

Sperm quality and pregnancy rate after COX-2 inhibitor therapy of infertile males with abacterial leukocytospermia

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BACKGROUND: Leukocytes are a frequent finding in seminal plasma of infertile males with abacterial inflammation. We evaluated the effects of treatment with rofecoxib, a cyclooxygenase-2 inhibitor, on sperm quality and pregnancy rate after intrauterine insemination (IUI) or monitored intercourse. **METHODS:** We selected 47 infertile patients referred to our sterility centre for semen analysis. Sperm evaluation was performed by light microscopy with Papanicolau and eosin staining, before and 1 month after therapy. Swim-up selection was carried out in two steps. Starting 6–8 weeks after the end of therapy, couples underwent different procedures of assisted fertilization according to their semen parameters. **RESULTS:** Semen analysis 30 days after the end of therapy showed a significant reduction in leukocyte concentrations with respect to baseline, an improvement of sperm motility and morphology, particularly the presence and shape of the acrosomal complex and tail structure. After monitored intercourse and IUI, pregnancy rate was 15.8 and 11.3%, respectively. **CONCLUSIONS:** Our results suggest that a decrease in leukocytospermia after rofecoxib therapy was associated with recovery of all seminal characteristics in basal and swim-up selected samples. This general improvement could justify the positive outcome of ART after anti-inflammatory therapy.

Key words: COX-2 inhibitor/infertility/leukocytospermia/pregnancy rate/sperm quality

Introduction

Leukocytes are a frequent finding in seminal plasma of infertile male patients, even in the absence of inflammatory symptoms. In many cases, leukocyte concentrations in seminal fluid are $\geq 1 \times 10^6 \text{ ml}^{-1}$, a pathological level according to the WHO (1999) criteria. Granulocytes are the most prevalent white blood cells (WBCs) in semen (50–60%), followed by macrophages (20–30%) and T-lymphocytes (2–5%). The frequency of leukocytospermia among infertile males is 30%, and in ~80% of leukocytospermic patients, no microbial infection can be detected in seminal fluid. The correlation between leukocytospermia and male infertility is controversial: some studies have failed to find any association (Fedder *et al.*, 1993; Curi *et al.*, 2003). However, there is evidence of sperm damage due to WBC: (i) seminal WBC numbers have been reported to be higher in infertile than in fertile male patients; (ii) leukocytospermia has been associated with decreased sperm concentration and motility; (iii) WBCs damage sperm function and are an important prognostic factor for IVF-ET failure (Wolff, 1995). Other studies have demonstrated teratoasthenozoospermia and necrozoospermia in

leukocytospermic men (Yanushpolsky *et al.*, 1996; Thomas *et al.*, 1997; Menkveld *et al.*, 1998). Aziz *et al.* (2004) highlighted a significant correlation between leukocytospermia and sperm defects at acrosome and tail level. WBCs are activated by different stimuli which generate an inflammatory response: the presence in the seminal fluid of inflammatory mediators might induce oxidative stress, alteration in sperm motility, acrosome reaction and DNA integrity (Whittington and Ford, 1999; Whittington *et al.*, 1999; Sharma *et al.*, 2001; Alvarez *et al.*, 2002). Different studies suggest a negative effect of leukocytes and reactive-oxygen species, primarily produced by leukocytes, on semen quality (Sharma *et al.*, 2001; Erenpreiss *et al.*, 2002; Saleh *et al.*, 2002).

There have been few reports of pharmacological treatment of leukocytospermia with anti-inflammatory drugs. Lackner *et al.* (2006) recently suggested that anti-inflammatory medication of 12 males with abacterial leukocytospermia improved sperm count and reduced leukocyte concentrations. Oliva and Multigner (2006) reported a significant increase in sperm motility and morphology after antihistamine-like drug treatment, with a significant decrease in leukocytes in semen.

Non-steroid anti-inflammatory drugs (NSAIDs) have been shown to inhibit the production of prostaglandins (PGEs) (Dawood *et al.*, 1993), which are synthesized from arachidonic acid by the action of cyclooxygenase (COX) enzymes. The principal mechanism of NSAIDs is COX inhibition. COX exists in two isoforms: COX-1 and COX-2. The former is constitutively expressed and is involved in PGE synthesis in many tissues and organs, such as the testis. COX-2 is induced by inflammatory cytokines, growth factors and endotoxins, and the inhibition of COX-2 is responsible for the anti-inflammatory effects of NSAIDs (Bolten, 1998). A newer class of COX-2 inhibitor drugs was developed for use in patients with osteoarthritis and/or acute pain. These drugs selectively inhibit COX-2 activity in a dose-dependent manner, lowering PGE concentrations. There is no significant inhibition of COX-1 activity. NSAIDs have been shown to have a positive effect in the co-treatment of inflammatory urogenital tract pathology, but their effect on sperm quality and the outcome of assisted reproductive procedures has never been investigated.

In infertile males with reproductive difficulties related to abacterial inflammation of the urogenital tract, we evaluated the effects of a COX-2 inhibitor on sperm quality and pregnancy rate after intrauterine insemination (IUI) or monitored intercourse.

Materials and methods

Patients

Among patients referred to our sterility centre between September 2005 and May 2006 for semen analysis after 12–18 months of sexual intercourse without conception, we selected 47 males on the basis of leukocyte count ($>1\,000\,000\text{ ml}^{-1}$) in seminal plasma frequently associated with increased ejaculation volume ($\sim 6\text{ ml}^{-1}$). Azoospermic patients were excluded from the study. In order to confirm the diagnosis of leukocytospermia, two semen analyses were performed at 1 month interval and the last one was used for statistical analysis. Medical history, physical examination and serum concentrations of hormones [total testosterone, follicle-stimulating hormone (FSH), luteinizing hormone, estradiol (E_2), and β -inhibin] were evaluated. In all patients, microbiological analysis of seminal fluid was performed at the Unit of Microbiology, Siena Hospital, in order to detect common bacteria, such as *Mycoplasma*, *Trichomonas vaginalis* and *Chlamydia trachomatis*. Bacterial cultures were considered positive if the number of colony-forming units of pathogenic bacteria resulted $\geq 1 \times 10^3\text{ ml}^{-1}$.

The female partners underwent complete infertility workup, including hysterosalpingography. No health or fertility problems were found. None of the women had untreatable hormonal irregularities.

Pharmacological treatment

Selected patients without seminal-fluid bacterial infection associated with leukocytospermia were treated with a daily dose of 25 mg of rofecoxib in capsules, for an uninterrupted period of 30 days.

Semen analysis

Light microscopy

For semen analysis, patients were required to observe 4 days of sexual abstinence. Semen samples were examined 30 days before therapy, at the beginning of therapy and 30 days after the end of therapy, applying

WHO-recommended methods (1999), using an inverted polarized Olympus IXS21 microscope, equipped with Hoffmann lens and plate at 37°C . Eosin Y staining was used to detect necrotic sperm.

In each step of therapy, morphological analysis of 100 sperm per sample was performed using the Papanicolaou staining method (Oettle, 1986). Morphological characteristics of sperm organelles (nucleus, acrosomal and postacrosomal region and tail) were evaluated according to the WHO (1999) criteria. We compared statistically the two steps: at the beginning and the end of therapy.

Peroxidase staining

Leukocytes were counted by the method of Politch *et al.* (1993) adapted by Endtz (1974). Briefly, 0.0375% H_2O_2 was added to 4-ml benzidine stock solution (0.0125% w/v benzidine, Sigma Aldrich, in 50% ethanol). Twenty microlitres of ejaculate was mixed with 20 μl fresh benzidine- H_2O_2 solution. After 5 min of incubation, 160 μl of PBS was added and peroxidase-positive (round, brown stained) and peroxidase-negative (unstained) cells were counted in a Mackler chamber using a phase-contrast microscope.

Swim-up procedure

Swim-up selection was performed before and after therapy to compare sperm recovery in the two steps. Aliquots (1 ml) of samples were gently mixed with an equal volume of sperm medium (COOK, Italy) and centrifuged at 300 g for 10 min. The supernatant was discarded and the pellet layered with 600–1000 μl fresh sperm buffer (COOK) and incubated for 40 min at 37°C in tubes inclined at an angle of 45° . Finally, 0.3–0.5 ml of the upper layer was aspirated and analysed.

Medically assisted procreation

Starting 6–8 weeks after the end of therapy, couples underwent different procedures of assisted fertilization according to semen parameters, as suggested by Milingos *et al.* (1996).

Monitored intercourse

Female partners were treated with clomiphene citrate for 5 days starting from day 3 of the menstrual cycle and with E_2 for 5 days starting from day 8. Ultrasound examination was performed on days 10 and 12 to evaluate follicular growth. The couple had sexual intercourse on the day that ovulation was monitored.

Intrauterine insemination

Female partners were treated with 50 IU of recombinant follicle-stimulating hormone (FSH) for 12–14 days starting on the first day of the menstrual cycle and follicle growth was monitored every 2-days. When at least one and no more than three follicles were 19 mm in mean diameter, we planned the day of insemination. About 35 h before IUI, the women were injected with 5000-IU chorionadotrophin alpha. IUI was performed in couples whose semen characteristics fulfilled the minimum criteria suggested by Milingos *et al.* (1996). About 400 μl of upper swim-up selected sperm were inseminated using the Insemicath IUI (COOK) catheter.

Statistical analysis

The data were collected and analysed by means of the commercial software GraphPad Prism4 (GraphPad Software Inc., San Diego, CA). Results were expressed as mean \pm SD. The signed rank test was used for comparisons. Statistical significance was set at $P < 0.05$.

Table I. Sperm parameters evaluated before and after therapy in basal and swimup-selected samples

	Spermogram		Swim-up	
	Before therapy (n = 47)	After therapy (n = 47)	Before therapy (n = 47)	After therapy (n = 47)
Sperm count (10^6 ml^{-1})	25.1 ± 7.6	29 ± 9.9	8.2 ± 4.1	17.6 ± 4.5
Progressive motility (%)	23.3 ± 10.1	$46.9 \pm 17.1^*$	48 ± 8.6	$76.7 \pm 15.1^{**}$
Volume (ml)	5.6 ± 2.1	3.6 ± 1.1	0.4	0.4
Leukocytes (10^6 ml)	1.6 ± 1.1	$0.4 \pm 0.2^*$	0	0
Normal sperm morphology (%)	19 ± 4.8	$34.1 \pm 7.3^{**}$	28 ± 6.2	$52 \pm 12.2^*$
Eosin staining (positive %)	54.7 ± 14.1	$28.4 \pm 8.9^{**}$	Not performed	Not performed

*Signed rank test: $P \leq 0.05$.**Signed rank test: $P \leq 0.01$.

Results

Among infertile males referred to our infertility centre, semen analysis detected leukocytosis in 27% of cases. The diagnosis was confirmed by two semen analyses at 1-month interval. A total of 47 male patients enrolled in this study did not reveal any anomalies in medical history, physical examination or endocrine profile. Microbiological analysis of seminal fluid performed to detect common bacteria, such as *Mycoplasma*, *Trichomonas vaginalis* and *Chlamydia trachomatis*, were negative in all selected patients.

Semen analysis before therapy revealed asthenoteratozoospermia associated with leukocytosis (Table I). In this group of selected patients, the mean ejaculated volume was 5.6 ± 2.1 ml, indicating a tendency to hypervolaemia, which we registered in ~45% of patients with leukocytospermia. Peroxidase staining was used to quantify leukocytes (Table I). The mean number of leukocytes per millilitre was higher than normal ($2.6 \pm 1.1 \times 10^6$). In these leukocytospermic patients without general symptoms of inflammation, eosin Y staining showed an elevated mean percentage (54.7 ± 14.1 ; Table I) of sperm with broken plasma membranes, a sign of necrosis.

Microscope analysis of sperm morphology after staining by the Papanicolaou method showed that the most frequent sperm anomalies in basal samples were: the acrosome was absent in $45.2 \pm 7.8\%$ of cells and reduced or displaced in $49.6 \pm 5.9\%$; $55.1 \pm 7.8\%$ of sperm showed malformed nucleus; rolled-up or altered tail was detected in $63.9 \pm 6.8\%$ of cells. The plasma membrane was damaged in ~50% of sperm.

In order to evaluate the fertility potential of semen samples, swim-up selection was performed before therapy. When compared with basal samples, swim up selected samples showed fewer sperm per millilitre, as expected, and slightly more sperm with progressive motility (Table I). The mean percentage of selected sperm with normal morphology before therapy was 28 ± 6.2 .

All selected patients were treated with rofecoxib and none of them complained of side effects. Then, analysis of all semen parameters was repeated 30 days after the end of therapy. The number of leukocytes decreased from a mean of $1.6 \pm 1.1 \times 10^6$ to $0.4 \pm 0.2 \times 10^6$. Ejaculation volume was also reduced somewhat smaller after treatment, decreasing from a mean of 5.6 ± 2.1 ml to 3.6 ± 1.1 ml. Eosin staining indicated a significant reduction in dead sperm, to a mean percentage of 28.4 ± 8.9 . We did not observe any difference

in sperm count, whereas sperm motility and morphology were significantly improved after therapy. Papanicolaou staining for analysis of morphology showed a general improvement in sperm characteristics, particularly the presence ($67.2 \pm 8.1\%$) and normal shape ($45.2 \pm 7.9\%$) of the acrosomal complex, as well as tail structure, which was normal in $58.9 \pm 6.5\%$ of observed cells.

When swim-up selection was repeated 30 days after the end of anti-inflammatory treatment, there was a significant increase in the percentage of sperm with rapid progressive motility and normal morphology (Table I).

The positive effect of therapy on sperm quality enabled patients to become eligible for assisted reproductive procedures including monitored intercourse and IUI, according to the seminal parameters suggested by Milingos *et al.* (1996). Starting 6–8 weeks after the end of therapy, IUI was performed in 28 couples.

Sperm concentration, progressive motility and morphology in swim-up selected samples used for IUI were comparable to the values observed after rofecoxib therapy. Leukocyte concentration was lower than 1×10^6 in all basal samples.

A total of 62 IUI cycles were performed and 7 clinical pregnancies were obtained (pregnancy rate per cycle 11.3%). The other 19 couples with treated male partners performed monitored sexual intercourse and three clinical pregnancies (15.8%) were obtained (Table II).

Discussion

In evaluating the effects of anti-inflammatory treatment on sperm parameters of infertile men with abacterial inflammation of the urogenital tract, we found a slight positive effect. The effects of NSAIDs on inflammatory seminal fluid pathology are not well documented. In a recent study, Lackner *et al.* (2006)

Table II. Pregnancy rate after intrauterine insemination (IUI) or monitored intercourse starting 6–8 weeks after the end of therapy of male partner

Number of patients	47
Male age (years)	29–41
Female age (years)	27–35
Number of couples treated with IUI	28
Number of cycles	62
Pregnancy rate per IUI cycle (%)	11.3
Number of couples performing monitored intercourse	19
Pregnancy rate (%)	15.8

demonstrated a positive effect of an anti-COX-2 drug on seminal parameters of a small number of patients after only 2 weeks of therapy.

Our study suggests that the COX-2 inhibitor has positive effects on sperm parameters of patients with asthenoteratozoospermia associated with leukocytospermia and, frequently, with increased ejaculate volume. These characteristics of seminal fluid could be responsible for the long duration of infertility. Inflammation of the urogenital tract, associated or otherwise with microbial infection, is often a cause of reduced fertility, although there is no consensus about whether and how male genital-tract inflammation affects sperm fertilizing potential. Leukocyte concentration is normally $<1\,000\,000\text{ ml}^{-1}$, but it increases in inflammatory conditions. As granulocytes are the main constituent of WBC in semen, Reinhardt *et al.* (1997) demonstrated that granulocyte elastase, determined by an enzyme immunoassay, is a very specific marker of male genital inflammation and Zorn *et al.* (2004) correlated the presence of seminal elastase–inhibitor complex, a marker of urogenital inflammation, with negative outcome of *in vitro* fertilization (IVF), in patients with asymptomatic inflammation. The influence of leukocytes on seminal fluid is controversial, despite its high incidence among infertile men, ranging from 15 to 28%, whereas in fertile men it is 10% (Zorn *et al.*, 2000). Few studies failed to detect a negative effect of leukocytes on sperm quality: Tomlinson *et al.* (1992) even suggested that they have a role in the removal of abnormal spermatozoa from the ejaculate, increasing the percentage of sperm with normal morphology, and Kaleli *et al.* (2000) have not observed sperm damage during leukocytospermia. On the contrary, many other authors have found evidence that WBCs are significant cofactors of male infertility, influencing sperm number, motility and functions in a negative way (Wolff *et al.*, 1990; Yanushpolsky *et al.*, 1996; Thomas *et al.*, 1997; Aziz *et al.*, 2004), as also demonstrated by the hamster-ovum penetration test, an important prognostic factor for IVF (Wolff, 1995).

Because of the absence of clinical symptoms, the origin of the leukocytes is unclear. Normally, most leukocytes appear to originate from the epididymis because vasectomized men show very few leukocytes in semen. Sperm damage by WBC can also be mediated by reactive-oxygen species, proteases and cytokines. Moreover, Erenpreiss *et al.* (2002) suggested that leukocytes induce DNA damage in a cascade-like manner, particularly in sperm with poor morphology and motility. DNA fragmentation also affects the outcome of assisted reproductive technologies.

Our results clearly indicated a decrease in leukocytospermia associated with recovery of all seminal parameters including motility and morphology in basal and swim-up selected samples. This general improvement could justify the positive outcome of assisted reproduction procedures carried out after anti-inflammatory therapy. The resulting percentage of clinical pregnancy in our treated couples did not differ from that reported for the general population of infertile couples. Nevertheless anti-inflammatory treatment improved semen quality, allowing these couples to become eligible for less invasive treatment of assisted reproduction, as monitored intercourse

or IUI. Inflammation reduces evidently the chance of pregnancy by impairing the fertilizing potential of sperm.

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