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Inhibition of Human Sperm Motility by Contraceptive Anti-Eppin Antibodies from Infertile Male Monkeys: Effect on Cyclic Adenosine Monophosphate[1]

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ABSTRACT

Epididymal protease inhibitor (eppin [official symbol, SPINLW1]) is of interest as a male contraceptive target because of its specificity and location on the human sperm surface. We have examined the effect of anti-eppin antibodies from infertile male monkeys and the effect of recombinant human semenogelin on human sperm motility. Anti-eppin antibodies significantly decreased the progressive motility of human spermatozoa as measured by decreased total distance traveled, decreased straight-line distance, and decreased velocity. Anti-eppin treatment of spermatozoa significantly increased the amount of cAMP present in nonprogressive spermatozoa; however, approximately 25% of antibody-treated spermatozoa could be rescued by the addition of cAMP-acetoxymethyl ester, indicating that anti-eppin-treated spermatozoa have a compromised ability to utilize cAMP. Addition of recombinant human semenogelin has a concentration-dependent inhibitory effect on progressive motility (increased tortuosity and decreased velocity). We tested the hypothesis that anti-eppin antibodies bound to eppin would subsequently block semenogelin binding to eppin. Anti-eppin antibodies from infertile monkeys inhibited eppin from binding to semenogelin. Addition of affinity-purified antibodies made to the dominant C-terminal epitope of eppin had an inhibitory effect on progressive motility (increased tortuosity, decreased velocity, and straight distance). Our results suggest that the eppin-semenogelin binding site is critical for the removal of semenogelin in vivo during semen liquefaction and for the initiation of progressive motility. We conclude that the eppin-semenogelin binding site on the surface of human spermatozoa is an ideal target for a nonsteroidal male contraceptive.

cAMP, contraception, eppin, gamete biology, semenogelin, seminal plasma, sperm, spermatozoa

INTRODUCTION

Eppin (epididymal protease inhibitor [official symbol, SPINLW1]) is of interest as a male contraceptive target because of its specificity and location on the human sperm surface. Previous work on eppin demonstrated that male monkeys immunized with recombinant human eppin to a high serum titer (>1:1000) and sustained over several months achieved an effective level of contraception (100%) that was reversible [1]. Contraceptive investigations in nonhuman primates demonstrated that eppin has an essential role in fertility and provided the first observations of reduced sperm motility that coincided with the appearance of an anti-eppin titer in the semen (see Fig. S3 [monkey sample no. 28309] in Supporting Online Material in O'Rand et al. [1]).

Eppin is a member of the whey acidic protein (WAP)-type four-disulfide core gene family located in a telomeric cluster on human chromosome 20q12-q13 and is the archetype of *WFDC* genes characterized by encoding Kunitz-type and WAP-type four-disulfide core protease inhibitor consensus sequences [2]. The eppin protein is specific to male reproductive tissue; secreted by Sertoli cells and epididymal epithelial cells [2, 3], eppin becomes localized on the surface of ejaculated spermatozoa in a complex of proteins containing lactotransferrin, clusterin, and semenogelin [3]. The eppin protein complex [3, 4] modulates prostate-specific antigen (PSA) protease activity [5] and provides antimicrobial protection for spermatozoa in the ejaculate coagulum [6]. Activated PSA cleaves semenogelin by hydrolysis immediately after ejaculation, liquefying the coagulum [7] and freeing the spermatozoa for motility and capacitation [8, 9].

To understand the essential role of eppin in fertility, we have conducted investigations on eppin function, which led to the demonstration that in seminal plasma eppin is bound to semenogelin I [4] and that on human spermatozoa following ejaculation eppin is present in a protein complex [3]. Moreover, the mechanism of action of the anti-eppin antibody seems to be to prevent normal eppin-semenogelin interaction [5], subsequently inhibiting the motility of ejaculate spermatozoa. To extend these observations to human spermatozoa, we have examined the effect of anti-eppin antibodies from infertile male monkeys [1], as well as the effect of recombinant human semenogelin (SEMG1) on human sperm motility. The contraceptive anti-eppin antibodies cause inhibition of progressive motility, which could be rescued in approximately 25% of antibody-treated spermatozoa by the addition of cAMP-acetoxymethyl ester (cAMP-AM). Our results suggest that the eppin-semenogelin binding site is critical for the removal of semenogelin from spermatozoa in vivo during semen liquefaction and for the initiation of progressive motility. These observations identify an ideal target on the surface of human spermatozoa, namely, the eppin-semenogelin binding site, for a nonsteroidal male contraceptive.

MATERIALS AND METHODS

Reagents and chemicals were molecular biology grade purchased from Sigma-Aldrich (St. Louis, MO). Human semen samples were obtained from the Department of Obstetrics and Gynecology, University of North Carolina at Chapel Hill, and this study was approved by the Committee on the Protection of the Rights of Human Subjects at the School of Medicine, University of North Carolina at Chapel Hill. Affinity-purified rabbit antibodies to the C-terminal of eppin (amino acids 103–123) were produced by Bethyl Laboratories, Inc.

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TABLE 1. Percentage motility of human spermatozoa treated with affinity purified anti-eppin antibodies from infertile male monkeys.[a]

	Motility range (%)[b]			
Monkey sample no.	0 min	30 min	60 min	120 min
0/656 (control)	100–75	100–75	100–75	100–75
145/610	100–75	100–75	100–75	25–0
145/656	100–75	100–75	50–25	25–0
145/657	100–75	100–75	75–50	0
523/602	100–75	100–75	25–0	0
523/619	100–75	100–75	25–0	0
523/625	100–75	75–50	50–25	50–25
523/679	100–75	100–75	75–50	0

[a] Sperm concentration = 2.04×10^6 sperm/ml; all antibody-treated spermatozoa completely or partially lost progressive motility by 120 min.
[b] Motility range was determined by counting >100 spermatozoa in each of six randomly selected fields for each time point.

(Montgomery, TX) to the peptide SMFVYGGAQGNNNNFQSKANC (antibody S21C), in which alanine was substituted for cysteine 110. Student *t*-test was used to test the null hypothesis that the means of the two populations are equal.

Preparation of Spermatozoa

Semen samples were collected from fertile donors at the UNC North Carolina Memorial Hospital infertility clinic, allowed to liquefy for 30 min, and given a standard semen analysis to determine acceptability as an anonymous semen donor. Samples from acceptable donors were frozen immediately in test-yolk buffer and stored in liquid nitrogen. Samples for study were thawed at 37°C, washed twice in 37°C sperm washing medium (modified human tubal fluid; Irvine Scientific, Santa Ana, CA), and collected by centrifugation at 300 × *g* for 5 min, and the supernatant was removed. A 1-ml aliquot of 37°C medium containing 25 mM sodium bicarbonate (M16; Sigma) was layered over the pellet, and spermatozoa were allowed to "swim up" into the medium in a CO_2 incubator. After 1 h, the M16 supernatant layer was removed and centrifuged at 300 × *g* for 5 min to collect the spermatozoa. Aliquots of the swim-up population were taken to determine percentage motility and sperm concentration.

Analysis of Sperm Motility

The objective of this study was to determine the change in progressive sperm motility in control and treated experimental groups of swim-up spermatozoa. Therefore, we used the following experimental parameters. For motility analysis, a 5-μl aliquot of the swim-up sperm sample was evenly distributed in a 20-μm glass chamber slide (Leja Products B.V., Nieuw Vennep, The Netherlands) and viewed immediately (within 3 min) with a Plan-Apochromat 20×/0.8 phase 2 (diameter width, 0.55 mm) objective or a Plan-Neofluar 10×/0.3 phase 1 objective on a Zeiss Axiophot microscope (all from Carl Zeiss, Thornburg, NY). At least four random fields in each chamber were selected, and sperm motility was recorded with an AxioCam HSc high-speed camera (Carl Zeiss). Recordings were for 1 sec at frame rates varying from 60 to 103 frames/sec and with pixel windows varying from 660 × 492 to 328 × 248, depending on the experiment. In some experiments, recordings were also made with an AxioCam MRc high-speed charge-coupled device camera (Zeiss) for 10 or 15 sec at frame rates varying from 6 to 18 frames/sec and with pixel windows varying from 1300 × 1030 to 256 × 256. Sperm tracks were analyzed with Zeiss Cell Observer time-lapse and tracking software (AxioVs40 V4.6.3.0; Carl Zeiss). As many sperm as possible were tracked in each field, with a total of 25–100 tracks analyzed from semen samples from at least two different donors for any experimental determination. Although these cameras and the analysis software do not provide the same motion analysis as a computer-aided sperm analysis system (limited to 60 frames/sec), they allow more flexibility for high-resolution observation of the forward-swimming progression of individual sperm over longer times and distances, with variable frame rates and pixel window sizes.

Affinity Purification of Monkey Anti-Eppin Antibodies

Monkey anti-eppin antibodies were obtained during our previous study [1] in which seven male monkeys immunized with recombinant human eppin were found to be infertile. As indicated in our previous study, these antibodies were obtained following protocols approved by our local Institutional Animal Care and Use Committee and by the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India. The serum from each male monkey was heat inactivated at 60°C for 40 min and precipitated with 40% ammonium sulfate. Pellets were washed three times and dialyzed into PBS (pH 7.4). Eppin affinity beads were made in bulk by binding His-tagged eppin to Ni^{2+}-affinity (NTA) resin (Qiagen, Valencia, CA) and by adding an equal volume of Sephadex G-50 (Sigma) to improve flow rate and packing. Beads were washed with several column volumes of buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 8.0) and buffer C (same as buffer B, pH 6.3), followed by extensive washing in Tris-buffered saline (TBS) (50 mM Tris [pH 7.4] and 150 mM NaCl). An aliquot of ammonium sulfate-precipitated monkey preimmune serum was applied to the beads to block nonspecific binding, and beads were washed again in TBS. Aliquots of these beads were taken to affinity purify each monkey's serum separately. Ten milligrams of each monkey's serum was applied to an aliquot of the eppin-Ni-NTA beads and washed with TBS. Anti-eppin antibodies were eluted with IgG elution buffer (Pierce, Rockford, IL), dialyzed in PBS, and concentrated. The following sera from the previous study [1] were used: group 1 (squalene adjuvant [Day 523 sera] in monkey sample nos. 602, 619, 625, and 679) and group 2 (complete Freund adjuvant [Day 145 sera] in monkey sample nos. 610, 656, and 657). Preimmune serum from monkey sample no. 656 was used for the control and was prepared as IgG. The protein concentration of each antibody was determined and adjusted so that they were all approximately equal (mean ± SD, 0.9 ± 0.1 mg/ml). Human sperm were prepared as swim-up sperm and assessed for forward motility. Only samples with >90% forward motility were used in the assay. The sperm concentration was adjusted to $\sim 2 \times 10^6$ sperm/ml. Equal volumes of antibody (in the same buffer used for the spermatozoa) and spermatozoa were mixed and incubated at 37°C (5% CO_2). Aliquots were taken at various times to assess motility.

Cyclic AMP

Cyclic AMP levels were measured in spermatozoa using an AlphaScreen cAMP assay kit (Perkin Elmer, Boston, MA) in a competitive immunoassay between endogenous cAMP and exogenous biotinylated cAMP. Treated and control spermatozoa were washed with PBS containing 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) (I5879; Sigma) and extracted in acidified ethanol, and 400-μl aliquots of supernatant were evaporated to dryness. Samples were dissolved in 50 μl of stimulation buffer (1× Hanks balanced salt solution, 0.5 mM IBMX, 0.1% BSA, 5 mM Hepes), and the pH was adjusted to ~7 with 5 μl of 1 M Hepes. Readings were taken in a Biotek Synergy 2 Multimode Microplate reader (Biotek Instruments, Winooski, VT). For reactivation experiments, spermatozoa were loaded to a final concentration of 60 μM cAMP-AM (Sigma) in 0.25% pluronic acid in M16 media [10].

TABLE 2. Inhibition of human sperm motility treated with affinity purified anti-eppin antibodies from an infertile male monkey.[a]

Parameter	Buffer control M16 (N = 110)[b]	Preimmune control (N = 31)[b]	Antibody 523/619 (N = 70)[b]	*P* value (antibody vs. preimmune control)	Percentage change (antibody vs. preimmune control)	*P* value (antibody vs. buffer control)
Total distance (μm)	131.4 ± 36	111.4 ± 41	78.96 ± 58	1.99×10^{-3}	71	6.26×10^{-10}
Straight distance (μm)	28.1 ± 11	17.8 ± 9	10.2 ± 9	1.63×10^{-4}	57	5.37×10^{-25}
Velocity (μm/sec)	131.7 ± 38	117.7 ± 44	83.4 ± 61	1.99×10^{-3}	71	3.96×10^{-8}
Tortuosity	6.0 ± 5	8.4 ± 6	11.7 ± 8	2.46×10^{-2}	1.4× increase	1.74×10^{-6}

[a] Sperm were treated with affinity purified anti-eppin antibody from monkey 523/619 or preimmune control IgG for 60 min; only spermatozoa with some discernable movement were measured.
[b] N = Number of tracks analyzed.

Eppin-Semenogelin Binding Assay

Triplicate samples of antibody dilutions preincubated with 500 ng of FLAG-eppin were applied to His-Select Nickel High Sensitivity 96-well plates (Sigma) previously coated with 100 ng/well of His-SEMG1 (semenogelin) and blocked in TBST (TBS + 0.05% Tween 20/1× casein [Vector Laboratories, Burlingame, CA]). After overnight incubation, plates were washed and incubated with 1:1000 anti-FLAG M2 antibody followed by horseradish peroxidase-labeled secondary antibody. The reaction was developed with TMB substrate (tetramethylbenzidine; KPL, Gaithersburg, MD), stopped, and the optical density (OD) was read at 450 nm. Positive controls for the assay included preimmune sera and buffer only. In the absence of anti-eppin antibodies, the assay gives an OD at 450 nm of approximately 1.0 ± 0.1. In the absence of FLAG-eppin, the assay gives an OD at 450 nm of approximately 0.2 ± 0.1. Consequently, as the concentration of anti-eppin antibody increases, the OD decreases.

TABLE 3. Inhibition of human sperm motility by semenogelin.

Treatment (N)	Tortuosity (curvilinear distance/straight line distance)	*P* value (SEMG1 vs. control)
Semenogelin buffer control (44)[a]	1.64 ± 1.2 SD	
Semenogelin (34)[b]	4.4 ± 17.7 SD	0.3
Semenogelin (38)[c]	7.4 ± 14.1 SD	0.0164

[a] Sperm were treated with M16 media.
[b] Sperm were treated with rSEMG1 (2.2 μg/10^6 sperm) for 60 min after "swim up" and evaluated for motility.
[c] Sperm were treated with rSEMG1 (6.5 μg/10^6 sperm) for 60 min after "swim up" and evaluated for motility.

RESULTS

Contraceptive anti-eppin antibodies from the seven infertile eppin-immunized male monkeys [1] were purified on eppin affinity columns and dialyzed against PBS, and their effect on the progressive motility of human spermatozoa was evaluated. All seven of the antibodies caused a loss of progressive motility within 1 h after addition to the spermatozoa; the spermatozoa began to swim more slowly and finally were unable to make forward progress and only twitched in place. No agglutination of spermatozoa was observed. Two hours after addition, four samples completely stopped the forward progress of the spermatozoa, two samples resulted in a reduction to 25% motility, and 1 sample resulted in a reduction to 50% motility (Table 1). Spermatozoa treated with preimmune control IgG remained motile after 2 h.

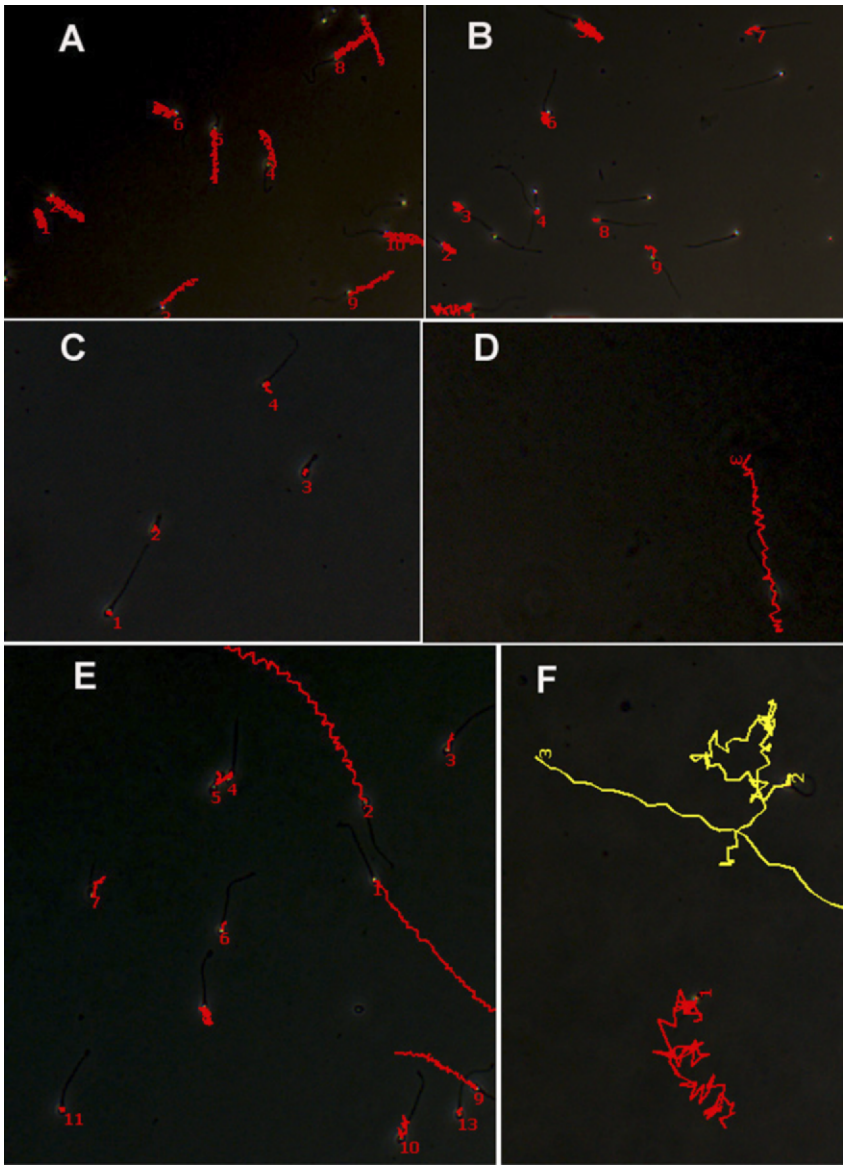

FIG. 1. Video frames of human sperm swimming patterns. **A**) Control spermatozoa from a normal swim-up sample from a fertile donor (still image from Supplemental Movie 1 [movie is presented in black and white]). Sperm tracks were recorded for 1 sec at 103 frames/sec with a 10× objective. **B**) Spermatozoa treated with anti-eppin antibodies from monkey sample no. 523/619 (still image from Supplemental Movie 2 [movie is presented in black and white]). Sperm tracks were recorded for 1 sec at 103 frames/sec with a 10× objective. **C**) Spermatozoa treated with anti-eppin antibodies from monkey sample no. 523/602 without cAMP-AM added. Sperm tracks were recorded for 10 sec at 7.4 frames/sec, 650 × 514 frame size, and no binning with a 10× objective. **D**) Spermatozoa treated with anti-eppin antibodies from monkey sample no. 523/602 with added cAMP-AM. Sperm track was recorded for 10 sec at 7.4 frames/sec, 650 × 514 frame size, and no binning with a 10× objective. **E**) Spermatozoa treated with recombinant semenogelin, 6.5 μg/10^6 sperm. Sperm tracks were recorded 1 h after treatment for 10 sec at 8 frames/sec with a 10× objective. **F**) Control spermatozoa from a normal swim-up sample from a fertile donor. Sperm tracks were recorded for 10 sec at 8 frames/sec with a 10× objective. Each number represents a sperm track as it is counted by the computer.

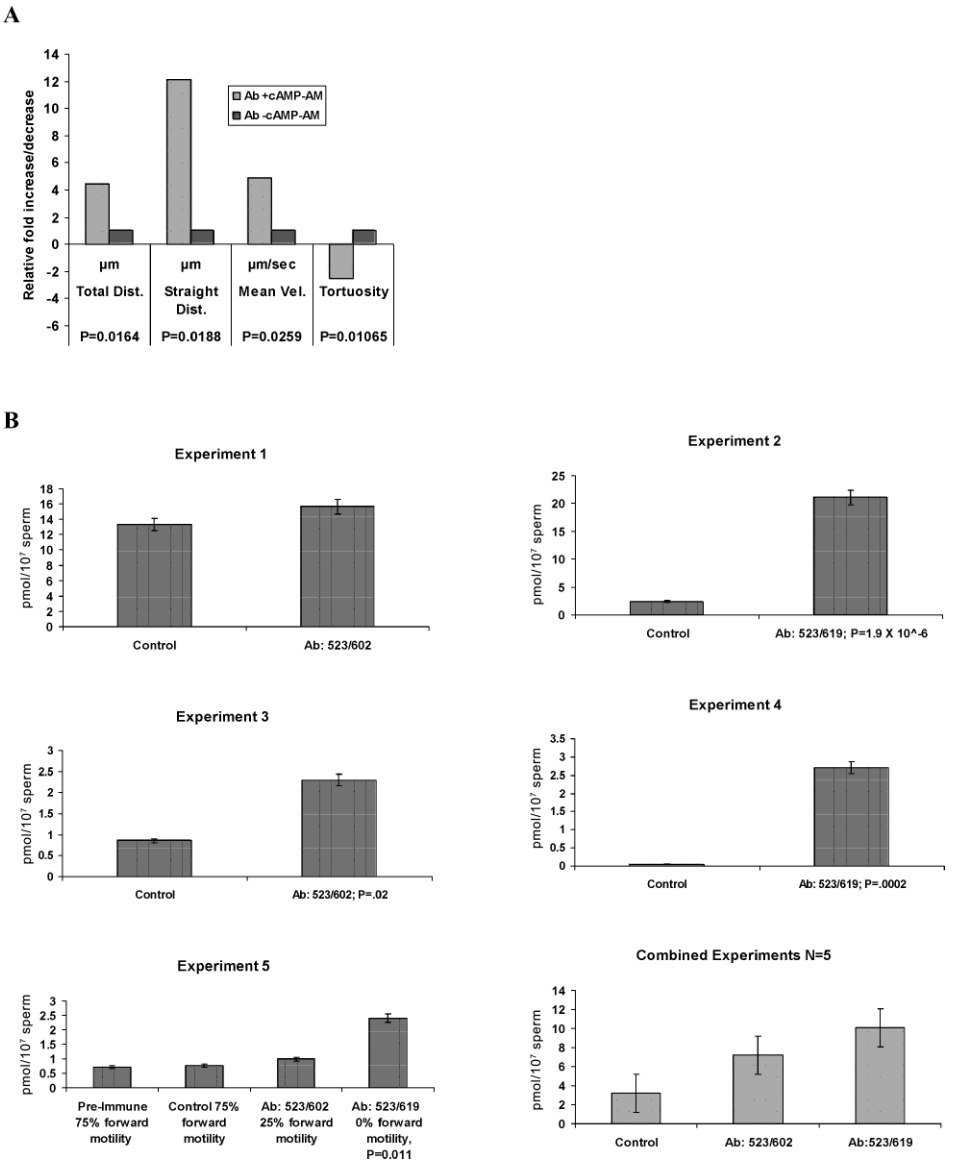

FIG. 2. Cyclic AMP in human spermatozoa treated with anti-eppin antibodies from infertile male monkeys. **A**) Recovery of motility by anti-eppin antibody-treated spermatozoa in the presence of added cAMP-AM. Total distance (Total Dist.), straight distance (Straight Dist.), and velocity (Mean Vel.) increase as tortuosity decreases. Data are from a single ejaculate collected from a typical experiment from different donors (n = 3). **B**) Five normal swim-up samples from fertile donors were treated with monkey sample no. 523/602 or 523/619 anti-eppin antibodies. After 2 h, spermatozoa had lost their motility (except for antibody from monkey sample no. 523/602 in experiment 5) and were measured for picomoles of cAMP per 10^7 spermatozoa (± SD). Antibody-treated samples that lost their motility were significantly different from buffer control or preimmune-treated samples (except for antibody from monkey sample no. 523/602 in experiments 1 and 5). Buffer control and preimmune samples are not significantly different from one another. Combined experiments graph (n = 5) shows (± SEM) that both antibodies are significantly different from controls. Ab, antibody; P, *P* value.

A more detailed analysis was undertaken using image analysis software (Zeiss Cell Observer). Figure 1A shows a typical video frame of human sperm-swimming patterns (tracks) found in a normal swim-up sample from a fertile donor. The field of view shows sperm tracks recorded for 1 sec at 103 frames/sec with a 10× objective. To determine relative forward-swimming progression, the tortuosity was calculated [11] as the curvilinear distance:straight-line distance ratio of the path. Fertile donor spermatozoa most often exhibited progressive straight tracks, with slight circular trajectories, over longer periods. Samples of spermatozoa measured at 103 frames/sec from sperm donors selected at random had a mean ± SD tortuosity of 6.0 ± 5.0 (range, 2.2–24.2; linearity, 17%; n = 110). When measured at 8 frames/sec for 10 sec, samples of spermatozoa from five semen donors selected at random had a mean ± SD tortuosity of 1.7 ± 1.1 (range, 1.0–5.8; linearity, 59%; n = 105). A tortuosity of 1.8 (linearity, 55%; n = 205) was measured for identical donor samples at 60 frames/sec using a computer-aided sperm analysis system. These results indicate that image sampling frequencies affect the values measured, as reported previously [12–14]; however, normal fertile spermatozoa exhibit identical swimming patterns (progressive straight tracks with slight circular trajectories) regardless of the number of frames per second.

Antibodies from the seven infertile eppin-immunized male monkeys were analyzed using image analysis software for their effect on sperm motility. Analysis was performed on each sample, and a typical result from monkey sample no. 523/619 is given in Table 2 and in Figure 1B. Results were similar for semen samples from several different donors. Compared with the control (preimmune IgG), there was a significant difference in the progressive motility of human sperm after treatment with anti-eppin antibody as measured by a decrease in the relative total distance traveled by 71% ($P = 1.99 \times 10^{-3}$), a decrease in the relative straight-line distance by 57% ($P = 1.63 \times 10^{-4}$), and a decrease in the velocity by 71% ($P = 1.99 \times 10^{-3}$). As shown in Table 2, the antibodies have the effect of decreasing the progressive motility; as progressive distance decreases, tortuosity increases by 39% ($P = 2.46 \times 10^{-2}$). Figure 1B shows a typical field of sperm tracks; sperm treated with preimmune IgG swim in progressive straight lines (similar to those shown in Fig. 1A), whereas sperm treated with anti-eppin antibodies lose their progressive motility (see online Supplemental Movies 1 and 2 available at www.biolreprod.org).

Human sperm require adenylyl cyclase (*ADCY10*), cAMP, Ca^{2+}, and the appropriate intracellular pH for normal forward motility [15–17], implying that one or more of these components may have been disrupted by the antibody treatments. Moreover, disruption of the mouse gene for soluble adenylyl cyclase (*Adcy10*) or the sperm-specific Na^+/H^+ exchanger (*Slc9a10*) results in spermatozoa that exhibit impaired motility that could be rescued by cAMP [10, 16]. Therefore, we tested whether anti-eppin-treated spermatozoa could be rescued by cAMP. Approximately 25% of spermatozoa that have lost their motility from anti-eppin antibody treatment can be rescued. If spermatozoa are preloaded with cAMP-AM for 20 min and then treated with monkey anti-eppin antibody, their loss of forward progress is delayed 30–45 min (antibody from monkey sample no. 523/619: mean ± SD tortuosity, 2.2 ± 1.2 for cAMP-AM loaded vs. 10.6 ± 12.4 for nonloaded control). In additional experiments, spermatozoa are loaded with cAMP-AM 30 min and 60 min after being treated with monkey anti-eppin antibody, and their motility was examined 75 min after the initial antibody treatment. These spermatozoa exhibit significant signs of recovery. As shown in Figure 2A, monkey anti-eppin antibody-treated spermatozoa treated with cAMP-AM increased their total distance traveled 4.4-fold, their straight distance traveled 12.1-fold, and their velocity 4.9-fold, all significant signs of resumed forward motility. Similarly, their tortuosity decreased 2.5-fold ($P = 0.01$). Their resumed forward motility can be seen in the sperm tracks shown in Figure 1, C and D, where the lack of forward progress after antibody treatment for 1 h (Fig. 1C) is replaced with a straight-line track (Fig. 1D) with the addition of cAMP-AM. These results indicate that anti-eppin antibodies bound to eppin on the sperm surface block the ability of the sperm to regulate its motility; however, addition of exogenous cAMP-AM can rescue the motility of some spermatozoa within the time frame that would otherwise produce immotile sperm.

To examine why this was not the case for a larger percentage of the population of spermatozoa, we measured cAMP levels in spermatozoa 2 h after anti-eppin antibody treatment. As shown in Figure 2B, five experiments from different semen donors revealed that anti-eppin treatment of spermatozoa significantly increased the amount of cAMP present in 10^7 spermatozoa (combined experiments, n = 5). After treatment with either of these two monkey anti-eppin antibodies, none of the spermatozoa remain motile (except for monkey sample no. 523/602 in experiment 5 (25% motile). Taken together, these cAMP data demonstrate that anti-eppin-treated spermatozoa have variable cAMP levels (25% of which can be rescued), implying that disruption of their progressive motility inhibits their ability to utilize cAMP.

Eppin has previously been shown to bind the seminal fluid protein semenogelin on the sperm surface in vivo and recombinant semenogelin in vitro [4, 18]. During contraception [1], the monkey anti-eppin antibodies would have been expected to bind to eppin in the epididymis before the spermatozoa were exposed to seminal fluids. Therefore, we tested the hypothesis that anti-eppin antibodies bound to eppin would subsequently block semenogelin binding to eppin. We used an in vitro assay to measure eppin-semenogelin binding (see *Materials and Methods*), and Figure 3A shows the effect of affinity-purified rabbit anti-recombinant eppin on eppin-semenogelin binding. As the antibodies are diluted, the binding of eppin to semenogelin increases. Similarly, Figure 3B shows that anti-eppin antibodies from two infertile monkeys, monkey sample nos. 523/625 and 145/656, inhibit eppin from binding to semenogelin. As the antibody is diluted, more eppin binds to semenogelin, and the OD increases.

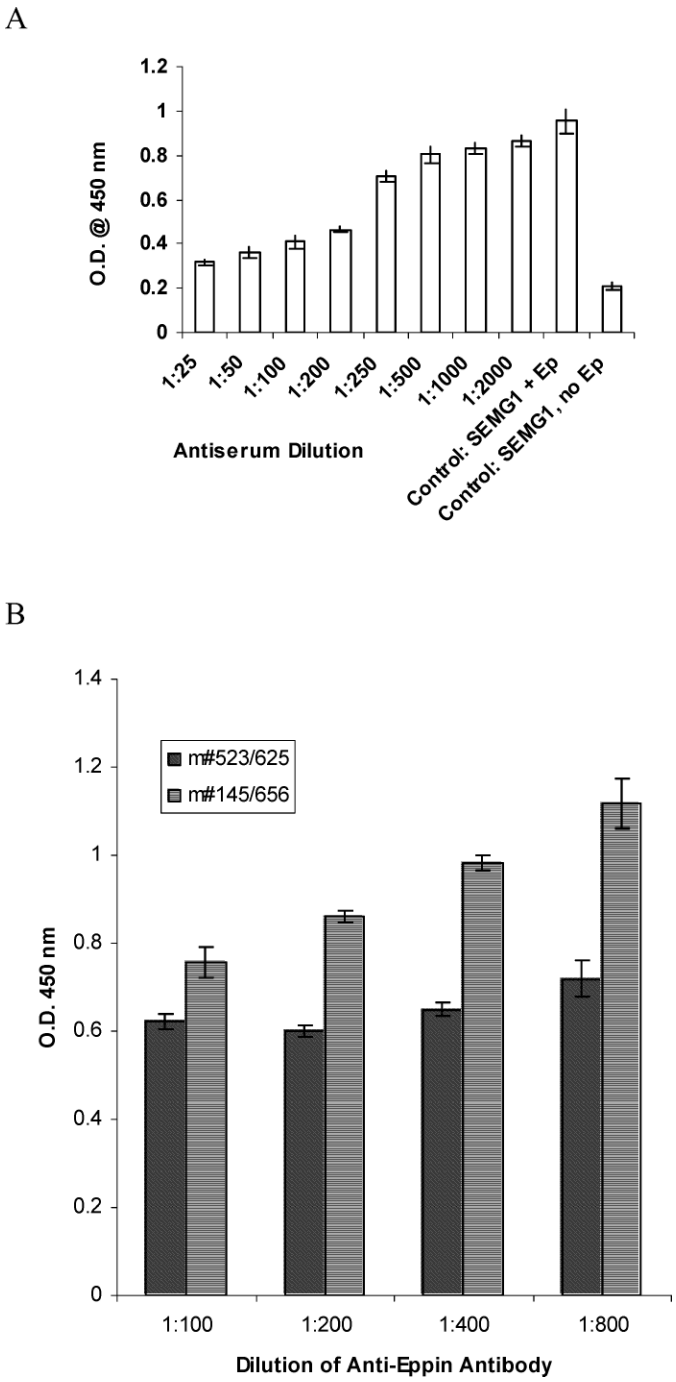

FIG. 3. **A**) In vitro assay of eppin-semenogelin binding. Rabbit affinity-purified anti-eppin antibody inhibits semenogelin (SEMG1) binding to eppin. The positive control was SEMG1 with eppin with no antibody present. The negative control was SEMG1 without eppin with no antibody present. As the antibody is diluted, eppin-SEMG1 binding increases. **B**) Contraceptive antibodies from infertile male monkeys (monkey sample nos. 523/625 and 145/656) inhibit eppin-SEMG1 binding; as the antibody is diluted, eppin-SEMG1 binding increases. Error bars indicate ± SD. ep, eppin; m, monkey; O.D., optical density.

Semenogelin contains a sperm motility inhibitory factor, which has been identified as amino acids 45–136 of the N-terminal of semenogelin [19–24]. Cleavage of semenogelin by PSA during liquefaction removes semenogelin from the sperm surface, resulting in progressive sperm motility. As summarized in Table 3, addition of semenogelin (containing amino

acid residues 5–365) to progressively motile spermatozoa has a concentration-dependent inhibitory effect on human sperm progressive motility as measured by increased tortuosity (7.4 vs. 1.6 in the control, $P = 0.0164$). Velocity decreases ranged from 13% to 53% for treatment of sperm from different donors ($n = 3$; range, $P = 0.01$ to $P = 2.4 \times 10^{-13}$). Figure 1E shows a typical video frame of sperm tracks 60 min after treatment with recombinant human semenogelin. Recorded at 8 frames/sec for 10 sec, progressive straight trajectories and nonprogressive (very short) path lengths can be seen, presumably because individual spermatozoa must bind a critical amount of recombinant human semenogelin (Table 3) to reduce the distances traveled and to decrease their velocities.

Tracks from anti-eppin antibody-treated sperm and semenogelin-treated sperm can be distinguished from progressive hyperactivated sperm tracks. Recording at slower frame rates for longer periods allows better visualization of the hyperactivated pattern. Two hyperactivated sperm tracks are shown in Figure 1F, recorded at 8 frames/sec for 10 sec. These can be distinguished from the progressive straight track in the center of the field. Hyperactivated tracks are usually a combination of sharp directional changes and tight circles [25, 26]. In Figure 1F, the straight-line track has a tortuosity value of 1.05 (track 3) and may be compared with the progressive hyperactivated tracks (tracks 1 and 2), which have tortuosity values of 7.91 (linearity, 13%) and 8.41 (linearity, 12%) respectively, similar to those reported previously for human hyperactivated spermatozoa [12].

Monkey anti-eppin and rabbit anti-eppin antibodies inhibit eppin-semenogelin binding as already described, and semenogelin has been shown to bind to the C-terminal amino acids 76–133 of eppin [4]. Moreover, the dominant specific epitopes of these anti-eppin antibodies have been determined [1, 5]; the contraceptive C-terminal epitope of eppin was found to be amino acids 101–125 (TCSMFVYGGCQGNNNNFQ SKANCLN). Therefore, we prepared affinity-purified rabbit antibodies to amino acids 103–123 in this epitope (antibody S21C) to test whether it would have effects similar to those of treatment with recombinant human semenogelin. Similar to the addition of recombinant human semenogelin to progressively motile spermatozoa, addition of antibody S21C had an inhibitory effect on human sperm progressive motility as measured by increased tortuosity (2.5 vs. 1.5 in the control, $P = 0.006$, two experiments from two different donors, $n = 160$), decreased velocity (18%, $P = 5.42 \times 10^{-5}$, $n = 160$) and by decreased straight distance (18%, $P = 0.007$, $n = 160$).

DISCUSSION

Contraceptive anti-eppin antibodies from male monkeys in our previous study [1] inhibit human sperm progressive motility. The antibodies significantly decrease sperm velocity and the distance they travel, increasing the curvilinear distance:straight distance ratio (tortuosity). The antibodies from individual infertile monkeys are polyclonal, with each individual's antibodies recognizing slightly different eppin epitopes yet all having common dominant epitopes [1]. This may explain the individual differences in the abilities of the antibodies to inhibit progressive motility, as summarized in Table 1. Future studies will focus on specific epitopes.

The contraceptive anti-eppin antibodies seem to disrupt the cAMP regulatory pathway of the sperm. The production of cAMP by adenylyl cyclase (soluble adenylyl cyclase [27]) requires HCO_3^- and Ca^{2+} [15, 17, 28], and loss of progressive motility can be directly correlated with loss of cAMP from inactivation of the mouse *Adcy10* gene [10]. Similarly, disruption of the mouse gene for the sperm-specific Na^+/H^+ exchanger (*Slc9a10*) results in spermatozoa with impaired motility [16]. Progressive motility may be recovered after inactivation of the *Adcy10* or *Slc9a10* gene by loading spermatozoa with cAMP-AM [10, 16, 29]. In the present study, we found that treatment of human spermatozoa with anti-eppin antibodies resulted in a significant increase in cAMP levels in the treated population of spermatozoa, although approximately 25% of these spermatozoa could be rescued by addition of the cAMP analogue cAMP-AM. Increased cAMP is generally associated with increased motility [17, 30]; therefore, the loss of progressive motility after anti-eppin treatment must have been the result of a downstream disruption in the cAMP pathway, resulting in an inability to utilize the cAMP. Inhibition of phosphodiesterase activity would result in increased cAMP levels; however, this would be predicted to increase motility [30]. Increased cAMP levels should have evoked increased Ca^{2+} entry, which also would be predicted to increase motility [30]. Although the three critical components required for motility, namely, cAMP, Ca^{2+}, and pH, may have been disrupted by the anti-eppin treatment, the finding that cAMP levels were elevated for the population of treated spermatozoa without subsequent increased motility suggests the intriguing possibility that coating the surface of the spermatozoon with antibody may cause loss of regulation of intracellular pH. If the sperm-specific Na^+/H^+ exchanger has a role in the compartmentalized control of flagellar pH as suggested by Wang et al. [31], then the slow decline in progressive motility in antibody-treated sperm may reflect a slow decrease in flagellar pH. This idea is supported by the observations that 1) outer dynein arm activity is pH dependent and requires alkalinization, as well as Ca^{2+} and cAMP-dependent phosphorylation [32, 33]; 2) flagella bending is pH dependent [26]; and 3) cAMP is necessary for flagella bending in a 7.9 pH environment [34]. In addition, it is possible that the free diffusion of lactate into the surrounding medium following conversion from pyruvate [35] might be blocked by antibody binding, decreasing the flagellar pH. Further experiments will be necessary to test this hypothesis.

The role of semenogelin during ejaculation has been reviewed by Robert and Gagnon [8]. In the efferent ducts and epididymis before ejaculation, eppin is localized on the entire surface of spermatozoa [2]. Upon ejaculation, semenogelin is added from the seminal vesicles and binds to eppin within the coagulum and on the sperm surface [4]. Eppin completely covers the sperm surface, and most recently we have shown that an eppin protein complex containing lactotransferrin, clusterin, and semenogelin is localized on the sperm tail [3]. To our knowledge, monkey anti-eppin antibodies have not been used to detect eppin by immunofluorescence on human spermatozoa. During liquefaction, semenogelin must be removed from the sperm surface [8] because it is a natural inhibitor of human sperm progressive motility [19–24, 36], and in this study we demonstrated that recombinant human semenogelin inhibits human sperm progressive motility. Using semenogelin I purified from semen, Yoshida et al. [36] demonstrated that treatment of spermatozoa with semenogelin decreased the straight-line velocity and linearity of human spermatozoa. Our previous observation that reduction and carboxymethylation of the only cysteine of semenogelin inhibit its binding to eppin [4] has been confirmed by mutation of the semenogelin amino acid cysteine; mutant recombinant human semenogelin does not bind eppin and removes the ability of recombinant human semenogelin to inhibit progressive motility (A. Mitra, personal communication).

Our demonstration that contraceptive antibodies to eppin that inhibit sperm motility also inhibit semenogelin binding to eppin (Fig. 3) suggests that eppin-semenogelin binding is a key step in regulating sperm motility. The inhibition of motility by anti-eppin antibodies directed toward the C-terminal contraceptive epitope of eppin on the surface of spermatozoa confirms the importance of this site in regulating motility. Consequently, we conclude from our studies that the eppin-semenogelin binding site is the key interaction site for the loss of semenogelin binding and for the initiation of progressive motility in vivo after liquefaction. It is also the key site for the inhibition of progressive motility by anti-eppin antibodies. This epitope is at least partially engaged in binding semenogelin because mutant semenogelin that does not bind eppin does not affect progressive motility. Our results (Fig. 1 and Table 2) support our previous hypothesis [5] that the effect of having anti-eppin antibodies on the surface of spermatozoa mimics the physiological effect of having the sperm motility inhibitor semenogelin bound to the surface. As a result of our analysis of the contraceptive effect of anti-eppin antibodies on human spermatozoa, we conclude that the eppin-semenogelin binding site on the surface of human spermatozoa is an ideal target for a nonsteroidal male contraceptive.

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