Journal of Andrology, Vol. 23, No. 4, July/August 2002

Cytofluorographic Identification of Activated T-cell Subpopulations in the Semen of Men With Spinal Cord Injuries

SARMISTHA BASU,* CHARLES M. LYNNE,*† PHILLIP RUIZ,‡ TEODORO C. ABALLA,* SEAN M. FERRELL,* AND NANCY L. BRACKETT*†

*From *The Miami Project to Cure Paralysis, the †Department of Urology, and the ‡Department of Pathology, University of Miami School of Medicine, Miami, Florida.*

ABSTRACT: The semen of most men with spinal cord injury (SCI) contains an abundance of leukocytes. It is not known if this leukocytospermia contributes to the abnormally low sperm motility observed in many of these men. Our study used flow cytometry to identify the leukocyte population in the semen of 12 men with SCI compared to 8 healthy age-matched control subjects. The results showed that, compared to control subjects, the semen of men with SCI had increased numbers of mature granulocytes and lymphocytes. The largest proportion of the leukocytes consisted of lymphocytes, and immunophenotypic analysis showed that the greater fraction were T cells, many of which coexpressed human leukocyte antigen HLA-DR and CD25, suggesting they were in an ''activated'' state. No significant B-cell population was evident. Our finding of immunologically active leukocytes is a significant step in understanding the relationship of leukocytospermia and decreased sperm motility in the semen of men with SCI.

Key words: Sperm, ejaculation, leukocytospermia, fertility, paraplegia, pyospermia, lymphocytes.

J Androl 2002;23:551–556

More than 10 000 cases of spinal cord injury (SCI) occur annually in the United States. The majority of the injured are men in the child-rearing age group (Stover et al, 1995). Men with SCI have impaired sexual function and impaired fertility mainly due to: 1) autonomic and neuromuscular dysfunction that hampers erection and ejaculation and 2) poor semen quality with sperm showing very low motility (Linsenmeyer and Perkash, 1991; Ohl et al, 1992; Sonksen and Biering-Sorensen, 1992; Brackett et al, 1996a,b, 1997b). Potential soluble factors accounting for low sperm motility may be toxic secretions from the prostate or seminal vesicles, which are the major contributors to the formation of semen (de Lamirande et al, 1986; Linsenmeyer, 1991; Brackett et al, 2000).

The macroscopic and microscopic appearance of semen in men with SCI is abnormal. Often, the semen is yellow or brown in color and contains numerous nonspermatozoon cells (Wieder et al, 1999), many of which are leukocytes (Aird et al, 1999). According to the World Health Organization (1999), concentrations of leukocytes greater than 1 million per milliliter in the ejaculate are considered abnormal. The significance of an increased number of leukocytes in the semen of men with SCI remains controversial (Aird et al, 1999). In non-SCI populations, increased leukocytes are related to low sperm motility (Wolff et al, 1990). An association between increasing seminal granulocyte concentrations and poor semen parameters has been previously reported (Aird et al, 1999). The purpose of this study was to identify different leukocyte populations by flow cytometry in the semen of men with SCI and to compare these results with those of normal control subjects. The sperm parameters between the 2 groups were also compared.

Materials and Methods

Subjects

Subjects were participants in the Male Fertility Research Program of The Miami Project to Cure Paralysis at the University of Miami School of Medicine, Miami, Fla. All subjects were in good health with no active urinary tract infections. SCI subjects were past the acute phase of injury, and their mean years postinjury (SE of the mean) equaled 6.2 plus or minus 1.1. Their mean age was 30.2 plus or minus 1.2 years. Levels of injury as assessed by the University of Miami Neurospinal Index (Klose et al, 1980) were C4 to C7 (5 patients), T3 to T4 (4 patients), and T9 to T11 (3 patients). SCI men who were azoospermic were excluded from this study. Controls were healthy men with normal semen analysis and no history of infertility (8 subjects,

Supported by the State of Florida Specific Appropriations and the Miami Project to Cure Paralysis.

Correspondence to: Dr Nancy L. Brackett, The Miami Project to Cure Paralysis, University of Miami School of Medicine, PO Box 016960, R-48, Miami, FL 33101 (e-mail: nbrackett@miami.edu).

Received for publication June 11, 2001; accepted for publication February 18, 2002.

mean age 30.3 plus or minus 1.8 years). No SCI or control subject had taken any medication known to affect semen quality within 6 months prior to this study.

Semen Collection and Analysis

In this study, only antegrade fractions obtained via penile vibratory stimulation (PVS) were used (Brackett et al, 1997a; Brackett, 1999; Brackett and Lynne, 2000). Retrograde fractions and specimens obtained by electroejaculation were not used, since these approaches may alter semen quality (Brackett et al, 1998a; Brackett and Lynne, 2000). For PVS, subjects were seated comfortably on an examination table, and a vibrator (Sunbeam model 1850-1, FERTI CARE personal or FERTI CARE clinic, Multicept, Hoersholm, Denmark) was applied to the dorsum of the glans penis or the frenulum until ejaculation occurred. In subjects with SCI, the period of abstinence from ejaculation ranged from 1 to 8 weeks. Control subjects collected semen by masturbation after 2 days of abstinence from ejaculation.

Semen analysis was performed on all specimens by manual microscopic methods according to World Health Organization criteria (1999). Each specimen was first allowed to liquefy (15–20 minutes) at room temperature. An analysis was done by placing 6 mL semen on a disposable semen analysis chamber (Cell-Vu, Fertility Technologies, Natick, Mass). All sperm parameters, including sperm motility, were assessed by the same person.

Flow Cytometry Studies

The analysis of semen took place between 1 and 2 hours after collection from the subject. Whole-semen specimens were diluted with an equal volume of Roswell Park Memorial Institute Medium (RPMI-1640) (Invitrogen, San Diego, Calif) (Moore and Hood, 1993) supplemented with 0.3% human serum albumin (ie, ''complete media''). A manual cell count and differential was performed for the leukocyte population, and the percentage of viable cells was quantified using the standard trypan blue exclusion method (Altman et al, 1993; Suttiyotin and Thwaites, 1994; Reno et al, 1997). The sample was then overlaid on Ficoll-Hypaque (equal volumes) in a 15-mL conical tube and centrifuged at 1500 rpm for 30 minutes at room temperature. The interface was aspirated, and the pellets were washed twice with 5 mL of complete media. The cells were then counted and resuspended in complete media for staining with antibodies.

Several combinations of monoclonal antibodies were used for 4-color immunophenotyping: 1) CD45-peridinin chlorophyll (PerCP)/CD11c-allophycocyanin (APC)/CD23-phycoerythrin (PE)/CD10-fluorescein isocytothiocyanate (FITC); 2) isotypic controls: mouse immunoglobulin G $(IgG)_1$-PerCP/mouse IgG_1-APC/mouse IgG_1-PE/mouse IgG_1-FITC; 3) CD14-PerCP/CD38-APC/CD13-PE/CD64-FITC; 4) CD3-PerCP/CD4-APC/CD56-PE/CD8-FITC; 5) CD42a-PerCP/CD34-APC/CD7-PE/CD33-FITC; 6) CD20-PerCP/CD5-APC/CD22-PE/TCR-α/β-FITC; 7) human leukocyte antigen (HLA-DR)-PerCP/CD69-APC/CD25-PE/CD2-FITC; 8) (2-color) CD19-PE/κ-FITC; and (9) (2-color) CD19-PE/λ-FITC.

CD45 is a pan leukocytic marker able to detect granulocytes, lymphocytes, and macrophages. The specificities of the remaining markers are listed in Table 1. Gating was performed on cells of lymphocytes (with characteristic forward [FS] and side scatter [SS] features and high CD45 staining), monocytes, and granulocytes. The designation of positive cells was based on values compared to cells stained with irrelevant isotype controls and conjugated to the same fluorochrome (see above) (Ruiz and Berho, 1998). The antibodies were attached to the cells after a washing step, incubated at 4°C for 30 minutes, and then washed twice. The cells were fixed with 2% paraformaldehyde and analyzed less than 2 hours after staining on a flow cytometer (Facs caliber, Beckman Coulter, San Jose, Calif). Listmode data on 5000 gated cells were collected with 1024 channel resolution and were analyzed using Cell Quest software version 2.0 (Becton Dickinson, Cockeysville, Md). Backgating of listmode files was utilized.

Data Analysis

Sperm parameters and leukocyte cell types were determined for each subject. For each sperm parameter and cell type, an SCI group mean and a control group mean were calculated. Differences between group means were compared by analysis of variance.

Results

Sperm Parameters

Consistent with previous reports, mean sperm concentration was similar, whereas mean sperm motility and viability were significantly lower in semen from men with SCI than from controls (Table 2) (Linsenmeyer and Perkash, 1991; Ohl et al, 1992; Sonksen and Biering-Sorensen, 1992; Brackett et al, 1996a,b, 1997b).

Leukocyte Immunophenotyping

The semen of men with SCI contained a significantly higher number of total leukocytes than the semen of control subjects (Figure 1). Immunophenotypic analysis of those leukocytes was performed by flow cytometry (Ruiz and Berho, 1998), and gating of the cell population was performed on the basis of FS and SS characteristics. The gates were divided into cells bearing low FS/low SS (''lymphocytes''), moderate FS/moderate SS (''monocytes''), and high FS/high SS (''granulocytes''). In men with SCI, a majority of the events in these gates were hematopoietic cells (ie, CD45 positive). Figure 2 shows the relative proportions of each physically defined subpopulation compared to normal men. As noted before, control semen had an overall lower leukocyte count, which sometimes hindered analysis and prevented an acquisition of total events compared to SCI-derived semen.

Our analysis of these cells in the 3 gates for their surface antigenic profile yielded an overall different profile in semen from SCI subjects compared to that from controls. An evaluation of the cells in the ''lymphocyte'' gate (Figure 3) revealed that the majority of the cells were T lymphocytes that had appropriate lineage antigens (CD2, CD3, CD5, and CD7), with most bearing α/β T-cell re-

Table 1. *Antigens analyzed on semen leukocytes*

Clone Name/Antigen*	Fluorochrome†	Specificity‡
2D1/CD45	PerCP	Pan leukocyte
MOP9/CD14	PerCP	Monocyte/macrophage
L138/CD13	PE	Pan myeloid
P67.6/CD33	FITC	Pan myeloid
10.1/CD64	FITC	Monocyte/macrophage
S-HCL-3/CD11c	APC	Monocytes
L243/HLA-DR	PerCP	MHC class II
W8E7/CALLA/CD10	FITC	Granulocytes, atypical lymphocytes
HB-7/CD38	APC	T activation/plasma cells
2A3/CD25	PE	Activation marker
L78/CD69	APC	Activation marker
BEB1/CD42a	PerCP	Platelet-associated antigen
MY31/CD56	PE	Natural killer cells
8G12/CD34	APC	Hematopoietic, progenitor
S5.2/CD2	FITC	Pan T cell
L17F12/CD5	APC	Pan T cell, some B cells
SK7/CD3	PerCP	Pan T cell
SK3/CD4	APC	T helper/inducer
SK1/CD8	FITC	T suppressor/cytotoxic
MT701/CD7	PE	Pan T cell
WT31/TCR	FITC	Pan T cell
4G7/CD19	PE	Pan B cell
L27/CD20	PerCP	Pan B cell
SHCL-1/CD22	PE	Early B cells
EBVCS-5/CD23	PE	B-cell subset
TB28-2/κ	FITC	B-cell subset
1-155-2/λ	FITC	B-cell subset

* The clone name refers to the particular hybridoma cell line source for the antibody.

† PerCP (peridinin chlorophyll), FITC (fluorscein isocytothiocynate), PE (phycoerythrin), and APC (allophycocyanin) are fluorochromes with non-overlapping spectra, thereby allowing 4-color analysis.

‡ MHC indicates major histocompatibility complex.

Table 2. *Sperm parameters for control and SCI subjects**

		Ct/mL × 10^6	% Motility	% Rapid Linear	% Viability
Control:	1	21.25	65	50	84
	2	21.25	44	20	62
	3	31.89	73	69	5
	4	74.62	49	40	45
	5	99.49	67	59	71
	6	112.25	63	57	93
	7	704.08	76	66	96
	8	130.74	65	60	81
Mean ± SEM		149.4 ± 80.6	62.8 ± 3.89	52.63 ± 5.66	67.13 ± 10.7
SCI:	1	12.75	17	9	2
	2	149.24	63	55	28
	3	89.92	40	30	13
	4	15.94	14	9	8
	5	75.26	35	22	20
	6	63.78	0	0	2
	7	69.52	36	31	5
	8	107.15	41	33	21
	9	71.43	52	45	17
	10	160.08	20	15	29
	11	15.94	11	6	5
	12	91.84	35	31	5
Mean ± SEM		79.9 ± 13.8	30.3 ± 5.27	23.8 ± 4.81	12.9 ± 2.85
Significance		NS	$P < .001$	$P < .001$	$P < .001$

* Ct/mL × 10^6 indicates millions of sperm per milliliter of ejaculate; % Motility, percentage of sperm with forward progression; % Rapid Linear, percentage of sperm with rapid linear motion; % Viability, percentage of viable sperm; NS, not significant; and SCI, spinal cord injury.

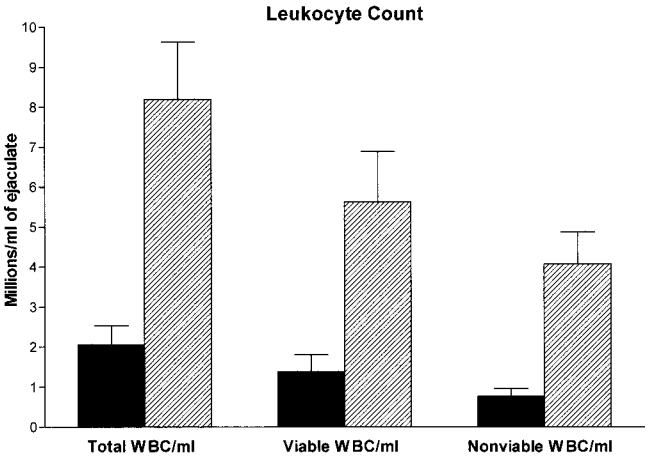

Figure 1. Total leukocyte count in the semen of spinal cord injury (SCI) and control subjects as determined by the trypan blue exclusion method.

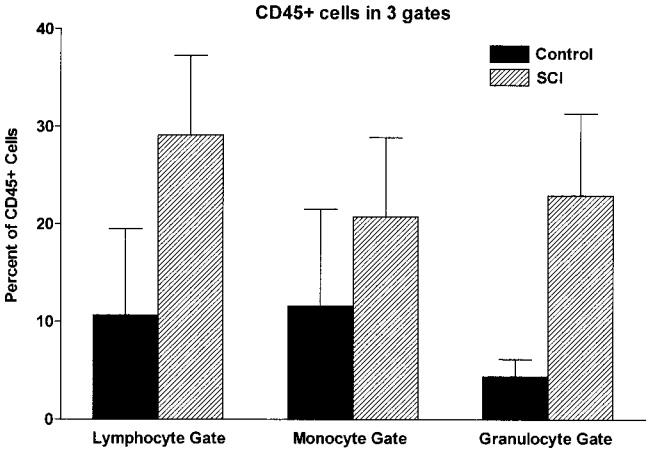

Figure 2. The percentage of CD45+ cells in spinal cord injury (SCI) and control subjects was determined in 3 gated areas on the basis of forward (FS) and side scatter (SS) characteristics (lymphocytes, monocytes, and granulocytes); see ''Materials and Methods.''

ceptors. The subpopulations of the T cells were skewed toward a predominance of CD4-positive cells (T-helper cells), although some CD8-positive cells were also evident. Multicolor analysis further demonstrated that a high proportion of the T cells coexpressed CD25 (interleukin [IL]-2 receptor) and HLA-DR, both associated in human T cells with an ''activated'' state. Minor B-cell populations were present, along with some granulocytes. An analysis of ''monocyte''- and ''granulocyte''-sized gated cells (data not shown) showed that a predominance of those cells were of myelomonocytic lineage.

Discussion

It is well known that men with SCI have abnormal semen quality (Brackett et al, 1996b, 1997b, 1998a). The most impaired parameter is sperm motility. The cause of this asthenozoospermia is unknown. This condition does not seem to be related to frequency of ejaculation (Sonksen et al, 1999), elevated scrotal temperature (Brackett et al, 1994), number of years postinjury (Brackett et al, 1998b), method of bladder management (Ohl et al, 1992), or method of ejaculation (Brackett et al, 1997a; Ohl et al, 1997). The problem may be related to abnormalities in the seminal plasma of these men. For example, the seminal plasma from men with SCI inhibits the motility of sperm in normal men (Brackett et al, 1996a), and in men with SCI, sperm from the vas deferens has higher motility than ejaculated sperm (Brackett et al, 2000). The question is, What factor or factors in the seminal plasma cause low sperm motility in men with SCI? It has been previously noted that semen from SCI men contains numerous nonspermatozoon cells (Wieder et al, 1999), many of which are leukocytes (Aird et al, 1999).

Numerous epidemiological (Harrison et al, 1991; Wang et al, 1994), clinical (Berger et al, 1982), and experimental studies (Wolff et al, 1990; Kovalski et al, 1992) report that leukocytospermia has been linked with poor semen quality and damaged sperm function. A few reports suggest little or no correlation between leukocyte concentration and semen parameters (Aitken et al, 1991, 1994). Studies on semen samples in patients with male accessory gland infection showed that asthenozoospermic samples with increased number of white blood cells (WBCs) showed even lower sperm motility than asthenozoospermic samples without WBCs (Depuydt et al, 1996). Overall, the association between leukocytospermia and decreased sperm motility is strong.

WBCs can have an effect on sperm quality by producing reactive oxygen species (ROS) and/or cytokines, each of which may have a deleterious effect on sperm motility. Studies suggest that cytokines and ROS may interact in mediating toxic effects on inflammation (Rajasekaran et al, 1995). ROS is required for sperm capacitation, and human spermatozoa can generate ROS (de Lamirande and Gagnon, 1993). Our group (Padron et al, 1997), as well as another group (de Lamirande et al, 1995), found that SCI patients had higher levels of ROS in their semen than did infertile men. It was also noted that the level of ROS in specimens from men with SCI correlated negatively with sperm motility and positively with WBC concentration.

In addition to ROS, a number of other substances have been found to differ significantly in the semen of SCI men compared to healthy controls. For example, prostate-specific antigen concentrations are lower (Brasso et al, 1998; Lynne et al, 1999); concentrations of fructose, albumin, glutamic oxaloacetic transaminase, and alkaline phosphatase are lower; concentrations of chloride are higher (Hirsch et al, 1991); and concentrations of somatostatin

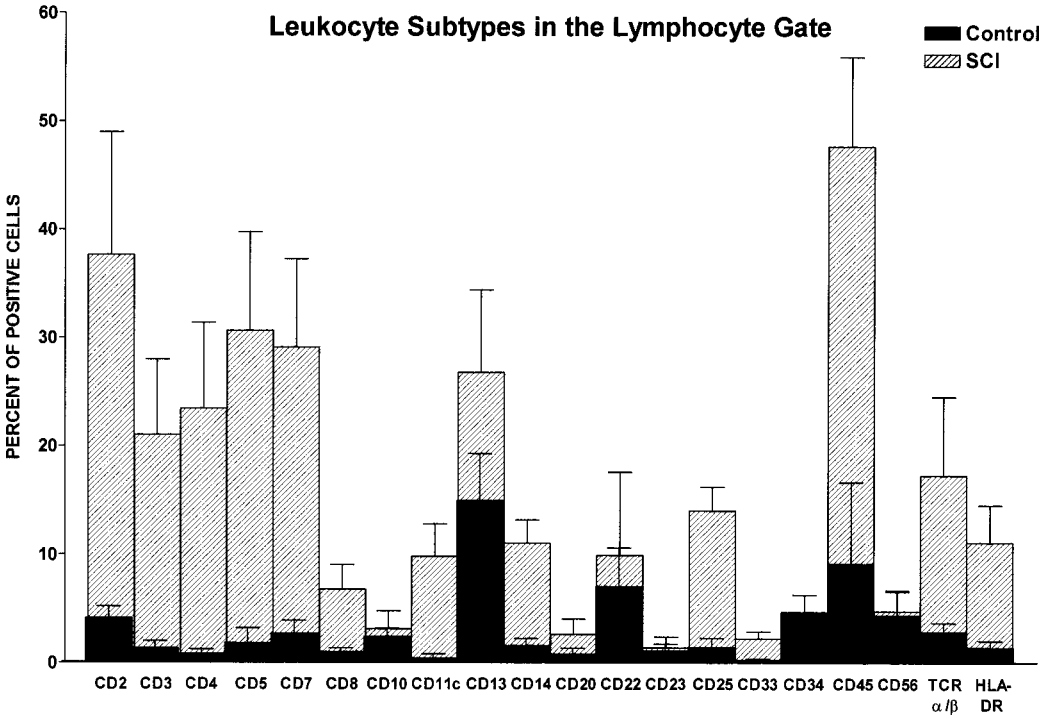

Figure 3. Relative percentages of hematopoietic cell subpopulations present in semen analyzed by flow cytometry.

are lower in patients T6 and above (Odum et al, 1995). It is not known, however, if abnormal seminal concentrations of these substances are causing, or simply coinciding with, the asthenozoospermia observed in the majority of these patients. Leukocytospermia is a ubiquitous condition in these patients, regardless of the period of abstinence. An important step in determining the mechanism causing asthenozoospermia in these men is to identify the leukocyte subtypes in their semen.

Various cytokines may be found routinely in the semen of normal men and in abnormal concentrations in the semen of infertile men (Depuydt et al, 1996; Gruschwitz et al, 1996; Dousset et al, 1997; Naz and Evans, 1998). Their origin from seminal leukocytes (polymorphonuclear leukocytes and lymphocytes) is implied, as is their detrimental effect on sperm motility. This issue has not been investigated extensively. We have found that the largest proportions of the leukocytes in the semen of SCI men appear to be lymphocytes, which, at this point, can be subtyped to T cells, many of which are activated or immunologically active and the majority of which are CD4 (helper) cells. For the moment, it is reasonable to assume that most of the abnormal levels of cytokines in the semen can be attributed to the abnormal levels of cytokine-producing cells (leukocytes) found in these patients. The source of these cells is not known, although our own studies (Brackett et al, 2000, unpublished data) have eliminated the epididymis and prostate gland as a source. Of the remaining structures along the ejaculatory pathway, the seminal vesicles are most suspect, since they contribute so much in volume and secretory products to the ejaculate, but they have not been investigated systematically for this problem. Further, it is not clear if this complex relationship of cells, ROS, and cytokines is a response to a local inflammatory or infectious conditions in these SCI patients or another manifestation of the poorly understood immune dysregulation that can be seen in SCI patients (Nash, 2000).

Conclusion

Our study showed that, compared to controls, men with SCI had significantly higher numbers of mature granulocytes and lymphocytes. The largest proportion of leukocytes seen were lymphocytes. Immunophenotypic analysis by flow cytometry showed that the greater fraction were T cells, many of which were in an activated state. No significant B-cell population was evident.

References

Aird IA, Vince GS, Bates MD, Johnson PN, Lewis-Jones DI. Leukocytes in semen from men with spinal cord injuries. *Fertil Steril.* 1999;72: 97–103.

Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol.* 1991;164:542–551.

Aitken RJ, West K, Buckingham D. Leukocytic infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. *J Androl.* 1994;15:343–352.

Altman SA, Randers L, Rao G. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol Prog.* 1993;9:671–674.

Berger RE, Karp LE, Williamson RA, Koehler J, Moore DE, Holmes KK. The relationship of pyospermia and seminal fluid bacteriology to sperm function as reflected in the sperm penetration assay. *Fertil Steril.* 1982;37:557–564.

Brackett NL. Semen retrieval by penile vibratory stimulation in men with spinal cord injury. *Hum Reprod Update.* 1999;5:216–222.

Brackett NL, Bloch WE, Lynne CM. Predictors of necrospermia in men with spinal cord injury. *J Urol.* 1998a;159:844–847.

Brackett NL, Davi RC, Padron OF, Lynne CM. Seminal plasma of spinal cord injured men inhibits sperm motility of normal men. *J Urol.* 1996a;155:1632–1635.

Brackett NL, Ferrell SM, Aballa TC, Amador MJ, Lynne CM. Semen quality in spinal cord injured men: does it progressively decline post-injury? *Arch Phys Med Rehabil.* 1998b;79:625–628.

Brackett NL, Lynne CM. The method of assisted ejaculation affects the outcome of semen quality studies in men with spinal cord injury: a review. *Neurorehabilitation.* 2000;15:89–100.

Brackett NL, Lynne CM, Aballa TC, Ferrell SM. Sperm motility from the vas deferens of spinal cord injured men is higher than from the ejaculate. *J Urol.* 2000;164:712–715.

Brackett NL, Lynne CM, Weizman MS, Bloch WE, Padron OF. Scrotal and oral temperatures are not related to semen quality or serum gonadotropin levels in spinal cord-injured men. *J Androl.* 1994;15:614–619.

Brackett NL, Nash MS, Lynne CM. Male fertility following spinal cord injury: facts and fiction. *Phys Ther.* 1996b;76:1221–1231.

Brackett NL, Padron OF, Lynne CM. Semen quality of spinal cord injured men is better when obtained by vibratory stimulation versus electroejaculation. *J Urol.* 1997a;157:151–157.

Brackett NL, Santa-Cruz C, Lynne CM. Sperm from spinal cord injured men lose motility faster than sperm from normal men: the effect is exacerbated at body compared to room temperature. *J Urol.* 1997b; 157:2150–2153.

Brasso K, Sonksen J, Sommer P, et al. Seminal plasma PSA in spinal cord injured men: a preliminary report. *Spinal Cord.* 1998;36:771–773.

de Lamirande E, Gagnon C. Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. *Fertil Steril.* 1993;59:1291–1295.

de Lamirande E, Leduc BE, Iwasaki A, Hassouna M, Gagnon C. Increased reactive oxygen species formation in semen of patients with spinal cord injury. *Fertil Steril.* 1995;63:637–642.

de Lamirande E, Sherins RJ, Gagnon C. The presence of a motility inhibitor within spermatozoa may explain the poor sperm motility of some infertile men. *J Androl.* 1986;7:215–219.

Depuydt CE, Bosmans E, Zalata A, Schoonjans F, Comitini G. The relation between reactive oxygen species and cytokines in andrological patients with or without male accessory gland infection. *J Androl.* 1996;17:699–707.

Dousset B, Hussenet F, Daudin M, Bujan L, Foliguet B, Nabet P. Seminal cytokine concentrations (IL-1beta, IL-2, IL-6, sR IL-2, sR IL-6), semen parameters and blood hormonal status in male infertility. *Hum Reprod.* 1997;12:1476–1479.

Gruschwitz MS, Brezinschek R, Brezinschek HP. Cytokine levels in the seminal plasma of infertile males. *J Androl.* 1996;17:158–163.

Harrison PE, Barratt CL, Robinson AJ, Kessopoulou E, Cooke ID. Detection of white blood cell populations in the ejaculates of fertile men. *J Reprod Immunol.* 1991;19:95–98.

Hirsch IH, Jeyendran RS, Sedor J, Rosecrans RR, Staas WE. Biochemical analysis of electroejaculates in spinal cord injured men: comparison to normal ejaculates. *J Urol.* 1991;145:73–76.

Klose KJ, Green BA, Smith RS, Adkins RH, MacDonald AM. University of Miami Neuro-Spinal Index (UMNI): a quantitative method for determining spinal cord function. *Paraplegia.* 1980;18:331–334.

Kovalski NN, de Lamirande E, Gagnon C. Reactive oxygen species generated by human neutrophils inhibit sperm motility: protective effect of seminal plasma and scavengers. *Fertil Steril.* 1992;58:809–816.

Linsenmeyer TA. Male infertility following spinal cord injury. *J Am Paraplegic Soc.* 1991;14:116–121.

Linsenmeyer TA, Perkash I. Infertility in men with spinal cord injury. *Arch Phys Med Rehabil.* 1991;72:747–754.

Lynne CM, Aballa TC, Wang TJ, Rittenhouse HG, Ferrell SM, Brackett NL. Serum and seminal plasma prostate specific antigen (PSA) levels are different in young spinal cord injured men compared to normal controls. *J Urol.* 1999;162:89–91.

Moore GE, Hood DB. Modified RPMI 164 culture medium. *In Vitro Cell Dev Biol Anim.* 1993;29A:268.

Nash MS. Known and plausible modulators of depressed immune function following spinal cord injuries. *J Spinal Cord Med.* 2000;23:111–120.

Naz RK, Evans W. Decreased levels of interleukin-12 are not correlated with leukocyte concentration and superoxide dismutase activity in semen of infertile men. *Arch Androl.* 1998;41:91–96.

Odum L, Sonksen J, Biering-Sorensen F. Seminal somatostatin in men with spinal cord injury. *Paraplegia.* 1995;33:374–376.

Ohl DA, Denil J, Fitzgerald-Shelton K, McCabe M, McGuire EJ, Menge AC, Randolph JF. Fertility of spinal cord injured males: effect of genitourinary infection and bladder management on results of electroejaculation. *J Am Paraplegic Soc.* 1992;15:53–59.

Ohl DA, Sonksen J, Menge AC, McCabe M, Keller LM. Electroejaculation versus vibratory stimulation in spinal cord injured men: sperm quality and patient preference. *J Urol.* 1997;157:2147–2149.

Padron OF, Brackett NL, Sharma RK, Lynne CM, Thomas AJ Jr, Agarwal A. Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. *Fertil Steril.* 1997;67:1115–1120.

Rajasekaran M, Hellstrom WJ, Naz RK, Sikka SC. Oxidative stress and interleukins in seminal plasma during leukocytospermia. *Fertil Steril.* 1995;64:166–171.

Reno F, Falcieri E, Luchetti F, Burattini S, Papa S. Discrimination of apoptotic cells in flow cytometry using trypan blue and FDA. *Eur J Histochem.* 1997;41:115–116.

Ruiz P, Berho M. Flow cytometry analysis of chronic B-lymphocyte malignancies. *BioMedicina.* 1998;1:350–354.

Sonksen J, Biering-Sorensen F. Fertility in men with spinal cord or cauda equina lesions. *Semin Neurol.* 1992;12:106–114.

Sonksen J, Ohl DA, Giwercman A, Biering-Sorensen F, Skakkebaek NE, Kristensen JK. Effect of repeated ejaculation on semen quality in spinal cord injured men. *J Urol.* 1999;161:1163–1165.

Stover SL, DeLisa JA, Whiteneck GG, eds. *Spinal Cord Injury: Clinical Outcomes From the Model Systems.* Gaithersburg, Md: Aspen Publishers Inc; 1995.

Suttiyotin P, Thwaites CJ. Effect of storage of smears on the staining of ram spermatozoa by eosin or trypan blue. *Aust Vet J.* 1994;71:87.

Wang AW, Politch JA, Anderson D. Leukocytospermia in male infertility patients in China. *Andrologia.* 1994;26:167–172.

Wieder JA, Lynne CM, Ferrell SM, Aballa TC, Brackett NL. Brown-colored semen in men with spinal cord injury. *J Androl.* 1999;20: 594–600.

Wolff H, Politch JA, Martinez A, Haimovici F, Hill JA, Anderson DJ. Leukocytospermia is associated with poor semen quality. *Fertil Steril.* 1990;53:528–536.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucous Interactions.* Cambridge, United Kingdom: Cambridge University Press; 1999.