

Relationship of interleukin-6 with semen characteristics and oxidative stress in vasectomy reversal patients

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Summary

This prospective study was performed to evaluate the relationship between interleukin-6 (IL-6), oxidative stress and sperm function following vasectomy reversal. We included 22 patients who underwent vasectomy reversal and 15 healthy sperm donors (controls) with normal sperm morphology criteria. Levels of IL-6 in the semen were measured by the enzyme-linked immunosorbent assay, and levels of reactive oxygen species (ROS) and total antioxidant capacity were measured by the chemiluminescence assay. The mean sperm concentration in the vasectomy reversal group was significantly lower than control group (45.3 ± 39.1 versus 63.1 ± 28.5 ; $P = 0.02$). Motility was also significantly lower in the vasectomy reversal group (32.1 ± 19.9 versus 54.6 ± 18.9 ; $P = 0.001$). Levels of IL-6 were significantly higher in the vasectomy reversal group (2.09 ± 0.87 versus 0.99 ± 0.97 ; $P = 0.007$) as were mean ROS levels (2.25 ± 0.97 versus 1.2 ± 0.7 ; $P = 0.009$). Significant positive correlation was observed between the IL-6 and ROS levels in vasectomy reversal patients compared with donors ($r = 0.41$, $P = 0.05$ versus $r = 0.38$, $P = 0.15$). We conclude that patients who undergo vasectomy reversal have elevated levels of IL-6 and ROS, which may contribute to decreased sperm motility and concentration and possibly lead to sub-fertility.

Introduction

It is estimated that approximately 2–6% of men who undergo a vasectomy for sterilization ultimately want to restore their fertility and request to have the procedure reversed (Anderson & Baird, 2002). Patency rates after vasectomy reversal range from 30% to 97% (Anderson & Baird, 2002). However, up to 72% of men remain infertile (Fuchs & Burt, 2002). The reason for this is unclear.

Various studies have reported elevated levels of reactive oxygen species (ROS) in infertile men (Sharma & Agarwal, 1996). High levels of ROS induce oxidative stress, which in turn damages spermatozoa and causes sperm dysfunction (Sharma & Agarwal, 1996). Interleukin (IL)-6 is a multifunctional cytokine found in seminal fluid, and levels of IL-6 correlate with the secretory activity of Sertoli cells (Legue *et al.*, 2001). Several studies have also found elevated levels of cytokines (e.g. IL-6 and IL-8) in

men with inflammation of the genital tract (Eggert-Kruse *et al.*, 2001). Like ROS, cytokines can negatively affect sperm function. Although the aforementioned studies hint at a connection between IL-6, oxidative stress, and infertility, no study to date has examined the relationship between these parameters in men after vasectomy reversal. We therefore undertook this study to examine whether any such relationship exists.

Materials and methods

Patients

This study, was approved by our Institutional Review Board, consisted of 37 participants – 15 healthy donors (control group) and 22 consecutive patients (fertile before vasectomy) who had undergone vasectomy reversal were enrolled through a male infertility clinic at a tertiary care teaching hospital. A thorough history and physical

examination was performed by our male infertility specialist (AJT) to rule out organic causes of infertility. The time interval between vasectomy and reversal and any other possible female factors were noted. None of the patients had either history of genital tract infection or surgeries. None of the patients had varicocele before or after surgery. Control group comprised of healthy volunteers with normal semen analysis according to the World Health Organization criteria (WHO, 1999), who had initiated a pregnancy within the previous 2 years. Semen samples were collected 6 months after surgery in the vasectomy reversal group. Men with azoospermia and/or leucocytospermia were excluded. Leucocytospermia was defined as the presence of at least 1×10^6 WBC ml^{-1} of semen sample. Semen samples were obtained by masturbation after at least 48 h of sexual abstinence. The samples were collected in sterile containers and allowed to liquefy at 37 °C for 30 min before they were analysed for sperm concentration, per cent motility, and sperm morphology (Menkveld *et al.*, 1990; WHO, 1993).

Semen analysis

Semen analysis was performed using computer-assisted semen analysis on all specimens (Motion Analysis VP 50; Motion Analysis Corporation, Santa Rosa, CA, USA). For each measurement, a 5 μl aliquot was loaded on a counting chamber (Micro Cell; Conception Technologies, La Jolla, CA, USA). Four to eight representative fields containing 200 or more spermatozoa were examined. Samples were analysed for concentration and per cent motility. The presence of granulocytes in semen specimens was assessed by a myeloperoxidase (Endtz) test (Shekarriz *et al.*, 1995).

Sperm morphology

For morphological evaluation, seminal smears were stained with Giemsa stain (Diff-Quik; Baxter Healthcare Corporation, McGraw Park, IL, USA), and the percentage of sperm with normal morphology was assessed using WHO (1993) guidelines and Tygerberg's strict criteria (Menkveld *et al.*, 1990).

Measurement of IL-6

Levels of IL-6 were measured by a double antibody 'sandwich assay' (enzyme-linked immunosorbent assay) using monoclonal antibody specific for IL-6 (Quantikine Kit; R & D Systems, Minneapolis, MN, USA) (Nallella *et al.*, 2004). The immobilized end product was read at 410 nm. The intensity of colour was proportional to the absorbance of AChE, which in turn was proportional to IL-6 levels (pmol/ml).

Measurement of oxidative stress

Reactive oxygen species: Aliquots of liquefied semen were centrifuged at $\times 300 \text{ g}$ for 7 min. Seminal plasma was aliquoted and frozen at $-20 \text{ }^\circ\text{C}$ for later measurement of total antioxidant levels. The sperm pellet was washed twice with phosphate-buffered saline, pH 7.4, and resuspended in the same medium at a concentration of 20×10^6 sperm ml^{-1} . ROS production was measured by the chemiluminescence assay method, using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St Louis, MO, USA) as the probe. Ten microlitres of 5 mM luminol prepared in dimethyl sulphoxide (Sigma Chemical Co.) was added to 400 μl of the washed sperm suspension. Levels of ROS were determined by measuring chemiluminescence with a luminometer (Autolumat LB 953; Berthold Technologies, Bad-Wildbad, Germany) in the integrated mode for 15 min. The results were expressed as $\times 10^4$ counted photons per minute (cpm) per 20×10^6 sperm (Nallella *et al.*, 2004).

Total antioxidant capacity: Total antioxidant capacity (TAC) was measured in seminal plasma using the enhanced chemiluminescence assay (Hendin *et al.*, 1999). Aliquots of the seminal plasma stored at $-20 \text{ }^\circ\text{C}$ were thawed at room temperature and immediately assessed for their antioxidant capacity as follows. Seminal plasma was diluted 1 : 10 with deionized water (dH_2O) and filtered through a 0.20 μm Millipore filter (Allegiance Healthcare Corporation, McGaw Park, IL, USA). Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Buckinghamshire, England). Twenty microlitres of horseradish peroxidase-linked immunoglobulin (HRP-linked Ig; Amersham Life Science) was added to 4.98 ml dH_2O . This was further diluted 1 : 1 to give a working solution with the desired luminescence output (3×10^7 cpm). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was added as the standard at concentrations between 50 and 150 μM .

With the luminometer set in the kinetic mode, 100 μl of signal reagent and 100 μl of HRP were added to 700 μl of dH_2O and mixed. The solution was then equilibrated to the desired level of chemiluminescence output (between 2 and 3×10^7 cpm) for 100 s. One hundred microlitres of the prepared seminal plasma was added to the signal reagent and HRP, and the chemiluminescence measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence was recorded. Antioxidant capacity was expressed as molar Trolox equivalents.

Statistical analysis

Pair wise comparisons were performed using Wilcoxon rank-sum tests. A *P*-value of <0.05 was considered statistically significant using the two-tailed test. Correlation between the IL-6 and sperm parameters was calculated with Spearman rank-correlation coefficient. Data analysis was done by GRAPH PAD Software (version 3.20 1998; GraphPad Software, Inc., San Diego, CA, USA).

Results

Sperm donors were younger than the vasectomy reversal patients (31.1 ± 2.1 years versus 43.35 ± 1.65). The mean duration between vasectomy and reversal was 10.37 ± 1.1 years. The mean sperm concentration in the vasectomy reversal group was significantly lower than that of the healthy donors (45.3 ± 39.1 versus 63.1 ± 28.5; *P* = 0.02). Motility was also significantly lower in the vasectomy reversal group (32.1 ± 19.9 versus 54.6 ± 18.9; *P* = 0.01). No difference was observed in sperm morphology according to WHO criteria (vasectomy reversal group 32.1 ± 19.9 versus donors 54.6 ± 18.9; *P* = 0.26) or Tygerberg's strict criteria (10.0 ± 3.3 versus 11.8 ± 2.9; *P* = 0.24).

Levels of IL-6 [\log_{10} (IL-6 + 1)] in the vasectomy reversal group were significantly higher than that of the

sperm donors (2.1 ± 0.9 versus 0.99 ± 0.97; *P* = 0.007). Mean ROS levels [\log_{10} (ROS + 1)] were also higher in the vasectomy reversal group (2.3 ± 0.97 versus 1.2 ± 0.7; *P* = 0.009). No significant difference was found in TAC between the two groups (Table 1).

Significant positive correlation was observed between the IL-6 and ROS levels in vasectomy reversal patients compared with the healthy donors (*r* = 0.41, *P* = 0.05 versus *r* = 0.38, *P* = 0.15). The negative correlation observed between the IL-6, sperm concentration and sperm motility in vasectomy reversal patients, was not significant (*r* = -0.24, *P* = 0.26 versus *r* = -0.27, *P* = 0.22) (Table 2).

Discussion

In this study, we evaluated the relationship between the levels of IL-6, oxidative stress, and sperm function after vasectomy reversal. Our analysis revealed that IL-6 and ROS levels were significantly higher in the vasectomy reversal patients. The high levels of IL-6 and ROS may be a result of continued inflammation after vasectomy reversal (Nallella *et al.*, 2004). However, the time period required for the inflammation to resolve completely is unclear. The presence of abnormal sperm parameters (concentration and motility) in the vasectomy reversal

Table 1 Comparison of semen characteristics, ROS, TAC, and IL-6 levels between healthy sperm donors and patients who underwent vasectomy reversal

Variables	Normal donors (<i>n</i> = 15)		Vasectomy reversal (<i>n</i> = 22)		<i>P</i> -value*
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	
Concentration (×10 ⁶ ml ⁻¹)	63.1 ± 28.5	31.3 (9–168)	45.3 ± 39.1	66.3 (18.2–164.4)	0.02
Motility (%)	54.6 ± 18.9	33.5 (3–71.0)	32.1 ± 19.9	52.0 (49–81)	0.001
WHO morphology (% normal)	39.1 ± 9.9	31 (18–51.0)	33.3 ± 10.1	38 (25–60)	0.26
Tygerberg strict morphology (% normal)	11.8 ± 2.9	10 (5–17)	10 ± 3.3	13 (7–16)	0.24
IL-6 (pmol/ml)	–	109.7 (0.0–895.0)	–	4.4 (0.0–210.6)	
\log_{10} (IL-6 + 1)	0.99 ± 0.97	–	2.1 ± 0.9	–	0.007
ROS (×10 ⁴)	–	105 (3.7–18030.0)	–	13.4 (0.9–218.2)	
\log_{10} (ROS + 1)	1.2 ± 0.7	–	2.3 ± 0.97	–	0.009
TAC (Trolox equivalents)	1,556.4 ± 468.1	1486.5 (834–3586.0)	1719.8 ± 758.6	1482.4 (881.5–2461.0)	0.69

SD, standard deviation; ROS, reactive oxygen species; IL-6, interleukin 6; TAC, total antioxidant capacity.

**P* < 0.05 was considered statistically significant.

Table 2 Correlation between interleukin-6 with semen characteristics and oxidative stress

Subjects	Concentration		Motility		ROS	
	<i>r</i> -value	<i>P</i> -value*	<i>r</i> -value	<i>P</i> -value	<i>r</i> -value	<i>P</i> -value
Control	-0.35	0.23	-0.47	0.07	0.38	0.15
Vasectomy reversal (Overall)	-0.24	0.26	-0.27	0.22	0.41	0.05

r, Spearman rank-correlation coefficient.

**P* < 0.05 was considered statistically significant.

patients may be explained due to high IL-6 and ROS levels in this group.

Interleukin-6 is a multifunctional cytokine found in seminal fluid that is produced by various types of cells in the genital tract. Levels of IL-6 correlate with the secretory activity of Sertoli cells (Naz & Kaplan, 1994). Currently, little is known about IL-6 levels in seminal plasma of men characterized according to the aetiological diagnosis of infertility. Significantly elevated IL-6 levels were seen in vasectomy reversal patients, compared with normal healthy men in our study.

The precise relation between IL-6 and sperm damage has not been established. However, high IL-6 levels have been associated with male infertility. These pro-inflammatory cytokines (IL-6) may modulate the pro-oxidant activities in male genital tract. The correlation between both IL-6 and ROS levels in vasectomy reversal patients and their relationship with sperm parameters is not well understood. However, the elevated levels of seminal ROS have been shown to decrease the fertilization rates *in vitro* by causing lipid peroxidation of sperm membrane (Sukcharoen *et al.*, 1995; Kolettis *et al.*, 1999). The positive correlation observed in our study suggests a common underlying pathophysiology that causes the dysfunction of spermatozoa. Lack of significant correlation between the IL-6 and sperm parameters may be due to small sample size of the study. Our study did not address whether elevated seminal IL-6 levels and ROS are the cause of spermatozoal dysfunction in men with vasectomy reversal. However, this should serve as a benchmark study for the future research to establish the relationship between the IL-6 and sperm parameters and infertility.

In conclusion, patients who undergo vasectomy reversal have elevated levels of IL-6 and ROS, which may contribute to decreased sperm motility and concentration and possibly lead to sub-fertility. These findings should be confirmed by large randomized controlled trials.

References

- Anderson RA, Baird DT (2002) Male contraception. *Endocr Rev* 23:735–762.
- Eggert-Kruse W, Boit R, Rohr G, Aufenanger J, Hund M, Strowitzki T (2001) Relationship of seminal plasma interleukin (IL)-8 and IL-6 with semen quality. *Hum Reprod* 16:517–528.
- Fuchs EF, Burt RA (2002) Vasectomy reversal performed 15 years or more after vasectomy: correlation of pregnancy outcome with partner age and with pregnancy results of *in vitro* fertilization with intracytoplasmic sperm injection. *Fertil Steril* 77:516–519.
- Hendin BN, Kolettis PN, Sharma RK, Thomas AJ Jr, Agarwal A (1999) Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Urol* 161:1831–1834.
- Kolettis PN, Sharma RK, Pasqualotto FF, Nelson D, Thomas AJ Jr, Agarwal A (1999) Effect of seminal oxidative stress on fertility after vasectomy reversal. *Fertil Steril* 71:249–255.
- Legue F, Guitton N, Brouazin-Jousseau V, Colleu-Durel S, Nourgalieva K, Chenal C (2001) IL-6 a key cytokine in *in vitro* and *in vivo* response of Sertoli cells to external gamma irradiation. *Cytokine* 16:232–238.
- Menkveld R, Stander FS, Kotze TJ, Kruger TF, van Zyl JA (1990) The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod* 5:586–592.
- Nallella KP, Allamaneni SS, Pasqualotto FF, Sharma RK, Thomas AJ Jr, Agarwal A (2004) Relationship of interleukin-6 with semen characteristics and oxidative stress in patients with varicocele. *Urology* 64:1010–1013.
- Naz RK, Kaplan P (1994) Interleukin-6 enhances the fertilizing capacity of human sperm by increasing capacitation and acrosome reaction. *J Androl* 15:228–233.
- Sharma RK, Agarwal A (1996) Role of reactive oxygen species in male infertility. *Urology* 48:835–850.
- Shekarriz M, Sharma RK, Thomas AJ Jr, Agarwal A (1995) Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. *J Assist Reprod Genet* 12:70–74.
- Sukcharoen N, Keith J, Irvine DS, Aitken RJ (1995) Predicting the fertilizing potential of human sperm suspensions *in vitro*: importance of sperm morphology and leukocyte contamination. *Fertil Steril* 63:1293–1300.
- World Health Organization (1993) Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction. Cambridge University Press, New York.
- World Health Organization (1999) Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction. Cambridge University Press, New York.