Cytokine Levels in the Seminal Plasma of Infertile Males

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ABSTRACT: Cytokines released by various cell subsets in the male urogenital tract are capable of markedly influencing sperm function and fertility. We determined the cytokine content in the seminal plasma of patients with unexplained infertility and correlated the results with urogenital infections and sperm parameters. Routine sperm parameters, bacterial culture of seminal plasma and blood follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were obtained from 14 infertile males and 8 healthy control subjects. Interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNFα) levels in the seminal plasma were measured by enzyme-linked immunosorbent assay (ELISA). IL-1β, IL-6, and TNFα levels in the seminal plasma were negatively correlated with the number of progressively motile sperm, but there was no correlation with total sperm counts, viability, pH, morphological alterations, type of abnormality, and hormone parameters. Cytokine levels were significantly elevated in seminal plasma exhibiting bacterial or mycoplasmal infections of the urogenital tract. Urogenital infections lead to an release of inflammatory cytokines, most probably by immunocompetent cells of the lymphocyte/macrophage origin. Cytokines such as IL-1, IL-6, and/or TNFα might influence sperm motility via direct or indirect effects, resulting in reduced mucosa penetration properties. Therefore, our data suggest that cytokines may be involved in reduced male fertility.

Key words: Infertility, semen, cytokines, human sperm, urogenital infection, IL-1, IL-1β, IL-6, TNFα.

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Sperm motility is an important factor in allowing male gametes to reach the fertilization site and to penetrate the zona pellucida (Majumder et al, 1990). Asthenozoospermia is therefore considered one of the major causes of male infertility. Additionally, it has been shown that sperm with abnormal morphology (teratozoospermia) also show reduced motilities (Marchini et al, 1991). The reasons for low sperm motility might have testicular (Eddy, 1988), epididymal (Cooper, 1986), and/or seminal origin (De Lamirande et al, 1986).

In endometriosis, commonly associated with infertility, several products of immunocompetent cells, including interleukin 1 (IL-1) (Fakih et al, 1987) and tumor necrosis factor alpha (TNFα) (Eisermann et al, 1988), have been found in the peritoneal fluid. In male patients, chronic urogenital infections or humeral immune responses (sperm antibodies) (Bronson et al., 1984) have been suggested to play a critical role in reduced sperm quality and fertility. Cytokines released by various cell populations are involved in proliferative and differentiating responses of germinative cell subsets and are capable of markedly influencing the biological activities of these cells (Hill et al,

1987). In addition, proinflammatory cytokines such as IL-1, interleukin 6 (IL-6), and TNFα are capable of reducing the ova-penetrating ability of spermatozoa. Nevertheless, cellular immune responses are largely unexplored in reproduction.

In our study we determined the proinflammatory cytokine repertoire in the seminal fluids of male patients with unexplained infertility. Results were correlated with the presence of urogenital infections, the reduction of motility (asthenozoospermia), and the amount (oligozoospermia) of spermatozoa, as well as with alterations of sperm morphology (teratozoospermia).

Materials and Methods

Material

After 4 days of ejaculatory continence, fresh semen was obtained from 17 males with unexplained infertility. Semen samples from eight healthy fertile persons served as controls. Semen samples were obtained and processed as recommended in the *WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction* (World Health Organization, 1995). The infertile men did not reveal the presence of antisperm antibodies in their seminal plasma or sera (measured by immunobead technique) (Bronson et al, 1985). Clinical data, sperm and hormone parameters, and results of sperm culture are presented in Table 1.

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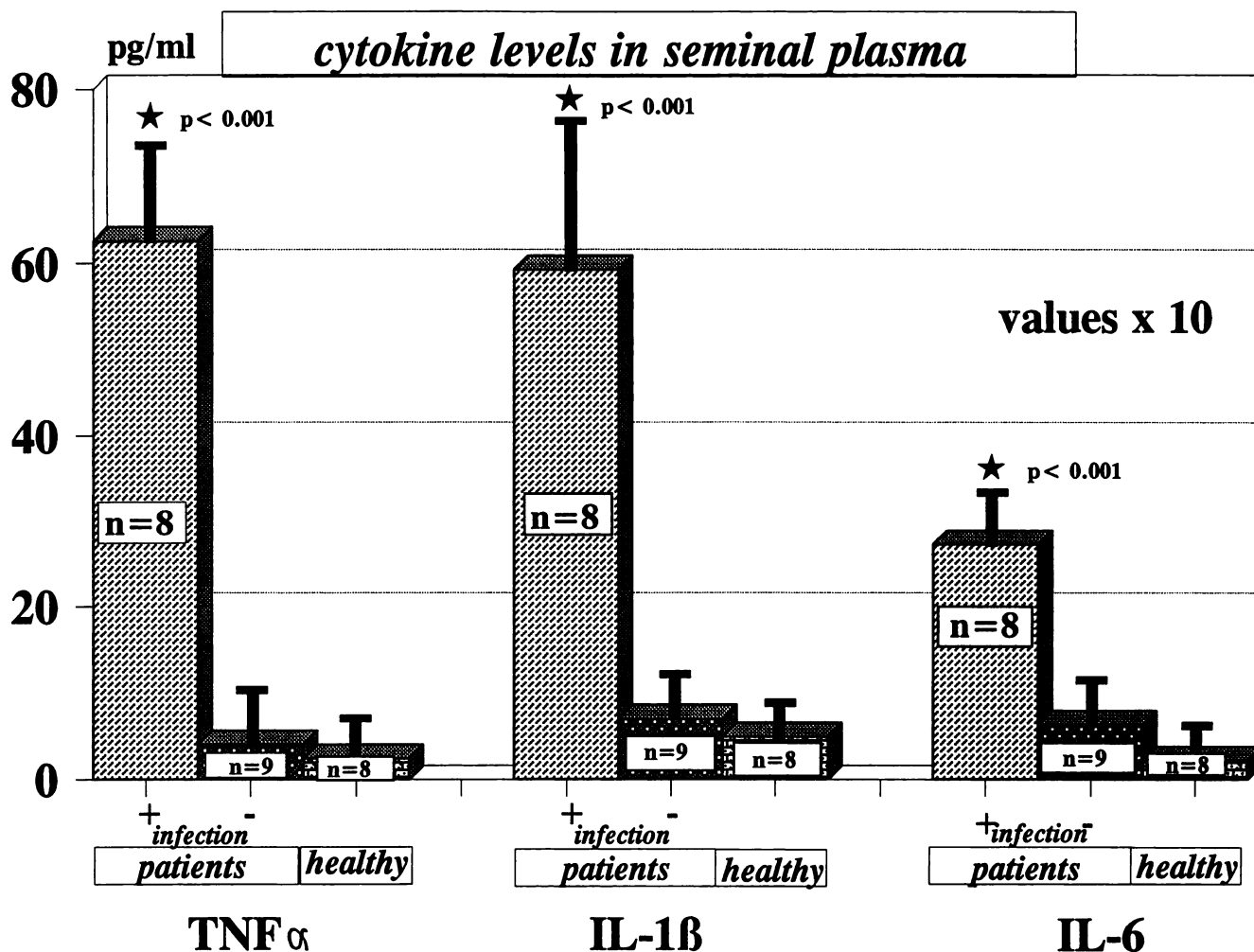
cytokine levels in seminal plasma

FIG. 1. Cytokine levels in seminal plasma in patients with and without bacterial or mycoplasmal infections. Values are expressed as mean ± SEM. Significant differences ($P < 0.001$) are marked by an asterix.

Methods

Sperm Parameters—Semen analysis was performed by determination of pH, liquefaction time, volume, total and progressive motility at 60 minutes after ejaculation, sperm/round cell/germ cell/leukocyte counts, and the total and specific head/midpiece/tail percentage of abnormal forms. Morphology was determined on methanol-fixed (2.5 minutes) and Giemsa-stained (10 minutes) sperm smears by light microscopy and by two independent observers (M.S.G., R.B.). Viability of sperms was measured by eosin Y staining. The concentration of polymorphonuclear neutrophil white blood cells was measured by means of peroxidase staining. Additionally, semen culture to determine bacterial infections and tests for chlamydia (Syva MicroTrak; Syva, Maidenhead, England) and for mycoplasma/ureaplasma urealyticum (transport medium and culture procedure obtained by the Department of Microbiology, University of Erlangen) were obtained.

Cytokine Enzyme-linked Immunosorbent Assay (ELISA)—Seminal plasma was collected from 100 μl of liquified semen by centrifugation. Additionally, a small part of the semen samples

was centrifuged (2,000 rpm, 5 minutes) to pellet the spermatozoa immediately after ejaculation. The protease inhibitor phenylmethylsulfonylfluoride (PMSF; 0.5 mM) was added to both types of fresh semen samples (Naz and Kaplan, 1994a). Supernatants were aspirated, diluted in assay buffer, and stored at $-90^\circ C$ until use for ELISAs.

High-sensitivity ELISAs were used to determine seminal plasma levels of IL-1β (DLB50, Biermann Diagnostika, Bad Nauheim, Germany/R&D Systems, Minneapolis, Minnesota; range 10–500 pg/ml, sensitivity 0.3 pg/ml), IL-6 (D6050; Biermann/R&D; range 31–2,000 pg/ml, sensitivity 0.35 pg/ml), and TNFα (DTA50; Biermann/R&D; range 500–32,000 fg/ml, sensitivity 170 fg/ml), as described elsewhere (Grau, 1990). Cross-reactivity with other related cytokines was less than 0.1%. After enzymatic reaction color intensity was determined spectrophotometrically by an ELISA reader (Multiscan Plus; Flow, ELIAS), the levels of various cytokines were expressed in pg/ml of seminal plasma.

Hormone Parameters—Testosterone serum levels were obtained by radioimmunoassay (RIA) (Biermann GmbH, Bad Nauheim, Germany); levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined by appropriate

Table 1. Clinical data, sperm parameters, and seminal cytokine levels of infertile men and healthy control subjects

Patient no.	Age (years)	Diagnoses*	Sperm count/ml ($\times 10^6$)	Total sperm count ($\times 10^6$)	% Progressively motile sperm	Viability	Morphological abnormalities	Infection	Leukocytes	TNFα (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
						Patients with positive sperm culture (n = 8)						
1	31	AT	35	120	67	94	+	+, ureaplasma	+	40.2	44	213
2	29	OA	11	39	31	87	−	+, chlamydia	−	40.8	43	837
3	29	AT	24	100	43	96	+	+, chlamydia	+	41.1	36	158
4	33	A	50	230	41	88	−	+, enterococcus	−	63.0	88	113
5	30	OAT	17	134	34	89	+	+, ureaplasma	+	83.1	62	574
6	28	OAT	1	10	16	78	+	+, ureaplasma	+	88.0	24	51
7	40	Az	0	0	0	—	−	+, proteus	−	104.3	141	168
8	32	Az	0	0	0	—	+	+, ureaplasma	−	39.5	36	61
									Mean ± SEM	62.5 ± 9.2	59.3 ± 13.6	272 ± 100
						Patients with negative sperm culture (n = 9)						
9	24	OAT	8	48	25	81	+	−	−	3.7	9.0	68
10	31	A	42	237	60	92	−	−	−	9.1	14.0	44
11	33	OAT	20	16	40	95	+	−	+	5.9	9.3	39
12	19	OA	6	12	65	93	−	−	−	1.2	7.0	63
13	23	(A)	40	240	64	96	−	−	−	4.5	2.0	48
14	26	OA	6	22	50	89	−	−	−	3.0	5.0	55
15	41	OAT	1	<1	0	52	+	−	−	1.6	8.6	81
16	24	C	1	<1	0	59	−	−	−	6.0	6.0	52
17	27	OAT	8	49	64	88	+	−	−	1.1	1.8	118
									Mean ± SEM	4.0 ± 0.9	7.0 ± 1.3	63.1 ± 8.1
						Healthy control subjects (n = 8)						
18	28	N	34	109	66	99	−	−	−	0.9	1.2	12.2
19	31	N	42	214	53	97	−	−	−	4.8	6.1	9.3
20	32	N	76	296	59	93	−	−	−	3.2	4.2	38.3
21	27	N	53	148	72	95	−	−	−	1.2	2.9	16.1
22	39	N	128	576	58	91	−	−	−	1.1	10.5	17.0
23	20	N	44	290	80	90	−	−	−	2.8	5.9	35.1
24	33	N	81	490	83	91	−	−	−	3.0	6.7	20.5
25	37	N	80	320	68	96	−	−	−	2.3	1.8	23.7
									Mean ± SEM	2.4 ± 0.5	4.9 ± 1.1	21.5 ± 3.7

A, asthenozoospermia; T, teratozoospermia; O, oligozoospermia; Az, azoospermia; C, cryptozoospermia.

ELISAs (Synelisa, Elias Medizintechnik GmbH, Freiburg, Germany).

Statistical Analysis

For statistical analysis, the non-parametric Kruskal-Wallis one-way analysis of variance rank test was used. The correlation coefficient (r) between seminal parameters was determined by analyzing for linear regression.

Results

Cytokine Levels in the Seminal Plasma

In seminal plasma, IL-1β, IL-6, and TNFα could be detected in different quantities. Individual cytokine levels of each patient and mean values (mean ± standard error of the mean [SEM]) of each group are shown in Table 1. No significant differences could be detected in samples that were frozen immediately after ejaculation or after complete liquefaction. In contrast to infertile men without urogenital infection (n = 9) and healthy control subjects (n = 8), seminal plasma from patients with positive sperm culture for microbiological agents (n = 8) showed significantly elevated (P < 0.001) cytokine levels for IL-1β (mean ± SEM: 7.0 ± 1.3; 4.9 ± 1.1 vs. 59.3 ± 13.6 pg/ml), IL-6 (mean ± SEM: 63.1 ± 8.1; 21.5 ± 3.7 vs. 271.9 ± 99.7 pg/ml), and TNFα (mean ± SEM: 4.0 ± 0.9; 2.4 ± 0.5 vs. 62.5 ± 9.2 pg/ml) (Fig. 1). There were no significant differences between values from infertile men without infections and those from healthy control subjects.
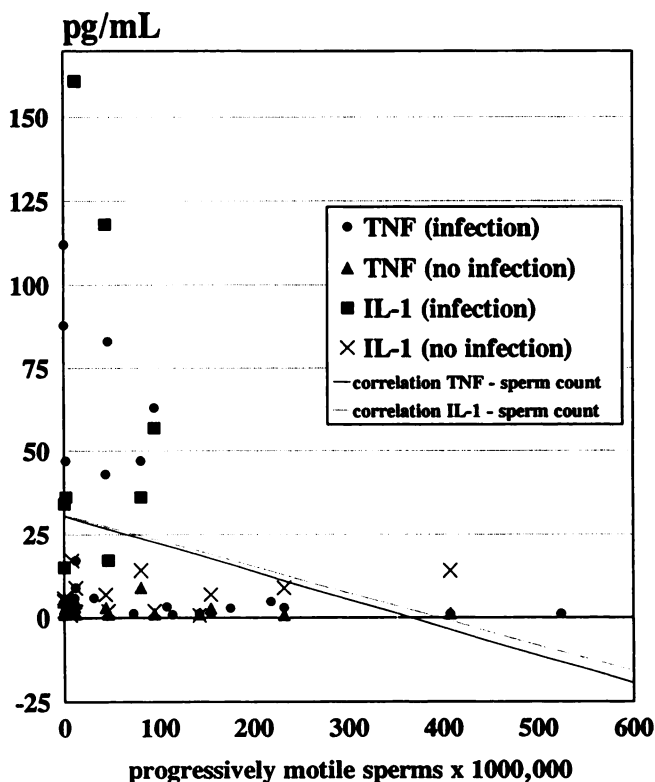
pg/mL

FIG. 2. Negative correlation between cytokine contents in seminal plasma and the number of progressively motile sperm.

Correlation of Seminal Cytokine Levels With Sperm Parameters

Proinflammatory cytokine levels were significantly elevated in seminal plasma exhibiting positive bacterial or mycoplasma/ureaplasma culture (Fig. 1). There were no differences in cytokine production, either between these two kind of infections or between different bacterial spe-

cies. We found a negative correlation between cytokine contents and the number of progressively motile sperm (Fig. 2). There was no relationship between absolute sperm counts and cytokine levels. Furthermore, no significant correlation of cytokine levels in the seminal plasma and viability, pH, morphological alterations, type of abnormality, and hormone parameters could be found.

Correlation With Hormone Parameters

No correlation between serum levels of FSH, LH, or testosterone and cytokine contents in the seminal plasma could be obtained. Enhanced serum levels of FSH and LH were correlated with decreased total sperm counts (primary testis failure) (Table 2).

Discussion

The involvement of cytokines in reproductive function and cytokine interactions with the hypothalamic and pituitary hormones as well as with embryonic development have been recently reviewed (Ben-Rafael and Orvieto, 1992). Factors secreted from immunocompetent cells were shown to affect the immune–endocrine communication that links the immune and reproductive system (Wegmann, 1990).

The majority of the immune responses are probably local, and cytokines released by immunocompentent cells during the defense of bacterial infections act generally in a paracrine or autocrine manner. These potent regulatory factors released in the reproductive tracts were able to influence sperm function directly or indirectly. The defense strategies of the immune system include the release of proinflammatory cytokines, especially IL-1, IL-6, and TNFα (Gately et al, 1986; Paul and Seder, 1994) as primary or secondary signals. Although cells of the immune

Table 2. *Hormone parameters in the sera of infertile men and healthy control subjects*

Patients with positive sperm culture (n = 8)						Patients with negative sperm culture (n = 9)						Healthy control subjects (n = 8)					
Patient no.	Age (years)	Diag-nosis*	Testos-terone (μg/L)†	FSH (IU/L)	LH (IU/L)§	Patient no.	Age (years)	Diag-nosis	Testos-terone (μg/L)	FSH (IU/L)	LH (IU/L)	Patient no.	Age (years)	Diag-nosis	Testos-terone (μg/L)	FSH (IU/L)	LH (IU/L)
1	31	AT	7.9	2.0	0.1	9	24	OAT	5.9	3.2	2.5	18	28	N	5.1	3.2	1.4
2	29	OA	8.5	6.7	5.2	10	31	A	6.6	3.2	1.6	19	31	N	6.9	2.8	1.3
3	29	AT	5.2	4.3	7.9	11	33	OAT	16.7	5.0	8.3	20	32	N	7.2	1.9	2.7
4	33	A	5.0	3.2	2.4	12	19	OA	5.2	5.3	2.7	21	27	N	4.8	3.3	0.9
5	30	OAT	6.7	10.0	12.0	13	23	(A)	10.0	5.3	1.9	22	39	N	5.7	0.4	0.1
6	28	OAT	5.3	4.5	0.8	14	26	OA	7.6	2.7	0.9	23	20	N	6.5	2.0	1.9
7	40	Az	4.9	23.5	6.5	15	41	OAT	3.3	13.0	2.7	24	33	N	8.0	1.8	1.7
8	32	Az	6.2	6.1	2.6	16	24	C	9.5	17.4	2.5	25	37	N	8.8	4.1	2.2
						17	27	OAT	5.0	2.0	2.1						

* O, oligozoospermia; A, asthenozoospermia; T, teratozoospermia; N, normozoospermia; C, cryptozoospermia; Az, azoospermia.
† Normal range: 4.0–9.5 μg/L (pathological values are bold).
‡ Normal range: 0.6–6.0 IU/L (pathological values are bold).
§ Normal range: 0.6–5.5 IU/L (pathological values are bold).

system may be the major source of these cytokines, other cells in the reproductive tract might also be capable of cytokine expression. Furthermore, in the prognosis of fertility, soluble factors secreted by the maternal reproductive tract must be taken into account.

In our study we were interested in the direct correlation of proinflammatory cytokine contents in seminal plasma and sperm parameters. In previous studies, many endogenous and/or exogenous components of sperm were demonstrated to influence sperm motility (Didolkar and Roychowdhury, 1980; De Lamirande et al, 1986; Gagnon et al, 1986; Siegel et al, 1986; Majumder et al, 1990). Recently, the presence of IL-1 in human seminal plasma and its influence in membrane peroxidation and fertility has been reported (Buch et al, 1994; personal communication). The direct influence of cytokines such as IL-1 and TNFα on sperm motility has been a topic of controversy (Hill et al, 1987; Wincek et al, 1991; Haney et al, 1992). The in vitro studies of Wincek et al. (1991) have shown that recombinant TNFα does not affect human sperm motility, hamster ova penetration, mouse in vitro fertilization, and embryo development. Indirect effects via activation of macrophages and enhanced phagocytosis and sperm–macrophage interactions could not be ruled out.

Two independent investigators could not demonstrate any influence of recombinant TNFα on sperm function (Soldati et al, 1989; Haney et al, 1992), whereas the effects of TNFα on sperm motility, sperm penetration, and mouse embryo development could be shown (Hill et al, 1987). Other studies also demonstrate that TNFα is able to decrease sperm motility (Fakih et al, 1987; Hill et al, 1987; Eisermann et al, 1989). Neither in patients without seminal infections nor in healthy control subjects were seminal cytokine levels significantly elevated. More recent studies were not able to detect IL-1β or TNFα in the seminal plasma, either in fertile or infertile men (Naz and Kaplan, 1994a). Infertile men had statistically significant higher IL-6 levels in the seminal plasma, but an association with the presence of microbacterial agents was not ruled out. Various studies have shown that IL-1 has no adverse effect on sperm motility and fertilization except in very high concentrations (Naz, 1985; Anderson and Hill, 1988). In contrast, recent studies demonstrated an inverse correlation between the sperm number and motion parameters and seminal IL-6 levels (Naz and Kaplan, 1994a). Furthermore, investigations by Comhaire et al (1994) demonstrated that seminal concentrations of IL-6 and IL-1β were unrelated to sperm concentration, motility, and morphology, but IL-1β and IL-6 were higher in cases with accessory gland inflammation.

In contrast to these studies, we were able to detect IL-1β, IL-6, and TNFα in seminal plasma in different quantities. Although we have only investigated a limited number of patients, our data clearly demonstrate a positive correlation between seminal cytokine levels and the presence of urogenital infections. In four out of eight patients with positive sperm culture, leukocytes could also be detected, but all of these patients exhibited elevated seminal plasma levels of proinflammatory cytokines.

Various studies have indicated an increased number of leukocytes in the ejaculate of infertile men, compared to fertile men, correlating with enhanced IL-6 levels (Naz, 1985; Naz and Kaplan, 1994a). In contrast, TNFα was detected in high concentrations in human semen plasma and was related to sperm parameters or to the presence of bacterial agents (Hussenet et al, 1993). Another study has shown a positive correlation between the number of leukocytes and IL-1β and IL-6 levels (Comhaire et al, 1994). Therefore, cytokine content in the seminal plasma represents a better diagnostic parameter for urogenital infections than the presence of inflammatory cells. Those patients exhibiting increased levels of IL-1β, IL-6, and/or TNFα showed a significantly reduced amount of progressively motile spermatozoa. Former investigations have shown an influence of IL-6 on various motility characteristics but not on the percent motility of sperm cells (Naz and Kaplan, 1994b).

No relationship between total sperm counts and cytokine contents in the seminal plasma could be found, pointing to a local effect of soluble mediators on sperm in the draining reproductive tract rather than in sperm-producing testicles.

Our data suggest that urogenital infections may lead to elevated levels of inflammatory cytokines in the seminal plasma. Data collected by Zolti et al. (1991) have shown that spermatocytes in vitro are not able to secrete detectable amounts of IL-1, IL-6, or TNFα. Furthermore, it was shown that IL-6 did not affect the fertilizing capacity of human sperm cells (Naz and Kaplan, 1994b). These soluble factors are most probably released into the draining reproductive tract or adnexes by immunocompetent cells involved in the defense against microbiological agents. Infectious agents might be an important factor in these mechanisms, because we observed a decrease in cytokine levels and improvement of sperm motility in two of three patients treated with antibiotics for 2 weeks. These cytokines may result in decreased sperm motility and, therefore, in reduced ova-penetrating properties. The mechanisms of cytokine action on sperm motility remain speculatory because the sperm membrane seems to stay unchanged. The cytotoxic effects of TNFα are normally mediated through membrane receptor mechanisms linked to protein synthesis. Nevertheless, many effects are mediated by activation of the TNFα-receptor pathways, including NFκB (nuclear factor kappa B) activation and induction of apoptotic processes. Furthermore, it remains to be determined whether IL-1β and IL-6 can directly effect sperm functions or act via alterations of cytokine

and hormonal networks. Our data suggest a cytokine-mediated influence on structural and/or metabolic components of the motility apparatus of spermatocytes. In conclusion, our data provide further evidence that cytokines may be critically involved in male infertility.

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