

Inflammatory Cytokine Concentrations Are Elevated in Seminal Plasma of Men With Spinal Cord Injuries

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ABSTRACT: The semen of most men with spinal cord injury (SCI) contains sperm with abnormally low motility. Studies suggest that the seminal plasma is the source of this condition. The seminal plasma of men with SCI contains an abnormally high number of white blood cells (WBC), specifically, activated T cells. It is known that activated T cells secrete cytokines and elevated concentrations of cytokines can be harmful to sperm. It is not known if the seminal plasma of men with SCI contains elevated concentrations of cytokines. The purpose of this study was to determine if the seminal plasma of men with SCI contained elevated concentrations of cytokines. Using the method of enzyme-linked immunosorbant assay (ELISA), ten cytokines were measured in the seminal plasma of men with SCI as well as healthy non-SCI control subjects. The cytokines

of interest were grouped according to Th1 effector functions: interleukin 1 beta, interleukin 2, interleukin 12, tumor necrosis factor alpha, tumor necrosis factor beta, interferon gamma (IL1 β , IL2, IL12, TNF α , TNF β , INF γ , respectively) and Th2 effector functions: interleukin 4, interleukin 6, interleukin 10, transforming growth factor beta 1 (IL4, IL6, IL10, TGF β 1, respectively). The results showed a predominance of Th1 versus Th2 cytokine production in the seminal plasma of men with SCI compared with that of control subjects. This finding suggests an immunologic basis for infertility as a possible avenue of investigation in these men.

Key words: Sperm, infertility, semen, T cells, Th1, Th2.

J Androl 2004;25:250–254

Men with SCI have a unique type of infertility. They suffer from neurogenic ejaculatory dysfunction and abnormal semen parameters. Their semen often contains an abnormally low concentration of motile sperm (Ohl et al, 1992; Sonksen and Biering-Sorensen, 1992; Brackett et al, 1998). The cause of this condition in men with SCI remains unexplained, and no specific remedies have been developed. Wheelchair confinement, bladder catheterization, endocrine imbalances, the inability to ejaculate, or the years postinjury all appear unrelated to the etiology of this semen abnormality (Brackett et al, 1996b). Interestingly, a soluble factor in the seminal plasma of men with SCI inhibits sperm motility of normal men within five minutes of contact (Brackett et al, 1996a). Furthermore, when the sperm of men with SCI have not encountered the seminal plasma (ie, the sperm have been extracted from the vas deferens), the motility is near normal (Brackett et al, 2000). The nature and source of the factor in the seminal plasma that causes the drop in sperm motility of SCI men is uncharacterized. Accompanying

this finding is our additional observation that the semen of men with SCI contains an abnormally high number of white blood cells, specifically, activated T cells (Basu et al, 2002). It is known that activated T cells secrete cytokines, and elevated concentrations of cytokines can be harmful to sperm (Doussset et al, 1997; Matalliotakis et al, 1998a; Matalliotakis et al, 1998b; Abbas et al, 2000). It is not known if the seminal plasma of men with SCI contains elevated concentrations of cytokines. The purpose of this study was to determine if the seminal plasma of men with SCI contains elevated concentrations of cytokines that might be associated with a Th1 versus Th2 immune pathway. Th1 and Th2 are two subsets of helper T cells that are characterized by the production of cytokine groups, which determine or augment different immune responses. For example, IFN γ is a key cytokine associated with Th1 differentiation and a cell-mediated immune response, while IL4 and IL5 are associated with Th2 differentiation and a humoral or antigen-mediated immune response (ie, stimulating and augmenting B-cell activity) (Abbas et al, 2000).

Supported by the Christopher Reeve Paralysis Foundation, the State of Florida Specific Appropriations, and the Miami Project to Cure Paralysis.

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Received for publication July 9, 2003; accepted for publication September 30, 2003.

Materials and Methods

Subjects

Subjects were 31 men with spinal cord injury in good general health. All were research volunteers participating in an IRB-

approved protocol of the Male Fertility Research Program of the Miami Project to Cure Paralysis at the University of Miami School of Medicine, Miami, Florida. As assessed by the University of Miami Neurospinal Index (Klose et al, 1980), the levels of injury were C3 to C7 in 15 patients, T1 to T5 in 10 patients, and T6 to T10 in 6 patients. Eighty percent of the participants had incomplete lesions, and 20% had complete lesions. Their mean age was 32 ± 2.1 years (range 27–42). Their mean years postinjury was 8.1 ± 0.9 (range 2–19). Control subjects were 12 healthy, non-SCI men, with no history of infertility. Their mean age was 30.7 ± 3.6 years (range 25–47 years). No SCI or control subject had taken any medication known to interfere with fertility within 6 months prior to their participation in this study.

Semen Analysis

A semen analysis was performed on each fresh semen specimen using World Health Organization (World Health Organization, 1999) criteria. All semen analyses were performed by the same technician.

Semen Collection

In SCI subjects, antegrade semen specimens were collected by the standard method of penile vibratory stimulation (Brackett, 1999). Retrograde ejaculates and electroejaculates were not used in this study because these procedures have been shown to alter semen quality (Brackett and Lynne, 2000). When possible, up to 3 semen specimens were collected from each SCI subject to achieve a final volume of ≥ 2 mL semen per subject, which was the minimum volume necessary to perform all cytokine assays. Multiple semen collections from the same SCI subject occurred at 2- to 4-week intervals, with no additional ejaculations between semen collections. Each semen specimen was centrifuged at $400 \times g$ for 10 minutes at room temperature to obtain the seminal plasma fraction. The protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF), was added to the seminal plasma to a final concentration of 0.5 mM (Gruschwitz et al, 1996). The PMSF-treated seminal plasma fractions were then stored in 1.5 mL microfuge tubes at -80°C until use. Control subjects collected their semen specimens by masturbation following 3 to 7 days of abstinence from ejaculation. Semen specimens from control subjects were processed in the same manner as semen specimens from SCI subjects.

Cytokine Determination in Seminal Plasma

The following 10 cytokines were measured in the seminal plasma of SCI and control subjects: IL1 β , IL2, IL12, TNF α , TNF β , INF γ , IL4, IL6, IL10, TGF β 1. Because the volume of ejaculate available from each subject was limited, in some instances multiple ejaculates from the same subject were collected and pooled. Even so, since duplicate determinations of each assay were performed, it was not possible to assay all 10 cytokines in each subject. Frozen seminal plasma was thawed at room temperature immediately prior to cytokine determination. Quantitation of these cytokines was done using ELISA kits (R&D Systems, Minneapolis, Minn). Briefly, a solid phase enzyme immunoassay employing the multiple antibody sandwich principle was applied. The microtiter plate was coated with antihuman monoclonal an-

Comparison of sperm count and motility between men with spinal cord injury (SCI) and control subjects

Parameter	Total Sperm Count in Antegrade Ejaculate ($\times 10^6$)	Percent Sperm Motility
Control subjects	243.2 ± 55.8	66.9 ± 4.0
SCI subjects	60.8 ± 12.2	29.6 ± 4.8
Significance	$P < .001$	$P < .001$

tibody specific for the cytokines. The immobilized antibody binds any cytokine present in the seminal plasma. A goat polyclonal antibody was added to which streptavidine-peroxidase was bound to biotin. The chromogen used was tetramethyl benzidine, the reaction stopped by using 2N H₂SO₄. Optical density readings were performed at 450 nm and corrected at 540 nm using a Wallac ELISA reader.

Cytokine Determination in Blood Serum

As a follow-up experiment to finding elevated semen cytokine concentrations in SCI subjects, cytokine concentrations were measured in the blood serum of SCI subjects to determine if the elevations were a local or systemic effect. Blood serum was not collected from control subjects since elevated semen cytokine concentrations were not observed in control subjects.

One serum specimen was collected from each of 12 randomly selected SCI subjects. Blood was collected by venipuncture into vacutainer tubes, allowed to clot for 15 minutes, then centrifuged at $400 \times g$ for 15 minutes to obtain serum. Serum specimens were treated similarly to seminal plasma, that is, serum was treated with PMSF, then frozen at -80°C until use. The presence of cytokines IL1 β , IL6, and TNF α was determined using ELISA as described above for cytokine determination in the seminal plasma.

Statistical Analysis

Group means were compared by analysis of variance. Fisher exact test was used in one instance to assess the significance of the finding that control subjects contained no IFN γ in their seminal plasma versus SCI subjects who contained a broad range of this cytokine (0–141.13 pg/mL) in their seminal plasma.

Results

Semen Analysis

Similar to numerous previous studies (Linsenmeyer, 1991; Ohl et al, 1992; Sonksen and Biering-Sorensen, 1992; Brackett et al, 1998), semen parameters were impaired in men with SCI compared with control subjects (Table).

Cytokine Determination in the Seminal Plasma

Of the cytokines typically elaborated by the Th1 subset of helper T cells, concentrations of IL1 β , IL12, and TNF α were significantly elevated in the seminal plasma of SCI

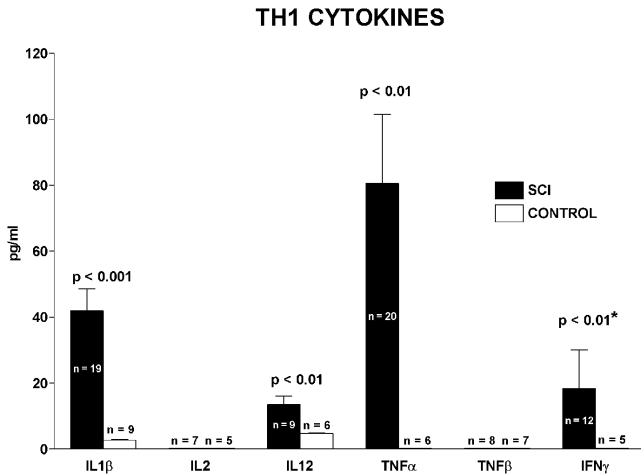


Figure 1. Mean concentrations of Th1 cytokines were compared in the seminal plasma of SCI subjects and control subjects. n = number of subjects analyzed in each group for each cytokine. * = Fisher exact test (see "Results" section).

subjects compared with control subjects (Figure 1). IL2 and TNF β were not present in the seminal plasma of SCI or control subjects. Likewise, IFN γ was not present in the seminal plasma of any control subject but was present in the seminal plasma of two thirds of the SCI subjects. Although the difference in mean concentrations of IFN γ in SCI versus control subjects did not reach statistical significance (probably due to the wide range of IFN γ concentrations in the SCI group: 0.0–141.13 pg/mL), the occurrence of this finding was significant when analyzed by the Fisher exact test ($P < .05$).

Of the cytokines typically elaborated by the Th2 subset of helper T cells, only IL6 was significantly elevated in the seminal plasma of SCI subjects compared with control subjects (Figure 2). There was no difference between SCI and control subjects in the concentrations of IL10. Concentrations of IL4 and TGF β 1 were significantly decreased in the seminal plasma of SCI versus control subjects.

Cytokine Concentrations in Blood Sera

IL1 β , IL6, and TNF α were not detected in the serum of men with SCI (data not shown).

Discussion

The results of this study show that the seminal plasma concentrations of IL1 β , IL12, IL6, and TNF α were significantly higher, whereas concentrations of IL4 and TGF β 1 were significantly lower in the seminal plasma of men with SCI compared with healthy non-SCI men. The presence of IFN γ in the seminal plasma of SCI men where none was found in controls was likewise signifi-

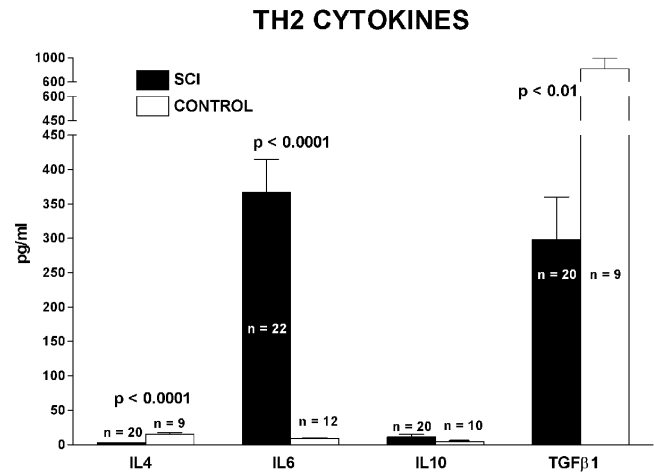


Figure 2. Mean concentrations of Th2 cytokines were compared in the seminal plasma of SCI subjects and control subjects. n = number of subjects analyzed in each group for each cytokine.

cant. Proinflammatory cytokines have been detected in the semen of men with inflammation-related infertility (Shimoya et al, 1993; Naz and Kaplan, 1994; Rajasekaran et al, 1995; Huleihel et al, 1996); however, these studies have been conducted only in infertile non-SCI men. Our study examined the SCI male population. As with previous studies, semen parameters were found to be abnormal in the SCI group. No detectable levels of IL1 β , IL6, or TNF α were found in the blood sera of SCI subjects, suggesting that the observed inflammatory response was restricted to the urogenital tract.

Cytokines rarely act in isolation, but rather in a network of other cytokines, many of which may be inhibitory or immunoregulatory. Two such cytokines, IL4 and TGF β 1 were found to be lower in the seminal plasma of SCI men. TGF β is a cytokine with autocrine and paracrine actions in the testis with potent immunoregulatory and anti-inflammatory activity (Loras et al, 1999). IL4 inhibits the production of a wide range of cytokines from stimulated monocytes (Mire-Sluis and Thorpe, 1998). One study (Naz and Evans, 1998) noted that IL12 was higher in fertile versus infertile men and was correlated with sperm count, but not sperm motility. Our study, however, showed increased concentrations of IL12 in seminal plasma of men with SCI (12.5 pg/mL) compared with those of controls (5.0 pg/mL).

Studies by Estrada et al (1997) and Paradisi et al (1996) showed that IFN γ had a somewhat detrimental effect on sperm motility and viability. In our study, IFN γ was present in the seminal plasma of 8 of the 12 SCI men tested for this cytokine and in none of the control subjects tested ($P < .05$).

The concentrations of IL1 β , IL6, and TNF α were greatly elevated in seminal plasma of men with SCI. Presence of IL1 in human seminal plasma and its influence in

membrane peroxidation and fertility has been reported (Buch et al, 1994). Various studies have shown that IL1 in very high concentrations can have an adverse effect on sperm motility and fertilization (Naz, 1985; Anderson and Hill, 1988). However, the direct influence of cytokines such as IL1 β and TNF β is a topic of controversy (Hill et al, 1987; Haney et al, 1992). Studies have also shown an inverse correlation between sperm number and motion parameters and seminal IL6 levels (Naz and Kaplan, 1994). IL6 is a pleiotropic cytokine produced by different types of cells. According to Matalliotakis et al, the prostate appears to be the main site for origin of IL6 in the semen (Matalliotakis et al, 1998b).

In vitro studies have shown that recombinant TNF α does not affect human sperm motility (Wincek et al, 1991). The effects of TNF α on sperm motility, sperm penetration, and mouse embryo development was shown by Hill et al (1987). The cytotoxic effects of TNF α are normally mediated through membrane receptor mechanisms linked to protein synthesis. Many effects are mediated by activation of the TNF α receptor pathways, including nuclear factor kappa B (NF κ B) activation and induction of apoptotic processes. Our data showed that TNF α was not found in detectable levels in control subjects. Even though its correlation with sperm motility and viability could not be determined due to absence of TNF α in control subjects, its presence in the seminal plasma of SCI subjects suggests a possible role in infertility.

Another mechanism of interference with sperm quality may be an adverse effect on sperm membrane properties such as lipid peroxidation (Buch et al, 1994). Increased oxidative stress may additionally modulate the concentration of these cytokines (Rajasekaran et al, 1995). Reactive oxygen species (ROS) might also play a role in reducing sperm motility along with cytotoxic cytokines. It has been shown that semen samples of SCI patients showed high levels of ROS, which was inversely related to sperm motility and positively related to polymorphonuclear neutrophils (Padron et al, 1997).

The role of abnormal sperm storage in the seminal vesicles of SCI men (Ohl et al, 1999) in contributing to the abnormal levels of cytokines, WBC, and overall sperm quality is unclear. It is tempting to assign all the abnormalities to this possibility. However, repeated frequent ejaculations (which should minimize the effects of abnormal storage) do not seem to change semen quality in these men (Sonksen et al, 1999).

Whereas cells of the immune system are the major sources of these cytokines, other cells in the reproductive tract might be capable of cytokine expression. It has been suggested that the prostate gland is the site of origin of IL6 (Matalliotakis et al, 1998b). Studies have suggested that there are both endogenous and exogenous factors that might affect sperm motility (Majumder et al, 1990; de

Lamirande and Gagnon, 1993). The precise origin of the cytokines, however, remains unclear. Presumably they are produced by WBC present in abnormally high numbers in the semen of men with SCI (Basu et al, 2002; Trabulsi et al, 2002). The origin of these WBC is unclear and may not be related to chronic infection or inflammation. For example, many men post retroperitoneal lymph node dissection (RPLND) share the same abnormal sperm profiles and ejaculatory dysfunction with men who have SCI but not the stigmata of bladder dysfunction, UTI, and so forth. Their commonality with SCI men is autonomic disinnervation of the accessory sex glands, a condition whose role is unclear with respect to immune function and regulation. Treatment of UTIs does not seem to make a major difference in the sperm parameters of men with SCI (Ohl et al, 1992). A biopsy study of the prostate glands of SCI versus control subjects showed no evidence of any significant acute or chronic inflammatory changes in the prostate glands of SCI men (Randall et al, 2003).

A previous study by our group showed that seminal plasma from men with SCI rapidly and profoundly reduced sperm motility of normal men (Brackett et al, 1996a). Additionally, sperm obtained from the vas deferens of men with SCI had significantly higher motility than that of their ejaculates (Brackett et al, 2000). Taken together, these studies indicate that when sperm of men with SCI comes into direct contact with their seminal plasma, sperm motility decreases. Further experiments will be required to prove the exact involvement of cytokines in reducing sperm motility. However, identification of the exact cytokines involved in this response will lead to the development of strategies to neutralize, eliminate, or otherwise combat these substances and their noxious effects.

Conclusion

This study demonstrates elevated concentrations of the cytokines IL1 β , IL6, IL12, IFN γ , and TNF α , and lower concentrations of IL4 and TGF β 1 in the seminal plasma of men with SCI compared with non-SCI healthy control subjects. Many of the cytokines were either not detectable or not present in the seminal plasma of control subjects. This finding suggests a cell mediated versus a humoral immunologic basis for infertility as a possible avenue of investigation in these men.

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