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Hyperactivation Enhances Mouse Sperm Capacity for Penetrating Viscoelastic Media[1]

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ABSTRACT

A movement pattern known as hyperactivation has been observed among sperm recovered from the periovulatory oviduct of several species. In culture medium, hyperactivated sperm swim in a pattern that is far less progressive than that of freshly ejaculated sperm. In the oviduct, sperm encounter highly viscoelastic substances, such as mucus and the cumulus matrix. We have previously reported that hyperactivated hamster sperm become more progressive in vitro when the viscosity of medium is increased. In the present study, we tested the effect of increasing the viscosity and viscoelasticity of the medium on the swimming progressiveness of mouse sperm. Caudal epididymal sperm were incubated in a medium that produced hyperactivated motility in 60 min. Swimming velocities of sperm incubated for 60 min were compared with those of fresh sperm after addition of one of the following to culture medium: solutions of 1.8% methylcellulose (high viscosity), 1.8% long chain polyacrylamide (high viscoelasticity), or culture medium alone (low viscosity). In culture medium, hyperactivated sperm had significantly lower mean straight-line velocities than fresh sperm ($p = 0.004$); this difference disappeared in methylcellulose ($p = 0.085$) and was reversed in polyacrylamide ($p = 0.004$). This and other velocity measurements indicated that hyperactivated mouse sperm penetrate viscoelastic media more efficiently than fresh sperm and therefore may be more efficient at penetrating oviductal mucus and cumulus matrix in vivo.

INTRODUCTION

Sperm recovered from the oviducts of several species during the periovulatory period exhibit a vigorous swimming pattern known as hyperactivation [1, 2]. Although this pattern appears to be nonprogressive in culture medium on microscope slides, it may be quite progressive in vivo, enabling sperm to move up the oviduct and penetrate the vestments of the oocyte. Unlike the flat surfaces of microscope slides, the mucosal surface of the oviduct is intricately structured due to extensive folding of the mucosa and twisting of the tube itself (see anatomical review by Hunter [3]). Unlike low-viscosity culture medium, the fluids sperm encounter in the oviduct contain mucous secretions [4–7], a viscoelastic extracellular cumulus matrix [8–10], and the elastic fibrillar network of the zona pellucida [11]. Under some of these varied and complex conditions in vivo, the flagellar beating pattern of hyperactivation may be advantageous for the sperm. We have demonstrated that hyperactivated hamster sperm maintain progressive motility to a greater extent than fresh sperm when the viscosity of the medium is increased through the addition of Ficoll, a nonionic synthetic polymer of sucrose [12].

The cumulus matrix is both viscous and highly elastic [8, 9], and sperm could behave differently when faced with a viscoelastic barrier than when faced with a simple viscous barrier. Therefore, in this paper, we describe the effect of increasing the viscosity of the medium through the addition of methylcellulose and increasing the viscoelasticity of medium through the addition of long-chain polyacrylamide. We chose these relatively unreactive substances to test for the independent effects of high viscosity and viscoelasticity in order to isolate such effects on sperm movement from chemical interactions (including charge attractions) between sperm and their environment.

While hyperactivated golden hamster sperm undergo the increases in flagellar bend amplitude and asymmetry that are characteristic of hyperactivation, their various movement patterns are qualitatively different from those of human sperm and the sperm of many farm animals. In the present work, the effect of increasing viscoelasticity was tested in mouse sperm. Mouse sperm are more similar to human sperm in terms of size [13] and thickness of dense fibers in the tail [14] than are hamster sperm. Freshly activated epididymal mouse sperm roll as they swim in slide chambers, as do ejaculated human and farm animal sperm, while freshly activated hamster sperm do not [15]. Neither human sperm nor farm animal sperm are suitable for these experiments because, to the best of our knowledge, only low percentages can be induced to hyperactivate in vitro [16, 17].

The results provide new information about the functional advantages of hyperactivation, in that mouse sperm were able to penetrate the viscoelastic medium more effectively after hyperactivation.

MATERIALS AND METHODS

Materials

The medium consisted of 110 mM NaCl, 2.68 mM KCl, 0.36 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.49 mM $MgCl_2$, 2.4 mM

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$CaCl_2$, 25 mM HEPES buffer, 5.56 mM glucose, 1.0 mM pyruvic acid, 0.006% Na penicillin-G, and 20 mg/ml of Fraction V BSA [18] (12659; Calbiochem, La Jolla, CA), 290–300 mOsm. The pH was adjusted to 7.6 with NaOH; then the medium was sterilized by filtration through a 0.22-μm Millex-GV filter (Millipore, Bedford, MA). All organic chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), except for HEPES buffer and Fraction V BSA, which were from Calbiochem. Viscoelastic medium was made by dissolving straight-chain polyacrylamide (6 000 kDa, Scientific Polymer Products Inc., Ontario, NY) directly in medium. Methylcellulose (143 kDa; Sigma, M-0512) was also dissolved directly in medium.

Slide chambers were made using slides and coverslips coated with a thin film of 0.4% agar [12]. Two strips of a single layer of Parafilm (American Can Co., Greenwich, CT) were used to support the coverslips. The resulting chamber was approximately 22 mm wide and 60 μm deep.

Sperm Preparation and Experimental Treatments

Retired breeder outbred ICR mice were obtained from Harlan Sprague Dawley (Kingston, NY) and maintained under a 14L:10D diurnal cycle. Sperm were collected as described previously [18]. Briefly, mice were killed by cervical dislocation and placed in a 37°C chamber. Their caudal epididymides were transferred to a 35-mm petri dish (Falcon 1008, Becton Dickinson, Oxnard, CA) containing 3 ml of medium. Each epididymis was punctured with a 27-gauge needle and the dish was incubated in the dark at 37°C. After 10 min, 1 ml of medium containing highly motile sperm was aspirated and placed in a prewarmed 1.5-ml microcentrifuge tube (USA/Scientific Plastic, Ocala, FL), where sperm numbers were adjusted to 2×10^6/ml by further addition of medium. The tube was placed in a 37°C incubator with an atmosphere of 5% CO_2 in air. After 0 and 1 h of incubation, 3 μl of sperm was added to prewarmed slide chambers and covered with 27 μl of 2% polyacrylamide gel or 2% methylcellulose in medium. Controls were covered with 27 μl of medium alone. According to the manufacturers, the viscosity of a 2% aqueous solution of either methylcellulose or polyacrylamide at room temperature is 4 000 centipoise. Collection of sperm motion data began within 10 sec following addition of control or experimental media to the chamber. This experiment was replicated 5 times. A different mouse was used each time and the contents of both epididymides were pooled.

Videomicroscopy and Image Analysis

The slides were placed on a 37°C heated stage of a Zeiss Axiovert microscope (Carl Zeiss Inc., Thornwood, NY) and the sperm in the chambers were videotaped through a 20× phase-contrast objective using a black-and-white CCD camera (CCD 72, Dage MTI, Michigan City, IN). The light source was a xenon stroboscope (Chadwick Helmuth Model 10030; El Monte, CA); the lamp flashed with a duration of 15 μsec/flash and a frequency of 30 flashes/sec. The light was passed through a red filter to minimize damage to sperm. Elapsed time in 0.01-sec intervals was recorded simultaneously with the image of the sperm via a Panasonic AG 7300 Super VHS video cassette recorder. At least 20 microscope fields were recorded for 2–3 sec each to ensure that 20 fields would be available for analysis. For analysis, 1 sec of videotape from each recorded field was digitized using 4 Epix Silicon Video Mux frame grabbers (Z Trends, Claremont, FL). The first sperm in focus to enter each microscope field was analyzed. On the digitized images, a cross hair was positioned over the head/midpiece junction of the sperm, and the x/y coordinates of the positions in 30 successive frames (equivalent to 1 sec of videotape) were used to calculate velocities, using an image analysis system (ESIMAGE, Elektron System Inc., St. Petersburg, FL; modified by Stephen Varosi with permission of the company).

The following velocity variables were determined for each sperm: straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP) [19]. VSL was calculated as the straight-line distance traveled by the head/midpiece junction over time. VCL was calculated as the total distance covered by the head/midpiece junction over time. VAP was the velocity over the average path of the sperm calculated as a five-point running average [20]. The linear index (VSL/VAP) [20], a measure of swimming straightness, was also determined.

The data were analyzed using Systat software (Systat Inc., Evanston, IL). Means were obtained from the 20 sperm measured for each treatment (medium control, methylcellulose, and polyacrylamide) and time point (fresh and hyperactivated). The means were used to test for effects of treatment and time, using a repeated measures ANOVA model. Post-hoc comparisons were made using paired *t*-tests, with the Bonferoni correction applied for multiple comparisons [21]. Differences with a probability of chance occurrence below 0.05 before the Bonferoni correction were considered significant.

RESULTS

The 2% solution of methylcellulose in medium was visibly viscous, as was the 2% solution of polyacrylamide. The polyacrylamide solution exhibited far greater elasticity. It could be drawn out into elastic strings about 1.5–2.0 cm in length, while the methylcellulose solution could not be drawn out more than 1 mm.

Freshly collected epididymal sperm exhibited the vigorous progressive motility of sperm activated from storage in the epididymis [22]. Samples of mouse sperm that had been incubated for 60 min exhibited the overall increase in flagellar bend amplitude and beat asymmetry characteristic of hyperactivation [22]. They exhibited frequent directional changes, as previously shown for hyperactivated mouse

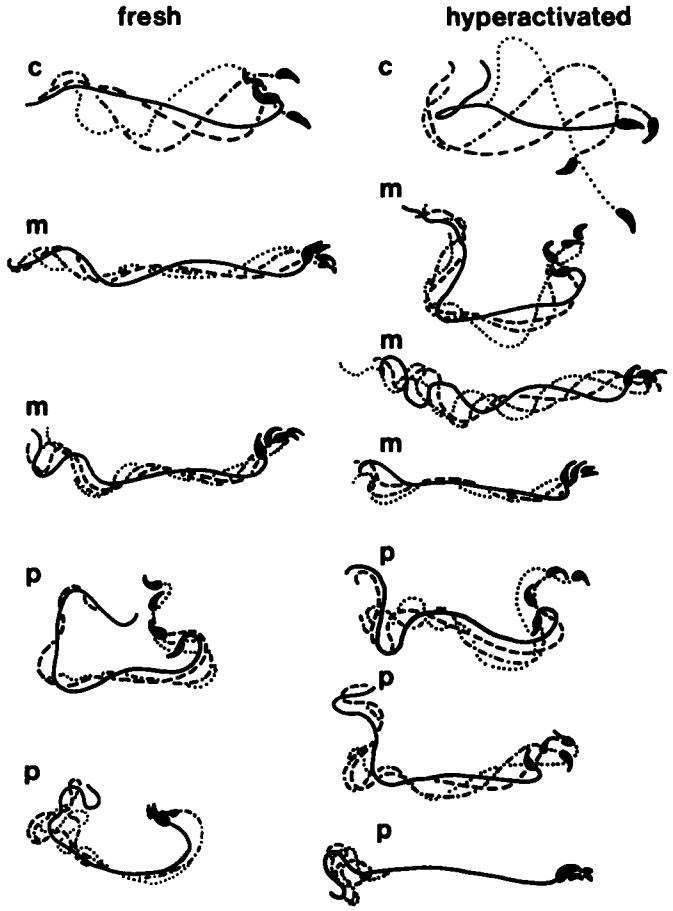

FIG. 1. Flagellar bending patterns of fresh and hyperactivated mouse sperm in control medium (c) and solutions of methylcellulose (m) and polyacrylamide (p) in medium. These are tracings from individual videoframes. Chronologically, the first tracing is represented by a solid line, the second by a dashed line, the third by a dashed/dotted line, and the fourth by a dotted line. The four tracings of each sperm were selected to cover about one beat cycle, beginning at the point of its initiation. For fresh sperm, the tracings were taken at every frame for the top three sperm (total time elapsed = 0.1 sec), every fifth frame for the fourth sperm (time elapsed = 0.5 sec), and every tenth frame for the bottom sperm (time elapsed = 1 sec). For hyperactivated sperm, the tracings were taken every frame for the top sperm (time elapsed = 0.1 sec), every other frame for the second and third sperm (time elapsed = 0.2 sec), every frame for the fourth sperm (time elapsed = 0.1 sec), and every fifth frame for the bottom 3 sperm (time elapsed = 0.5 sec).

sperm [20]. In some samples these changes were continuous, creating a circular movement pattern. We did not attempt to categorize individual sperm as hyperactivated or not, because we have found these distinctions difficult to make for mouse sperm as compared with hamster sperm. In reporting and discussing results, we will refer to sperm that had been incubated for 60 min as hyperactivated, meaning that the predominant type of motion observed among sperm in the sample was a vigorous nonlinear movement involving deep flagellar bends; sperm prior to incubation will be referred to as fresh.

Addition of solutions of methylcellulose and polyacrylamide had a marked effect on the movement patterns of both fresh and hyperactivated sperm (Fig. 1). In methylcellulose, the flagellar bend amplitude and wavelengths of fresh sperm were markedly decreased, but the sperm remained progressive, snaking through the solution. Helical or rolling movement, commonly seen in sperm swimming in medium alone, was rarely seen in the sperm in methylcellulose or polyacrylamide solutions. In polyacrylamide, however, most fresh sperm became nonprogressive, arching backwards to swim slowly in circles. Some did move forward, but slowly. The addition of methylcellulose produced similar movement patterns in hyperactivated and in fresh sperm; that is, the swimming became progressive in most sperm. When polyacrylamide was added to the medium containing hyperactivated sperm, most snaked straight through the solution. The flagellar waves were dampened in the midpiece, even to the point where they were barely perceptible, but then grew in amplitude in the principal piece. The appearance of these sperm was similar to that of fresh human and bovine sperm swimming in estrous cervical mucus [2, 23].

Quantitative analyses of sperm velocities also revealed a difference in the response of fresh and hyperactivated sperm to polyacrylamide and methylcellulose. Additions of methylcellulose and polyacrylamide each resulted in significantly lowered mean VSL, VCL, and VAP. The overall effect of treatment as measured by ANOVA was highly significant ($p < 0.0001$), and all the t-tests comparing the addition of each agent to medium indicated a significant reduction in

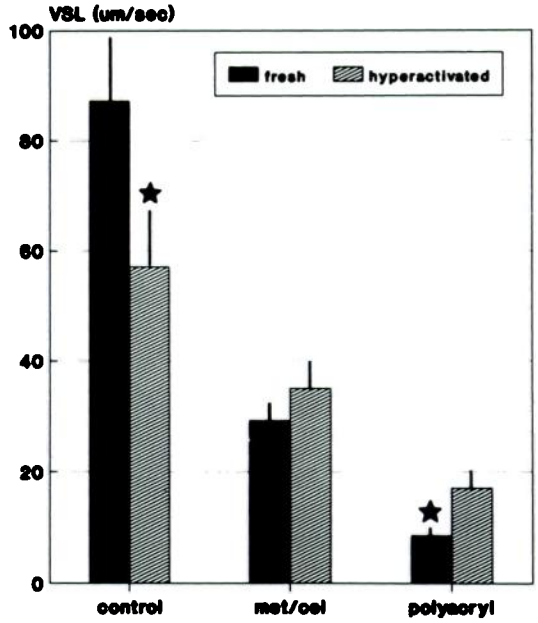

FIG. 2. Mean VSL for fresh and hyperactivated mouse sperm swimming in control medium, methylcellulose, and polyacrylamide. For both fresh and hyperactivated sperm, paired t-tests indicated that the mean VAP were significantly lower in methylcellulose than in control medium, and in polyacrylamide than in methylcellulose (highest p value = 0.007). The star indicates a significant difference between hyperactivated and fresh sperm within the treatment ($p < 0.004$).

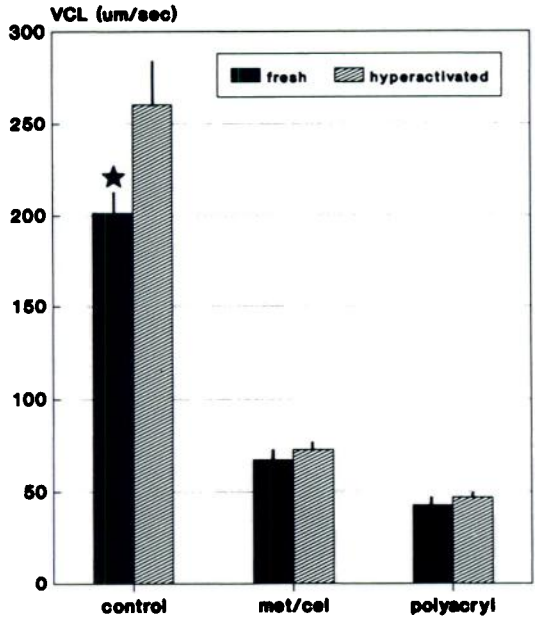

FIG. 3. Mean VCL for fresh and hyperactivated mouse sperm swimming in control medium, methylcellulose in medium, and polyacrylamide in medium. For both fresh and hyperactivated sperm, paired *t*-tests indicated that the mean VCL were significantly lower in methylcellulose than in control medium, and in polyacrylamide than in methylcellulose (highest p value = 0.0007). The star indicates a significant difference between hyperactivated and fresh sperm within the treatment ($p < 0.002$).

all three velocity variables (no p value exceeded 0.007). Samples of fresh sperm in medium alone had significantly higher mean VSL values (Fig. 2) and significantly lower mean VCL values (Fig. 3) than hyperactivated samples, while the mean VAP values (Fig. 4) did not differ significantly. In the viscous solutions of methylcellulose, VSL, VAP, and VCL did not differ between fresh and hyperactivated samples. In the viscoelastic solutions of polyacrylamide, the mean VSL and VAP values for hyperactivated sperm were actually significantly higher than for fresh sperm, while there was no detectable difference in VCL.

As shown in Table 1, the mean linear index for hyperactivated sperm in the control medium was significantly lower than for fresh sperm, as would be expected. In contrast, addition of polyacrylamide significantly lowered the mean linear indices for fresh sperm but raised linear indices for hyperactivated sperm. In polyacrylamide, the mean linear indices of hyperactivated sperm were actually higher than for fresh sperm. Thus, the path of hyperactivated sperm was straightened in the viscoelastic medium. The effect of the methylcellulose was intermediate, bringing the mean linear index of hyperactivated sperm up to that of fresh sperm.

DISCUSSION

Each of the velocity measurements signifies a different aspect of sperm movement. The VCL measures the velocity of the head/midpiece junction and does not distinguish between lateral movements caused by flagellar wave initiation (i.e., the tail wagging the dog) and the directional movements resulting from flagellar wave propagation. The VCL is dependent upon flagellar beat frequency, wavelength, and amplitude, and can be considered a measure of the instantaneous swimming speed [19]. The VAP represents an attempt to measure directional movement of the sperm cell, that is, the velocity of the sperm cell along its trajectory, by eliminating the lateral movements of the head [20, 24]. VAP was determined objectively, by calculating a five-point running average of the position of the head [20]. VSL is the progressive swimming speed.

Given these interpretations of the velocity measurements, the following conclusions could be drawn about the results of the data analysis. The VCL data, as a measure of instantaneous velocity or activity, indicate that while hyperactivated sperm thrashed about more rapidly than fresh sperm in medium, the difference disappeared in viscous and viscoelastic media. The translation of this activity into progressive movement (VSL) indicates that hyperactivated sperm progressed more efficiently in viscoelastic medium than did fresh sperm, even though they progressed far less efficiently in medium alone. The VAP data indicate that the translation of flagellar beats into directional movement of the whole cell was approximately equal for hyperactivated and fresh sperm in medium and in methylcellulose, but

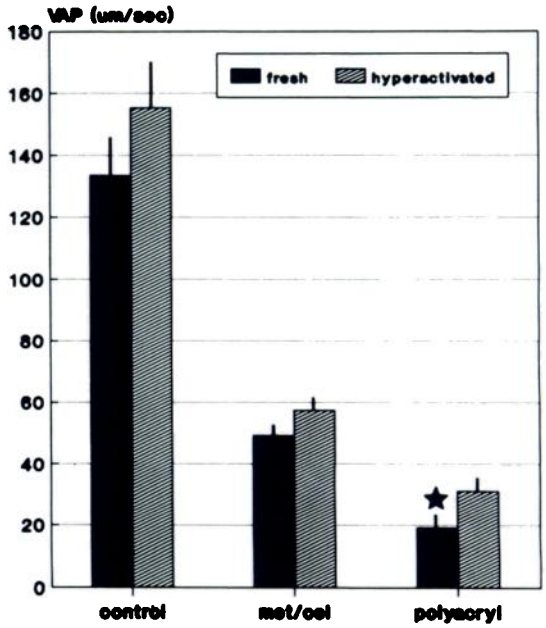

FIG. 4. Mean VAP for fresh and hyperactivated mouse sperm swimming in control medium, methylcellulose, and polyacrylamide. For both fresh and hyperactivated sperm, paired *t*-tests indicated that the mean VAP were significantly lower in methylcellulose than in control medium, and in polyacrylamide than in methylcellulose (highest p value = 0.0003). The star indicates a significant difference between hyperactivated and fresh sperm within the treatment ($p = 0.005$).

TABLE 1. Mean linear indices (VSL/VAP) of fresh and hyperactivated sperm swimming in control medium, methylcellulose, and polyacrylamide (n = 20 sperm/sample); standard deviations are indicated in parentheses. Five experiments were performed.

Sperm	Control	Methylcellulose	Polyacrylamide
Fresh	0.597 (0.085)	0.579 (0.204)	0.494 (0.132)
	0.622 (0.127)	0.612 (0.122)	0.444 (0.230)
	0.736 (0.164)	0.586 (0.134)	0.414 (0.130)
	0.586 (0.134)	0.582 (0.169)	0.425 (0.153)
	0.755 (0.185)	0.671 (0.198)	0.442 (0.119)
Mean	0.659*[a] (0.080)	0.606[a] (0.039)	0.444*[b] (0.031)
Hyper	0.389 (0.153)	0.545 (0.104)	0.548 (0.102)
	0.373 (0.176)	0.677 (0.143)	0.567 (0.158)
	0.375 (0.145)	0.643 (0.165)	0.555 (0.149)
	0.294 (0.137)	0.612 (0.128)	0.590 (0.106)
	0.450 (0.187)	0.662 (0.157)	0.501 (0.114)
Mean	0.376*[a] (0.056)	0.628[b] (0.052)	0.552 *[b] (0.033)

*$p < 0.01$ for comparison within column.
[a,b]Means with different superscripts indicate that $p < 0.01$ for comparisons within the row.

detectably superior for hyperactivated sperm in polyacrylamide.

It was apparent through observation that hyperactivated sperm were more progressive in polyacrylamide solutions than were fresh sperm. Since the mean VSL and VAP values were higher for hyperactivated sperm than for fresh sperm in polyacrylamide, while the mean VCL values were not different, these measures also indicated that hyperactivated sperm were more progressive than fresh sperm. The linear indices suggested that the hyperactivated sperm swam in straighter trajectories in the highly viscous and viscoelastic media than in a low viscosity medium, and swam straighter than even fresh sperm in highly viscoelastic media. While quantitative measurements of velocity and progressiveness are meant to impart objectivity to analysis of sperm movement, they can be blind to some important aspect or characteristic of sperm movement. However, in these experiments, our visual impressions agreed with the objective data. It was obvious that sperm movement was depressed when viscosity and viscoelasticity were increased and also that the hyperactivated sperm moved more quickly and progressively through the polyacrylamide than did the fresh sperm.

In these experiments, we attempted to compare the effects of increased viscosity with those of increased viscoelasticity. While it is difficult to measure separately the viscosity and elasticity of aqueous solutions [25], we attempted to start with equally viscous solutions (according to the manufacturers' information) that exhibited an obvious visual and tactile difference in elasticity. Methylcellulose and the selected polyacrylamide are both unbranched chain polymers (the polyacrylamide was not cross-linked). The polyacrylamide chains have a molecular mass of about 6 000 kDa, whereas that of the methylcellulose is only 143 kDa [26]. Elasticity has been found to be dependent upon chain length [27]. Since the viscosities of the two substances were equal at 2% according to the manufacturers, theoretically the elastic component of the polyacrylamide should be greater. Therefore, to the best of our knowledge, the differences observed between the effects of methylcellulose and polyacrylamide are attributable to the increased elasticity of polyacrylamide.

If hyperactivated sperm are more efficient at penetrating viscoelastic media, one might ask why sperm are not hyperactivated at ejaculation in species for which sperm must penetrate cervical mucus. Perhaps the cumulus matrix and the mucous secretion in the oviductal isthmus present greater viscoelastic barriers to sperm than does estrous cervical mucus. Yudin and coauthors [28] have examined the ultrastructure of both cumulus matrix [9, 10] and cervical mucus [28] and have noted that the fibrillar elements of the cumulus matrix are more regularly interconnected, implying stronger elastic behavior for the cumulus matrix than for estrous cervical mucus [28]. Overstreet and Katz [29] have proposed that pericoital pressure changes assist sperm transport into and through cervical mucus so that sperm at the external cervical os may have assistance not available to sperm at the periphery of the cumulus. The oviductal mucus may also provide a more viscoelastic barrier than the cervical mucus. We have found that mucus produced by bovine and porcine oviductal epithelia in culture is nearly impenetrable by fresh bull and boar sperm [7, 17, 30]. Thus, hyperactivation may be necessary in the oviduct but not in the cervix.

In previous experiments with hamster sperm, Ficoll was used to increase viscosity of the medium [12]. Methylcellulose was used instead in our experiments because over 70% of mouse sperm became immobile in Ficoll solutions. We observed in five preliminary experiments that methylcellulose had an effect similar to that of Ficoll on hamster sperm movement patterns and therefore used methylcellulose for the mouse sperm. Methylcellulose has been used extensively to test the effects of viscosity on sperm movement [26, 31–33]; it was chosen for these experiments also because it is an unbranched chain, as is the polyacrylamide. The movement patterns of mouse sperm in methylcellulose differed from those of hamster sperm in Ficoll and in methylcellulose. In mouse sperm, the flagellar wave increased in amplitude at the distal end of the tail (Fig. 1), while in hamster sperm it dampened or increased only very slightly [12]. This difference may be attributable to the differences in cross-sectional area of the outer dense fibers in the tail: the fibers of hamster sperm are much thicker than those of mouse sperm [14, 23].

In summary, hyperactivated mouse sperm can swim through highly viscoelastic medium more quickly than fresh mouse sperm. While hyperactivated movement is nonlinear in a medium of low viscoelasticity, the trajectory of hyperactivated mouse sperm becomes straighter when the sperm enter highly viscous or viscoelastic environments. Thus hyperactivation may be advantageous for sperm moving

through viscous oviduct fluid, viscoelastic oviductal mucus, and the viscoelastic cumulus matrix.

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