Article

DNA fragmentation of spermatozoa and assisted reproduction technology

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Dr Ralf Henkel

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Abstract

Despite the ever-increasing knowledge of the fertilization process, there is still a need for better understanding of the causes of sperm DNA fragmentation and its impact on fertilization and pregnancy. For this reason, human sperm DNA fragmentation was investigated by means of the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) assay and the production of reactive oxygen species (ROS) in the ejaculate and in the spermatozoa themselves. These data were correlated with fertilization and pregnancy data from IVF and intracytoplasmic sperm injection (ICSI) patients. Sperm DNA fragmentation did not correlate with fertilization rate, but there was a significantly reduced pregnancy rate in IVF patients inseminated with TUNEL-positive spermatozoa. ICSI patients exhibited the same tendency. This implies that spermatozoa with damaged DNA are able to fertilize an oocyte, but at the time the paternal genome is switched on, further development stops. The determination of ROS in the ejaculate and the percentage of ROS-producing spermatozoa revealed markedly stronger correlations between sperm functions (i.e. motility) and the percentage of ROS-producing spermatozoa. The influence of seminal leukocytes, known to produce large amounts of oxidants, on sperm DNA fragmentation should not be neglected.

Keywords: DNA fragmentation, human spermatozoa, IVF, leukocytes, pregnancy failure, ROS production

Introduction

There is still little known regarding the causes of human sperm DNA fragmentation and its impact on fertilization and pregnancy after IVF or intracytoplasmic sperm injection (ICSI). Recently, scientists have been focusing on the impact and the involvement of DNA fragmentation on male fertility. It has been shown repeatedly that DNA damage in ejaculated spermatozoa as determined by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) or Comet assays is significantly and negatively correlated with fertilization and pregnancy in IVF (Sun et al., 1997; Morris et al., 2002; Henkel et al., unpublished data), intracytoplasmic sperm injection (ICSI) (Lopes et al., 1998a) and intrauterine insemination (Duran et al., 2002) patients. Reports of an early and massive wave of programmed spermatogenic cell death, necessary for control and development of normal spermatogenesis (Rodriguez et al., 1997), and the observation of annexin V binding to the spermatozoa (Oosterhuis et al., 2000) lead to the assumption that sperm DNA fragmentation is a result of apoptotic events (Sakkas et al., 1999; Francavilla et al., 2002). In addition, active caspase-3, an enzyme that is associated with apoptosis, has been detected in human spermatozoa (Weng et al., 2002). However, DNA fragmentation can also be induced by reactive oxygen species (ROS; Lopes et al., 1998b; Irvine et al., 2000), either produced by leukocytes (Aitken and West, 1990) or by the sperm cells themselves (Holland et al., 1982; Henkel et al., 1997). Moreover, ROS have been shown to be strongly associated with male infertility (Iwasaki and Gagnon, 1992).

Although there is increasing knowledge about the mechanisms and factors involved in the fertilization process, one out of six couples of reproductive age still suffer from infertility (Hull et al., 1985). About 50% of infertility cases are male factor, while nearly 50% of infertile men are classified as idiopathic.
(Sherins, 1995). A large number of patients exhibit leukocytospermia, but this is not a reliable indicator of asymptomatic urogenital tract infections (Barratt et al., 1990). Thus, the impact of these cells and their products, ROS, on male fertility potential is still poorly understood.

This study investigated human sperm DNA fragmentation by means of the TUNEL assay and ROS production in the ejaculate as well as by the spermatozoa themselves and correlated these data with fertilization and pregnancy in both an IVF and an ICSI programme.

### Materials and methods

To investigate the possible causes and relationships of sperm DNA fragmentation to fertility, the TUNEL assay was performed and ROS production determined in the spermatozoa and the ejaculate respectively, in 65 randomly selected patients undergoing standard IVF \((n = 45)\) and ICSI treatment \((n = 20)\). Sperm concentration and motility were analysed according to WHO guidelines (1999), while normal sperm morphology was performed after Shorr stain according to the Düsseldorf classification (Hofmann, 1987). The data were also correlated with sperm concentration, motility, sperm morphology, fertilization, embryo fragmentation and pregnancy. In addition, the number of peroxidase-positive cells in the ejaculate was determined by means of the standard techniques suggested by WHO (1999). These new data \((n = 65)\) were combined with data from a previous study (Henkel et al., unpublished data) and results from the TUNEL assay, fertilization and pregnancy rates were correlated with fertilization and pregnancy; IVF \((n = 163)\) and ICSI \((n = 34)\). A total of 208 IVF and 54 ICSI patients were investigated. To ensure comparability of the data, strict adherence to the same IVF and ICSI insemination procedures and TUNEL assay protocol was followed.

### IVF procedure

Ovarian stimulation was performed according to a standardized protocol with human menopausal gonadotrophin \((\text{HMG}; \text{Menogon, Ferring, Kiel, Germany})\) and Supercit\(^{\circledast}\) \((\text{Hoechst, Frankfurt, Germany})\). Ovulation was induced by means of 10,000 IU human chorionic gonadotrophin \((\text{HCG}; \text{Predalon 5000; Organon, Oss, The Netherlands})\) and follicles were aspirated by means of standard techniques 35 h after HCG administration. Human tubular fluid \((\text{HTF}; \text{medium (Quinn et al., 1985)})\) supplemented with 5 IU/ml heparin \((\text{Roche, Basel, Switzerland})\), 20 mmol/l HEPES \((\text{Sigma, St Louis, USA})\) and 0.2% serum albumin \((\text{Behring, Marburg, Germany})\) was used to flush the follicles. Motile spermatozoa were isolated by means of a standard swim-up procedure from washed spermatozoa in HTF medium containing 0.3% serum albumin. Co-culture of the oocytes with spermatozoa \((\text{approximately 100,000 spermatozoa per oocyte})\) was performed in HTF medium supplemented with 0.3% serum albumin at 37°C under an atmosphere of 5% \(\text{CO}_2\). After 18 h, oocytes were checked for fertilization.

### ICSI procedure

In brief, for ICSI, ovarian stimulation and follicle aspiration were performed according to the same protocol as described for IVF. Oocytes were then denuded in Ham’s F10 medium supplemented with 0.3% serum albumin and 20 mmol/l HEPES \((\text{Sigma})\) and 80 IU/ml hyaluronidase type IV \((\text{Sigma})\). Motile spermatozoa were isolated by means of a migration-sedimentation technique, a two-phase density gradient centrifugation with SilSelect \((\text{Janssen, Hamburg, Germany})\) or with a single wash. For sperm separation, Ham’s F10 medium \((\text{Sigma})\) supplemented with 0.3% serum albumin was used. For immobilization of spermatozoa, PVP medium from MediCult \((\text{Jyllinge, Denmark})\) was used. Standard ICSI procedure was performed with a Narishige micromanipulator \((\text{Narishige, Tokyo, Japan})\) mounted to a Nikon inverted microscope \((\text{Nikon, Düsseldorf, Germany})\), holding and injection pipettes were from Humagen \((\text{Charlottesville, VA, USA})\). Culture of the injected oocytes was performed in universal IVF medium \((\text{MediCult})\) supplemented with 0.3% serum albumin at 37°C under an atmosphere of 5% \(\text{CO}_2\). After 16–18 h, oocytes were checked for fertilization.

### TUNEL assay

For the determination of sperm DNA fragmentation, a detection kit \((\text{Apoptosis Detection System Fluorescein; Promega, Mannheim, Germany})\) was used. Sperm suspensions were centrifuged for 10 min at 300 \(g\) and 4°C. The supernatant was discarded and the remaining pellet was washed in PBS, pH 7.4, \((\text{Oxoid, Hampshire, UK})\). A droplet of this sperm suspension was smeared onto Superfrost\(^{®}\) slides \((\text{Menzel, Braunschweig, Germany})\), air-dried and fixed by immersing in freshly prepared 4% methanol-free formaldehyde in phosphate-buffered saline \((\text{PBS})\) for 25 min at 4°C. Afterwards, the slides were washed in fresh PBS for 5 min at room temperature, treated with 0.2% Triton X-100 in PBS for 5 min and rinsed twice in PBS for another 5 min at room temperature. Excess liquid was removed by tapping the slides. The final steps of the procedure were performed according to the manufacturer’s instructions. After final rinses, excess water was drained off, a drop of Anti-Fade solution \((\text{Molecular Probes, Eugene, OR, USA})\) was added, cover slip applied, and 300 randomly selected spermatozoa were immediately analysed with an epifluorescence microscope \((\text{Zeiss, Oberkochen, Germany})\) at a \(\times 1000\) magnification. The percentage of spermatozoa showing green fluorescence \((\text{TUNEL-positive})\) was determined. Negative controls without TdT enzyme were prepared for each batch of analysed slides.

### Determination of ROS production in the spermatozoa

To determine ROS production within the spermatozoa, 100 \(\mu\)l native ejaculate were diluted 1:2 with PBS \((\text{Oxoid})\) and centrifuged at 300 \(g\) for 10 min. The supernatant was discarded and the remaining pellet resuspended to a final sperm count of 20–30 \(\times 10^6/\mu l\). A 20 \(\mu l\) aliquot of a 20 \(\mu mol/l\) dihydroethidium solution \((\text{Molecular Probes})\) was added to 180 \(\mu l\) of the cell suspension and incubated for 15 min at 37°C. A droplet of this suspension was smeared on a micro slide and the percentage of red-orange fluorescing spermatozoa was calculated. The intracellular oxidation of dihydroethidium to ethidium, which...
emits red fluorescence after excitation with light of 488 nm (Rothe and Valet, 1990).

**Determination of ROS production in the ejaculate**

ROS activity was measured as a chemiluminescent reaction using an MTP reader (Hamamatsu Photonics, Herrsching, Germany). Semen aliquots of 100 μl were diluted 1:5 with HTF–HSA and centrifuged at 300 g for 10 min. The pellets were re-suspended in 400 μl HTF–HSA. Afterwards, 20 μl of 4 mmol/l luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Serva, Heidelberg, Germany), a non-specific probe, was added. Three separate 100 μl aliquots were read in white microtitre plates (Dynatech, Chantilly, VA, USA). The chemiluminescent signal was recorded and integrated over a period of 15 min. A medium control was run in parallel and background chemiluminescence was subtracted from the test values. The concentration of ROS was expressed as counts per million viable spermatozoa.

**Statistical evaluation**

All statistical calculations were performed with the appropriate tests after checking normal distribution of the data by means of the MedCalc programme (version 7.0.0.2; MedCalc Software, Mariakerke, Belgium).

**Results**

**Sperm DNA fragmentation and IVF**

Table 1 shows correlations and significance of TUNEL-positive spermatozoa with other sperm parameters measured. Some highly significant negative correlations were found (i.e. sperm motility, progressive motility in the ejaculate). The percentage of TUNEL-positive spermatozoa was also negatively correlated with normal sperm morphology \((r = -0.4423; P = 0.0026)\), which itself was positively correlated with motility \((r = 0.4818; P = 0.0008)\) and progressive motility \((r = 0.4545; P = 0.0017)\). There was no direct correlation between the percentage of TUNEL-positive spermatozoa and fertilization rate \((r = 0.0113; P = 0.8718)\), embryo fragmentation rate \((r = 0.0406; P = 0.5855)\) and pregnancy \((r = -0.0889; P = 0.2016)\) for IVF data (Table 1). After performing ROC analysis (Table 2) and using the calculated cut-off value of 36.5% for the percentage of TUNEL-positive spermatozoa for the distinction of groups, a significant difference between TUNEL-positive (>36.5% TUNEL-positive spermatozoa) and TUNEL-negative (<36.5% TUNEL-positive spermatozoa) ejaculates could be found for pregnancy \((P = 0.0218)\), but not for the fertilization rate \((P = 0.4484; Figure 1)\). While the mean pregnancy rate for TUNEL-negative ejaculates was 34.7%, only 18.7% of the patients fell pregnant if the percentage of TUNEL-positive spermatozoa in the ejaculate was higher than 36.5%. The incidence of TUNEL-positive ejaculates (>36.5% TUNEL-positive spermatozoa) amongst the IVF patients was 31.3%.

**Sperm DNA fragmentation and ICSI**

As observed for IVF, there were also no direct correlations between DNA fragmentation and fertilization rate, embryo fragmentation rate or pregnancy for ICSI. In addition, no correlation was observed with sperm count and motility (Table 1). After performing a ROC analysis, a cut-off value of 24.3% TUNEL-positive spermatozoa was calculated (Table 2). There was no difference in the percentage of patients showing good fertilization between the TUNEL-positive and the TUNEL-negative group, but there was a tendency \((P = 0.0799)\) towards a lower percentage of pregnant women in the TUNEL-positive group (22.2 versus 48.0%; Figure 1).

**Determination of ROS and correlation with other parameters**

The determination of ROS-producing spermatozoa (Figure 2) and any significant correlations with other sperm parameters are shown in Table 3. A significant positive correlation was found with the percentage of TUNEL-positive spermatozoa. ROS production in the ejaculate (Table 1) showed similar correlations, but on a lower level of significance for global

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**Table 1. Relationship between the percentage of TUNEL-positive spermatozoa and different parameters in IVF \((n = 208)\) and ICSI \((n = 54)\).**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation coefficient ((r))</th>
<th>Probability ((P\text{-value}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count</td>
<td>-0.2707</td>
<td>-0.1425</td>
</tr>
<tr>
<td>Concentration of motile sperm</td>
<td>-0.3249</td>
<td>n/a</td>
</tr>
<tr>
<td>Concentration of progressively motile sperm</td>
<td>-0.3321</td>
<td>n/a</td>
</tr>
<tr>
<td>Motility</td>
<td>-0.6026</td>
<td>0.1536</td>
</tr>
<tr>
<td>Progressive motility</td>
<td>-0.5662</td>
<td>n/a</td>
</tr>
<tr>
<td>Motility after swim–up</td>
<td>-0.4843</td>
<td>n/a</td>
</tr>
<tr>
<td>Normal sperm morphology</td>
<td>-0.4423</td>
<td>n/a</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>0.0113</td>
<td>-0.2678</td>
</tr>
<tr>
<td>Embryo fragmentation rate</td>
<td>0.0406</td>
<td>0.1666</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>-0.0889</td>
<td>-0.0523</td>
</tr>
</tbody>
</table>

n/a = not applicable.
motility, progressive motility and normal sperm morphology. No relationship for ROS and sperm count or motility after sperm separation was found. Interestingly, the correlation coefficient and the probability for the analysed parameters were always better for the ROS production within the spermatozoa than for the ROS production in the ejaculate (Table 3: Figure 3). The most powerful correlations are depicted in Figure 3. The ROS production in the ejaculate and the percentage of ROS-producing spermatozoa are markedly less related (r = 0.363; P = 0.0160). On the other hand, the percentage of TUNEL-positive spermatozoa was significantly correlated with both the ROS production in ejaculate and spermatozoa at the same comparably low level (r = 0.391; P = 0.0104 versus r = 0.385; P = 0.0116) (Table 4).

A differentiation in ROS-positive (cut-off for the percentage of ROS-producing spermatozoa: 43.2%; cut-off for ROS in the ejaculate: >645.5 counts/10⁶ spermatozoa/15 min) and ROS-negative ejaculates after ROC analysis with a subsequent comparison of the mean fertilization and pregnancy rates showed no differences for IVF (P > 0.05), either for fertilization or for pregnancy. As the number of patients investigated after ICSI was only 20, such a calculation was not performed for this group.

### Table 2. ROC analysis for the TUNEL assay in IVF and ICSI.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IVF (n = 208)</th>
<th>ICSI (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off value</td>
<td>36.5</td>
<td>24.3</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>80.6</td>
<td>66.7</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>34.9</td>
<td>63.6</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>34.5</td>
<td>50.0</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>81.0</td>
<td>77.8</td>
</tr>
</tbody>
</table>

Figure 2. Determination of ROS-producing spermatozoa by means of dihydroethidium. (A) Phase-contrast and (B) fluorescence image of the same field of vision. The arrows indicate spermatozoa that produce reactive oxygen species, the arrowheads highlight spermatozoa that do not produce ROS.

Relation of the number of peroxidase-positive cells in the ejaculate to other parameters

A comparison of the number of peroxidase-positive cells, the percentage of TUNEL-positive spermatozoa and ROS production in the spermatozoa and ejaculate among each other revealed a highly significant positive correlation between the number of peroxidase-positive cells and the ROS production in the ejaculate (r = 0.602; P = 0.0001), which is less with the ROS production in the sperm cells (r = 0.318; P = 0.0394). However, no significant correlation was observed between the number of peroxidase-positive cells and the percentage of TUNEL-positive spermatozoa (r = 0.227; P = 0.1468) (Table 4).
Table 3. Relationships for ROS production in the spermatozoa and ROS production in the ejaculate with different parameters. Principally, the correlations for ROS production in the ejaculate and in the spermatozoa showed the same pattern. However, for ROS production in the ejaculate, the level of significance was lower, and significance was not reached for sperm count and motility after sperm separation (n = 45).

<table>
<thead>
<tr>
<th></th>
<th>ROS production in spermatozoa</th>
<th>ROS production in ejaculate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count</td>
<td>$r = -0.3369, \ P = 0.0236$</td>
<td>$r = -0.262, \ P = 0.0822$</td>
</tr>
<tr>
<td>Normal sperm morphology</td>
<td>$r = -0.5000, \ P = 0.0009$</td>
<td>$r = -0.440, \ P = 0.0035$</td>
</tr>
<tr>
<td>Global motility</td>
<td>$r = -0.5393, \ P = 0.0001$</td>
<td>$r = -0.415, \ P = 0.0059$</td>
</tr>
<tr>
<td>Progressive motility</td>
<td>$r = -0.5503, \ P = 0.0001$</td>
<td>$r = -0.398, \ P = 0.0083$</td>
</tr>
<tr>
<td>Motility after sperm separation</td>
<td>$r = -0.3331, \ P = 0.0333$</td>
<td>$r = -0.112, \ P = 0.4803$</td>
</tr>
<tr>
<td>Percentage of spermatozoa with DNA fragmentation</td>
<td>$r = 0.4312, \ P = 0.0035$</td>
<td>$r = 0.391, \ P = 0.0104$</td>
</tr>
</tbody>
</table>

Discussion

The present study confirms previous data on the impact of sperm DNA fragmentation on IVF (Henkel et al., unpublished data) and also the findings of Oosterhuis et al. (2000) regarding sperm count. Significant negative correlations were also found for normal sperm morphology and motility, in agreement with Sun et al. (1997). Although not significant, the tendency in ICSI patients seems to be the same as for IVF: no difference for fertilization but half the pregnancy rate for patients with TUNEL-positive ejaculates. This result may not be as significant as for IVF because of the size of the study population. According to these data, it seems obvious that spermatozoa with DNA fragmentations are able to form the pronuclear stage after IVF and ICSI (Twigg et al., 1998), thus fertilizing the oocytes. When the paternal genome is switched on at the 4- to 8-cell stage, further development seems to stop and results in early embryo death (Jurisicova et al., 1996). In this study, the incidence of high DNA fragmentation was about one-third, which is in accordance with data published by Larson et al. (2000) after using the sperm chromatin structure assay.

Causes of DNA fragmentation could be internal influences like apoptosis or ROS production of the spermatozoa, or external inducers like leukocytes. ROS production in the ejaculate by leukocytes seems to have a small influence on sperm DNA fragmentation. However, as even low amounts of ROS are harmful to sperm DNA integrity (Zorn et al., 2000), causality between leukocytes in the ejaculate and DNA fragmentation should not be neglected (Henkel et al., 2003). These cells play an important role in immunosurveillance in the ejaculate and produce high amounts of oxidants, including hydrogen peroxide (Aitken et al., 1994) and it has been shown that this oxygen metabolite accounts for most human sperm damage (Iwasaki and Gagnon, 1992). In addition, because it is not charged, hydrogen peroxide can easily penetrate plasma membranes, enter the spermatozoa and damage DNA integrity.

When comparing the different sources of ROS, i.e. peroxidase-positive cells in the ejaculate and ROS-producing spermatozoa, there is a strong and highly significant correlation between the number of peroxidase-positive cells and ROS production in the ejaculate. This result is expected because leukocytes are the main source of ROS in the ejaculate (Aitken et al., 1994). The percentages of ROS-producing spermatozoa and ROS production in the ejaculate are more weakly and less significantly correlated. The same applies to the relationship of peroxidase-positive cells with the percentage of ROS-producing spermatozoa, where significance is even lower. These findings clearly show that spermatozoa are only a minor source of ROS production in the ejaculate.

It remains questionable as to where leukocytes affect spermatozoa after ejaculation, during epididymal maturation or during spermatogenesis. Due to the limited and short-term exposure, a direct and immediately mediated effect of leukocytes appears unlikely. This might also explain the poor correlation between the percentage of TUNEL-positive spermatozoa and the ROS production in the ejaculate, and the non-significant relationship between the number of peroxidase-positive cells and the percentage of TUNEL-
positive spermatozoa respectively. Nevertheless, it should be noted that this leukocyte-mediated sperm damage gains importance when spermatozoa are separated in vitro and when the seminal plasma, which contains scavengers for ROS (Iwasaki and Gagnon, 1992), is being eliminated. During epididymal maturation of spermatozoa, the time of exposure to the cells and its products is much longer, so that a direct influence is more probable. A strong correlation between the percentages of TUNEL-positive spermatozoa with ROS-producing spermatozoa would support this idea. During spermatogenesis, Sertoli cell function can be affected, thus resulting in poor morphogenesis of spermatozoa. It is well known that poor morphology, especially excess residual cytoplasm, significantly affects sperm fertilizing potential (Keating et al., 1997). Spermatozoa that have cytoplasmic residues have a higher content of cytoplasmic enzymes, i.e. glucose-6-phosphate dehydrogenase (Gomez et al., 1996), that is thought to stimulate the generation of ROS in the

Figure 3. Relationships between the percentage of ROS-producing spermatozoa and ROS in the ejaculate with the percentage of normal sperm morphology (A, B), progressive motility (C, D) and the percentage of TUNEL-positive spermatozoa (E, F). Significant negative correlations were found for normal sperm morphology and progressive motility, while a positive correlation was found for the percentage of TUNEL-positive spermatozoa in the ejaculate. In addition, the correlation between ROS production in the ejaculate and the respective parameters was weaker and less significant (see also Table 3).
Table 4. Relationships between number of peroxidase-positive cells, percentage of TUNEL-positive spermatozoa and ROS production in ejaculate and spermatozoa. The number of peroxidase-positive cells was strongly correlated with ROS production in the ejaculate, but less strongly with ROS production in spermatozoa. There was no significant correlation between the number of peroxidase-positive cells and the percentage of TUNEL-positive spermatozoa. Correlation coefficients for the relationship between the percentage of TUNEL-positive spermatozoa and ROS production in ejaculate and spermatozoa were both significant and approximately the same (n = 45).

<table>
<thead>
<tr>
<th>Parameters correlated</th>
<th>Correlation coefficient (r)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase-positive cells/ROS production in the ejaculate</td>
<td>0.602</td>
<td>0.0001</td>
</tr>
<tr>
<td>Peroxidase-positive cells/% ROS-producing spermatozoa</td>
<td>0.318</td>
<td>0.0394</td>
</tr>
<tr>
<td>Peroxidase-positive cells/% TUNEL-positive spermatozoa</td>
<td>0.227</td>
<td>0.1468</td>
</tr>
<tr>
<td>ROS production in the ejaculate/% ROS-producing spermatozoa</td>
<td>0.363</td>
<td>0.0160</td>
</tr>
<tr>
<td>% TUNEL-positive spermatozoa/ROS production in the ejaculate</td>
<td>0.391</td>
<td>0.0104</td>
</tr>
<tr>
<td>% TUNEL-positive spermatozoa/% ROS-producing spermatozoa</td>
<td>0.4312</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

spermatozoa themselves (Gomez et al., 1996; Aitken et al., 1997). The clinical importance of this is underlined by the considerably stronger correlations of the percentage of ROS-producing spermatozoa with the different parameters measured in this study.

Finally, it is important to mention the consequences of fertilization of oocytes with spermatozoa deriving from an ejaculate containing a high incidence of DNA fragmentation in IVF and especially in ICSI patients. According to present knowledge, sperm DNA fragmentation might not only cause impaired embryonic development and early embryonic death (Asch et al., 1995; Jurisicova et al., 1996; Simerly et al., 1997), but also an increased risk of childhood cancer in the offspring (Ji et al., 1997; Aitken et al., 1998). The latter is due to the vulnerability of human sperm DNA during late stages of spermatogenesis and epididymal maturation. At this stage, DNA repair mechanisms have been switched off, resulting in a genetic instability of the male germ cells (Aitken and Krausz, 2001), especially on the Y chromosome resulting in male-specific cancers (McElreavey and Quintana-Murci, 2003). Therefore, the pathophysiology of ROS and the impact of leukocytes on spermatozoa and DNA integrity should be better understood.

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