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Effect of Pentoxifylline on the Intrinsic Swimming Forces of Human Sperm Assessed by Optical Tweezers

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ABSTRACT: It is still controversial whether in vitro exposure of sperm to pentoxifylline increases sperm motility and force, which is defined as the product of velocity by beat frequency of the tail. Laser optical tweezers have been successfully used in the past to evaluate sperm force in basal conditions. The aim of this prospective study was to determine whether exposure of human sperm to pentoxifylline has any effect on sperm intrinsic forces. Twelve healthy subjects undergoing routine semen analysis were enrolled in the study. Ten exhibited normal semen parameters, 2 exhibited asthenozoospermia. Each semen specimen was washed and, after swim-up, resuspended in human tubal fluid (HTF) and divided into 2 aliquots. One aliquot was incubated with pentoxifylline for 30 minutes (final concentration = 3.6 mM); the second aliquot, without pentoxifylline, served as a control. After 30 minutes the pentoxifylline-treated aliquot was divided into 2 portions, 1 of which was washed to remove the pentoxifylline, the other was left in prolonged coincubation with the chemical. The main outcome was the measurement of sperm intrinsic force in milliwatts (mW), which was assessed by means of a noninvasive infrared laser optical trap created by a continuous wave, 1064-nm Nd:YAG laser beam directed in an inverted microscope. Exposure of sperm to pentoxifylline consistently increased sperm relative escape force from the laser optical trap. The increase ranged from 33% to 154% over baseline force compared with controls. The average absolute increase in sperm force rose from 37 mW to 79 mW ($P < .05$). Specimens with sperm having an initial low relative escape force gained the highest relative increase. The effect of pentoxifylline on sperm force, already apparent after 5 minutes, reached a peak at 30 minutes and persisted for up to 3 hours in sperm that were left in coincubation and in those on which the pentoxifylline had been washed off. In conclusion, pentoxifylline significantly increases sperm intrinsic relative force in normozoospermic and asthenozoospermic samples. This experiment confirms that optical tweezers can provide an accurate determination of sperm force in in vitro conditions. Clinical data must now establish whether a documented increase in sperm force is an important parameter for assessing sperm fertilizing capacity.

Key words: Male infertility, in vitro fertilization, sperm biochemistry, sperm biophysics, sperm stimulants.

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In recent years, a number of groups have reported on pentoxifylline and its use as a chemical sperm stimulant (Sikka and Hellstrom, 1991; Tesarik et al, 1992; Lewis et al, 1993, 1994; Pang et al, 1993). Pentoxifylline is a phosphodiesterase inhibitor that allows accumulation of sperm intracellular adenosine triphosphate and, hence, the enhancement of sperm motility. Some authors have suggested that pentoxifylline increases sperm velocity in both normal and asthenospermic samples or in asthenozoospermic samples only (Sikka and Hellstrom, 1991; Tesarik et al, 1992). In a recent study in which sperm motion parameters were assessed by computer analysis, it was argued that the addition of pentoxifylline to normospermic samples increases neither the number of progressively motile sperm nor their progressive velocity; it increases only the curvilinear velocity and the amplitude of lateral head displacement of sperm that are already motile (Lewis et al, 1993, 1994). Yet another study using computer analysis found an increase in velocity and vigor when sperm were exposed to pentoxifylline (Tournaye et al, 1994); however, the parameter of intrinsic swimming force in sperm after exposure to pentoxifylline has not yet been investigated through measurement.

Relative intrinsic sperm forces can be objectively and accurately measured using the optical forces produced within an optical laser trap (optical tweezers), in which a highly focused laser beam is used to optically confine motile sperm samples. The minimum power of a trapping laser beam at which sperm can escape from the optical field becomes a direct measure of the sperm intrinsic relative escape force (Tadir et al, 1989, 1990) because the applied optical force is directly proportional to the intrin-

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sic forces of the cell sample. In this study, we evaluated the intrinsic force of sperm from 10 normozoospermic men and 2 asthenospermic men before and after in vitro exposure to pentoxifylline and the duration of the effect for different time intervals.

Materials and Methods

Subject Selection

Semen specimens were obtained by masturbation from 12 healthy donors. Ten volunteers fulfilled the minimal criteria defined by World Health Organization (WHO) guidelines (World Health Organization, 1992) and were classified as normozoospermic nonsmokers and as antisperm-antibody negative. The remaining 2 donors, also nonsmokers and antisperm-antibody negative, were classified as asthenozoospermic (sperm motility 28%, and 32%, respectively). Each subject provided a sample after 48 hours of sexual abstinence. This research was approved by the Investigational Review Board (Protocol # 95-35) at the Beckman Laser Biology Institute, University of California at Irvine.

Preparation of Sperm Samples

Following liquefaction, each sperm sample was examined by light microscopy for count, percentage motility, progression (scale 0–4), and percentage of normal forms. Each semen sample was washed in a capacitation medium of HEPES-buffered human tubal fluid (HTF; Irvine Sciences, Irvine, Calif) supplemented with 0.3% human serum albumin, and centrifuged at 300 × *g* for 10 minutes. After discarding the supernatant, the pellet was gently tapped and resuspended in 1 mL HTF medium. Motile spermatozoa were isolated using the swim-up procedure at 37°C for 45 minutes. The supernatant was removed and stored at room temperature. The sample from each subject was then divided into 2 equal aliquots. One was incubated with pentoxifylline for 30 minutes (1 mg/mL = 3.6 mM concentration; used clinically as the sperm stimulant). The second aliquot served as the control and was mixed with an equal amount of HTF medium. After 30 minutes, the pentoxifylline-treated aliquot was divided in half. One portion was washed to remove the pentoxifylline, the other remained in a prolonged coincubation with pentoxifylline. Samples from the controls were likewise washed and centrifuged. For analysis, sperm samples were loaded into a microchamber for the laser-trapping microscope. Swimming force measurements were carried out up to 3 hours after the initial incubation period. A minimum of 80 sperm were analyzed from each specimen.

Experimental System for Optical Trapping and Sperm Force Analysis

Figure 1 depicts the experimental system that was used to create an optical laser trap (ie, optical tweezers) to noninvasively confine and measure the swimming force of sperm samples. The system consists of a continuous wave Nd:YAG laser beam that is directed into an inverted optical microscope. The laser emits up to 500 milliwatts (mW) in the TEM_{00} mode at 1064 nm. The beam is first polarized using a linear polarizer, moderately focused using a converging lens, and then highly focused to a near-diffraction-limited spot size using a high magnification and large numerical aperture oil-immersion microscope objective (MO; Zeiss Neofluar 100×, 1.3 N.A.; Zeiss, Jena, Germany). This process creates an optical trap in the microscope object plane and fixes the trap position at a specific height above the bottom cover slide of the sample chamber. Typically, this height was kept at 5 μm in order to avoid changes in trapping efficiency during measurement. A half-wave plate (λ/2 plate) located in front of the linear polarizer was used to continuously vary the incident laser power. A portion of the incident beam (~10%) was deflected by a beam splitter onto a photodetector so that the actual amount of laser power delivered into the sample chamber could be continuously monitored and calibrated. Another beam splitter placed just below the MO was used in conjunction with a video camera and monitor to visualize the sample. A sample was then loaded into a microchamber that had been maintained at room temperature and monitored using a thermocouple probe. The heating effect on the medium in the vicinity of the laser trapping beam is about 10.3°C, and its effect on sperm intrinsic force is, therefore, considered negligible.

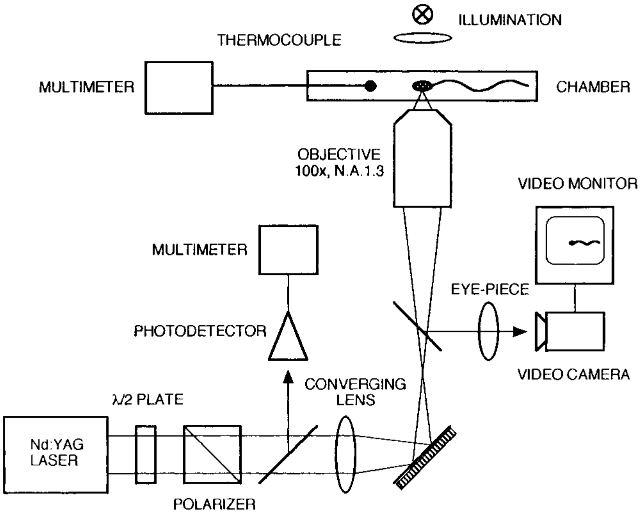

Figure 1. Schematic of the laser trapping system used to measure swimming forces produced by chemically treated human spermatozoa. A trapping beam is derived from a continuous wave Nd:YAG laser that emits light at 1064 nm in the fundamental TEM_{00} mode. The output laser power can be varied between 0 and 500 mW by rotating a half-wave plate against a fixed polarizer. The beam is brought to focus at a given depth within the sample chamber using a 100× (1.3 N.A.) oil-immersion microscope objective. Sperm trapping is visualized with a video camera and monitor.

The laser trapping power was initially set to a value of about 250 mW during experiments. This created an optical trap that caught sperm that swam within a 5-μm radius of the trap without causing physical harm or thermal damage to them. After a sperm cell was caught, the laser power was gradually reduced by adjusting the λ/2 plate until the cell could no longer be held by the optical beam, and swam away. The power at which the cell escaped the trap was then recorded. Because the optical power was directly proportional to the mechanical swimming force of the spermatozoa, any change to the intrinsic force of the sample in response to a physical, chemical, or biological forcing factor

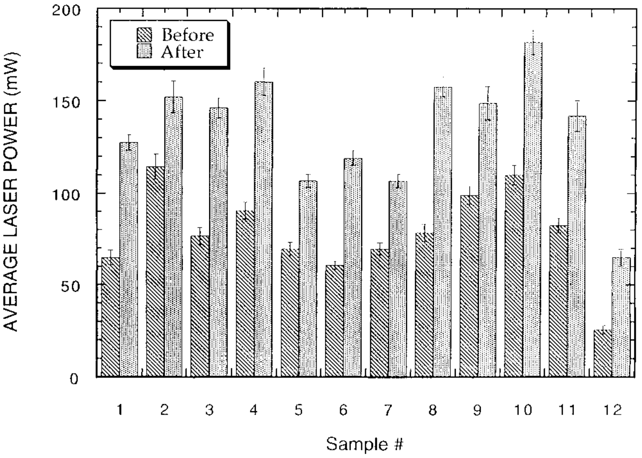

Figure 2. Histogram of the average laser power required for trapping human sperm before and after treatment with pentoxifylline. Twelve different sample groups were tested. For all groups, a consistently higher laser trapping power was required to confine sperm after chemical treatment, indicating an increase in intrinsic swimming force. Numbers 6 and 12 indicate subjects with asthenozoospermia.

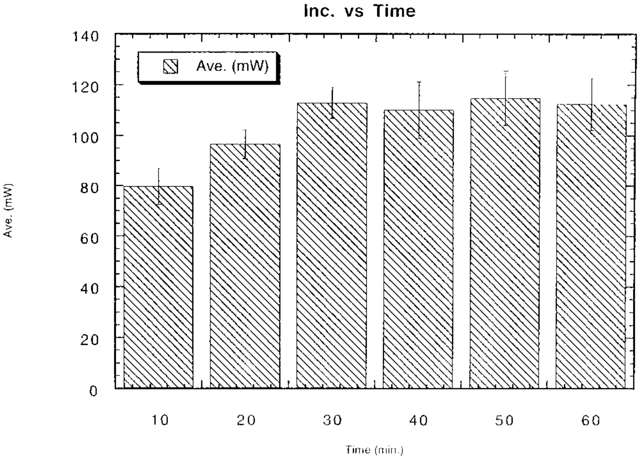

Figure 3. Relationship between average laser power and pentoxifylline incubation time. The average laser power (ie, swimming force) required to confine swimming sperm reached a maximum after 30 minutes of chemical incubation.

would appear directly as either an increase or decrease in the amount of power required for trapping.

Statistical Analysis

The changes in sperm relative escape force with and without drug exposure at each time point were recorded for each subject and each experiment. Statistical analysis of differences was carried out by paired *t*-test.

Results

In vitro incubation with pentoxifylline consistently resulted in an increase in the average amount of laser power required to confine sperm samples derived from all 12 donors. These results are shown in the histogram (Figure 2), which compares the trapping power of samples from the control group (untreated samples) with those that underwent treatment with pentoxifylline. In all cases, the optical power needed to confine sperm increased, indicating that sperm intrinsic swimming forces increased. In Sample 1, for example, an average power of 65 mW was required to confine untreated sperm, whereas a power of nearly 130 mW was needed to confine sperm that had been exposed to pentoxifylline. This represents an increase of 100% ($P < .05$). For all 12 patients, the percentage increase in laser trapping power ranged from about 33% up to 155%; in absolute measurements the increase varied from about 37 mW to 79 mW.

The relationship between laser trapping power and incubation time for pentoxifylline was also examined (Figure 3). Sperm samples were measured for drug incubation times that ranged between 5 and 60 minutes. Measurements were made as soon as 5 minutes after pentoxifylline was introduced into the sample medium, and every 10 minutes thereafter. The minimum laser power required to confine sperm in the control group was 60 mW. Within the first 10 minutes of pentoxifylline addition, the minimum trapping power increased to 80 mW, and 95 mW at 20 minutes. At 30 minutes, sperm escape force reached a maximum of 113 mW. The critical exposure of sperm to pentoxifylline stimulated the highest sperm intrinsic swimming forces. In another set of experiments, a comparison was made between sperm that had remained in the pentoxifylline medium beyond the initial 30-minute incubation period and those that were washed immediately after 30 minutes of incubation. No obvious differences in the minimum amount of trapping laser power could be observed; therefore, the effects of pentoxifylline stimulation were both maximized and saturated after 30 minutes.

Discussion

Pentoxifylline significantly increases sperm relative escape force in normospermic samples. A similar effect was also observed in the 2 samples with asthenozoospermia. The increase in relative escape force in normospermic samples contrasts with observations (Lewis et al, 1993; Yovich et al, 1994) that pentoxifylline had no effect on progressively motile sperm in normal samples; yet, tends to agree with reports stating that pentoxifylline *does* increase sperm velocity and vigor (Fuse et al, 1993; Tournaye et al, 1994; Tarlatzis et al, 1995). Each sperm sample reached maximal stimulation potential relative escape force within 30 minutes. Furthermore, the effect of pentoxifylline exposure was observed for up to 3 hours in specimens that were kept in the medium containing pentoxifylline as well as in those from which the chemical was removed. Maintenance of increased sperm force for this period of time may suggest that sperm have an adequate chemical reserve to sustain such metabolic activity.

Any significant modification in sperm cell response as a result of sample heating induced by the laser trap can be ruled out for this study. Recent work has shown that, at 1064 nm, the increase in temperature of an optically confined sperm cell is less than 1°C per 100 mW of laser power (Liu et al, 1995). In the present study, the temperature of the medium was constantly monitored by means of a thermocouple that was located about 100 μm away from the center of the laser trap. A temperature variation of less than 0.3°C was observed during all experiments; therefore, changes in sample or medium temperatures do not appear to be factors that affect intrinsic sperm force. Furthermore, because the laser power was reduced, within a few seconds of initial confinement to a level that would facilitate escape of sperm from the trap, it is likely that any side effects derived from the trap itself are negligible.

We have directly analyzed the effects of the drug in terms of minimum laser power required to hold motile sperm rather than in terms of intrinsic swimming force. The absolute intrinsic force of a spermatozoon is difficult to determine because such a force depends on the optical properties of the sperm head at 1064 nm and a detailed knowledge of the forces and beating patterns produced by the flagellum. However, these factors affect only the efficiency of the optical trapping process. The trapping force is still linearly proportional to the applied laser power. Hence, the least amount of laser power needed to optically confine a sperm cell can be used to effectively evaluate relative intrinsic swimming force. As an estimate, these intrinsic forces are several tens of pico-newtons, assuming that the trapping efficiency for the sperm in the given optical system is about 10%.

In conclusion, the use of optical tweezers allows an objective and accurate determination of sperm force. Optical tweezers offer several advantages in the analysis of drug effects such as those of pentoxifylline. They can provide data on relative intrinsic sperm forces with great accuracy because fluctuations in laser power, which represent the main source of measurement error, can be experimentally controlled to better than 1%. The measurement technique is also noninvasive and, essentially, a damage-free tensiometric method (Colon et al, 1992; Liu et al, 1995). Many more studies of sperm force response to chemical stimulants can be carried out with this model. Clinical data have now to establish if sperm force and its subsequent increase after pentoxifylline exposure—more than sperm velocity or motility—is an important parameter by which to assess sperm fertilization capacity in vitro.

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