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Effects of leucocytospermia on semen parameters and outcomes of intracytoplasmic sperm injection

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Summary

Leucocytes are present throughout the male reproductive tract but the clinical significance of leucocytic infiltration in the human ejaculate is controversial. The World Health Organization (WHO) defines leucocytospermia as the presence of peroxidase-positive leucocytes in concentrations of $\geq 1 \times 10^6$/mL of semen. The goals of this study were to clarify the relationship between leucocytospermia and semen parameters including sperm concentration, progressive and total motility before and after semen preparation, and intracytoplasmic sperm injection (ICSI) outcomes, including fertilization, embryo development, embryo morphology, cleavage and pregnancy rates. We compared the semen parameters and ICSI outcome of 34 leucocytospermic and 36 non-leucocytospermic control couples who were undergoing ICSI because of male factor infertility including oligo and/or astheno and/or teratozoospermia. Semen parameters including progressive motility rate (1.5% vs. 3%) and sperm concentrations (12 vs. 29 million/mL) were significantly lower in the leucocytospermic group compared with the control group. Other semen parameters were not affected by the presence of leucocytes. ICSI outcome, including fertilization (82% vs. 87%) and embryo development rates (79% vs. 86%) were significantly lower in the leucocytospermic group compared with the control group although there were no statistical difference for embryo quality, embryo cleavage and pregnancy rates. These results indicate that some semen parameters and the outcome of ICSI were negatively affected by the presence of leucocytospermia.

Keywords: embryo, intracytoplasmic sperm injection, leucocytospermia, semen

Introduction

Leucocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (El Demiry *et al.*, 1987). They are thought to play an important role in immunosurveillance (Kiessling *et al.*, 1995) and phagocytic clearance of abnormal sperm (Tomlinson *et al.*, 1992). The leucocyte count in semen is one of the classical measures of semen quality and presence of leucocytes in abnormally high concentrations ($\geq 10^6$/mL) (WHO, 1999) is defined as leucocytospermia or leucospermia (Fedder, 1996). Increased concentrations of leucocytes in semen provide an important clinical indicator of genital tract infection or inflammation (Comhaire *et al.*, 1980). The incidence of leucocytospermia is high among infertile patients (Wolff, 1995).

There are different opinions about the role of leucocytospermia in the pathogenesis of male infertility in the

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literature. Several reports on leucocytospermia have been published indicating negative effects on semen parameters and reproduction outcome (Wolff *et al.*, 1990; de Geyter *et al.*, 1994; Sukcharoen *et al.*, 1995; Vicino *et al.*, 1999; Arata de Bellabarba *et al.*, 2000; Diemer *et al.*, 2003) whereas others have found leucocytes to have no negative influence (Tomlinson *et al.*, 1993; Rodin *et al.*, 2003) A few papers indicated a positive role for seminal leucocytes by elimination of morphologically abnormal spermatozoa via phagocytosis (Kiessling *et al.*, 1995; Kaleli *et al.*, 2000). The traditional view is that high concentrations of seminal leucocytes are detrimental to sperm function (Aitken & Baker, 1995) because leucocytes are an important source of oxidative stress (Aitken & Fisher, 1994; Sharma *et al.*, 2001; Menkveld, 2004).

Oxidative stress is a key factor in the aetiology of male infertility and develops when levels of reactive oxygen species (ROS) production by leucocytes become high enough to overwhelm the antioxidant strategies present in semen (Sikka *et al.*, 1995). ROS are known to impair sperm function via different mechanisms involving lipid peroxidation, DNA damage, and protein inactivation resulting in the loss of fertilizing potential (Sharma & Agarwal, 1996). Spermatozoa are particularly susceptible to oxidative stress-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (Alvarez & Storey, 1995) and their cytoplasm includes low concentrations of scavenging enzymes (de Lamirande *et al.*, 1995).

Intracytoplasmic sperm injection (ICSI) is a widely used assisted reproduction technique to treat severe male infertility. There are few papers which study the effects of leucocytospermia on the assisted reproduction techniques and they focused especially on the fertilization rates in IVF (de Geyter *et al.*, 1994; Sukcharoen *et al.*, 1995). There is only one study in the databases, studying the effects of leucocytospermia on ICSI outcome (Vicino *et al.*, 1999). The aim of this study was to determine the effects of leucocytospermia on semen parameters and ICSI outcome in infertile patients undergoing ICSI.

Materials and Methods

Subjects

The ethical comity of Kadir Has University, Medical Faculty approved this study. The study group included 70 couples undergoing ICSI because of male factor infertility including oligo and/or astheno and/or teratozoospermia. The semen specimens were evaluated on the day of oocyte pick-up. Patients were divided into two groups according to the amount of leucocytes present in semen. Patients in group 1 had WHO-defined leucocytospermia $>1 \times 10^6$/mL ($n = 34$) and non-leucocytospermic patients in group 2 had seminal leucocytes fewer than 1×10^6/mL ($n = 36$).

Semen collection and analysis

Semen analysis was performed according to WHO criteria (WHO, 1999). Specimens were collected by masturbation after 3–7 days of sexual abstinence. Semen analysis was performed after liquefaction with a phase contrast (Nikon E-400, Kanagawa, Japan) microscope. Liquefaction time, appearance, volume, pH and viscosity of semen samples were determined. Semen volumes varied between 2 and 6 mL. Samples were analysed for sperm concentration, forwardly progressive sperm motility and total motility rate before and after semen preparation. Spermatozoa were scored with 40× objective for motility evaluation expressed as grades a to d. Total motility rate is calculated as the total of a, b and c motility. Round cells were also assessed by dropping 10 μL of the semen on Makler chamber.

Determination of leucocytes

Peroxidase staining which is a practical and reliable method recommended by WHO (1999) for determining leucocytes in the semen was used to differentiate leucocytes from other round cells. Leucoscreen (FertiPro, Beernam, Belgium) was used for this purpose. First, all round cells were counted on a Makler chamber and the round cell concentration was determined. Then the two contents of Leucoscreen, the hydrogen peroxide (30%) and the benzidine/cyanosine solution were mixed (3 and 100 μL respectively) and a solution is prepared. Of the prepared solution, 10 μL was mixed with 10 μL of liquefied semen on a slide and covered with a coverslip to be assessed under a phase contrast microscope (40× magnification). The brown stained cells were counted as peroxidase positive leucocytes and the pink stained cells were counted as all other cells. At least 100 cells were counted to determine the peroxidase positive leucocyte percentage. Leucocyte concentration was calculated by multiplying the percentage of peroxidase-positive leucocytes with the round cell concentration determined by a Makler counting chamber.

Semen preparation

The semen samples were prepared by two-layer (90% and 45%) discontinuous PureSperm (Nidacon, Mölndal, Sweden) gradient technique. The semen samples were divided into portions of 1 mL or less and the entire sample is used for semen preparation. For this purpose, 1 mL of semen was added on the top of two layers and centrifuged at 400 ***g*** for 20 min. Thereafter 400 μL of the pellet containing the selected spermatozoa were collected from the bottom of the conical-based tubes (Falcon 2095, Becton Dickinson, NJ, USA). All the pellets from the patient were collected in one tube and were suspended in 5 mL of GAMETE (Vitrolife, Kungsbacka, Sweden) medium pre-equilibrated at 37 °C and 6% CO_2. The suspension was centrifuged at 500 ***g*** for 6 min. The supernatant was discarded and centrifugation was repeated once more. Finally the supernatant

was discarded again and the pellet was suspended in 0.5 mL medium for further examination.

Ovarian stimulation and ICSI procedures

All female partners underwent ovulation induction using a short or long gonadotrophin-releasing hormone (GnRH) analogue suppression protocol or a GnRH antagonist protocol and human menopausal gonadotrophins or recombinant follicle-stimulating hormone (FSH). Oocyte-cumulus complexes (OCC) were recovered 36 h after the administration of 5000 or 10 000 IU of human chorionic gonadotrophin. The surrounding cumulus and corona cells were then removed using both enzymatic (Hyase 10× Vitrolife) and mechanic techniques. The nuclear maturation of the oocytes was assessed under an inverted microscope. Metaphase II oocytes and metaphase I oocytes that extrude their polar body in 4 h after the oocyte pick-up, were injected with a motile sperm. Micro-injection was performed as described previously by Van Steirteghem *et al.*, 1993.

Assessment of fertilization and embryo development

Further culture of injected oocytes was performed in 25 μL microdrops of culture medium (IVF, Vitrolife) under light paraffin oil (OVOIL, Vitrolife). Fertilization was confirmed after 16–18 h by the observation of two distinct pronuclei (2PN) and two polar bodies. The fertilization rate was calculated as the number of fertilized oocyte divided by total number of mature oocytes for each couple. Oocytes with 2PN were observed for embryonic development on day 2 and 3 after micro-injection. The embryo development rates were calculated as the number of embryos developed divided by the total number of mature oocytes for each couple. The cleavage status and the quality of the embryos were assessed under an inverted microscope with ×40 magnification according to the embryo quality criteria of Staessen *et al.*, 1989. A and B grade embryos were grouped as good quality embryos and grade C and D embryos were grouped as poor embryos. Good/poor embryo rates were calculated as the number of good/poor embryos developed divided by the total number of embryos for each couple. Embryos that cleaved to 4-cell in day 2 were grouped as normally cleaved embryos and embryos that cleaved less or more than 4-cell in day 2 were grouped as abnormally cleaved embryos. Normally/abnormally cleaved embryo rates were calculated as the number of normally/abnormally cleaved embryos developed divided by the total number of embryos for each couple. The mean rates for all the parameters were calculated by averaging rates calculated for each couple.

Embryo transfer and pregnancy assessment

Embryo transfers were performed by the same clinician on day 2 or 3 according to the number and quality of the embryos. Wallace (Smiths, Kent, UK) or TDT (Laboratoire C.C.D., France) catheter were used for embryo transfer. Twelve days after the embryo transfer, β-hCG level in the blood was measured. A ≥15 mIU/mL β-hCG value indicated a positive pregnancy.

Statistics

The results are expressed as median with 25th, 75th quartiles and as mean for pregnancy assessment. The data were analysed using SPSS (Statistical Package for Social Sciences) software (SPSS Inc., Chicago, IL, USA) for Windows 10.0. Mann–Whitney *U*-test was used for abnormally distributed variables and Student's-*t*-test for normally distributed variables. Chi-square test was used to compare the pregnancy rates between the groups. For all analyses, statistical significance was assessed at $p < 0.05$.

Results

The median age of the female partners was similar in the leucocytospermic (33 years) and the non-leucocytospermic group (31 years). There were no differences in the number of injected mature oocytes, total number of embryos and number of embryos transferred between the groups as represented in Table 1.

Effects on semen parameters

The median leucocyte concentration was found 2.68 million/mL in the leucocytospermic group and 0 in the non-leucocytospermic group.

Forward progressive motility rates (1.5% vs. 3%) and sperm concentrations (12 vs. 29 million/mL) were significantly lower in the leucocytospermic group compared with the non-leucocytospermic group ($p < 0.05$) (Table 1).

The total motility rates were similar between the groups (56% vs. 58%) ($p > 0.05$) (Table 1). There were also no difference for forward progressive motility (16% vs. 19%) and total motility rates (85% vs. 79%) after semen preparation between the groups ($p > 0.05$) (Table 1).

Effects on ICSI outcome

The two important parameters of ICSI outcome including fertilization and the embryo development rates were decreased by the presence of leucocytes. Fertilization rates (82% vs. 87%) and embryo development rates (79% vs. 86%) in the leucocytospermic group were significantly lower compared with the non-leucocytospermic group ($p < 0.05$) (Table 1).

There were no statistical difference between the leucocytospermic group and the non-leucocytospermic group for embryo quality (88% and 92% for good quality embryos and 11% and 3% for poor embryos respectively) ($p > 0.05$), embryo cleavage (50% and 50% for normally cleaved embryos and 50% and 50% for abnormally cleaved embryos respectively) ($p > 0.05$). Pregnancy rates were also not affected by the presence of leucocytes between the groups (30.3% and 30.6% respectively) ($p > 0.05$) (Table 1).

Table 1. Comparison of the leucocyte concentrations, semen parameters and ICSI outcome between the leucocytospermic and the non-leucocytospermic group

	Leucocytospermic ($n = 34$)	Non-leucocytospermic ($n = 36$)	p-value
Leucocyte concentration (million/mL)	2.68 (1.5, 6.1)	0 (0, 0.3)	0,000
Forward progressive motility (%)	1.5 (0, 9)	3 (0, 21)	0.020
Total motility (%)	56 (33, 75)	58 (38, 70)	0.711
Sperm concentration (million/mL)	12 (2, 31)	29 (4, 66)	0.008
Forward progressive motility after semen preparation (%)	16 (6, 34)	19 (9, 57)	0.293
Total motility after semen preparation (%)	85 (64, 92)	79 (64, 92)	0.549
Woman's age (years)	33 (30, 38)	31 (28, 36)	0.353
Mature oocytes	9 (7, 14)	9 (7, 13)	0.818
Total number of embryos	7 (4, 11)	8 (6, 12)	0.209
Number of embryos transferred	2 (2, 3)	2 (2, 3)	0.714
Fertilization (%)	82 (60, 88)	87 (81, 100)	0.000
Embryo development (%)	79 (58, 87)	86 (80, 98)	0.001
Good quality embryo (%)	88 (63, 100)	92 (70, 100)	0.671
Poor embryo (%)	11 (0, 36)	3 (0, 29)	0.593
Normally cleaved embryo (%)	50 (22, 76)	50 (33, 64)	0.669
Abnormally cleaved embryo (%)	50 (23, 77)	50 (35, 66)	0.660
Pregnancy (%)*	30.3*	30.6*	0.595

*Pregnancy rates were expressed as mean.
Results are presented as median with 25th, 75th quartiles. Mann–Whitney U and Student's-t-tests was used for the statistical analysis of the parameters and chi-square test was used to compare the pregnancy rates.
$p < 0.05$ was considered significant.

Discussion

As the aetiology of seminal leucocytes is unclear, it may be more useful to focus on their effects. The clinical significance of leucocytospermia in human ejaculate is controversial (Wolff, 1995). We investigated the effects of leucocytospermia on semen parameters and ICSI outcomes.

Forward progressive motility rates were significantly lower in the leucocytospermic group according to our results. This result seems to be in accordance with the findings of Diemer *et al.* (2003), who observed that incubation of normal sperms with leucocytes results in loss of progressive motile sperm rate. Sperm concentrations were also reduced in the leucocytospermic group. The result obtained for this parameter also confirms the findings of Aziz *et al.* (2004), who found a reduced sperm concentration in the leucocytospermic samples compared with the non-leucocytospermic samples. The negative effects observed for these semen parameters may be the result of the ROS released by leucocytes.

Wolff *et al.* (1990) and Aziz *et al.* (2004) observed that the total motility rate was also affected negatively by the presence of leucocytes but this parameter was not affected in our study. According to our results, it may be hypothesized that the period that sperms were incubated with the leucocytes was not enough to stop the sperms totally, the period was enough just to slow them down. Total motility and progressive motility rates after preparation of the semen were also not affected by the presence of leucocytes in our study reflecting that ROS produced by the leucocytes are washed away after the removal of the seminal plasma.

The effects of leucocytospermia varied for different semen parameters in previous studies and also in our study. This may be because of the different parameters involved in the effects of leucocytes on sperm functions like the number of cells involved, their state of activation, the level of free radical generation and the place they are added to the sperm suspension (Sharma & Agarwal, 1996).

The effects of leucocytes on ICSI are also questioned in this study. A reduced rate of fertilization in IVF cycles was reported when the number of leucocytes exceeded 6×10^6/mL of ejaculate (de Geyter *et al.*, 1994; Sukcharoen *et al.*, 1995). After these studies, Vicino *et al.* (1999), confirmed that leucocytes have negative effects on the fertilization process for both IVF and ICSI cycles. In this study, we investigated the effects of leucocytes for only ICSI cycles with a larger group. We found a significant reduced fertilization rate in the leucocytospermic group compared with non-leucocytospermic group.

Our results obtained for fertilization rates seems to be meaningful because leucocytospermic samples are shown to produce oxidative stress sufficient to damage DNA in sperms (Sharma *et al.*, 2001; Alvarez *et al.*, 2002) and the percentage of spermatozoa with fragmented DNA is suggested to have a negative correlation with fertilization rates in IVF (Sun *et al.*,

1997) and ICSI (Lopes *et al.*, 1998). Erenpreiss *et al.*, 2002 reported the average percentage of cells with impaired DNA to be more than doubled in sperm with leucocytospermia compared with sperm without leucocytospermia. When intrauterineinsemination (IUI) or IVF is used such DNA damage may not be important because, the peroxidative damage to the sperm plasma membrane ensures that fertilization cannot occur with a DNA-damaged sperm. However, when ICSI is used, this natural selection barrier is bypassed and a spermatozoon with damaged DNA may be directly injected into the oocyte (Twigg *et al.*, 1998). Such spermatozoa may not be capable of achieving fertilization and this may be the reason of reduced fertilization rates. In the light of these data, leucocyte contamination is suggested to be an important factor in determining the fertilizing potential of human spermatozoa in vitro (Sukcharoen *et al.*, 1995).

Our results indicate a reduced embryo development rate in the leucocytospermic group. Although we found no data about the correlation between leucocytospermia and the embryo development rate, reduced fertilization rate may be the reason of reduced embryo development.

Vicino *et al.* (1999) reported a low percentage of good quality embryos and a low embryo cleavage rate after ICSI in the presence of leucocytospermia. However, embryo quality and embryo cleavage rates of day 2 were not affected by the presence of leucocytes in our study. These results confirm the findings of Tesarik *et al.* (2004) who found that high percentages of spermatozoa with fragmented DNA did not cause any apparent impairment of zygote and cleaving embryo quality (cleavage speed and morphology) as the percentage of fragmented DNA increases in cases of leucocytospermia (Erenpreiss *et al.*, 2002).

The pregnancy rates were also not statistically different between the groups according to our results. There is no data about the effects of leucocytes on pregnancy rates of ICSI cycles in the databases so this is the first study that investigates the pregnancy rates in cases of leucocytospermia.

The effects of leucocytospermia for ICSI outcome also varied for different parameters. It may be concluded that leucocytospermia effects the fertilization and therefore embryo development rates but if fertilization occurs and an embryo develops, this situation has no adverse effect on embryo cleavage, embryo morphology and pregnancy outcome. Implantation and take-home baby rates should also be investigated in the future studies because this situation may result as repeated assisted reproduction treatment failures (Tesarik *et al.*, 2004).

Finally, according to our results, leucocytospermia seems to affect some of the semen parameters and ICSI outcome. The adverse effect of lipid peroxidation of the plasma membrane and damage to the nuclear genome of sperm as a result of leucocyte contamination could be hypothesized to explain this correlation. Further studies are needed in order to verify the role of oxidative stress produced by leucocytes on sperm functions and ICSI outcome. In addition, studies on long-term clinical effects of leucocytospermia will be useful to develop improved prevention and treatment strategies. Care should be taken about the short and long-term affects of leucocytospermia and couples should be counselled about this situation before undergoing an ICSI programme.

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