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Semen leukocytes and oxidative-dependent DNA damage of spermatozoa in male partners of subfertile couples with no symptoms of genital tract infection

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SUMMARY

The influence of seminal leukocytes on generation of oxidative damage to sperm DNA was here investigated on male partners of subfertile couples asymptomatic for a genital tract infection. The study included 111 ejaculates from men attending the Andrology Centre at University of L'Aquila. Semen leukocytes subset included round cells expressing pan-leukocyte CD45 antigen, monocyte/macrophage lineage antigen CD14, and activated macrophages HLA-DR antigen. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) expression identified spermatozoa with DNA oxidative adducts while terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling (TUNEL) assay detected spermatozoa with DNA fragmentation. Flow cytometry and immunocytochemistry was used for determinations. Main outcome measure was the association of semen leukocyte subpopulations with spermatozoa showing oxidative-related DNA damage and with routine semen parameters. Leukocyte subpopulations were strictly correlated ($p < 0.0001$), but no association was found between the concentration of leukocytes, semen parameters, the percentage of TUNEL-positive and of 8-OHdG-positive spermatozoa. The percentage of 8-OHdG-positive spermatozoa was positively correlated with the percentage of TUNEL-positive spermatozoa ($r = 0.48$; $p < 0.0001$) and negatively correlated with sperm concentration ($r = -0.44$; $p < 0.0001$). Sperm concentration and the percentage of TUNEL-positive spermatozoa independently contributed ($\beta = -0.25$, $p = 0.008$; $\beta = 0.23$, $p = 0.05$, respectively) to the variation in percentage of 8-OHdG-positive spermatozoa after adjusting for age, abstinence time, and smoking. In conclusion, oxidative-dependent DNA damage in spermatozoa was associated to poor semen quality but not to different leukocyte subpopulations in ejaculates of men asymptomatic for a genital tract infection.

INTRODUCTION

A concentration of leukocytes detected by peroxydase stain $>1 \times 10^6$/mL of semen (World Health Organization, 2010) is a marker of genital tract infection (Comhaire *et al.*, 1980). However, a relatively large number of men attending fertility clinics exhibit a high number of seminal leukocytes without any symptoms of genital tract infection (Wolff, 1995) and the concentration of semen leukocytes is not predictive for actual microbial infections (Trum *et al.*, 1998). The presence of a high number of leukocytes has been associated with couple subfertility and with decreased semen quality, although conflicting results stem from different methods used to assess the number of leukocytes in semen (Wolff, 1995). The peroxidase stain test proposed by World Health Organization (2010) detects granulocytes only and it may underestimate their number because some of these cells are in an activated state in human semen and therefore might have released peroxidase-positive granules (Aitken & West, 1990). Immunocytological staining test employing monoclonal antibodies against surface antigens of white blood cell (WBC) subpopulations is considered the gold standard of semen WBC test at least for clinical research (Ricci *et al.*, 2000).

Leukocytes seem to mediate sperm damage, including DNA fragmentation, through secretion of reactive oxygen species (ROS) (Ochsendorf, 1999; Whittington & Ford, 1999; Henkel *et al.*, 2005; Agarwal *et al.*, 2014). Sperm DNA fragmentation may also be the result of a persistence of DNA strand breaks (Sakkas *et al.*, 2002) and of abnormal chromatin condensation (De Iuliis *et al.*, 2009) during spermiogenesis and it may be oxidatively induced (Aitken *et al.*, 2014). This is substantially supported by the observation that sperm DNA fragmentation

measured by the terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling (TUNEL) or sperm chromatin dispersion assays is highly associated with the expression of sperm 8-hydroxy-2′-deoxyguanosine (8-OHdG) (De Iuliis *et al.*, 2009; Santiso *et al.*, 2010). The latter is a bio product specific for an oxidant-induced DNA damage (Kasai, 1997) and can be measured on a single-cell basis in ejaculated spermatozoa (De Iuliis *et al.*, 2009; Cambi *et al.*, 2013), therefore its determination may explore the relationship between semen infiltration of leukocytes and sperm DNA damage.

A simultaneous determination of different subpopulations of infiltrated leukocytes, in situ sperm DNA fragmentation and sperm 8-OHdG expression, was here carried out by immunocytochemistry coupled with cytofluorimetric analysis in ejaculates of men attending a fertility clinic, asymptomatic for a genital tract infection. The primary outcome objective was to define the association between the presence of specific leukocyte subset and the level of oxidative-related DNA damage of ejaculated spermatozoa. The secondary outcome objective was to better define the association between oxidative-related DNA damage and quality of ejaculated spermatozoa.

MATERIALS AND METHODS

Samples and semen analysis

Semen samples were obtained through masturbation following 3–5 days of sexual abstinence from consecutive 111 men attending the Andrology Clinic at the University of L'Aquila, as a result of couple subfertility for more than 1 year. None of the participants had taken antioxidant supplements or medications affecting the immune system in the last 3 months. Patients reporting fever or a history of genital tract infections in the past 3 months were preliminarily excluded from the study. The study was approved by the local Institutional Review Board, and all subjects signed an informed consent statement.

All samples were produced at the clinic into sterile containers and, after complete liquefaction, underwent semen analysis, including a count of round nucleated cells, using a hemocytometer counting chamber, according to WHO recommendations (World Health Organization, 2010).

Determination of semen leukocytes by immunocytochemistry

According to WHO recommendations (World Health Organization, 2010), round cells were recognized as leukocytes by detecting the pan-leukocyte CD45 antigen with a monoclonal anti-CD45 antibody (Santa Cruz Biotechnology, DBA, Milan, Italy) and an avidin-biotin immunoperoxidase staining (Santa Cruz Biotechnology, DBA). Semen concentration of leukocytes (10^6/mL) was obtained by multiplying the percentage of CD45-stained round cells for the concentration of round cells in the ejaculate. After semen analysis, each sample was washed twice (400 ***g*** for 10 min) in PBS with 0.1% human serum albumin (HSA) and divided into three aliquots for subsequent flow cytometric assessment of leukocyte subpopulations, sperm DNA fragmentation, and sperm 8-OHdG expressions, respectively, as described below.

Identification of leukocyte subpopulations

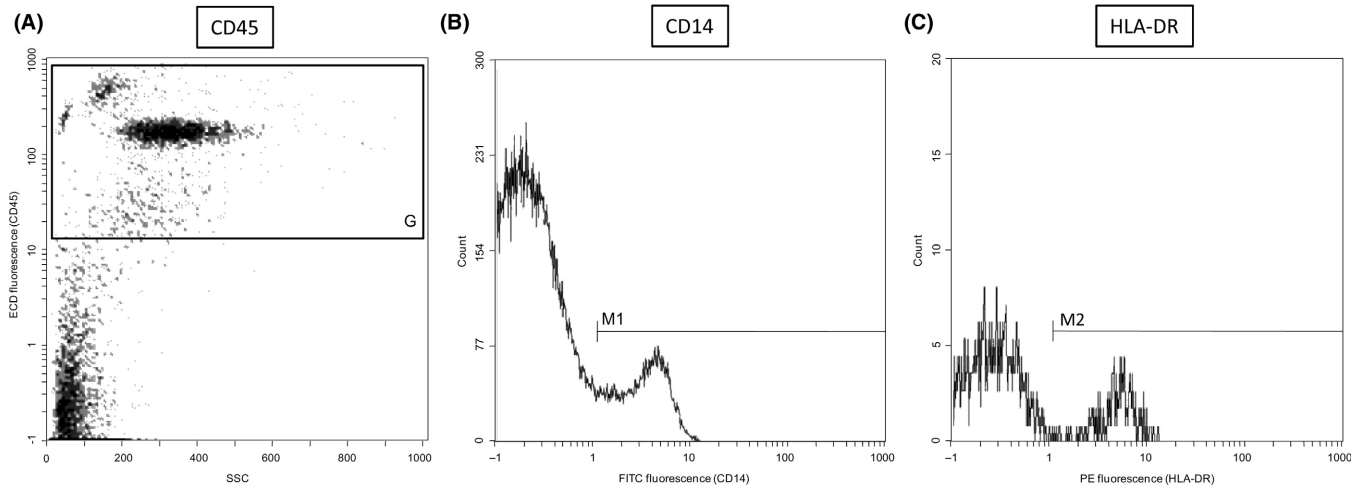
Leukocyte subpopulations were determined by flow cytometry, which allowed calculating percentages even at a very low cell concentration, by acquiring 10,000 events or more in each sample. As at the forward scatter (FSC) vs. side scatter (SSC) flow cytometric dot plot, round cells are not fully distinguishable from spermatozoa (Ricci *et al.*, 2000), it was impossible to accurately determine the percentage of round cells expressing CD45 by flow cytometry. Therefore, semen concentrations of macrophages and of activated macrophages were calculated by the flow cytometric percentages of CD45-positive cells expressing also CD14 (macrophages) and HLA-DR (activated macrophages), multiplied for the semen concentration of CD45-positive cells obtained with immunocytochemistry as described.

Cell suspensions (70 μL) were incubated for 30 min at room temperature (RT) with 10 μL of the following monoclonal antibodies (mAb): (i) ECD-conjugated anti-CD45 mAb diluted in a buffer-containing 0.2% bovine serum albumin (BSA) (Beckman Coulter, Rome, Italy), (ii) fluorescein isothiocyanate (FITC)-conjugated anti-CD14 mAb diluted in a buffer containing 5% BSA (Gen-probe, Diaclone, Pantec S.r.l., Turin, Italy), and (iii) phycoerithryn (PE)-conjugated anti-HLA-DR diluted in a buffer containing 1% BSA (Gen-probe, Diaclone, Pantec S.r.l.). After two washes in PBS (400 ***g*** for 7 min), cell suspensions were analyzed within a few minutes at a flow cytometer (Beckman Coulter's Epics XL-MCL; Beckman Coulter, Inc., Fullerton, CA, USA) equipped with a 15 mW argon-ion laser for excitation. For each sample, 10,000 events were recorded at a flow rate of 200–300 cells/sec. The FITC, PE and ECD fluorescence were measured in the FL-1, FL-2 and FL-3 channel, respectively. At the ECD fluorescence (*y*-axis) vs. SSC (*x*-axis) plot (Fig. 1A), events in the lower-left corner, representing debris and CD45-negative cells (spermatozoa and germ-line round cells), were excluded from the analysis, whereas events in the upper quadrant represented CD45-positive cells (leukocytes). The whole gated CD45-positive population was further analyzed on the FL-1 channel (Fig. 1B) to obtain the percentage of CD45-positive cells exhibiting FITC labeling of CD14 (monocytes/macrophages). Finally, CD14-positive cells were analyzed on the FL-2 channel (Fig. 1C) to calculate the proportion of monocytes/macrophages also exhibiting the red PE fluorescence of HLA-DR antigens (activated macrophages). The percentages of labeled cells were immediately evaluated, using the flow cytometer System II Version 3.0 software (Beckman Coulter, Inc.).

Assessment of sperm DNA fragmentation

Sperm DNA fragmentation was assessed by TUNEL/Propidium iodide (PI) assay, using the In Situ Cell Death Detection Kit, fluorescein, Roche Molecular Biochemicals, Milan, Italy, as previously described (Muratori *et al.*, 2008). Samples were fixed in paraformaldehyde (4% in PBS pH 7.4 for 30 min at RT) and permeabilized with 0.1% Triton X-100 in 100 μL of 0.1% sodium citrate for 4 min on ice. After washing twice, the labeling reaction was performed by incubating samples in 50 μL of labeling solution containing the TdT enzyme for 1 h at 37 °C in the dark. Finally, samples were washed twice, resuspended in PBS, stained with PI, (30 μg/mL in PBS) and incubated in the dark for 15 min at RT. For each test sample, a negative control was prepared by omitting the TdT enzyme. Positive controls were prepared by treating samples with DNAse I (Pharmacia Biotech Italia, Milan, Italy), 2 IU for 20 min at 37 °C. After labeling, samples were washed and resuspended in PBS for flow cytometric analysis (Fig. 2). The red fluorescence of PI was detected

Figure 1 Flow cytometric identification of leukocyte subpopulations. Seminal leukocytes are displayed in a dot plot on the basis of CD45-ECD fluorescence vs. side scatter (SSC) (A). The whole gated CD45-positive population (gate G in panel A) was analyzed on the FL-1 fluorescein isothiocyanate (FITC) channel (B) to obtain the percentage of CD14-positive cells (monocytes/macrophages). The CD14-positive cells were further analyzed on the FL-2 phycoerithryn (PE) channel (C) to obtain the percentage of HLA-DR-positive cells (activated macrophages). In preliminary experiments samples were stained with uncorrelated isotype monoclonal antibodies, as negative controls, to set regions excluding all FITC-negative (region M1, in panel B) and PE-negative (region M2, in panel C) events.



by a FL-2 (563–607 nm wavelength band) detector, while TUNEL green fluorescence was revealed by the FL-1 (515–555 nm wavelength band) detector. A total of 10,000 events were acquired for each sample. Based on the light scatter characteristics of swim-up selected spermatozoa, debris and round cells were gated out by establishing a region (R1) around the 'flame shaped' population of interest in the FSC/SSC dot plot (Fig. 2A). As previously described (Marchiani *et al.*, 2007), the whole population within the region R1 was analyzed on the FL-2 channel of PI fluorescence (Fig. 2B) and only nucleated (PI positive) elements (spermatozoa) were further analyzed on the FL-1 to calculate the percentage of spermatozoa exhibiting TUNEL green fluorescence (Fig. 2D).

Assessment of sperm 8-OHdG

The generation of the oxidized base adduct, 8-OHdG was detected as a biomarker for oxidative genomic damage (Kasai, 1997). For the assay, samples were washed twice in PBS, the pellet was then resuspended in dithiothreitol (2 mM) and incubated for 45 min at 37 °C. After centrifugation at 600 ***g*** for 5 min, samples were fixed in paraformaldehyde (4% in PBS for 15 min at 4 °C). Fixed cells were washed and split into two aliquots subsequently incubated (1 h at 37 °C) in 100 μL 0.1% sodium citrate/0.1% Triton X-100 containing a mouse anti-8OHdG monoclonal antibody (Santa Cruz Biotechnology), diluted in a PBS solution with 2% HSA (test sample, 2 μg/mL) or a mouse IgG2a (isotype control, 2 μg/mL). After washing twice, spermatozoa were incubated in the dark (1 h at RT) with a FITC-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology) diluted 1 : 100 in PBS. Then, samples were washed twice, resuspended in 500 mL PBS and stained with PI (50 mg/mL in PBS) for 15 min at RT in the dark before flow cytometric analysis (Fig. 3). Positive controls were prepared by incubating samples for 1 h at RT with H_2O_2 (2 mM) and FeCl2·4H_2O (1 mM) in a final volume of 200 μL of PBS, as previously described (Barbonetti *et al.*, 2014), before fixation procedure.

Statistical analysis

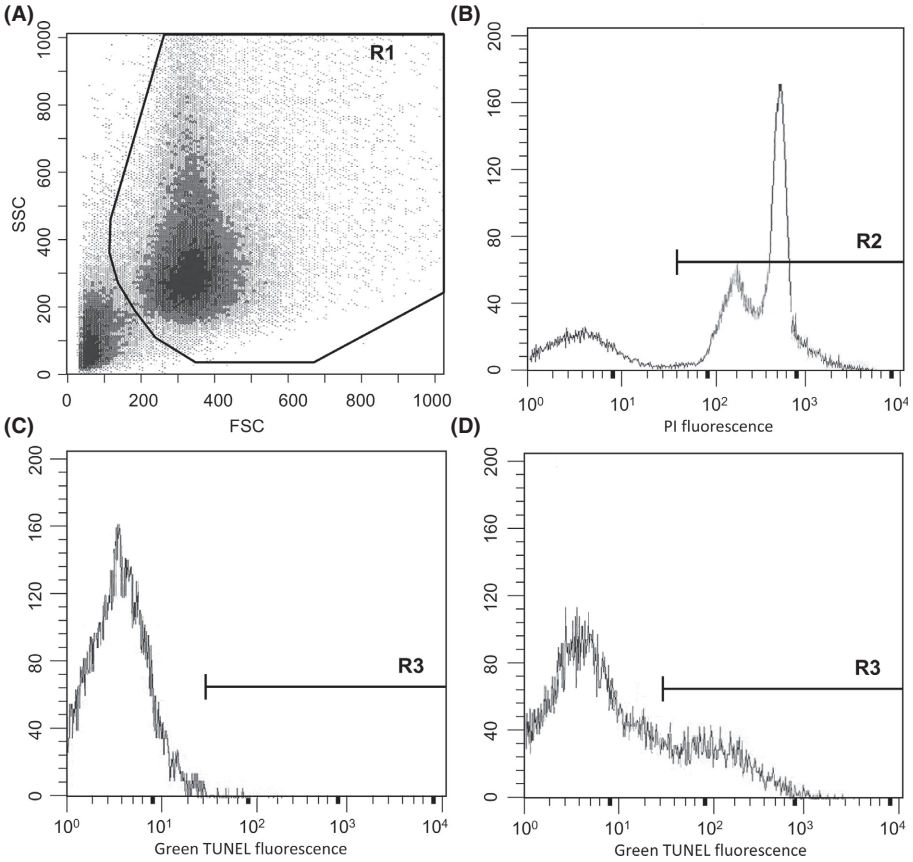
Statistical analysis was performed using the SAS statistical software (version 9.2, 2008; SAS Institute, Inc., Cary, NC, USA). Non-parametric statistics were used to protect against non-normal distribution of data. Values were presented as median and interquartile range. Spearman Rank Order coefficient was used to assess the relationship among the variables. Differences among groups were assessed by Kruskall Wallis one-way analysis of variance (ANOVA) by ranks, followed by Wilcoxon rank-sum test with a downward adjustment of the α level to compensate for multiple comparisons, to maintain the overall probability at a level of 0.05. Multivariate regression analysis of logarithmic-transformed values was applied to analyze the independent contribution of semen and of clinical parameters to predict the variation in 8-OHdG sperm expression.

RESULTS

Ejaculated leukocyte subset and semen parameters

Table 1 shows semen parameters of the 111 ejaculates from male partners of subfertile couples asymptomatic for a genital tract infection. Samples were compared accordingly to concentration of CD45-positive cells (Table 2). No relevant differences in clinical parameters and in semen variables including the percentage of TUNEL-positive spermatozoa and of 8-OHdG-positive spermatozoa were observed in ejaculates in the high quartiles compared to the low quartiles. Although a significant difference at ANOVA was observed in sperm morphology among groups, ejaculates in the high quartiles (III and IV), did not show a significant different sperm morphology compared to the quartile I. On the contrary, the concentration of CD14-positive and of HLA-DR-positive cells progressively and significantly increased throughout the lowest quartile to the highest quartiles of CD45-positive cell concentration ($p < 0.0001$). The concentration of CD45-positive cells was strictly correlated with the concentration of CD14-positive and of HLA-DR-positive cells ($r = 0.94$,

Figure 2 Flow cytometric assessment of sperm DNA fragmentation. In the forward scatter/side scatter (FSC/SSC) dot plot (A) obtained from fixed and permeabilized semen samples, a region (R1) was established to exclude debris. The whole population within the region R1 was analyzed on the FL-2 propidium iodide (PI) channel (B), to set a region (R2) excluding non-nucleated (PI negative) elements. Only PI-positive elements (spermatozoa) were further analyzed on the FL-1 channel of TUNEL green fluorescence: a marker (R3) was set on the FL-1 fluorescence histogram obtained by analyzing a negative control (C) in which the TdT enzyme was omitted; when this marker was translated in the corresponding test sample (D), all the events beyond the marker of the FL-1 channel were considered TUNEL-positive (spermatozoa with fragmented DNA).



$p < 0.001$ and $r = 0.74$, $p < 0.001$, respectively). No correlation was found between the concentration of different leukocyte subset and conventional semen parameters, the percentage of TUNEL-positive or the percentage of spermatozoa with oxidized DNA (8-OHdG-positive cells) (data not shown). The lack of an association between the infiltration of leukocytes and the percentage of 8-OHdG-positive spermatozoa, a bio product of oxidant-induced sperm DNA damage (De Iuliis *et al.*, 2009) prompted to explore the relationship between the percentage of 8-OHdG-positive spermatozoa, sperm DNA fragmentation and sperm quality.

Sperm 8-OHdG, sperm DNA fragmentation and semen parameters

Ejaculates were divided in 2 groups according to the median value of the percentage of 8-OHdG-positive sperm (Table 3). Age, the smoking frequency or abstinence were not different in the two groups. A higher rate of 8-OHdG-positive was associated to a higher rate of TUNEL-positive spermatozoa ($p < 0.0001$) and to a lower sperm concentration ($p = 0.002$), but no difference was detected for forward sperm motility or the percentage of spermatozoa with oval heads. As expected, leukocytes concentration was not different in the two groups. The percentage of 8-OHdG-positive spermatozoa was positively correlated with the percentage of TUNEL-positive spermatozoa ($r = 0.48$; $p < 0.0001$), and negatively correlated with sperm concentration ($r = -0.44$; $p < 0.0001$), or with total motile sperm count ($r = -0.36$; $p < 0.0001$) (Table 4). No correlation was found between the percentage of 8-OHdG-positive spermatozoa and age, sperm forward motility, sperm morphology, and the concentration of different leukocyte subpopulations. The percentage of spermatozoa with fragmented chromatin was negatively correlated with sperm concentration ($r = -52$; $p < 0.0001$), the percentage of spermatozoa with forward motility ($r = -31$; $p = 0.0008$), and with oval heads ($r = -30$; $p = 0.0011$) (Table 4). The multivariate regression analysis, after adjusting for age, abstinence time and smoking (Table 5), showed that the sperm concentration and the percentage of TUNEL-positive spermatozoa independently contributed ($\beta = -0.25$, $p = 0.008$, and $\beta = 0.23$, $p = 0.050$, respectively) to 19% of variation in the percentage of 8-OHdG-positive spermatozoa.

DISCUSSION

This study showed that the concentration of seminal leukocytes was unrelated to an oxidative sperm DNA damage as well as to sperm quality in ejaculates of men attending a fertility clinic and asymptomatic for a genital tract infection.

Figure 3 Flow cytometric assessment of sperm 8-hydroxy-2′-deoxyguanosine. In the forward scatter/side scatter (FSC/SSC) dot plot (A) obtained from fixed and permeabilized semen samples, a region (R1) was established to exclude debris. The whole population within the region R1 was analyzed on the FL-2 propidium iodide (PI) channel (B), to set a region (R2) excluding non-nucleated (PI negative) elements. Only PI-positive elements (spermatozoa) were further analyzed on the FL-1 fluorescein isothiocyanate (FITC) channel: a marker (R3) was set on the FL-1 fluorescence histogram of negative controls (C) obtained by incubating samples with a mouse IgG2a (isotype control) before the addition of a FITC-conjugated goat anti-mouse IgG antibody; when this marker was translated in the corresponding test sample (D), all the events beyond the marker of the FL-1 channel were considered 8-OHdG-positive (spermatozoa with oxidized DNA).

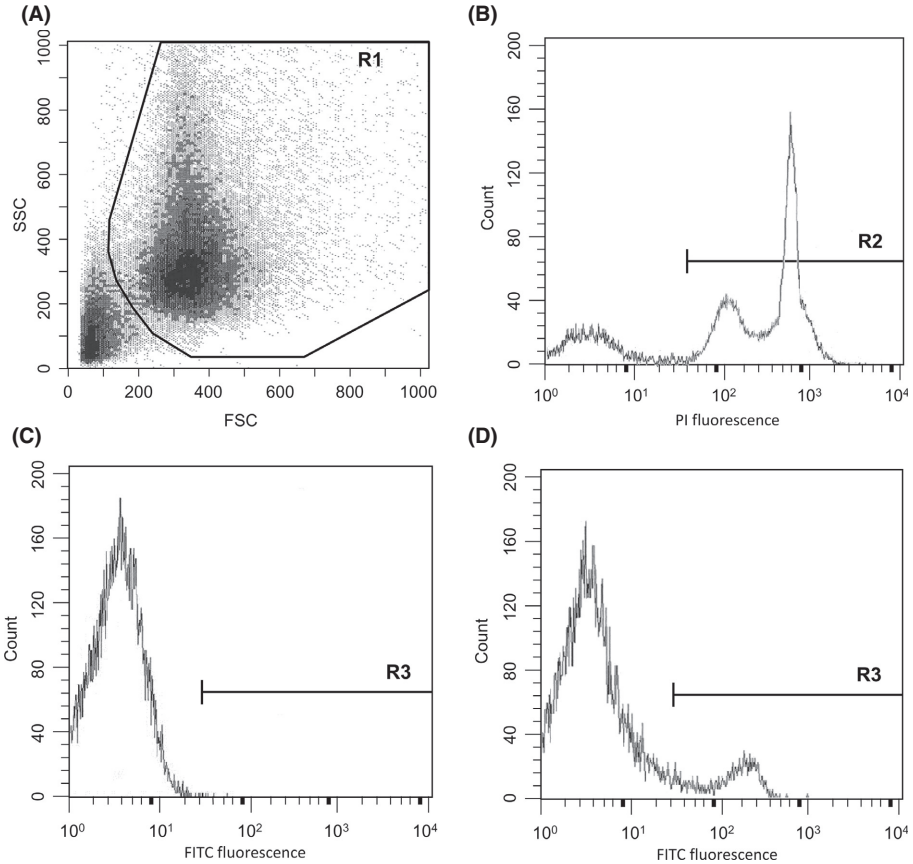


Table 1 Clinical and semen parameters in ejaculates of 111 male partners of subfertile couples with no symptoms of genital tract infection

Variables	Median (inter-quartile range)
Age, years	35 (32–40)
Smoke, number (%)	58 (52.5)
Abstinence, days	4 (2–5)
Volume, mL	3.3 (2.3–4.2)
Semen pH	7.6 (7.6–7.8)
Concentration, 1 × 10^6/mL	36 (12.5–75)
Forward motility, %	49 (35–59)
Total motile spermatozoa, million	50.8 (15.0–144.7)
spermatozoa Oval Heads, %	8 (3–15)
TUNEL positive spermatozoa, %	16 (8–31)
8-0HdG positive spermatozoa, %	6.4 (2.13–13.00)
CD 45 positive cells, 1 × 10^6/mL	0.74 (0.28–1.60)
CD14 positive cells, 1 × 10^6/mL	0.08 (0.02–0.50)
HLA-DR positive cells, 1 × 10^6/mL	0.01 (0.00–0.10)

8-0HdG, 8-Hydroxy-2′-deoxyguanosine.

The relationship between seminal leukocyte and sperm quality is still debated. According to the expression of monoclonal antibodies specific for all leukocytes such as CD45 (Charbonneau *et al.*, 1988), no association (Eggert-Kruse *et al.*, 1992; Tomlinson *et al.*, 1992a,b; Aitken *et al.*, 1994; Ricci *et al.*, 2000), or a negative association (Wolff & Anderson, 1988) was found between the number of leukocytes and sperm parameters. The present study agrees with previous findings suggesting that the concentration of CD45-positive cells is independent from conventional semen parameters in men asymptomatic for a genital tract infection. Here we also revealed no association between semen parameters and the concentration of seminal monocytes/macrophages, based on expression of CD14, a common marker for monocytes/macrophages lineage (Wright *et al.*, 1990), and of HLA-DR, a marker of the activated state of macrophages (Viksman *et al.*, 1997). This disagrees with previous findings of a negative correlation between the number of ejaculated HLA-DR-positive cells and semen parameters (Pelliccione *et al.*, 2011). The relative small size of study groups probably accounted for different findings, suggesting that a larger number of ejaculates should be evaluated to better explore the correlation between the number of activated macrophages and quality of semen analysis.

The potential effect of seminal leukocytes on male fertility remains elusive. The concentration of different subset of seminal leukocytes was not associated to the rate of conception in vivo (Tomlinson *et al.*, 1993), or to the IVF (Tomlinson *et al.*, 1992a) or ICSI outcome (Ricci *et al.*, 2015). The concentration of leukocytes expressing CD45 was, however, negatively correlated with

Table 2 Clinical and semen parameters in ejaculates compared accordingly to semen concentration of CD45-positive cells. Quartile I: ≤0.3 × 10^6/mL; Quartile II: >0.3 ≤ 0.7 × 10^6/mL; Quartile III: >0.7 ≤ 1.5 × 10^6/mL; Quartile IV: >1.5 × 10^6/mL

Variables	Quartile I *n* = 30	Quartile II *n* = 27	Quartile III *n* = 27	Quartile IV *n* = 27	Kruskal–Wallis *p*-value
Age, years	35 (30.5–38.5)	37.5 (34.5–42)	34 (30–39)	35 (26–37)	0.06
Abstinence, days	4 (3–4.5)	4 (3–5)	5 (3–5)	5 (3–5)	0.14
Volume, mL	3.25 (2.4–5.0)	3 (2.1–4.5)	3.5 (2.5–5.7)	3.5 (2–4.5)	0.78
Semen pH	7.6 (7.6–7.7)	7.7 (7.6–7.8)	7.6 (7.6–7.6)	7.8 (7.6–7.8)	0.08
Sperm concentration, 1 × 10^6/mL	29 (19.5–55.0)	14 (4.5–55)	38 (10–96)	62 (11–110)	0.18
Sperm forward motility, %	48.5 (35.5–58.5)	35 (24.5–48.5)	48 (38–61)	51 (34–58)	0.08
Total motile sperm, 1 × 10^6	45.7 (20.1–106.7)	12 (3.6–49.0)	76.1 (10.0–190.3)	76.5 (15.0–150.5)	0.09
Sperm oval heads, %	11 (3.5–22)[a]	2 (1–5.5)[b]	7 (5–13)	8 (4–14)	0.01
TUNEL positive spermatozoa, %	13.5 (7.9–30.4)	22.4 (14.5–39.0)	21 (16.0–36.4)	16.6 (5.7–31.0)	0.23
8-OHdG positive spermatozoa, %	6.8 (1.4–11.5)	2.7 (1.7–12.2)	6.4 (1.9–13.8)	5.0 (2.5–16.8)	0.73
CD 14 positive cells, 1 × 10^6/mL	0.01 (0.00–0.02)[c]	0.05 (0.04–0.08)[d]	0.17 (0.08–0.40)[e]	0.90 (0.52–25.9)	<0.0001
HLA-DR positive cells, 1 × 10^6/mL	0.00 (0.00–0.01)[d]	0.01 (0.00–0.02)[d]	0.03 (0.01–0.11)[e]	0.16 (0.07–0.41)	<0.0001

8-0HdG, 8-Hydroxy-2′-deoxyguanosine. Values are expressed as median (interquartile range); [a] through [e], $p < 0.05$ at adjusted post-hoc analysis: [a]compared to quartile II; [b]compared to quartile IV; [c]compared to all; [d]compared to quartiles III, IV; [e]compared to quartile IV.

Table 3 Clinical and semen parameters in ejaculates with a low or a high percentage of 8-0HdG positive spermatozoa

Variables	8-OHdG positive spermatozoa low *n* = 57	8-OHdG positive spermatozoa high *n* = 54	*p*[a]
Age, years	36 (32–39)	35 (31–40)	0.59
Smoke, *n* (%)	26 (45.6)	32 (60)	0.52
Abstinence, days	4 (3–5)	4 (3–5)	0.62
Semen volume, mL	3 (2.2–4.1)	3.5 (2.5–4.2)	0.42
Semen pH	7.6 (7.6–7.8)	7.6 (7.6–7.8)	0.97
Sperm concentration -1 × 10^6/mL	51 (28–104)	29 (8.4–54)	0.002
Sperm forward motility,%	48 (33–57)	50.5 (38–63)	0.32
Total motile sperm a, 1 × 10^6	67.7 (24–152)	38.7 (8.5–107.3)	0.04
Spermatozoa oval heads, %	8 (3–15)	8.5 (3–15)	0.54
TUNEL positive spermatozoa, %	12.2 (7–19)	23.3 (9.7–38.1)	0.0012
8-OHdG positive spermatozoa, %	2.1 (1.2–3.8)	13.4 (10.6–26.0)	<0.0001
CD 45 positive cells, 1 × 10^6/mL	0.73 (0.33–1.60)	0.84 (0.28–1.53)	0.98
CD14 positive cells, 1 × 10^6/mL	0.08 (0.02–0.41)	0.11 (0.03–0.65)	0.63
HLA-DR positive cells, 1 × 10^6/mL	0.01 (0.01–0.10)	0.02 (0.00–0.07)	0.50

Ejaculates with ≤6.4% were compare with those with >6.4% of 8-0HdG positive spermatozoa – the median value obtained in the whole study group. Values are expressed as median (interquartile range). 8-0HdG, 8-Hydroxy-2′-deoxyguanosine. [a]Wilcoxon Rank Sum Test.

Table 4 Correlation analysis between the percentage of 8-OHdG positive spermatozoa or the percentage of TUNEL positive spermatozoa and semen parameters of 111 men partners of subfertile couples with no symptoms of genital tract infection

Variables	8-OHdG positive spermatozoa %		Tunel positive spermatozoa %	
	r[a]	*p*[a]	*r*[a]	*p*[a]
Sperm concentration, 1 × 10^6/mL	−0.44	<0.0001	−0.52	<0.0001
spermatozoa forward motility, %	−0.03	0.73	−0.31	0.0008
Total motile spermatozoa, 1 × 10^6	−0.36	<0.0001	−0.53	<0.0001
spermatozoa oval heads, %	0.01	0.87	−0.30	0.0011
TUNEL positive spermatozoa, %	0.48	<0.0001	–	–
CD 45 positive cells, 1 × 10^6/mL	0.06	0.52	0.03	0.77
CD14 positive cells, 1 × 10^6/mL	0.14	0.19	0.14	0.19
HLA-DR positive cells, 1 × 10^6/mL	0.01	0.86	0.003	0.97

8-0HdG, 8-Hydroxy-2′-deoxyguanosine. [a]Spearman's rho non-parametric correlation.

Table 5 Multivariate analysis to predict the change in percentage of 8-Hydroxy-2′-deoxyguanosine (8-OHdG)-positive spermatozoa

Variables	β value	*p*
Sperm concentration, 1 × 10^6/mL	−0.25	0.008
TUNEL positive spermatozoa, %	0.23	0.050
Age, years	−0.37	0.445
Smoke, *n*	0.03	0.375
Abstinence, days	-0.23	0.469

the fertilization rate of metaphase II oocytes (Moilanen *et al.*, 1998). Although the association between the presence of leukocytes and semen quality or sperm fertility potential is at best questionable, the proposed detrimental effect of leukocytes on spermatozoa is suggested to rely on the ability of leukocytes to release ROS and inflammatory mediators (Aitken & West, 1990; Plante *et al.*, 1994; Whittington & Ford, 1999; Sharma *et al.*, 2001; Henkel *et al.*, 2005; Agarwal *et al.*, 2014; Hagan *et al.*, 2015). Accordingly, ROS are considered the main cause of sperm DNA damage as a consequence of leukocyte infiltration (Aitken *et al.*, 2014). Here we showed that the expression of 8-OHdG, a bio product specific for oxidant-induced DNA damage (Kasai, 1997) detected in spermatozoa at single-cell level using an immunofluorescence method coupled to flow cytometry (Cambi *et al.*, 2013), is strongly associated with sperm DNA damage detected by TUNEL assay. This confirmed previous data obtained with fluorescence microscopy or flow cytometry to measure a binding protein to 8-OHdG (OxyDNA assay) (De Iuliis *et al.*, 2009; Santiso *et al.*, 2010; Zribi *et al.*, 2011). All results are consistent with the contention that DNA damage in spermatozoa is mainly oxidatively induced (Aitken *et al.*, 2014).

In our hands, ejaculates of men attending a fertility clinic with no symptoms of genital tract infection showed that 8-OHdG immunofluorescence expression and DNA fragmentation in spermatozoa were associated to poor sperm quality but not to the presence of different leukocyte subset. This suggests that the

oxidative-dependent DNA damage in ejaculated spermatozoa in the study group was probably related to the altered sperm structure and function and is consistent with the hypothesis that sperm DNA damage depends mainly upon errors occurring during spermatogenesis. According to Aitken *et al.* (2014), a poor chromatin protamination during spermiogenesis creates a state of vulnerability in spermatozoa, which indeed show a poorly condensed chromatin in the testes with a defective spermatogenesis (Francavilla *et al.*, 2001). Subsequent exposure to exogenous ROS, or to endogenous ROS triggered by entry into the intrinsic apoptotic cascade, ultimately results in an enhanced oxidative damage and DNA fragmentation of vulnerable spermatozoa. Intriguingly, although the expression of 8-OHdG was significantly associated to sperm DNA fragmentation and to a lower sperm concentration (Table 4), both parameters explained 19% only of the variation in the percentage of 8-OHdG-positive spermatozoa at the multivariate analysis. Therefore, the presence of an oxidative-related DNA damage of ejaculated spermatozoa in a population of men attending a fertility clinic and asymptomatic for a genital tract infection was faintly associated to an altered semen quality as already suggested (Kodama *et al.*, 1997; Ni *et al.*, 1997; Shen *et al.*, 1999; Kao *et al.*, 2008; Zribi *et al.*, 2011; Cambi *et al.*, 2013; Guz *et al.*, 2013) and it should be present in alive and motile spermatozoa with no DNA fragmentation. An oxidative stress may result in the presence of 8-OHdG adducts in the DNA of ejaculated potentially fertile spermatozoa.

Limitations

The conclusion inferred from this study should be taken with caution as this was obtained in a relatively small number of ejaculates. Relevant methodological differences in the evaluation of semen leukocyte infiltration and sperm DNA fragmentation may account for the conflicting opinion between those who denied (Henkel *et al.*, 2005; Moskovtsev *et al.*, 2007; present study) or those who advocated (Agarwal *et al.*, 2014; Lobascio *et al.*, 2015) a positive association between sperm DNA fragmentation and the concentration of semen leukocyte. Furthermore, findings cannot be extended to ejaculates with a leukocyte infiltration associated to a symptomatic genital tract infection or to increased levels of inflammatory markers in the seminal plasma (Hagan *et al.*, 2015). These limitations are balanced by the effort to apply an objective method that coupled cytofluorimetry and immunocytochemistry. Larger studies should confirm and expand the present findings.

CONCLUSIONS

The availability of a reliable and objective cytofluorimetric method coupled with immunocytochemistry showed that the oxidative-dependent DNA damage in ejaculated spermatozoa was associated to a poor semen quality but not to the presence of different leukocyte subset in ejaculates of men asymptomatic for a genital tract infection. The application of this methodology in different populations of men attending an infertility clinic should unravel the effect of oxidative-dependent DNA damage of ejaculated spermatozoa in human reproduction.

DECLARATION OF INTEREST

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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