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Changes in sperm apoptotic markers as related to seminal leukocytes and elastase


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Abstract To elucidate the effects of inflammation on sperm quality, this study analysed classical sperm characteristics, leukocytes and elastase in neat semen, and sperm apoptotic markers, i.e. changes in plasma membrane phospholipid asymmetry, mitochondrial membrane potential (MMP), DNA integrity and intracellular reactive oxygen species (ROS), in semen prepared by density gradient using flow cytometry from 348 men of infertile couples. Increased leukocytes ($\geq 0.1 \times 10^6/\text{ml}$) were associated with a decreased sperm concentration, motility and normal morphology ($P \leq 0.001$). Sperm necrosis and DNA denaturation were increased (31.3 versus 26.6%, $P = 0.020$; 15.5 versus 11.5%, $P = 0.011$, respectively), whereas spermatozoa with normal MMP were decreased (64.1 versus 70.0%, $P = 0.004$). High leukocyte levels ($\geq 1 \times 10^6/\text{ml}$) were not associated with any of the observed sperm parameters. At low elastase concentration (100–290 $\mu\text{g/l}$), DNA denaturation was higher (16.1 versus 10.5%, $P = 0.024$) compared with very low elastase concentration ($<100 \mu\text{g/l}$). A high elastase concentration (290–1000 $\mu\text{g/l}$) was associated with higher ROS index compared with low elastase concentration (1.28 versus 1.01, $P = 0.016$). Slightly increased leukocytes and elastase are associated with slightly poorer sperm characteristics and/or increased sperm necrosis, DNA denaturation and intracellular ROS and decreased MMP. 

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Introduction

The number of infertile couples seeking infertility treatment in reproductive clinics is increasing and male factor has been estimated to affect at least 50% of them. Silent male genital tract inflammation represents a major cause of male infertility. According to the World Health Organization (WHO) (Rowe et al., 2000), silent genital tract inflammation is present in 20–30% male partners of infertile couples; a threshold of $>1 \times 10^6$ leukocytes/ml of ejaculate, the so-called-leukocytospermia, has been recommended as the main diagnostic marker for genital tract inflammation. Moreover, the diagnosis of male accessory gland infection, the most frequent cause of silent genital tract inflammation, is made only if the patient has abnormal semen quality.

During infection, activated granulocytes secrete elastase and chromatin; together they form neutrophil extracellular traps that kill bacteria (Fuchs et al., 2007). Increased concentrations of granulocyte elastase (≥ 250 – $290 \mu\text{g/l}$) in the seminal plasma are indicative of male genital tract infection (Jochum et al., 1986; Zorn et al., 2000).

Leukocytes are the main producers of reactive oxygen species (ROS), which are deleterious for sperm DNA and sperm membrane, therefore increased leukocytes are supposed to affect sperm quality negatively (Wolff et al., 1990). Also, increased elastase is frequently found in infertile men; an earlier study found a negative correlation between increased elastase concentrations and embryo development to the blastocyst stage (Zorn et al., 2004) in conventional IVF, suggesting a negative effect of genital tract inflammation on fertility potential.

On the other hand, Tomlinson et al. (1992) reported a positive relationship between leukocytes and sperm quality. Moreover, no correlation was found between elastase and standard sperm characteristics (Zöpfgen et al., 2000), and also no correlations were found between elastase, cytokines and resistin seminal concentrations (two other markers of inflammation) and sperm quality (Eggert-Kruse et al., 2007; Kratzsch et al., 2008).

The pathophysiology may explain these apparently contradictory findings. Specific inhibitors of elastase prevent the spread of phagocytosis. Experimentally, it has been found that the overexpression of elastase inhibitor increases cell survival in induced apoptosis (Altairac et al., 2003) and that the addition of recombinant elastase inhibitor restores sperm motility (Moriyama et al., 1998).

During recent years, flow cytometry (FCM) has been increasingly used to explore the integrity of different sperm cell compartments and functions such as the cytoplasmic membrane phospholipid asymmetry, mitochondrial membrane potential (MMP), DNA and intracellular reactive oxygen species (ROS) production. FCM is advantageously applied to measure apoptotic markers which evaluate physiologically-linked phenomena (Barroso et al., 2006). These apoptotic markers used alone or in combination have been proposed as prognostic factors of male fertility potential *in vivo* and *in vitro* (Troiano et al., 1998).

Apoptosis in ejaculated spermatozoa was first described in 1993 (Gorczyca et al., 1993). Sixteen years later, its existence, origin and role are still debated. Abnormal spermatozoa in the ejaculate may be due to abortive apoptosis in

relation with faulty nuclear remodelling during spermiogenesis in the testis (Paasch et al., 2003; Sakkas et al., 2003). The phenomenon of sperm apoptosis has also been observed during sperm cryopreservation or toxin exposure (Grizard et al., 2007). Furthermore, abnormal concentrations of apoptotic markers have been found present in the ejaculate of men with genital tract inflammation. ROS induce changes in cytoplasmic membrane symmetry (Villegas et al., 2005); moreover, increased leukocytes and ROS are related to lower MMP (Wang et al., 2003) and to increased DNA fragmentation (Alvarez et al., 2002; Barroso et al., 2000; Henkel et al., 2005; Saleh et al., 2003).

The present study was designed in order to analyse the extent of changes in apoptotic markers in prepared spermatozoa, used for assisted reproductive techniques, according to the levels of two mostly used markers of male genital tract inflammation, leukocytes and elastase. In male partners of infertile couples, this study looked for the relationships between seminal leukocyte and elastase levels and classical sperm characteristics of neat semen and between seminal leukocyte and elastase levels and apoptotic markers (changes in cytoplasmic membrane asymmetry, MMP, DNA integrity and ROS) in prepared semen.

Materials and methods

Study populations

From January 2004 to December 2006, 348 male partners of infertile couples attending the infertility outpatient clinic of the Department of Obstetrics and Gynecology, University Medical Centre Ljubljana, were consecutively enrolled. All were free of clinical signs of acute genital tract infection and had not received antibiotics for genitourinary infection in the preceding 12 months. Azoospermic men were excluded.

The study was approved by the national medical ethics committee; informed consent for the use of data for scientific evaluation was obtained from all enrolled men.

Sperm analysis by light microscopy

Semen was assessed according to the WHO guidelines (World Health Organization, 1999) with regard to volume, sperm concentration (normal = $\geq 20 \times 10^6$ spermatozoa/ml), rapid progressive motility or motility (normal = $\geq 25\%$) and normal morphology using strict criteria (normal = $\geq 14\%$). According to sperm characteristics, men were considered either normozoospermic if all sperm characteristics were normal or oligoasthenoteratozoospermic (OAT) if any characteristic was abnormal. Leukocytes were detected by peroxidase test using benzidine.

Determination of elastase in the seminal plasma

An aliquot of neat semen was first centrifuged at 450 g for 10 min and seminal plasma was separated from leukocytes, to prevent *in-vitro* secretion by leukocytes, and frozen (-20°C).

Elastase concentrations were measured in frozen–thawed cell-free seminal plasma, prepared in physiological

solution using homogeneous immunoassay Ecoline PMN elastase (Merck, Darmstadt, Germany). Elastase concentrations determined as low ($<289 \mu\text{g/l}$), high ($290\text{--}999 \mu\text{g/l}$) and very high ($\geq 1000 \mu\text{g/l}$), respectively, were used to indicate no, moderate or acute male genital tract inflammation (Wolff et al., 1990; Zorn et al., 2000). Further, the 'no inflammation' group was subdivided into two, i.e. in men with very low ($<100 \mu\text{g/l}$) and low ($100\text{--}289 \mu\text{g/l}$) elastase concentrations. Due to too small semen volumes provided, elastase assessment was not possible in 97 men (28%).

Preparation of spermatozoa and seminal plasma

Semen (1.5 ml) was layered on two-step discontinuous Pure-Sperm (Nidacon, International AB, Sweden) concentration gradient (100%/40%) and centrifuged at 160 g for 30 min. Then the sperm pellet from the 100% layer was washed with 2 ml of SpermPrep medium (MediCult, Jyllinge, Denmark), centrifuged at 220 g for 10 min and resuspended in 0.5 ml of SpermPrep medium. An aliquot of prepared spermatozoa at a final concentration of $1 \times 10^6/\text{ml}$ was used for FCM apoptotic markers screening. Cells were measured on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and analysed with FACSDiva software (Becton Dickinson Biosciences, San Jose, CA, USA).

Sperm apoptosis and ROS as detected by changes in cytoplasmic membrane asymmetry, MMP, DNA denaturation and hydroethidine transformation

Prepared spermatozoa were subjected to FCM analysis of phospholipid asymmetry in plasma membrane using fluorescein isothiocyanate-conjugated annexin V, MMP using 3,3'-dihexyloxacarbocyanine iodide ($\text{DiOC}_6(3)$; Molecular Probes, Eugene, Oregon, USA), DNA denaturation and ROS as revealed by hydroethidine (HE; Molecular Probes) transformation according to the methodologies described by Marchetti et al. (2002) and Evenson et al. (2002).

Detection of phospholipid asymmetry on the sperm plasma membrane

The loss of membrane asymmetry and presence of surface phosphatidylserine were detected using fluorescein isothiocyanate-conjugated annexin V (annexin V-FITC; Becton Dickinson Pharmingen, San Diego, CA, USA). Semen samples were diluted to 1×10^5 spermatozoa cells/ml in 100 μl binding buffer with 10 μg annexin V-FITC. The samples were stained simultaneously with 1 $\mu\text{g}/\text{ml}$ propidium iodide (PI; Molecular Probes). Unstained samples were used as negative fluorescence controls. Two replicate experiments were done for each sample and the average values were used in further analyses. According to their reactivity to annexin V and PI, spermatozoa were classified as normal (viable) (annexin V and PI both negative), apoptotic (positive annexin V and negative PI) and necrotic (annexin V and PI both positive). Changes in phospholipid asymmetry on the sperm membrane were expressed as percentages of normal, apoptotic and necrotic spermatozoa.

Assessment of sperm mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured by means of $\text{DiOC}_6(3)$ staining. PI was used as a supravital fluorescent stain.

Because $\text{DiOC}_6(3)$ has a single wave length emission, a normal MMP was attributed to cells with a high fluorescence signal. Spermatozoa were incubated in 1 ml $\text{DiOC}_6(3)$ (0.5 nmol/l) at a 37°C water bath for 20 min. Ten thousand cells were analysed by FCM. As a negative control, the sperm sample was also incubated with 1 mmol/l carbamoyl cyanide *m*-chlorophenylhydrazine, an uncoupling agent that abolishes the MMP. The analysis only used the percentage of viable spermatozoa with normal MMP.

Sperm DNA denaturation

Spermatozoa cells were treated with a low pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15 mol/l NaCl, and 0.08 N HCl for 30 s, and then stained with 0.02 mg/ml of purified acridine orange (AO; Molecular Probes) in a phosphate-citrate buffer, pH 6.0.

The samples were assessed twice using FCM; the results are presented as the mean value of the two FCM measurements. Under these conditions, when excited with a 488-nm light source, AO intercalating with double-stranded DNA emits green fluorescence and AO associated with single-stranded DNA emits red fluorescence. Thus, sperm chromatin damage can be quantified by FCM measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and is displayed as green (DNA stainability) versus red (denatured DNA) fluorescence intensity cytogram patterns. The analysis only used the percentages of spermatozoa with denatured DNA.

Determination of ROS

HE is oxidized by superoxide anion to ethidium bromide, emitting red fluorescence. Sperm cells were split into two aliquots of 155 μl of prepared semen and further incubated for 15 min either with or without 320 μl phorbol 12-myristate 13-acetate (PMA; Sigma Chemicals, Deisenhofen, Germany). The samples were washed twice and then labelled with HE. A final concentration of 10 $\mu\text{mol}/\text{ml}$ HE was added and incubated for 15 min at 37°C . The median fluorescent intensity (MFI), which reflects the overall production of ethidium bromide, was determined. The ROS index was determined as the ratio between MFI of PMA-stimulated cells and MFI of non stimulated cells. ROS was determined in 92 men.

Statistical analysis

Statistical Package for Social Sciences version 15.0 software (SPSS, Chicago, Illinois, USA) was used for statistical analysis. Data were expressed as mean \pm SD (range). Due to their abnormal distribution, the percentages of spermatozoa with

DNA denaturation and seminal elastase concentrations were \log_{10} transformed.

Relationships between the percentages of apoptotic markers in prepared spermatozoa, classical neat sperm characteristics and leukocyte and elastase levels were tested by Spearman correlation test. Mann–Whitney *U*-test and analysis of variance were used to test the relationships between the apoptotic markers in prepared spermatozoa according to neat sperm quality (normal versus abnormal spermatozoa).

Analysis of variance with Bonferroni correction was used to test the relationships between classical characteristics in neat spermatozoa and apoptotic markers in prepared spermatozoa and leukocyte ($0, 0.1\text{--}0.9$ and $\geq 1.0 \times 10^6/\text{ml}$) and elastase levels ($<100, 100\text{--}289, 290\text{--}999$ and $\geq 1000 \mu\text{g/l}$) in neat semen.

Differences in confounding factors (patients' age, history of cryptorchidism, testicular volume, presence of a varicocele and duration of abstinence) among the groups were checked using the chi-squared test. Statistical significance was set at $P < 0.05$.

Results

Table 1 presents the characteristics of the study population consisting of 348 male partners of infertile couples.

Relationships between classical characteristics in neat spermatozoa and membrane asymmetry changes, vital spermatozoa with normal MMP, DNA denaturation and ROS in prepared spermatozoa

Significant associations existed between classical sperm characteristics and sperm membrane asymmetry changes, and spermatozoa with normal MMP and DNA denaturation ($P < 0.001$; **Table 2**), whereas no significant correlation was found between classical sperm characteristics and ROS.

Significantly different percentages of normal, necrotic and apoptotic spermatozoa, vital spermatozoa with normal MMP and DNA denaturation were present in semen of normozoospermic in comparison with OAT men ($P < 0.001$; **Table 3**).

Relationship between seminal leukocytes and classical characteristics in neat spermatozoa and apoptotic markers in prepared spermatozoa

The relationships between seminal leukocyte levels, classical sperm characteristics and sperm apoptotic markers are presented in **Table 4**. There were no linear correlations found between leukocytes, classical characteristics and apoptotic markers. When dividing the men into those with $<0.1 \times 10^6$ leukocytes/ml (group 1, $n = 156$), those with $0.1\text{--}0.9 \times 10^6$ leukocytes/ml (group 2, $n = 114$) and those

Table 1 Relevant characteristics of the 348 male partners from infertile couples included in the study.

Characteristic	Value
Age (years)	33.5 ± 5.4 (21–56)
Duration of infertility (years)	2.3 ± 2.1 (1–15)
<i>Neat semen</i>	
Sperm concentration ($10^6/\text{ml}$)	61.3 ± 43.5 (6.0–325.0)
Sperm rapid progressive motility (%)	30.5 ± 9.3 (10.0–50.0)
Sperm normal morphology (%)	25.8 ± 13.2 (4.0–60.0)
Leukocyte concentration ($10^6/\text{ml}$)	0.57 ± 1.16 (0.0–11.2)
Without leukocytes ($<0.1 \times 10^6/\text{ml}$)	164 (48.0)
Low leukocytes ($0.1\text{--}0.9 \times 10^6/\text{ml}$)	118 (34.5)
Leukocytospermia ($\geq 1.0 \times 10^6/\text{ml}$)	60 (17.5)
Elastase concentration ($\mu\text{g/l}$)	289.6 ± 583.6 (0–4307)
Very low ($<100 \mu\text{g/l}$)	137 (54.6)
Low ($100\text{--}289 \mu\text{g/l}$)	50 (19.9)
High ($290\text{--}999 \mu\text{g/l}$)	48 (19.1)
Very high ($>1000 \mu\text{g/l}$)	16 (6.4)
<i>Spermatozoa prepared by density-gradient centrifugation</i>	
Sperm cytoplasmic membrane asymmetry	
Normal (%)	62.1 ± 19.4 (4.0–91.0)
Necrotic (%)	27.8 ± 15.8 (1.0–86.0)
Apoptotic (%)	10.1 ± 11.6 (0.2–78.0)
Vital with normal MMP (%)	68.9 ± 19.7 (2.0–99.0)
With DNA denaturation (%)	13.0 ± 11.2 (1.2–75.9)
ROS index	1.05 ± 0.23 (0.82–2.62)

Values are either mean \pm SD (range) or number (%).

MMP = mitochondrial membrane potential; ROS = reactive oxygen species.

Table 2 Relationships between sperm membrane asymmetry changes, vital spermatozoa with normal mitochondrial membrane potential, spermatozoa with DNA denaturation, reactive oxygen species index and classical sperm characteristics.

Spermatozoa prepared by density gradient	Neat spermatozoa			P-value
	Sperm concentration (10^6 /ml)	Sperm rapid progressive motility (%)	Sperm normal morphology (%)	
Normal (%)	0.478	0.550	0.466	<0.001
Apoptotic (%)	−0.309	−0.249	−0.239	<0.001
Necrotic (%)	−0.401	−0.514	−0.414	<0.001
Vital with normal MMP (%)	0.543	0.577	0.516	<0.001
With DNA denaturation (%)	−0.284	−0.452	−0.392	<0.001
ROS index	−0.050	0.020	−0.023	NS

Values are correlation coefficients.

MMP = mitochondrial membrane potential; NS = not statistically significant; ROS = reactive oxygen species.

Table 3 Apoptotic markers in prepared spermatozoa according to neat sperm quality.

Spermatozoa prepared by density-gradient centrifugation	Neat spermatozoa		P-value
	Normozoospermia (n = 229)	OAT (n = 119)	
Normal (%)	68.2 ± 16.9	50.6 ± 13.7	<0.001
Apoptotic (%)	8.1 ± 10.0	13.7 ± 13.2	<0.001
Necrotic (%)	23.6 ± 13.9	35.6 ± 16.0	<0.001
Vital with normal MMP (%)	76.3 ± 15.2	55.0 ± 19.6	<0.001
With DNA denaturation (%)	10.2 ± 8.6	18.3 ± 13.5	<0.001
ROS index	1.05 ± 0.26	1.03 ± 0.18	>0.05

Values are mean ± SD.

MMP = mitochondrial membrane potential; OAT = oligoasthenoteratozoospermia; ROS = reactive oxygen species.

with more than 1×10^6 leukocytes/ml (group 3, leukocytospermia, $n = 57$), a decrease in sperm concentration, rapid progressive motility and normal morphology was observed in group 2 compared with group 1. In group 3, all three sperm characteristics were normal (all $P < 0.001$).

The comparison of apoptotic markers in prepared spermatozoa among the groups showed a significantly higher percentage of spermatozoa with necrosis and DNA denaturation in group 2 ($P = 0.020$ and $P = 0.011$, respectively) versus group 1. A significantly lower percentage of vital spermatozoa with normal MMP was found in group 2 compared with group 3 ($P = 0.004$; **Table 4**). Among the groups, no significant differences in ROS concentrations were observed. The groups did not differ according to age, history of cryptorchidism, testicular volume, presence of a varicocele and duration of abstinence.

Relationship between seminal elastase and classical characteristics in neat spermatozoa and apoptotic markers in prepared spermatozoa

The relationships between seminal elastase concentrations, classical sperm characteristics and sperm apoptotic markers are presented in **Table 5**. No linear correlations were found

between seminal elastase concentrations and classical sperm characteristics or between seminal elastase and sperm apoptotic markers.

Also, when the men were divided according to the seminal elastase concentration into the very low elastase group ($<100 \mu\text{g/l}$, group 1, $n = 137$), low elastase group ($100\text{--}289 \mu\text{g/l}$, group 2, $n = 50$), high elastase group ($290\text{--}999 \mu\text{g/l}$, group 3, $n = 48$) and very high elastase group ($\geq 1000 \mu\text{g/l}$, group 4, $n = 16$), no difference was found among the groups in classical sperm characteristics. The comparison of sperm apoptotic markers among the groups showed a higher percentage of spermatozoa with DNA denaturation in group 2 compared with group 1 ($P = 0.024$; **Table 5**). Moreover, sperm ROS content was significantly higher in group 3 in comparison to group 1 (1.01 versus 1.28 , $P = 0.016$). These groups did not differ according to patients' age, history of cryptorchidism, testicular volume, presence of a varicocele and duration of abstinence.

Discussion

To further elucidate the pathophysiology of male genital tract inflammation as a cause of male infertility, sperm

Table 4 Relationships between neat semen leukocyte and elastase concentrations, classical sperm characteristics and prepared sperm apoptotic markers.

Characteristic	Leukocytes <0.1 × 10 ⁶ /ml (group 1, n = 156)	Leukocytes 0.1–0.9 × 10 ⁶ /ml (group 2, n = 114)	Leukocytes ≥1.0 × 10 ⁶ /ml (group 3, n = 57)
<i>Neat semen</i>			
Elastase (µg/l)	129.4 ± 385.8 ^a	306.6 ± 547.6	681.9 ± 859.3 ^a
Sperm concentration (10 ⁶ /ml)	72.0 ± 51.6 ^b	40.1 ± 22.5 ^{b,c}	72.7 ± 34.6 ^c
Sperm rapid progressive motility (%)	32.2 ± 8.3 ^d	27.1 ± 10.1 ^{d,e}	32.7 ± 8.2 ^e
Sperm normal morphology (%)	27.6 ± 13.9 ^f	22.3 ± 12.3 ^{f,g}	27.9 ± 11.6 ^g
<i>Spermatozoa prepared by density-gradient centrifugation</i>			
Apoptotic (%)	11.7 ± 13.9	9.1 ± 8.9	7.8 ± 8.6
Necrotic (%)	26.6 ± 15.5 ^h	31.3 ± 16.5 ^h	25.3 ± 14.6
Vital with normal MMP (%)	70.0 ± 20.0 ⁱ	64.1 ± 20.0 ^{i,j}	73.9 ± 16.9 ^j
With DNA denaturation (%)	11.5 ± 10.1 ^k	15.5 ± 12.9 ^k	11.1 ± 8.9
ROS index	1.01 ± 0.11	1.09 ± 0.36	1.08 ± 0.23

Values are mean ± SD.

^{a–k} Same letters indicate statistically significant differences ($P \leq 0.020$).

MMP = mitochondrial membrane potential; ROS = reactive oxygen species.

Table 5 Relationships between neat seminal elastase and leukocyte concentrations, classical sperm characteristics and prepared sperm apoptosis markers.

Characteristic	Very low elastase <100 µg/l (group 1, n = 137)	Low elastase 100–289 µg/l (group 2, n = 50)	High elastase 290–999 µg/l (group 3, n = 48)	Very high elastase ≥1000 µg/l (group 4, n = 16)
<i>Neat semen</i>				
Leukocytes (10 ⁶ /ml)	0.34 ± 0.95 ^{a,b}	0.59 ± 1.26 ^c	0.96 ± 0.93 ^{a,d}	2.15 ± 2.78 ^{b,c,d}
Sperm concentration (10 ⁶ /ml)	63.9 ± 47.6	61.9 ± 44.9	54.5 ± 36.6	55.8 ± 28.5
Sperm rapid progressive motility (%)	31.3 ± 9.2	29.0 ± 9.4	29.3 ± 9.7	32.5 ± 8.9
Sperm normal morphology (%)	26.0 ± 13.2	24.4 ± 15.0	25.5 ± 12.6	27.7 ± 11.6
<i>Spermatozoa prepared by density-gradient centrifugation</i>				
Apoptotic (%)	11.0 ± 12.9	9.0 ± 11.8	10.3 ± 10.1	9.8 ± 7.9
Necrotic (%)	27.0 ± 16.1	28.8 ± 16.9	28.8 ± 13.8	21.3 ± 12.6
Vital with normal MMP (%)	70.2 ± 19.3	69.3 ± 17.7	66.5 ± 20.0	77.9 ± 18.3
With DNA denaturation (%)	10.5 ± 9.8 ^e	16.1 ± 13.2 ^e	14.2 ± 9.7	12.1 ± 13.9
ROS index	1.01 ± 0.12 ^f	1.12 ± 0.38	1.28 ± 0.50 ^f	1.00 ± 0.08

Values are mean ± SD.

^{a–f} Same letters indicate statistically significant differences ($P < 0.05$).

MMP = mitochondrial membrane potential; ROS = reactive oxygen species.

quality using apoptotic markers was investigated. In men from infertile couples, increased incidence of spermatozoa with necrosis and DNA denaturation and decreased MMP were observed even when leukocyte and elastase levels were only marginally increased, below the classical thresholds for inflammation. However, the observed negative changes were not sufficient to cause changes in classical sperm characteristics. Furthermore, an improvement in sperm characteristics and sperm function, i.e. better sperm

quality and a higher percentage of spermatozoa with normal MMP, were observed above the threshold of $\geq 1 \times 10^6$ leukocytes/ml of ejaculate.

The study population included non-selected male partners of infertile couples; 40% of men had abnormal spermatozoa according to WHO criteria. Leukocytospermia was present in 17.5% of the men, which falls within the published values; 7% observed by Yanushpolsky et al. (1995), 13% by Comhaire et al. (1980) and 24% by Endtz (1974).

The incidence of moderate and acute male genital inflammation was present in 25% of men with elastase concentration above 290 $\mu\text{g/l}$, which is in accordance with the report by Jochum et al. (1986).

The incidence of abnormal apoptotic markers in spermatozoa prepared by density-gradient centrifugation was similar to that reported by Oosterhuis et al. (2000). Apoptotic markers, ROS excluded, highly correlated to all three classical parameters, which is in agreement with Zini et al. (1999) and Chen et al. (2006). The highest positive correlation was found when testing the percentage of spermatozoa with normal MMP.

Normozoospermics had less apoptotic cells than men with OAT (8.1% versus 13.7%, respectively), which is in accordance with Gandini et al. (2000). Sperm necrosis was present in 24% of normozoospermic men, whereas in men with abnormal spermatozoa it was about 30% higher, which is in concordance with the report by Ricci et al. (2009). The percentage of spermatozoa with normal MMP was about 75% in normal spermatozoa and about 50% in abnormal spermatozoa. The mean percentage of spermatozoa with DNA denaturation was 10% in normal spermatozoa and was twofold higher in abnormal spermatozoa; the same results have been reported by Zini et al. (2001).

A positive relationship was found between an increased percentage of spermatozoa with DNA denaturation and increased leukocyte and/or elastase levels.

The relationship between inflammation and spermatozoa with DNA denaturation has already been demonstrated (Alvarez et al., 2002). Other apoptotic changes observed concern sperm necrosis, MMP and sperm ROS content. Similar changes have been reported in inflammation due to the presence of various bacteria (*Chlamydia trachomatis* and *Escherichia coli*) resulting in increased phosphatidylserine membrane externalization (Satta et al., 2006; Villegas et al., 2005), necrosis (Collodel et al., 2005) and cellular death (Eley et al., 2005). In the current study, intracellular sperm superoxide anion, as assessed by HE, was positively correlated to elastase. As far as is known, this is the first report on a possible relationship between increased sperm intracellular ROS and genital tract inflammation. Henkel and Schill (1998) found that ROS activity in semen, determined by means of luminol chemiluminescence, was correlated to the concentration of neutrophil elastase. However, intracellular ROS concentrations were not related to the number of leukocytes. This is not surprising as leukocytes, stained by peroxidase, are not activated and automatically involved in the sperm cell damage induced by the inflammation process.

The changes in sperm classical characteristics and apoptotic markers were observed with $\leq 1 \times 10^6$ leukocytes/ml, which is not considered as representative of inflammation. Normally, semen is not contaminated by leukocytes; almost 50% of semen samples did not contain leukocytes. Therefore, a negative change in sperm quality, especially in motility due to an increase in ROS may be observed even with low leukocyte levels, as reported by Sharma et al. (2001). Henkel et al. (2005) justifiably questioned whether the threshold of 1×10^6 leukocytes/ml was not too high in the screening for genital tract inflammation and its consequences.

However, the changes in sperm apoptotic markers observed in men with increased leukocyte and elastase levels may not be clinically important. Spermatozoa were affected by a higher percentage of spermatozoa with necrosis and DNA denaturation and a lower percentage of spermatozoa with normal MMP, yet the quality parameters were still better compared with typical abnormal spermatozoa. Also, Gallegos et al. (2007) reported that an increase in sperm DNA fragmentation provoked by *C. trachomatis* was not strictly accompanied by changes in classical sperm characteristics. The current study found a relatively small change in DNA integrity in relation to the elastase concentration and this may explain why negative changes in classical sperm characteristics were not found. However, the DNA damage was observed in prepared semen, the fraction usually used in assisted reproductive techniques and might be underestimated in comparison with that observed in neat semen. Indeed, a significantly lower percentage of spermatozoa with DNA fragmentation and dysfunctional mitochondria and apoptotic or necrotic spermatozoa has been found in prepared spermatozoa than in neat semen samples by other groups (Donnelly et al., 2000; Ricci et al., 2009). Nevertheless, the negative changes of sperm DNA integrity in relation to inflammation observations are too small to exert negative effects on the fertility potential of men affected by silent genital inflammation. This study found 15% of spermatozoa with DNA denaturation, which is substantially lower than the threshold of $\geq 30\%$ proposed by Evenson and Wixon (2006) to be abnormal. Further studies are needed to demonstrate whether the DNA denaturation in less than 30% of spermatozoa, perhaps in combination with an increase in necrosis and a decrease in normal MMP expression, is a potential indicator of reproductive abnormalities. Sperm apoptosis is related to suboptimal oocyte penetration (Grunewald et al., 2008). Many reports have suggested that DNA fragmentation could be connected with embryo quality (Borini et al., 2006; Sakkas et al., 1998; Saleh et al., 2003; Virro et al., 2004; Zini et al., 2005). A previous study found an increased incidence of poor quality embryos conceived with spermatozoa originating from semen with increased elastase (Zorn et al., 2004).

Surprisingly, the percentage of vital spermatozoa with normal MMP was found to be higher in men with leukocytospermia compared with men with low leukocyte levels. When the leukocyte level exceeds $1 \times 10^6/\text{ml}$, antioxidants are present in larger quantities. Aitken et al. (1994) reported that leukocytes had no or little effect on sperm quality due to seminal antioxidants which protect spermatozoa from ROS injury.

An increased number of spermatozoa with normal MMP may also be related to elastase inhibitor intervention. After activation of elastase, an elastase inhibitor is produced that prevents negative effects of elastase on spermatozoa (Maegawa et al., 2001). This intervention might explain why, with elastase concentration $\geq 1000 \mu\text{g/l}$ and in the case of leukocytospermia, the percentages of apoptotic and necrotic sperm decrease, the percentage of spermatozoa with normal MMP increases and the process of DNA denaturation is reduced, as was observed in this study. Consequently, it is not surprising that many reports suggest that leukocytes

play a positive role in sperm quality and function (Ziyyat et al., 2008), possibly by eliminating apoptotic spermatozoa (Ricci et al., 2002).

Excess leukocytes may also be the consequence of a sudden delivery of leukocytes by the prostate. These leukocytes are in contact with spermatozoa for too short a time to provoke sperm alterations. In contrast, in chronic epididymitis, because of prolonged cohabitation of leukocytes and spermatozoa and absence of antioxidant secretion by abnormal epididymis, negative effects on sperm quality are more likely (Haidl et al., 2008).

Therefore, increased necrosis, DNA denaturation and ROS and decreased normal MMP can be observed in ejaculated spermatozoa at different stages of genital tract inflammation. Contrary to a chronic disease such as varicocele, genital tract inflammation is a dynamic and often a transient process, depending on the level of toxic ROS and sperm apoptosis.

Silent male genital tract inflammation in infertile men needs new diagnostic approaches. The markers of inflammation actually used have not been proven to be clinically useful. By using the recommended thresholds, changes in sperm quality and function due to inflammation may be overlooked. Moreover, suspected genital inflammation cannot be efficiently managed by classical sperm analysis only. The assessment of sperm apoptotic markers and intracellular ROS might be decisive.

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