

## Peritoneal fluid from women with moderate or severe endometriosis inhibits sperm motility: the role of seminal fluid components\*

Engin Oral, M.D.

Aydin Arici, M.D.

David L. Olive, M.D.

Gabor Huszar, M.D.†

*The Sperm Physiology Laboratory, Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Connecticut*

**Objective:** To examine the mechanism of sperm motility inhibition by peritoneal fluid (PF) from women with endometriosis.

**Design:** Prospective, randomized study.

**Setting:** University-based andrology laboratory.

**Patients:** Women with and without endometriosis.

**Interventions:** Fresh semen or Percoll-purified sperm fractions were combined with PF from women with endometriosis (n = 20), from fertile women without endometriosis (n = 10), or with physiological saline.

**Main Outcome Measure:** Sperm motility parameters were determined with computer-assisted semen analysis. Data were evaluated by the analysis of variance and the Student's *t*-test.

**Results:** Peritoneal fluid from women with minimal or mild endometriosis did not inhibit sperm motility in semen. Peritoneal fluid from women with moderate or severe endometriosis caused approximately 40%, 50%, and 80% declines in sperm motility and in percent progressive motile sperm, after 4, 7, and 24 hours, respectively. Sperm velocity was inhibited by approximately 30% and 60% after 7 and 24 hours, respectively. However, in the Percoll-purified sperm fractions the same PF did not inhibit sperm motility within the 4- to 7-hour time frame, and only a 17% to 42% inhibition occurred after the overnight incubation. Sperm velocity was not affected.

**Conclusion:** Cellular components of seminal fluid appear to mediate the inhibitory action of PF. Assuming that the leukocyte components of semen and PF are common, the cell-mediated inhibition of sperm motility is a likely contributor to endometriosis-related infertility.

Fertil Steril® 1996;66:787-92

**Key Words:** Endometriosis, peritoneal fluid, CASA, sperm motility

Endometriosis, a disease commonly seen in women of reproductive age, is associated with pelvic pain and infertility. It is estimated that 25% to 50% of infertile women have endometriosis, and among women with endometriosis, 30% to 50% are infertile (1). Although many factors are implicated in the un-

derlying pathophysiology, the exact mechanism by which endometriosis interferes with fertility is not known (2-4). It has been suggested that the peritoneal fluid (PF) from these patients may inhibit various sperm functions (5-7). Peritoneal fluid is the medium for ovum transport and its content also affects sperm motility and sperm-oocyte interaction in vivo. The adverse effects of PF from infertile women were reported by several laboratories (5, 6). However, some aspects are controversial, because of inconsistencies, such as in the definition of patient groups, in the time frame of observations, or in the use of selected sperm fractions versus whole semen. Another aspect of endometriosis yet to be understood

Received April 11, 1996; revised and accepted June 5, 1996.

\*Supported by grants HD-19505 and HD-32902 (to G.H.) from the National Institutes of Health, Bethesda, Maryland.

†Reprint requests: Gabor Huszar, M.D., Department of Obstetrics and Gynecology, Yale University School of Medicine, 333 Cedar Street FMB 331, New Haven, Connecticut 06510-8063 (FAX: 203-737-1200).

is the association between the increased numbers of peritoneal macrophages, which promote cell-mediated cytotoxicity, and sperm phagocytosis (8).

Because sperm motility is the best predictive parameter for fertilizing capacity (9), various studies have addressed the effect of PF on sperm motility, which was found to be reduced in infertile women with endometriosis (9–11). However, other reports did not confirm these findings (12, 13). The conflicting results may be due to differences in the quality and preparation of sperm and PF samples, to variations in incubation times, and to the methods of sperm motility assessment.

Several investigators have attempted to identify the PF constituents that are responsible for the inhibition of sperm motility. Cytokines such as interleukin-1, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$ , activated macrophages, prostanoids, and autoantibodies have all been considered (7, 14). Physiological levels of TNF- $\alpha$  inhibit sperm motility in a dose-related manner, and this inhibition was eliminated with the addition of anti-TNF- $\alpha$  antibody (7, 15).

Because of the importance of retention of sperm motility and viability for fertility, the goal of this study was to assess, objectively, the time-related effects of PF from women with minimal or mild and moderate or severe endometriosis on sperm motility and velocity in semen and in Percoll-purified sperm fractions.

## MATERIALS AND METHODS

### Peritoneal Fluid Collection

Peritoneal fluid samples were obtained from women undergoing diagnostic laparoscopy. All PF samples were collected on days 12 to 16 of the menstrual cycle to ensure uniformity. Informed consent was obtained according to the protocol approved by the Institutional Review Board of the Yale School of Medicine. Endometriosis was confirmed by direct laparoscopic visualization and/or by biopsy of lesions. The median age of the women was 31 years (range 26 to 39 years) for the endometriosis group and 34 years (range 31 to 40 years) for the control group. Symptoms of the endometriosis group ( $n = 20$ ) included infertility ( $n = 11$ ), pelvic pain ( $n = 6$ ), and dysmenorrhea ( $n = 3$ ). The severity of the disease was staged by the revised American Fertility Society classification (16) as patients with minimal or mild endometriosis ( $n = 10$ ) and patients with moderate or severe endometriosis ( $n = 10$ ). Control PF was collected from 10 fertile women undergoing laparoscopic tubal ligation for sterilization.

After insertion of the laparoscope and ancillary

trocars, PF was aspirated immediately to avoid blood contamination. Fluid from the anterior and posterior cul-de-sacs was collected into heparinized tubes and immediately was transported to the laboratory. Blood-free PF was centrifuged at  $600 \times g$  for 20 minutes at room temperature to remove cells. The supernatant was aliquoted and immediately stored at  $-80^{\circ}\text{C}$ . Peritoneal fluid samples to be tested were thawed and filtered through a  $0.22\text{-}\mu\text{m}$  Millipore filters (Falcon; Becton Dickinson Co., Lincoln Park, NJ) into plastic sterile tubes ((Falcon; Becton Dickinson) immediately before their addition to sperm sample.

### Experimental Design

Fresh normospermic (17) semen specimens were used from 20 men (concentration  $\geq 40 \times 10^6$  sperm per ejaculate, motility  $\geq 50\%$ , and  $\geq 30\%$  normal morphology) who presented for semen analysis at the Sperm Physiology Laboratory. Semen samples were collected by masturbation after 2 days of abstinence into sterile, plastic containers and were allowed to liquefy for  $\geq 60$  minutes. In the first set of experiments PF was combined with semen at 1:1 ratio (vol/vol) and duplicate aliquots were incubated at  $37^{\circ}\text{C}$ . In each experiment aliquots of the sperm samples were incubated separately with PF of women with minimal or mild endometriosis, of women with moderate or severe endometriosis with PF, of women with no disease and with normal saline.

In the second set of experiments the spermatozoa were separated from the seminal plasma by discontinuous Percoll (Sigma Co., St. Louis, MO) gradient centrifugation using a two-step gradient comprised of 2 mL 40% and 2 mL of 80% Percoll as described (18). The semen specimens were layered on top of the gradient, and the separation of spermatozoa from the seminal fluid was achieved by centrifugation at  $500 \times g$  for 20 minutes. All of the supernatant was removed, and the pellet was washed with 1 mL modified human tubal fluid with bovine serum albumin, 5 mg/mL, (Irvine Science Co., Irvine, CA). After a washing step ( $400 \times g$  for 5 minutes), the pellet was resuspended in the same medium to a sperm concentration of  $20 \times 10^6$  to  $50 \times 10^6/\text{mL}$ . The PF was combined with the Percoll-purified sperm fractions at 1:3 ratio (vol/vol) and duplicate aliquots of samples were incubated at  $37^{\circ}\text{C}$  with PF samples from the women undergoing tubal sterilization, with PF from women with moderate or severe endometriosis, and with the saline control.

### Sperm Motility Analysis

In both experiments the sperm motility parameters were determined at 0 time, and after 2, 4, 7

hours, and overnight incubations. Sperm motility in semen and in the Percoll sperm fractions were analyzed by computer-assisted sperm analysis (CASA) with the Hamilton Thorn Integrated Visual Optical System version 10.6t (Hamilton-Thorn Research, Beverly, MA). At each time a 6- $\mu$ L aliquot was placed on a prewarmed 20- $\mu$ m Cell-VU Chamber (Fertility Technologies Inc., Natick, MA). The CASA settings used were as follows: frame rate, 60 Hz; number of frames acquired, 30; minimum cell size, 4 pixels; low and medium average path velocity cutoff ( $\mu$ m/s), 7 to 25; and magnification factor, 1.95. For each determination, duplicate samples in three different fields were assessed in two separate drops (6 fields in all) by the following parameters: motility (%), percent progressive motile sperm (path velocity > 25  $\mu$ m/s) (%), straight line velocity (VSL,  $\mu$ m/s), curvilinear velocity (VCL,  $\mu$ m/s), path velocity (VAP,  $\mu$ m/s), beat cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH,  $\mu$ m), straightness (%), and linearity (%).

## Data Analysis

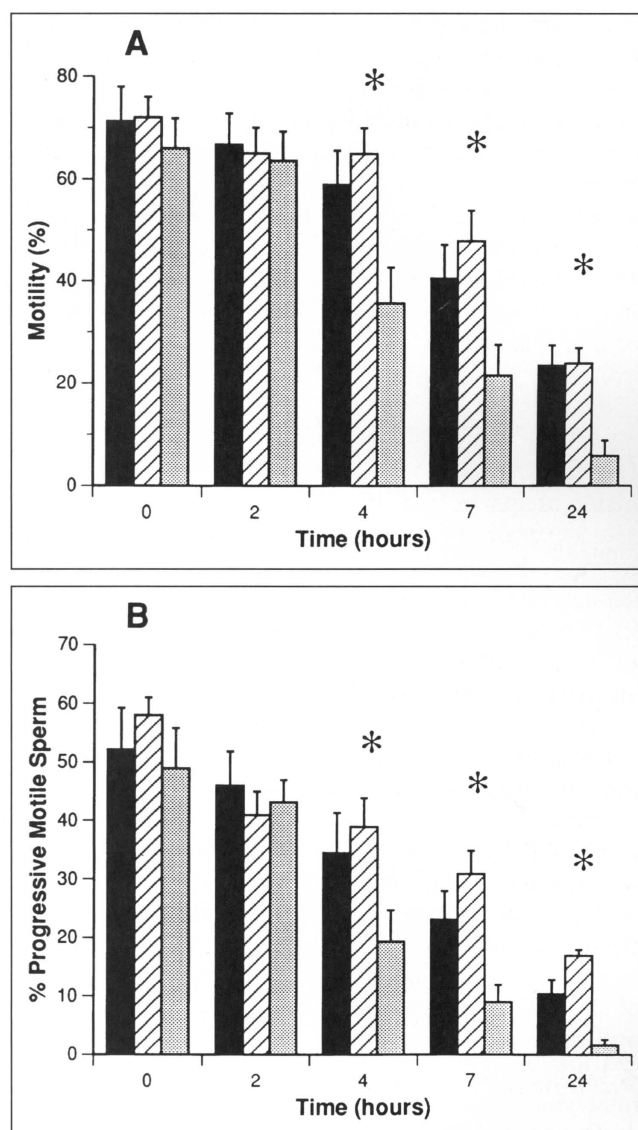
Data were expressed as means  $\pm$  SEM. Velocity parameters were distributed normally, the differences between the groups of PF were analyzed using one-way analysis of variance, followed by Student's *t*-test. *P* value < 0.05 was considered significant. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Version 6.0 for Windows (SPSS Inc., Chicago, IL).

## RESULTS

### Sperm in Semen

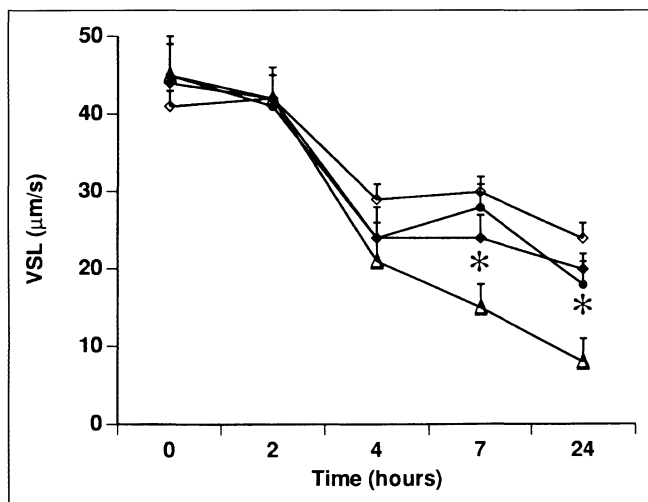
In the first set of experiments 10 normospermic semen samples were studied (concentration:  $72.2 \pm 7.4 \times 10^6$  sperm/mL, motility:  $74.1\% \pm 6.0\%$ , and percent progressive motile sperm:  $57.4\% \pm 6.6\%$ ). The effects of PF on sperm motility parameters from women with or without endometriosis as a function of time are summarized in Figure 1. No significant differences at any time points were observed in motility parameters when semen was incubated with PF from women with minimal or mild endometriosis compared with control, or under saline conditions. However, the motility and percent progressive motile sperm were decreased at 4 hours by 39% and 46%, respectively, in the group incubated with PF from women with moderate or severe endometriosis (*P* < 0.01). By 24 hours the respective inhibitions were 75% and 80% with PF from women with moderate or severe endometriosis, respectively (*P* < 0.005).

The velocity parameters VSL, VCL, and VAP also



**Figure 1** Effect of the PF on sperm motility in semen. (A) sperm motility; (B) percent progressive motile sperm. ■, PF from women without endometriosis; ▨, PF from women with minimal to mild endometriosis; ▤, PF from women with moderate or severe endometriosis. \**P* < 0.01, PF from women without endometriosis versus PF from women with moderate or severe endometriosis.

were diminished in response to PF from women with moderate or severe endometriosis versus the control samples after the 4-hour time point, and by 24 hours the inhibition of these reached the 60%, 55%, and 56% levels, respectively (see VSL in Fig. 2, *P* < 0.05). The patterns of decline in the VCL and VAP parameters were also similar (overnight incubation: VCL:  $33 \pm 3$  versus  $15 \pm 6$   $\mu$ m, *P* < 0.005; VAP:  $23 \pm 2$  versus  $10 \pm 4$   $\mu$ m, *P* < 0.05). This indicates that in addition to the retention of sperm motility in the samples, the velocity of the motile sperm population also is affected by PF from women with moderate or severe endometriosis.



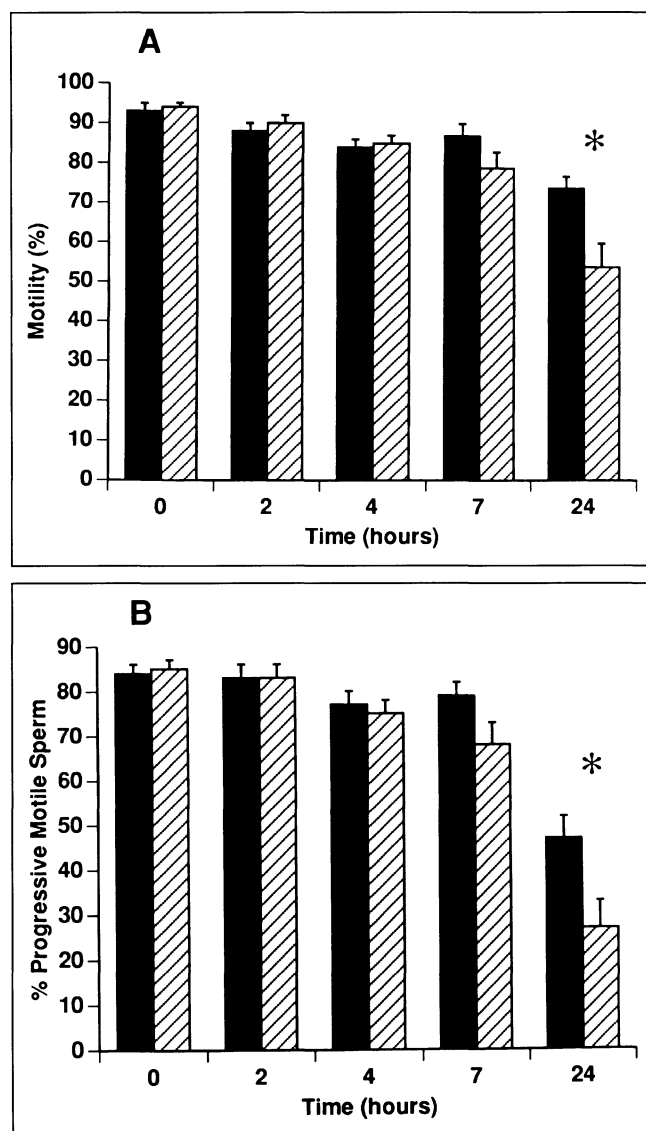
**Figure 2** Effect of PF on sperm velocity in semen.  $\diamond$ , physiological saline;  $\bullet$ , PF from women without endometriosis;  $\blacklozenge$ , PF from women with minimal or mild endometriosis;  $\triangle$ , PF from women with moderate or severe endometriosis. \* $P < 0.05$ , PF from women without endometriosis versus PF from women with moderate or severe endometriosis.

### Percoll-Purified Sperm Fractions

To evaluate whether the inhibition of sperm motility was due to the PF from women with moderate or severe endometriosis alone or if there were other semen factors that mediated this effect, we examined Percoll-purified sperm fractions (concentration:  $37.3 \pm 4.3 \times 10^6$  sperm/mL; motility:  $94.4\% \pm 2.1\%$ ; and percent progressive motile sperm:  $88.0\% \pm 1.9\%$ ,  $n = 10$ ). Peritoneal fluid from women with moderate or severe endometriosis inhibited motility and reduced the percent progressive motile sperm only after the overnight incubation (Fig. 3). The degree of inhibition was much less with Percoll-purified sperm fractions than with semen samples. The decline of sperm motility and percent progressive motile sperm was only 22% and 42% ( $P < 0.05$ ), respectively, substantially lower than the 75% and 80% inhibition in the presence of semen components ( $P < 0.01$ ). Another finding that suggests that the reason for this late and limited inhibition is different from that of the inhibition in semen was the lack of velocity loss in the sperm fraction that remained motile. The velocity parameters VSL, VCL, and VAP did not differ between PF from women without endometriosis and PF from women with moderate or severe endometriosis during 24 hours (data not shown). All other sperm motility parameters measured by CASA, including ALH, straightness, linearity, and BCF showed no consistent differences in either the semen or the Percoll-purified sperm fraction experiments.

### DISCUSSION

In addition to peritoneal adhesions, several factors that may alter the function of the fallopian tubes were suggested as potential causes underlying infertility associated with endometriosis. Peritoneal fluid components may adversely affect ovulatory function, gamete transport or survival, as well as early embryonic development and implantation (2–5). It is also an attractive idea that PF from women with endometriosis contains substances or cells that have a detrimental effect on sperm function. This issue was addressed by many investigators (5–7). In these studies, sperm motility (12, 19) and sperm functions directly related to fertilization, such as capacitation (20), acrosome



**Figure 3** Effect of the PF on sperm motility in the Percoll-sperm fractions. (A) sperm motility (B) percent progressive motile sperm.  $\blacksquare$ , PF from women without endometriosis;  $\hatched$ , PF from women with moderate or severe endometriosis. \* $P < 0.05$ .

reaction (6), zona-free hamster oocyte penetration assay (21), and sperm-oocyte interaction (5) were explored. Initial research in this area also was concerned with peritoneal macrophages from PF, which may have a high capacity to phagocytize sperm (8).

With respect to previous studies dealing with endometriosis and sperm motility, Stone et al. (13) inseminated patients just before laparoscopy and then collected cul-de-sac fluid. No difference in the recovered motile sperm density was found in women with mild endometriosis. Muse et al. found no difference in sperm motility when specimens were capacitated in Ham's F10 solution and incubated with PF from women with and without endometriosis (Muse K, Estes S, Vernon M, Zavos P, Wilson E, abstract). However, Burke (11) reported reduced sperm velocity in capacitated sperm when PF from patients with minimal or mild endometriosis was added to the medium. Oak et al. found a significantly reduced proportion of motile sperm in the presence of PF from women with mild or moderate endometriosis (10). Curtis et al. (12) observed, using CASA, significant reductions in the velocity parameters of sperm (but not in the percent motility) exposed to PF from women with minimal or moderate endometriosis.

In this study there was no inhibition of sperm motility or velocity by PF from women with minimal or mild endometriosis even after the 24-hour incubation, which we carried out to simulate the effects of PF within a time frame similar to that of the presence of spermatozoa in the Fallopian tubes. However, PF from women with moderate or severe endometriosis caused a significant decrease in sperm motility, in percent progressive motile sperm, and in the velocity of the sperm fraction that remained motile. The inhibitory effects of PF were proportional to the grade of endometriosis. Because the higher concentration of macrophages in PF of women with endometriosis may affect oviductal sperm transport and sperm survival, we investigated the impact of PF on Percoll-purified sperm fractions without seminal fluid, which are representative of sperm that reach the upper region of the female reproductive tract.

It is of great interest that the same PF from women with moderate or severe endometriosis did not inhibit the motility of Percoll-purified sperm fractions until after a 24-hour incubation, and even then the sperm velocity was unaffected. The differences in the semen and the Percoll-purified sperm fractions regarding the onset (4 versus 24 hours) and extent (75% versus 22%) of motility inhibition and in the decline of sperm velocity suggest that the mechanisms of inhibition in the presence or absence of semen are different. The lag period in inhibition is consistent with the idea that the components of

seminal fluid mediate the inhibitory action of PF. One possibility is that some cytokines that are known to be elevated in the PF of women with endometriosis (22) may activate the white cell population of semen to secrete factors that will decrease sperm motility and velocity; the lag period is necessary to achieve the inhibitory concentration in the assay. The relevance of our finding to endometriosis is further enhanced by the fact that the white blood cell components are common in the male and female reproductive tract; thus PF cells likely would affect sperm motility *in vivo* in women with endometriosis.

The delayed inhibitory effect of PF from patients with endometriosis also was observed by other investigators, who reported that the decline in sperm motility occurred after  $\geq 3$  hours of incubation (19). Two relevant *in vitro* studies addressed quantitatively the concentrations of PF sperm motility inhibitors: [1] There was a relationship between the inhibition of sperm motility and TNF- $\alpha$  concentrations in the medium (15); and [2] activated leukocyte cultures had to be maintained for 5 to 7 days to achieve lymphokine-monokine concentrations that inhibited sperm motility (7).

With respect to the attenuated inhibitory effect of sperm motility, but not velocity, in the Percoll-purified sperm fractions, a likely possibility is that it is not linked directly to specific components of PF, but rather to the proteolytic enzymes of PF, which diminish sperm membrane integrity and viability during the 24-hour incubation.

The data from the various laboratories clearly indicate the lack of agreement about the effects of PF from women with endometriosis on sperm motility and velocity. There are several factors that may account for this controversy. [1] In some studies semen was mixed directly with PF, whereas in others the investigators performed a sperm washing procedure, which eliminates seminal fluid but not the cellular elements of semen. In other studies swim-up sperm fractions were used exclusively. Our approach, using Percoll-purified sperm fractions, eliminated all cellular components, particularly macrophages that produce enzymes, cytokines, and other factors, in addition to those present in PF. [2] With respect to the PF, in some reports the endometriosis was not classified, whereas in others pooled PF samples, rather than PF originating in an individual patient, was tested. [3] The inhibitory action may be altered by "protective" effects of noncytokine components of PF, which are known to affect sperm motility and velocity in the female reproductive tract, such as hyaluronic acid and follicular fluid proteoglycans (23, 24). [4] Our data indicate that the PF-related inhibition of sperm motility and velocity occurs with a 3- to 4-hour time lag. Most studies investigated

the effects of PF in a shorter time period. [5] Most of the earlier studies assessed sperm motility and velocity by manual observation and not by objective CASA measurements. Indeed, even with other CASA measurements, the algorithms of the Hamilton-Thorn instrument, which allow the selection of percent progressive motile sperm and the classification of sperm populations by the VAP parameters, are of significant advantage.

In any case, we have demonstrated with objective CASA measurements, an inhibitory effect of PF from women with moderate or severe endometriosis on sperm motility, percent progressive motile sperm, and sperm velocity after a 4-hour lag period, in the presence of seminal fluid components. The same PF from women with moderate or severe endometriosis showed a much delayed and lower inhibitory effect on sperm motility and no effect on sperm velocity in the Percoll-purified sperm fractions. These data suggest that the non-sperm cellular components of seminal fluid mediate the inhibitory action. Because the white blood cell components of the male and female reproductive tracts are common, our findings are likely to be related to the pathogenesis of endometriosis-mediated infertility and to a functional idiopathic, or unexplained, infertility in the male partners of women with moderate or severe endometriosis.

## REFERENCES

1. Strathy JH, Molgaard CA, Coulam CB, Melton LJ III. Endometriosis and infertility: a laparoscopic study of endometriosis among fertile and infertile women. *Fertil Steril* 1982; 38:667-72.
2. Thomas EJ. Endometriosis and infertility. In: Thomas EJ, Rock J, editors. *Modern approaches to endometriosis*. Boston: Kluwer Academic Publishers, 1991:113-30.
3. Halme J, Becker S, Haskill S. Altered maturation and function of peritoneal macrophages: possible role in pathogenesis of endometriosis. *Am J Obstet Gynecol* 1987;156:783-9.
4. Halme J. Release of tumor necrosis factor-alpha by human peritoneal macrophages in vivo and in vitro. *Am J Obstet Gynecol* 1989;161:1718-25.
5. Coddington CC, Oehninger S, Cunningham DS, Hansen K, Sueldo CE, Hodgen GD. Peritoneal fluid from patients with endometriosis decreases sperm binding to the zona pellucida in the hemizona assay: a preliminary report. *Fertil Steril* 1992;57:783-6.
6. Arumugam K. Endometriosis and infertility: raised iron concentration in the peritoneal fluid and its effect on the acrosome reaction. *Hum Reprod* 1994;9:1153-7.
7. Hill JA, Haimovici F, Politch JA, Anderson DJ. Effect of soluble products of activated lymphocytes and macrophages (lymphokines and monokines) on human sperm motion parameters. *Fertil Steril* 1987;47:460-5.
8. Muscato JJ, Haney AF, Weinberg JB. Sperm phagocytosis by human peritoneal macrophages: a possible cause of infertility in endometriosis. *Am J Obstet Gynecol* 1982;144:503-10.
9. Aitken RJ, Best FSM, Richardson DW, Djahanbakhch O, Mortimer D, Templeton AA, et al. An analysis of sperm function in cases of unexplained infertility: conventional criteria, movement characteristics and fertilizing capacity. *Fertil Steril* 1982;38:212-21.
10. Oak MK, Chantler EN, Williams CAV, Elstein M. Sperm survival studies in peritoneal fluid from infertile women with endometriosis and unexplained infertility. *Clin Reprod Fertil* 1985;3:297-303.
11. Burke RK. Effect of peritoneal washings from women with endometriosis on sperm velocity. *J Reprod Med* 1987;32:743-6.
12. Curtis P, Jackson AE. Adverse effects on sperm movement characteristics in women with minimal and mild endometriosis. *Br J Obstet Gynaecol* 1993;100:165-9.
13. Stone SC, Himsl K. Peritoneal recovery of motile and nonmotile sperm in the presence of endometriosis. *Fertil Steril* 1986;46:338-9.
14. Leach RE, Arneson BW, Ball GD, Ory SJ. Absence of anti-sperm antibodies and factors influencing sperm motility in the cul-de-sac fluid of women with endometriosis. *Fertil Steril* 1990;53:351-3.
15. Eisermann J, Register KB, Strickler RC, Collins JL. The effect of tumor necrosis factor on human sperm motility in vitro. *J Androl* 1989;10:270-4.
16. The American Fertility Society. Revised American Fertility Society classification of endometriosis: 1985. *Fertil Steril* 1985;43:351-2.
17. World Health Organization. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. 3rd ed. New York: Cambridge University Press, 1992.
18. Huszar G, Vigue L. Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. *Mol Reprod Dev* 1993;34:292-8.
19. Drudy L, Lewis SEM, Kinsella CB, Harrison RF, Thompson W. The influence of peritoneal fluid from patients with minimal stage or treated endometriosis on sperm motility parameters using computer-assisted semen analysis. *Hum Reprod* 1994;9:2418-23.
20. Bielefeld P, Graf MA, Jeyendran RS, De Leon FD, Zaneveld LJD. Effects of peritoneal fluids from patients with endometriosis on capacitated spermatozoa. *Fertil Steril* 1993; 60:893-6.
21. Chacho JK, Chacho MS, Andersen BSD, Scommegna A. Peritoneal fluid in patients with and without endometriosis: prostanoids and macrophages and their effect on the spermatozoa penetration assay. *Am J Obstet Gynecol* 1986; 154:1290-9.
22. Arici A, Tazuke SI, Attar E, Kliman HJ, Olive DL. Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells. *Mol Hum Reprod* 1996;2:40-5.
23. Huszar G, Willetts M, Corrales M. Hyaluronic acid (Sperm Select) improves retention of sperm motility and velocity in normospermic and oligospermic specimens. *Fertil Steril* 1990;54:1127-34.
24. Eriksen GV, Malmström A, Ulldbjerg N, Huszar G. A follicular fluid chondroitin sulfate proteoglycan improves the retention of motility and velocity of human spermatozoa. *Fertil Steril* 1994;62:618-23.