Total reactive antioxidant potential and DNA fragmentation index as fertility sperm parameters

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SUMMARY

There is a growing evidence that oxidative stress play a major role in the etiology of defective sperm function including impaired morphology, motility, metabolism and fertility. The aim of the present study was to examine: 1/ total reactive antioxidant potential (TRAP) in seminal plasma; 2/ sperm DNA fragmentation index (DFI), 3/ sperm morphology and motility and 4/ cellular membrane integrity (hypoosmotic swelling test: HOS test) in patients attending in vitro fertilization/intracytoplasmatic sperm injection (IVF/ICSI) program. According to the DFI value, the men were divided into: group 1 with DFI ≤15% (n=38) and group 2 with DFI ≥15% (n=37). Significant differences between the two groups were found in TRAP, sperm motility, morphology and concentration as well as HOS test scores. In group 1, DFI was negatively correlated with sperm motility and HOS test scores (p<0.05). The sperm morphology was positively correlated with sperm motility and HOS test scores in both groups. There was no correlation between TRAP and sperm chromatin fragmentation. Our results suggest that seminal plasma TRAP level may be a DFI independent parameter of sperm fertility. Reproductive Biology 11 2: 35-144.

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Key words: TRAP, DFI, sperm morphology, sperm motility, sperm concentration, HOS test

INTRODUCTION
Many reports proved the standard sperm analysis including sperm morphology, concentration and motility is not a sufficient fertility diagnostics. Besides psychological, environmental and genetic factors influencing fertility, the overproduction of reactive oxygen species (ROS) and the failure of the antioxidant system seem to draw great attention from the scientific world. In comparison with fertile patients, the patients with idiopathic infertility generally present a significantly higher seminal ROS level and lower antioxidant potential [8]. Spermatozoa are very susceptible to damage by reactive oxygen species, and an oxidative stress status reflected by a balance between ROS and antioxidants may be necessary for the survival and normal functions of spermatozoa [9, 17, 20].

The production of ROS by spermatozoa is a normal physiological process required for the occurrence of the capacitation and acrosome reaction. However, the production of an abnormal ROS level is associated with human male infertility due, among others, to the high content of polyunsaturated fatty acids within plasma membranes and the low content of scavenging enzymes in the cytoplasm [7]. It was demonstrated that the imbalance between the production of ROS and the amount of ROS scavenged by antioxidants resulted in sperm damage and correlated with idiopathic infertility [19]. Moreover, male infertility has been linked with the excessive generation of reactive oxygen species by defective spermatozoa [11].

ROS overproduction may cause peroxidation of sperm cell membrane lipids, affecting structure of enzymes, receptors and/or transporting proteins [4]. Lipid peroxidation triggers the loss of membrane integrity and results in the increased cell electrolyte permeability. This may affect cellular energy metabolism and cause the depletion of ATP [2, 18]. Excessive ROS generation may also be involved in structural DNA damage [21]. High ROS levels mediate DNA fragmentation commonly observed in the spermatozoa of infertile patients. The percentage of sperm with DNA damage is negatively correlated with the fertilization rate. As a result, a normal cellular mechanism required for fertilization is impaired [13].
Although the importance of seminal plasma in the protection of spermatozoa against ROS is well documented, only a few studies have investigated the relationship between seminal plasma antioxidative properties and DNA damage or sperm morphology and motility or the hypo-osmotic swelling test HOS test scores [6, 10]. Therefore, the aim of this study was to assess sperm characteristics and function (HOS-test) as well as sperm DNA fragmentation index (DFI) and total reactive antioxidant potential (TRAP) in seminal plasma of male patients within an in vitro fertilization/intracytoplasmatic sperm injection (IVF/ICSI) program.

MATERIALS AND METHODS

Patients and standard semen analysis
Semen specimens were collected after three or four days of sexual abstinence from 75 patients aged 24-35 years (±28.5) attending an IVF/ICSI program at the Novomedica Center in Mysłowice, Poland. The semen samples (n=75) were assessed according to standard criteria recommended by WHO [24] and classified as follows: the cut-off value for normal sperm (normozoospermia: N) concentration at $\geq 20 \times 10^6$/ml, normal sperm morphology at $\geq 30\%$, and sperm motility at $\geq 50\%$ spermatozoa with rapid (category A) + slow (category B) progressive movement or $\geq 25\%$ with rapid progressive motility. The evaluation of sperm concentration and motility was performed using Makler counting chambers. According to the DFI value, the patients were divided into: group 1 with DFI $\leq 15\%$ (n=38) and group 2 with DFI $\geq 15\%$ (n=37). The DFI $\leq 15\%$ is a cut-off point for normal fertility potential [14]. The criteria for exclusion from the study group were infertility secondary to infection, medication or congenital defect and varicocele.

The hypo-osmotic swelling test (HOS-test)
The hypo-osmotic swelling test [6, 10] was performed to evaluate the functional integrity of the sperm plasma membrane with a diagnostic kit (FertiPro N.V., Beernem, Belgium). Sperm was considered normal when $\geq 50\%$ spermatozoa had swollen tails.
Sperm chromatin structure assay (SCSA)
The assessment of the sperm chromatin structure assay (SCSA) was performed using flow cytometry [23]. Briefly, after partial denaturation of the DNA (pH=1.5), the samples were stained with metachromatic fluorochrome: acridine orange (Ex/Em = 488/525 and 615 nm). The fluorescence in green (515-530 nm) and red (>630 nm) bands was measured using a flow cytometer (DAKO Galaxym DAKO, Denmark). The fluorescence bands corresponded to the intact double stranded DNA (green fluorescence) and fragmented, single stranded (red fluorescence) sperm DNA. Approximately 15 000-25 000 spermatozoa were acquired for each sample at a flow rate of 400-500 events/s. An artificial parameter alpha t (alpha t = red/green+red fluorescence) was calculated for each analyzed sperm cell and shown on a histogram (fig. 1). The cells with the abnormal chromatin structure showed a distinct shift of alpha t parameter value.

Figure 1. The results of sperm chromatin structure assay (SCSA) of two exemplary semen samples: A/ sperm with DFI ≤15%, B/ sperm with DFI >15%. R3: moderate DNA fragmentation; R4: high DNA fragmentation; counts: number of spermatozoa; FL3: Alfa t; Alfa t: red fluorescence/(green fluorescence +red fluorescence)

Total reactive antioxidant potential
Liquefied semen was centrifuged at 1000×g for 10 min. The seminal plasma was aspirated and kept frozen (-30ºC) until examination. The antioxidant capacity was performed according to procedure described by Smith et al. [22]. The luminescence signal was measured in an AutoLumat LB953 luminometer (Berthold, Bad Wildbad, Germany).
Statistical analysis
All variables were tested for the normality of distribution by the Kolmogorov Smirnov test. For a statistical analysis the non-parametric Mann-Whitney U test was used. The correlation rate was calculated using Spearman’s test. The Spearman rank correlation coefficient (r_s) was determined. All results were expressed as mean±standard deviation (mean±SD) and median.

RESULTS
The human subjects from group 1 (DFI ≤15%) had a significantly (p<0.001) lower DFI than those from group 2 (DFI ≥15%; tab. 1). The mean total antioxidant capacity and sperm concentration were higher (p<0.05) in group 1 compared to group 2. The sperm progressive motility (p<0.01) as well as the percentage of normal spermatozoa (p<0.001) were also higher in group 1 than in group 2. Finally, men from group 1 exhibited higher (p<0.01) HOS test scores in comparison to those from group 2 (tab. 1).

Table 1. Sperm parameters in human subjects with different DFI

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DFI≤15%, n=38</td>
<td>DFI&gt;15%, n=37</td>
</tr>
<tr>
<td>mean±SD</td>
<td>median</td>
<td>range (min-max)</td>
</tr>
<tr>
<td>DFI (%)</td>
<td>10.6±3.3***</td>
<td>11.0</td>
</tr>
<tr>
<td>TRAP (µmol TE/l)</td>
<td>316.3±98.0*</td>
<td>330.0</td>
</tr>
<tr>
<td>Morphology (% normal sperm forms)</td>
<td>65.5±13.3***</td>
<td>53.0</td>
</tr>
<tr>
<td>Progressive motility A+B (%)</td>
<td>51.6±17.8**</td>
<td>68.0</td>
</tr>
<tr>
<td>HOS test (%)</td>
<td>78.9±12.8**</td>
<td>81.0</td>
</tr>
<tr>
<td>Sperm concentration (ml/ml)</td>
<td>59.7±31.9*</td>
<td>62.0</td>
</tr>
</tbody>
</table>

DFI: DNA fragmentation index; TRAP: total reactive antioxidant potential; HOS test: hypoosmotic swelling test; *p<0.05; **p<0.01; ***p<0.001
There were no significant correlations between TRAP and any other measured parameter in both groups (tabs. 2 and 3). DFI was negatively correlated (p<0.05) with the sperm progressive motility (A+B; r_s=-0.56) and HOS test scores (group 1: r_s=-0.56, group 2: r_s=-0.48). A positive correlation (p<0.05) between sperm morphology and HOS test scores was found in both groups (group 1: r_s=0.66; group 2: r_s=0.55). In group 1, sperm concentration was positively correlated (p<0.05) with sperm motility (A+B; r_s=0.44) and HOS test scores (r_s=0.43), and negatively correlated (p<0.05) with the DFI (r_s=-0.47; tab. 2). In group 2, no significant correlations between sperm concentration and any other parameter were found (tab. 3).

Table. 2. The Spearman rank correlation coefficient (r_s) between the selected sperm parameters in group 1 (DFI ≤15%)

<table>
<thead>
<tr>
<th></th>
<th>TRAP</th>
<th>Progressive motility A+B</th>
<th>Sperm morphology</th>
<th>HOS test</th>
<th>Sperm concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFI</td>
<td>-0.25</td>
<td>-0.56*</td>
<td>-0.26</td>
<td>-0.56*</td>
<td>-0.47*</td>
</tr>
<tr>
<td>TRAP</td>
<td></td>
<td></td>
<td>0.02</td>
<td>-0.23</td>
<td>-0.19</td>
</tr>
<tr>
<td>Progressive motility A+B</td>
<td></td>
<td></td>
<td>0.71*</td>
<td>0.91*</td>
<td>0.44*</td>
</tr>
<tr>
<td>Sperm morphology</td>
<td></td>
<td></td>
<td>0.66*</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>HOS test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.43*</td>
</tr>
</tbody>
</table>

DFI: DNA fragmentation index; TRAP: total reactive antioxidant potential; HOS: test hypoosmotic swelling test; *p<0.05
**Table 3.** The Spearman rank correlation coefficient ($r_s$) between the selected sperm parameters in group 2 (DFI ≥15%)

<table>
<thead>
<tr>
<th></th>
<th>TRAP</th>
<th>Progressive motility A+B</th>
<th>Sperm morphology</th>
<th>HOS test</th>
<th>Sperm concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFI</td>
<td>-0.06</td>
<td>-0.56*</td>
<td>-0.40*</td>
<td>-0.48*</td>
<td>0.29</td>
</tr>
<tr>
<td>TRAP</td>
<td></td>
<td>-0.26</td>
<td>-0.09</td>
<td>0.04</td>
<td>-0.29</td>
</tr>
<tr>
<td>Progressive motility A+B</td>
<td></td>
<td></td>
<td>0.74*</td>
<td>0.86*</td>
<td>0.16</td>
</tr>
<tr>
<td>Sperm morphology</td>
<td></td>
<td></td>
<td></td>
<td>0.55*</td>
<td>0.37</td>
</tr>
<tr>
<td>HOS test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.12</td>
</tr>
</tbody>
</table>

DFI: DNA fragmentation index; TRAP: total reactive antioxidant potential; HOS: test hypoosmotic swelling test; *p<0.05

**DISCUSSION**

Several studies implied that sperm parameters such as motility, concentration, cellular membrane integrity are unreliable predictors of a successful outcome in assisted reproduction treatment (IVF/ICSI) and embryo transfer [4, 15]. These parameters do not identify subtle defects which may be associated with DNA fragmentation [4, 5, 7]. In contrast, the formation of reactive oxygen species and the ROS level correlate with sperm motility [8]. Aziz et al. [3] showed that the ROS production was positively correlated with a high proportion of sperm amorphous heads, damaged acrosomes, midpiece and tail defects as well as the occurrence of cytoplasmic droplets. The authors concluded that standard semen analysis (sperm motility, morphology and sperm deformity index) scores are useful tools in identifying infertile patients with high seminal ROS.

The results of our study showed more morphologically normal spermatozoa in group 1 (DFI ≤15%) than in group 2. However, in group 1 no correlation between DFI and sperm morphology was found. In contrast, Piasecka et al. [15] demonstrated that in patients with normal sperm parameters, DNA fragmentation might be associated with poor sperm morphology.
Despite significant differences in TRAP level between the two examined groups, no correlation between TRAP and the studied parameters was demonstrated. In contrast, Rao et al. [16] reported a positive correlation between lipid peroxidation and morphometric abnormalities of spermatozoa, primarily concerning a tail defect. Moreover, Smith et al. [22] showed that TRAP was positively correlated with rapid sperm motility and negatively correlated with abnormal sperm morphology. Lewis et al. [12] found that seminal plasma from infertile patients had a lower antioxidant level than that from fertile patients. As we did not find any correlation between the TRAP and any other parameter in the two studied groups, we suggest that the semen antioxidant capability is a biological trait not associated with other spermatozoa traits. Thus, the assessment of TRAP should be an independent measure of sperm fertility potential.

Previous studies have yielded some confusion regarding the interpretation of DFI data [1]. It was shown that lower DFI was associated with normal motility and high HOS test scores. De Lamirande et al. [5] showed a moderate positive correlation between sperm motility and SCSA results. Results of our study support the observation that SCSA and sperm motility are correlated predictors of male fertility. In summary, TRAP, sperm morphology, motility and concentration as well as the HOS test scores differed significantly between the groups with different DFI. In both groups, the DFI was negatively correlated with sperm motility. The sperm motility may therefore be a reliable sperm parameter. TRAP did not correlate with any of the examined parameters in either group. The rate of chromatin damage (DFI) and sperm progressive movement are the most reliable measures of sperm fertility.

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