The influence of peritoneal fluid from patients with minimal stage or treated endometriosis on sperm motility parameters using computer-assisted semen analysis

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The aim of this study was to determine the influence of peritoneal fluid from patients with minimal stage or treated endometriosis on sperm motility parameters. Peritoneal fluid aspirated at diagnostic laparoscopy for unexplained infertility from women during the luteal phase of the menstrual cycle (days 20–23) was incubated for 5 h with fresh semen samples obtained from men of recently proven fertility. Spermatozoa were prepared by a swim-up technique from unprocessed semen. Using computer-assisted semen analysis (Hamilton-Thorn Research, MA, USA), sperm motility and motion parameters were observed at 0, 120, 180 and 300 min. Compared with spermatozoa incubated in Earle’s balanced salt solution/human serum albumin, the percentage motility, percentage progressive motility and progressive velocity of spermatozoa incubated in peritoneal fluid from patients without visible endometriosis were significantly higher (P < 0.05). Maximal effect was observed at 3 h and maintained until 5 h. We conclude that in an in-vitro study, in contrast to peritoneal fluid from patients with minimal stage endometriosis, peritoneal fluid from patients with unexplained infertility and no visible endometriosis can improve sperm motility when compared with culture medium. 

Key words: computer-assisted motility analysis/endometriosis/peritoneal fluid/spermatozoa

Introduction

Of all the parameters measured in a typical semen analysis, motility has been considered the most important for predicting fertility (Aitken et al., 1982). Secretions of the female reproductive tract confer functional changes on spermatozoa (Bedford, 1983). Peritoneal fluid bathes the fimbrial end of the Fallopian tubes and thus comes in contact with both ova and spermatozoa. It has been implicated as a mediator of changes in sperm motion (Suarez et al., 1984; Soldati et al., 1989).

Endometriosis, which may be present in varying amounts in up to 30% of diagnostic laparoscopies for infertility (Harrison, 1991), may alter the composition of peritoneal fluid leading to adverse effects on sperm motility and hence fertility. Studies have shown that peritoneal fluid from fertile and infertile patients inhibits sperm motility (Oak et al., 1985; Burke, 1987; Soldati et al., 1989; Curtis et al., 1993). This is in contrast to the findings of other researchers (Stone and Hims, 1986). The conflict may be due to differences in the quality and preparation of spermatozoa and peritoneal fluid samples, the subjective assessment of sperm motion and/or the incubation times measured. In an attempt to evaluate the effects of peritoneal fluid on sperm motility, we addressed each of these problems by adopting stringent criteria in selecting normozoospermic semen donors, the preparation of semen and peritoneal fluid samples, experimental design and using computer-assisted semen analysis (CASA) for an objective measurement of sperm motility. During this study we documented sperm motility parameters in peritoneal fluid over a 5 h period.

Materials and methods

Subject selection

Male

Semen samples were obtained from nine male volunteers whose partners had recently achieved pregnancy following in-vitro fertilization (IVF). All participants had repeatedly demonstrated a normozoospermic semen analysis according to World Health Organization criteria (WHO, 1992; >20 x 106 spermatozoa/ml, >50% with normal progression). In addition, these men had no past or present history of systemic disease and had no known drug ingestion during the 6 months prior to the study. Subjects were asked to abstain from sexual activity for 48 h before the sample was produced. In the 12 h prior to attendance each subject was asked to refrain from any caffeine- or nicotine-containing drug. The sample was produced by masturbation into a wide-mouthed sterile plastic container.

Female

Peritoneal fluid was collected from 27 patients from a population of women scheduled for laparoscopy as part of their investigations for unexplained infertility. Hospital ethical committee approval and informed patient consent were obtained. Entry criteria included 2 years primary infertility, previous mid-luteal progesterone concentrations suggestive of ovulation, normal serum prolactin (<500 IU), and normal post-coital test and semen analysis by WHO standards. Laparoscopy was undertaken during the luteal phase (days 20–23) which had been calculated previously from menstrual data. Patients studied were from three groups on the basis of the presence or absence of visible
endometriosis: group PC, nine unexplained infertile patients with a mean age of 32 years and no visible endometriosis (control patients); group PE, nine unexplained infertile patients with minimal stage endometriosis (American Fertility Score of 1–6) of mean age 30 years; and group PT, nine patients of mean age 31 years who had presented with unexplained infertility, were diagnosed with minimal stage endometriosis at laparoscopy and were then treated for minimal stage endometriosis with nafarelin (200 μg intranasally twice daily) for 3 months.

Collection and preparation of peritoneal fluid
Peritoneal fluid was aspirated at laparoscopy in these three groups of patients and collected from the pouch of Douglas into heparinized tubes. The fluid was centrifuged at 350 g at 4°C for 10 min and the supernatant frozen at −20°C. At the time of the experiment, samples of peritoneal fluid were brought to room temperature and filtered using Millex GV filters.

Preparation of spermatozoa
Semen samples were allowed to liquefy and semen analysis was undertaken according to WHO criteria (WHO, 1992). On liquefaction, spermatozoa were prepared by a multiple tube swim-up separation method from unprocessed semen: 0.5 ml semen was added to each tube and gently layered with an equal volume of Earle’s balanced salt solution (EBSS) supplemented with human serum albumin (HSA) (Buminate, Travenol Laboratories) to give a final concentration equivalent to 10% serum. The tubes were inclined at 45% and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C for 1 h. The supernatant was then removed, taking care not to disturb the zone adjacent to the seminal fluid. The pooled supernatants were centrifuged at 300 g for 20 min and the resultant pellet re-suspended in EBSS/HSA. A final concentration of 10–50×10⁶ spermatozoa/ml was obtained, as recommended by Hamilton-Thorn Research (MA, USA) and similar to that suggested by Neuwinger et al. (1990).

To peritoneal fluid from patient groups PC, PE and PT, a 1+2 dilution of spermatozoa was prepared. As an additional control a 1+2 dilution of spermatozoa to EBSS/HSA was prepared as control (CO). Samples were then incubated in a humidified atmosphere of 5% CO₂ in air at 37°C until analysis.

Sperm motion analysis
Nine experiments were undertaken as described above; in each experiment an aliquot of one of the nine sperm samples was incubated separately with a peritoneal fluid sample from each of the three groups and in EBSS/HSA. Measurements were taken at 0, 30, 60 and 90 min. At each time measurement a 10 μl aliquot was placed on a pre-warmed micro-slide (Fertility Technologies Inc., Natick, MA, USA). A 20 μm deep micro-slide was used as this did not restrict sperm movement and minimized interaction with the chamber walls. The micro-slide was then placed onto the heated stage (37°C) of a Hamilton-Thorn Research Motility Analyser (HTM; Model 2030, version 7.2y). The settings used for the analysis were similar to those used by Burkman (1991): frame acquisition rate, 30 Hz; minimum contrast, 7; minimum size, 6; low gate size, 0.4; high gate size, 1.6; low intensity gate, 0.4; high intensity gate, 1.6; HTM magnification factor, 2.04.

Results
Effect of peritoneal fluid (PC) on sperm motion parameters
At 0 h, immediately after dilution of the spermatozoa with peritoneal fluid or EBSS/HSA, VSL of CO was significantly (P < 0.05) higher than that of PC using a paired t-test. By 2 h this trend had been reversed and the VSL of PC was significantly (P < 0.05) higher than that of CO. This effect was also observed at 3 h and the increased VSL led to an increase in linearities (Table I). At 3 and 5 h the percentage motility and percentage progressive motility in the PC group were enhanced compared with the CO group (Table I and Figure 1). There was no trend of differences in VCL between any patient group or CO at any time-point (Table I).

Effect of peritoneal fluid on sperm head movement
Similarly there were no significant effects on the BCF, except at 0 h when the BCF of spermatozoa incubated in PC and that of PT was lower than that of the spermatozoa incubated in EBSS/HSA (CO) (P < 0.5) (Table II). ALH was not significantly altered by any patient group or CO throughout the experiment (Table II).

Frequency distributions of VSL and VCL of spermatozoa incubated with peritoneal fluid (PC) and EBSS/HSA (CO) for 3 h
Progressive and curvilinear velocity distributions from each individual (4000 spermatozoa each for PC and CO) were examined at 3 h, the point where the greatest mean difference was observed. Although the shapes of the distribution curves were similar in each case (Figure 2A and B), the curve for PC had
shifted to the right (Table I). A large proportion of spermatozoa from CO moved at lower velocities (Figure 2A), with the majority of PC spermatozoa moving at higher velocities.

**Frequency distributions of ALH of spermatozoa incubated with control peritoneal fluid (PC) and EBSS/HSA (CO) for 3 h**

Here the curves overlapped almost completely, accounting for the insignificant difference and mean ALH (Table II and Figure 2C). However, a proportion of spermatozoa from both PC and CO displayed ALH values >5 µm, fulfilling one of the criteria for hyperactivation.

**Response of spermatozoa to individual peritoneal fluids**

Spermatozoa showed similar responses to the majority (7/9) of individual peritoneal fluids. Any inter-individual variability was not consistent over the four times measured.

**Effect of peritoneal fluid with minimal stage endometriosis (PE) or treated minimal stage endometriosis (PT) on sperm motion parameters**

The VCL in peritoneal fluid from treated endometriosis (PT) was significantly lower than that in CO (EBSS/HSA) at time zero. No other significant differences were observed in motility parameters when spermatozoa were incubated in the peritoneal fluid from minimal stage endometriosis patients (PE) or treated endometriosis patients (PT) compared with control peritoneal fluid (PC) at any of the time-points studied (P < 0.05).

**Effect of peritoneal fluid with minimal stage (PE) and treated endometriosis (PT) on sperm head movements**

No significant differences were observed in sperm head movements of spermatozoa incubated in peritoneal fluid from patients with minimal stage endometriosis (PE) or treated endometriosis (PT) compared with control peritoneal fluid (PC) over 5 h (P < 0.05).

**Discussion**

Many studies have shown that peritoneal fluid is a mediator of changes in sperm function. Some studies have shown an inhibition of sperm motility in peritoneal fluid from fertile and infertile patients (Oak et al., 1985; Burke, 1987; Soldati et al., 1989;
Influence of peritoneal fluid on sperm motility

Table II. Effect of peritoneal fluid from patients with unexplained infertility (PC), minimal stage endometriosis (PE), or treated endometriosis (PT) plus the effect of EBSS/HSA (CO) on sperm head movements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Time (h)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude of lateral head displacement (ALH, µm)</td>
<td>PC</td>
<td>4.6 ± 0.3</td>
<td>4.7 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>4.5 ± 0.2</td>
<td>4.8 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PT</td>
<td>4.7 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>4.4 ± 0.3</td>
<td>4.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>4.6 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Beat cross frequency (BCF, Hz)</td>
<td>PC</td>
<td>15.8 ± 0.5</td>
<td>15.7 ± 0.4</td>
<td>16.5 ± 0.4</td>
<td>16.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>15.5 ± 0.5</td>
<td>15.8 ± 0.5</td>
<td>16.1 ± 0.4</td>
<td>15.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PT</td>
<td>15.6 ± 0.4</td>
<td>16.1 ± 0.2</td>
<td>15.4 ± 0.8</td>
<td>16.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>16.7 ± 0.5</td>
<td>16.5 ± 0.3</td>
<td>15.8 ± 0.6</td>
<td>16.2 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 9).
*Significantly lower PC versus CO (P < 0.05, paired t-test).
*aSignificantly lower PT versus CO (P < 0.05, paired t-test).

Curtis et al., 1993). However, in a group of infertile patients no difference was observed in sperm motility in spermatozoa recovered by laparoscopy in patients with or without endometriosis (Stone and Himsl, 1986).

Soldati et al. (1989) suggested that the conflicting results might be due to differences in the quality and preparation of spermatozoa. Here, we used only normozoospermic samples, and to minimize biological variation even further, only samples from donors with recently proven fertility. In addition, we used a sperm preparation which involved spermatozoa swimming out of seminal plasma without washing so that proteolytic enzymes present in the plasma would not come into contact with the peritoneal fluid to alter it.

Although peritoneal fluid contains substances secreted by cells contained in the fluid, e.g. macrophages (Hill et al., 1987), peritoneal fluid is also the product of ovarian exudation which varies with the stage of the menstrual cycle. It is therefore important to standardize the time at which the fluid is collected. We ensured that all peritoneal fluid samples were collected at diagnostic laparoscopy on days 20—23 of each patient’s menstrual cycle to ensure uniformity and that the mean age of patients was similar. In addition, each sample was centrifuged to remove particulate matter and filtered before freezing. Measurements were taken over a range of times from 0 to 5 h in an attempt to observe any time-dependent effects of peritoneal fluid on sperm motility over the range of times during which spermatozoa may be present in the Fallopian tubes.

In this study we found, like Burke (1987) and Soldati et al. (1989), that spermatozoa incubated in peritoneal fluid from patients without endometriosis had the numbers of motile and progressively motile spermatozoa enhanced over those incubated in EBSS/HSA. However, our maximal effect was observed earlier than Soldati et al. (1989) at 3 h and maintained until 5 h. Immediately after dilution of the spermatozoa with peritoneal fluid or EBSS/HSA, VSL of CO was significantly (P < 0.05) higher compared with that of PC. By 2 h this trend had been reversed and the VSL of CO was significantly (P < 0.05) higher than that of PC. This effect was also observed at 3 h and the increased VSL led to an increase in linearity. At 3 and 5 h the percentage motility and percentage progressive motility of PC were enhanced compared with CO. This longer incubation time interval is important in that it probably reflects the in-vivo situation. Similarly, Soldati et al. (1989) and Burke (1987) found an initial decrease in VSL at 2.5 h followed by an increase at 5 h. In our study, the VSL of PC was initially lower than that of CO but this position was reversed at 2 and 3 h. This may be due to differences between peritoneal fluid and culture medium in the initial reaction when incubated with spermatozoa. Interestingly this increase in VSL was actually due to the influence of PC in maintaining the VSL with time, whereas the VSL of spermatozoa in CO dropped off with time. The trends observed in percentage motility and VSL were similar for the majority of sperm samples. Perhaps the sample dependence of the spermatozoa is more variable in a group diagnosed as normozoospermic by basic semen analysis but with unexplained infertility than with a group of normozoospermic samples from men with proven fertility, as in our study.

Our data also showed that peritoneal fluid from patients with minimal stage endometriosis did not significantly improve sperm motility when compared with CO, nor was it significantly worse than sperm motility in PC. Similarly, Stone and Himsl (1986) found no significant difference in the percentage of motile spermatozoa following peritoneal recovery from infertile patients with or without endometriosis. In the study by Stone and Himsl (1986) the control patients were undergoing diagnostic laparoscopy for infertility.

Peritoneal fluid did not appear to enhance hyperactivation. Taking the values of Mortimer and Mortimer (1990) as the criteria for hyperactivation, i.e. VCL ≥ 100 µm/s, linearity < 60%, ALH ≥ 5 µm, no significant differences were seen between patient groups and CO. The mean values for these parameters (Tables I and II) did not suggest that hyperactivation was occurring. This is hardly surprising since it can be a transitional phase, and more frequent measurements than employed in this study would be necessary to detect it. Looking at the distribution frequencies of VCL and ALH it is evident that a small proportion of spermatozoa are fulfilling one of the criteria for hyperactivation.

Sperm motion parameters in the human associated with the in-vivo interaction between spermatozoa and the normal female reproductive tract, oocyte binding and penetration have not been fully elucidated. Difficulties therefore arise in the assessment of
unexplained infertility in the mild stages of the disease (Muse and Wilson, 1987). Identical auto-antibody abnormalities have been reported in unexplained infertility and endometriosis (Gleicher et al., 1987). As no statistically significant differences were observed in this study between the three patient groups, our results may be in agreement with the theory that unexplained infertility precedes endometriosis.

In conclusion, our data show positive sperm motility parameters in peritoneal fluid over 5 h, suggest that peritoneal fluid is a favourable environment for sperm survival and demonstrate clearly that in an in-vitro study peritoneal fluid can improve sperm motility in comparison with culture medium. A deleterious effect of peritoneal fluid from patients with minimal stage endometriosis on sperm motility was not elucidated over the 5 h incubation period of this study. However, peritoneal fluid from patients with minimal stage endometriosis appears to lose some of the ability to improve sperm motility. Specific factors that contribute to the development and progression of endometriosis have not been identified conclusively. Early diagnosis of endometriosis is urgently needed to prevent further deterioration of fertility in patients with unexplained infertility. The interaction between spermatozoa and peritoneal fluid could be taken into consideration when improving gamete culture conditions for assisted conception. Further in-vivo studies on sperm motion parameters in peritoneal fluid at the peri-ovulatory stage of the menstrual cycle from patients with minimal stage endometriosis may therefore provide further insight into the pathophysiology of endometriosis in relation to infertility.

References


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