperm mitochondria (95%) in the PCP 200-μM treatment group were labeled inactive (green). No significant difference was observed when the percentage of different color-labeled sperm pre- and postactivation in each group was compared. Sperm treated with different levels of ACH in glucose or nonglucose media did not show significant difference in JC-1 staining, suggesting a lack of direct effect on mitochondria (Table 4).

Sperm Intracellular ATP Level

The sperm intracellular ATP level after ACH and PCP in the presence and absence of glucose is shown in Figure 4. The data are presented as percentages when compared to the TL-BSA control group (open bar). In glucose-containing media, sperm treated with ACH or PCP (closed bars) showed decreased intracellular ATP level compared to the TL-BSA control group (open bar). The most marked effect of ACH on ATP levels was seen after 5 mM ACH treatment where the change was found to be statistically significant ($P < 0.05$). In nonglucose media (patterned bars), the intracellular ATP level was initially much lower than in glucose-containing media but did not change with ACH treatments when compared to the TL-BSA control group. When comparing the sperm in TL-BSA with or without glucose, the sperm in nonglucose TL-BSA (TL-BSA-NG) showed a significant decrease ($P < 0.05$) of intracellular ATP level when compared to the sperm glucose-containing TL-BSA (open bar).

Percentage of Sperm with Protein Tyrosine Phosphorylation

The results of the sperm tyrosine phosphorylation labeling experiment are shown in Table 5. After 1 h of treatment with ACH or PCP in glucose-containing media, low levels of sperm tyrosine phosphorylation in the principal piece of the flagellum were measured. After 30 min of chemical activation, all groups except for the ACH 0.5-mM treatment group showed a large and statistically significant increase in the percentage of sperm with tyrosine phosphorylation. Sperm treated with ACH 0.5 mM failed to demonstrate any change in protein tyrosine phosphorylation after treatment with dbcAMP.

Sperm-Zona Binding and Zona-Induced Acrosome Reaction

Sperm treated with ACH and PCP in glucose-containing media showed decreased numbers of sperm bound to the zona pellucida compared to the TL-BSA control group; however, the differences were not statistically significant (Table 6). The percentage of acrosome-reacted sperm induced by the zona

pellucida in all treatment groups was similar to the TL-BSA control group.

DISCUSSION

The ability of sperm to swim to the oocyte is crucial to achieve fertilization. The source of energy to drive flagellar movement of sperm has been thought to be from the mitochondria, as oxidative phosphorylation from mitochondrial respiration is more efficient than glycolysis for ATP production. However, there is debate about whether ATP produced by the mitochondria can be effectively delivered from the mitochondria supply the entire flagellum [7–9]. In order to address alternate possibilities, studies have focused on glycolysis as a possible energy source. In the present study, by using ACH and PCP, a glycolysis inhibitor and an oxidative phosphorylation inhibitor, we have demonstrated that glycolysis is the major energy source and more important than oxidative phosphorylation to support sperm motility, hyperactivation, and protein tyrosine phosphorylation in rhesus monkey sperm.

ACH has a structure similar to glycerol and is oxidized to 3-chloroactaldehyde by a NADP-dependent dehydrogenase that converts glycerol to glyceraldehyde [35]. 3-Chloroaceataldehyde inhibits GAPDH most likely because of its structural similarity to the GAPDH substrate, glyceraldehyde 3-phosphate, and consequently inhibits ATP production via glycolysis [36]. Sperm treated with ACH in vitro showed reduced fertilizing capacity in rats and mice [37]. Oral administration of ACH for 10 days resulted in reduced GAPDH activity in proximal and distal epididymal sperm in mice [38]. An acute study with ACH found inhibition of GAPDH activity not only in sperm but also in other tissues, although the inhibitory effect in sperm was greater than in other tissues [25]. Studies in vitro with mouse or rat sperm have also shown that without decreasing the percentage of motile sperm, 1 mM ACH decreased sperm motion parameters such as VCL, VSL, and LIN as well as glycolytic enzyme activities such as GAPDH and triosephosphate isomerase after 2 h of incubation [23, 39]. However, previous studies showed that the level of ATP was not decreased significantly in rhesus monkey sperm when treated with 0.5 mM ACH for 1 h in glucose-containing medium [40, 41]. In contrast with the later rodent studies [23, 39], an earlier study found that ACH did not decrease the ATP content in rat sperm [42]. Our results showed that the ATP level was lower in the 0.5-mM ACH treatment group (closed bar) when compared to the TL-BSA control group (open bar) while glucose was present; however, the decrease was not statistically significant, which is consistent with the earlier studies in rhesus monkey sperm [40, 41]. It is possible that active mitochondria seen to be intensely labeled with MitoTracker Red in the 0.5-mM ACH treatment group partially compensated for ACH-dependent loss of ATP (Fig. 3). An in vivo study in rhesus macaque also showed that ACH increased sperm oxygen consumption, which supports our finding in mitochondrial labeling intensity [43]. Although ATP was not significantly depleted at the 0.5-mM dose level, there was a marked effect on motility (Table 2), suggesting that mitochondrial ATP might not be delivered in sufficient quantity along the length of the flagellum to sustain normal sperm motility (Table 2) [7–9]. A earlier study had shown that a glycolytic product is required for whiplash motility during fertilization in mice [44]. We found that without affecting the percentage of motile sperm (Fig. 2), ACH impaired not only the whiplash motility (ALH, BCF) but also the velocity of motility (VAP,

TABLE 2. Computer-assisted semen analysis of sperm treated with ACH or PCP in glucose-containing media (data are presented as mean ± SEM; N = 3).

Treatment	VAP (μm/sec)	VSL (μm/sec)	VCL (μm/sec)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)	HYP (%)
CASA analysis after 1 h treatment								
TL-BSA	164.17 ± 4.76^{a}	157.07 ± 5.57^{a}	203.07 ± 2.28^{a}	6.40 ± 0.21^{a}	33.63 ± 1.36^{a}	94.00 ± 0.58^{a}	76.67 ± 1.45^{a}	12.39 ± 1.15^{a}
ACH 0.5 mM	70.63 ± 6.69^{b}	65.30 ± 7.15^{b}	99.60 ± 5.55^{b}	3.80 ± 0.15^{b}	26.83 ± 1.72^{b}	91.33 ± 2.33^{a}	66.33 ± 4.26^{a}	1.59 ± 1.33^{b}
PCP 50 μM	155.00 ± 10.32^{a}	145.13 ± 9.85^{a}	198.57 ± 13.47^{a}	6.47 ± 0.44^{a}	32.83 ± 0.62^{a,b}	91.00 ± 1.00^{a}	72.00 ± 1.53^{a}	17.03 ± 4.95^{a}
PCP 200 μM	102.70 ± 8.63^{c}	95.07 ± 7.31^{c}	137.97 ± 11.66^{c}	5.10 ± 0.44^{c}	28.60 ± 0.83^{a,b}	90.33 ± 0.88^{a}	68.33 ± 0.88^{a}	10.21 ± 2.31^{a,b}
CASA analysis after activation								
TL-BSA	129.97 ± 12.20^{a}	96.10 ± 10.74^{a,b}*	225.50 ± 10.89^{a}	9.23 ± 0.61^{a}	26.60 ± 2.16^{a}	72.67 ± 1.86^{a}*	44.33 ± 2.96^{a}*	62.57 ± 5.35^{a}*
ACH 0.5 mM	73.20 ± 5.48^{b}	67.60 ± 4.68^{b}	110.00 ± 9.69^{b}	4.47 ± 0.32^{b}	29.70 ± 0.89^{a}	90.33 ± 0.33^{a,b}	62.00 ± 1.15^{a}	4.42 ± 2.49^{b}
PCP 50 μM	134.73 ± 9.93^{a}	107.87 ± 7.96^{a}*	220.57 ± 8.46^{a}	8.80 ± 0.32^{a,c}	28.17 ± 0.65^{a}	79.33 ± 2.91^{a,b}	50.33 ± 2.03^{a}*	57.99 ± 3.08^{a}*
PCP 200 μM	113.43 ± 16.98^{a}	100.07 ± 17.32^{a,b}	175.63 ± 19.46^{c}	7.17 ± 0.32^{c}*	27.93 ± 3.06^{a}	85.67 ± 3.38^{b}	57.00 ± 6.24^{a}	34.89 ± 5.80^{c}*

^{a,b,c} $P < 0.05$ when comparing the mean differences between the treatment groups.
* $P < 0.05$ when comparing mean differences between pre- and post-activation.

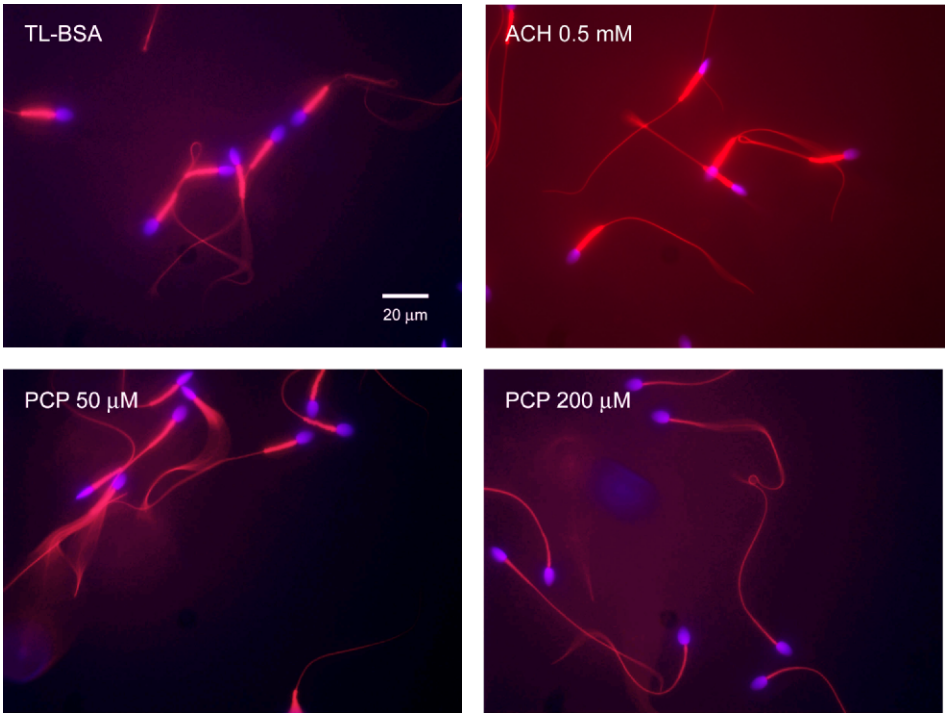

FIG. 3. Mitochondria integrity after 1 h of treatment in glucose-containing media. Sperm were stained with MitoTracker Red and mounted with Vectashield mounting medium with DAPI to examine mitochondria integrity. Sperm in the TL-BSA control and PCP 50-μM treatment groups showed strong and similar level of MitoTracker Red labeling in the midpieces, while sperm in the PCP 200-μM treatment group showed no MitoTracker Red labeling. In contrast, sperm in the ACH 0.5-mM treatment group showed intensive MitoTracker Red labeling, indicating that the mitochondria were very active.

VSL, VCL) after 1 h of treatment in glucose-containing media (Table 2).

In the presence of glucose, ACH also inhibited hyperactivation of rhesus monkey sperm that is induced by the chemical activators dbcAMP and caffeine (Table 2). Studies have shown that ATP production through glycolysis is required for hyperactivated sperm motion in humans and mice [44–46]. Those studies support our findings that when glycolysis is inhibited, despite the presence of chemical activators, the ATP level in sperm maintained by highly active mitochondria is not adequate to support the high energy demand needed for hyperactivation.

Protein tyrosine phosphorylation is one of the characteristics that sperm acquire during capacitation and has been correlated to sperm hyperactivation in humans, nonhuman primates, and other species [47–49]. Our study showed that ACH also inhibits protein tyrosine phosphorylation after chemical activation (Table 5). Other investigators have demonstrated that ATP produced by glycolysis is required for protein tyrosine phosphorylation in mouse sperm [17], which is consistent with our finding that inhibiting glycolysis with ACH prevents protein tyrosine phosphorylation after chemical activation. Apparently, without displaying hyperactivity and tyrosine phosphorylation, the sperm can still bind to the zona pellucida and undergo zona-induced acrosome reaction (Table 6), which suggests that sperm-zona interaction and sperm hyperactivity have different energy requirements. Our previous study in cynomolgus macaques has shown that sperm-zona binding ability and zona-induced acrosome reaction are independent of sperm hyperactive motility; when treated with caffeine or dbcAMP only, increased numbers of sperm bound to the zona pellucida, and an increased percentage showed a zona-induced acrosome reaction yet without displaying hyperactive motility [50]. A study in human sperm has shown that protein tyrosine phosphorylation is correlated with sperm-zona binding but not with the zona-induced acrosome reaction [51]. In our study, the number of sperm bound to the zona pellucida was decreased but not statistically significant compared to the TL-BSA control, and the zona-induced acrosome reaction was not affected (Table 6). Previous studies in cynomolgus macaques and mice have shown that when coincubating sperm and oocytes in culture medium without glucose, the binding of sperm to the oocytes was retarded [20, 52]. With the support of previous findings [20, 44–46], our

TABLE 3. Percentage of sperm mitochondria labeled with bright orange, faint orange, and green JC-1 after ACH or PCP treatment.[a]

Treatments	Bright orange (%)	Faint orange (%)	Green (%)
After 1 h treatment			
TL-BSA	85.00 ± 4.16	3.67 ± 1.33	11.67 ± 3.18
ACH 0.5 mM	86.00 ± 3.79	6.33 ± 0.88	7.67 ± 3.38
PCP 50 μM	79.33 ± 4.06	5.67 ± 1.45	15.00 ± 5.13
PCP 200 μM	2.33 ± 1.20*	15.67 ± 6.77	81.67 ± 7.31*
After activation			
TL-BSA	90.00 ± 0.58	2.33 ± 0.67	7.67 ± 0.33
ACH 0.5 mM	75.67 ± 6.74	9.00 ± 4.36	14.33 ± 3.38
PCP 50 μM	84.67 ± 2.60	2.33 ± 0.33	13.00 ± 2.31
PCP 200 μM	0.33 ± 0.33*	4.33 ± 0.67	95.33 ± 0.33*

[a] Data are presented as mean ± SEM; N = 3.
* $P < 0.05$ when comparing the mean differences between the treatment groups.

TABLE 4. Percentage of JC-1 labeled sperm treated with ACH in glucose or non-glucose media.[a]

Treatment	Bright orange (%)	Faint orange (%)	Green (%)
With glucose			
TL-BSA	88.25 ± 3.82	5.25 ± 1.93	6.50 ± 2.33
ACH 0.1 mM	92.00 ± 1.47	1.75 ± 0.75	6.25 ± 1.31
ACH 0.5 mM	89.00 ± 2.42	2.25 ± 1.11	8.75 ± 1.44
ACH 1 mM	91.00 ± 2.08	2.25 ± 1.31	6.75 ± 2.50
ACH 5 mM	90.50 ± 3.01	2.00 ± 1.08	7.50 ± 3.33
Without glucose			
TL-BSA	90.75 ± 1.11	2.50 ± 0.50	6.75 ± 0.95
ACH 0.1 mM	87.00 ± 2.12	4.25 ± 1.03	8.75 ± 2.21
ACH 0.5 mM	89.50 ± 2.40	1.25 ± 0.25	9.25 ± 2.29
ACH 1 mM	92.25 ± 1.18	1.00 ± 0.71	6.75 ± 1.75
ACH 5 mM	91.75 ± 1.18	2.50 ± 1.26	5.75 ± 0.85

[a] Data are presented as mean ± SEM; N = 4.

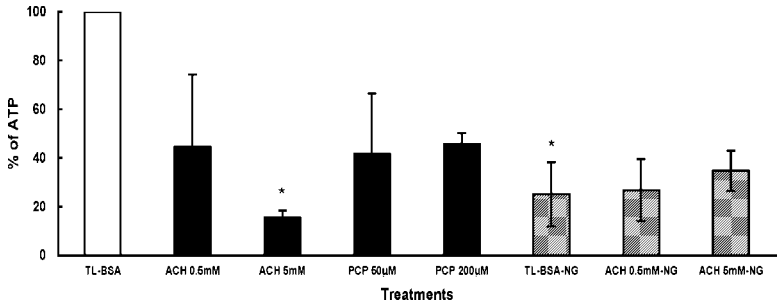

FIG. 4. Intracellular ATP level after 1 h of treatment. The level of ATP is presented as percentage, and the TL-BSA with glucose is presented as 100% (open bar). The other treatments with glucose-containing media are shown in closed bars. Sperm treated in media without glucose (NG) are shown in patterned bars. Data are from three males and presented as % ± SEM. *, $P < 0.05$ when comparing mean differences with the TL-BSA control group.

results suggest that glycolysis is not required for sperm-zona binding, zona-induced acrosome reaction, or capacitation but is essential for hyperactivated motility and protein tyrosine phosphorylation in rhesus monkey sperm.

PCP is an uncoupler of oxidative phosphorylation and inhibits respiratory enzymes to prevent ATP production in the mitochondria [53]. The PCP 50-μM treatment showed no effect on the motion parameters of sperm after 1 h of treatment and after activation (Table 2), and the mitochondria remained intact after treatment (Fig. 3 and Table 3). When treated with a higher level of PCP (200 μM), sperm mitochondrial activity was completely disrupted and displayed no MitoTracker Red labeling after 1 h incubation (Fig. 3); when labeled with JC-1, the majority of mitochondria (81%) showed inactive staining (green) (Table 3). Surprisingly, without active mitochondria, the sperm were still motile and showed no difference in the percentage of motile sperm compared to the TL-BSA treatment group (Fig. 2). Although the sperm motion parameters in the PCP 200-μM treatment group showed significant changes compared to the TL-BSA treatment group after 1 h of treatment, the changes were much smaller than those in the ACH treatment group (Table 2). A study has shown that when treated with another oxidative phosphorylation chemical, carbonyl cyanide m-chlorophenylhydrazone (CCCP), mouse sperm motility was not impaired [54]. Our results and these other studies suggest that the ATP produced via glycolysis alone is sufficient to support sperm motility.

After chemical activation, sperm in both PCP treatment groups displayed the characteristic motion of hyperactivity, such as increased VCL and ALH and decreased BCF, STR, and LIN. Sperm treated with 50 μM PCP showed no difference in sperm motion parameters when compared to the TL-BSA control group. Sperm treated with 200 μM PCP also showed some degree of hyperactivity (although lower HYP) after chemical activation despite the fact that the sperm motility was affected by 1 h of PCP treatment; only VCL, ALH, and STR showed significant differences compared to the TL-BSA control group after activation (Table 2). These results suggest that the sperm mitochondria may play a secondary role in maintaining normal sperm motility and hyperactive motility because PCP impairment of sperm motility can be rescued after chemical activation. Although sperm treated with 200 μM PCP showed some impairments of hyperactivity, the ability of sperm to bind to the zona pellucida was not significantly affected, and the sperm could undergo zona-induced acrosome reaction (Table 6). In addition, sperm showed normal protein tyrosine phosphorylation after chemical activation (Table 5), which is consistent with the finding in mouse sperm that when treated with oxidative phosphorylation uncouplers and electron transfer inhibitors, protein tyrosine phosphorylation was not inhibited [55]. Our findings provide further support that glycolysis but not oxidative phosphorylation is essential for protein tyrosine phosphorylation in rhesus monkey sperm.

There appears to be a species difference in sensitivity of sperm mitochondria to PCP. In rats, 0.1 μM of PCP significantly disrupted mitochondrial membrane potential [26]. In our study, the mitochondria were affected only when treated with 200 μM PCP (Fig. 3), indicating that monkey sperm are more tolerant to PCP. The EC_{50} value to affect bull sperm motility, velocity, and viability are 43, 27, and 360 μM, respectively; however, the ATP level was not affected when treated with 100 μM PCP [27]. Our study showed that the percentage of motile sperm was not changed even with PCP 200-μM treatment. The velocity of sperm motion was decreased in the 200-μM PCP treatment group (Table 2), which is a much higher dose level than that producing an effect in bull sperm. These species sensitivity differences may be a confounding factor in previous studies that have evaluated whether oxidative phosphorylation or glycolysis is more important for energy production to support sperm function.

It is hypothesized that incubating sperm with ACH in glucose-containing medium would increase the concentration of glycolytic intermediates that bind most of the phosphate in the sperm, consequently reducing the availability of phosphate for oxidative phosphorylation and decreasing of sperm motility, and this is likely to increase rather than decrease mitochondrial membrane potential by reducing the availability of free phosphate for ATP synthesis [18]. Sperm treated with ACH showed intensive MitoTracker Red staining, suggesting that the mitochondrial membrane potential was increased, perhaps because of the lack of free phosphate for ATP synthesis (Fig. 3); however, some studies propose that an

TABLE 5. Percentage of sperm with tyrosine phosphorylation after 1 h of treatment with ACH or PCP in glucose-containing media.[a]

Treatments	After 1 h treatment	After activation
TL-BSA	3.00 ± 0.58	87.00 ± 2.00[†]
ACH 0.5 mM	0.33 ± 0.33	1.00 ± 0.58*
PCP 50 μM	6.33 ± 1.45	86.33 ± 2.40[†]
PCP 200 μM	9.00 ± 3.61	72.67 ± 12.33[†]

[a] Data are presented as mean ± SEM; N = 3.
* $P < 0.05$ when comparing the mean differences between the treatment groups.
[†] $P < 0.05$ when comparing the mean differences between pre- and post-activation.

TABLE 6. Number of sperm bound to zona and percentage of acrosome reaction after 1 h of treatment with ACH or PCP in glucose-containing media.[a]

Treatments	Total bound	Acrosome reacted (%)
TL-BSA	40.14 ± 6.49	31.15 ± 5.79
ACH 0.5 mM	30.90 ± 7.85	26.52 ± 6.01
PCP 50 μM	30.38 ± 5.31	28.72 ± 7.07
PCP 200 μM	25.28 ± 5.19	25.90 ± 6.60

[a] Data are presented as mean ± SEM; N = 6.

increase of membrane potential might indicate a higher rate of mitochondrial ATP synthesis, which may also suggest a more active mitochondria perhaps to compensate for diminished ATP production [56, 57]. Previous studies in rhesus monkey sperm have shown that ATP level was maintained and oxygen consumption was increased when treated with ACH [40, 43], supporting the possibility that insufficient delivery of ATP diminished sperm motion after ACH treatment despite the active mitochondria. In addition, our PCP data strongly suggest that without functional mitochondria, monkey sperm still displayed hyperactivated motility.

We conclude that glycolytic pathways play an important role in supplying ATP to support sperm motility in rhesus monkey sperm. While ATP from mitochondrial sources does contribute to sperm motility, our data indicate that the mitochondria appear to be less important for hyperactivated motility. Despite the absence of hyperactive motility after chemical activation, sperm treated with either ACH or PCP can bind to the zona pellucida and undergo zona-induced acrosome reaction, indicating that the ATP from either glycolysis or oxidative phosphorylation alone is sufficient to provide the energy required for sperm to capacitate and bind to the zona pellucida then acrosome react, at least in the in vitro conditions. Sperm mitochondria have been used to assess the viability of sperm for decades. With the inconsistency of mitochondrial activity and sperm motility in this study and others [54], mitochondrial integrity is not necessarily the best primary index of sperm viability.

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