Evaluation of correct endogenous reactive oxygen species content for human sperm capacitation and involvement of the NADPH oxidase system

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BACKGROUND: Generation of controlled amounts of reactive oxygen species (ROS) and phosphorylation of protein tyrosine residues (Tyr) are two closely related changes involved in sperm capacitation. This study investigated the effect of altered endogenous ROS production on Tyr-phosphorylation (Tyr-P), acrosome reaction (AR) and cell viability during sperm capacitation. The possible origin of the altered ROS production was also evaluated by apocynin (APO) or oligomycin (Oligo) addition.

METHODS: A total of 63 samples of purified sperm were analysed for ROS production by enhanced chemiluminescence, Tyr-P pattern by immunocytochemistry, and AR and viability by fluorochrome fluorescein isothiocyanate (FITC)-labelled peanut (Arachis hypogaea) agglutinin and propidium iodide positivity, respectively.

RESULTS: Samples were divided into four categories depending on the ability of sperm to produce ROS, expressed as Relative Luminescence Units (RLU), in capacitating conditions: low ROS production (LRP), range about 0.0–0.05 RLU; normal (NRP), 0.05–0.1 RLU; high (HRP), 0.1–0.4 RLU; very high (VHRP) 0.4–2.0 RLU. In NRP sperm heads, capacitation induced Tyr-P in 87.9 ± 4.3%, and the AR occurred in 62.5 ± 5.4% of cells; in LRP, HRP and VHRP Tyr-P labelling rarely spread over the head, acrosome-reacted cells only accounted for a small number of sperm, and the non-viable cells (NVC) were increased. The addition of APO, but not Oligo, drastically decreased ROS production in analysed samples.

CONCLUSIONS: This study proposes the optimal threshold for endogenous ROS production for correct sperm viability and functioning, and indicates the direct involvement of APO-sensitive NADPH oxidase in ROS production.

Key words: sperm ROS generation / Tyr-phosphorylation / capacitation / acrosome reaction

Introduction

Capacitation is a set of alterations leading to the acrosome reaction (AR), an exocytotic process by which hydrolytic enzymes (e.g. acrosin) are released to allow sperm to fertilize the oocyte (Olds-Clarke, 2003). Several cellular changes occur at specific times and locations during sperm capacitation, including an increase in membrane fluidity due to lipid modifications, an influx of Ca²⁺, generation of controlled amounts of reactive oxygen species (ROS) and phosphorylation of proteins on serine, threonine and tyrosine (Tyr) residues (de Lamirande and Gagnon, 1993, 1995; de Lamirande et al., 1997; Aitken et al., 1998; O’Flaherty et al., 2003, 2005; Liguori et al., 2005). During sperm capacitation, the stimulation of Tyr-phosphorylation (Tyr-P) has been demonstrated to be a redox-regulated event induced by endogenous ROS generation (Aitken et al., 1995, 1998; Leclerc et al., 1997).
Infertility is frequently associated with excessive generation of ROS, and ROS content in infertile patients is correlated with the percentage of both apoptotic and necrotic sperm (Pasqualotto et al., 2000; Agarwal et al., 2003; Moustafa et al., 2004).

ROS may also be generated by sperm itself, and enhanced ROS production has been observed in infertile men (Aitken and Clarkson, 1987; Alvarez et al., 1987; Pasqualotto et al., 2001). Routine evaluation of an infertile couple often reveal no detectable abnormalities in sperm count, morphology or motility, thus leading to the diagnosis of idiopathic or unexplained infertility. Recent studies have suggested the opportunity of introducing ROS evaluation in the semen of infertile patients as a new parameter for better understanding and diagnosis of male factor infertility (Agarwal et al., 2006). Defective antioxidant defences may be responsible for the increase in oxidative stress (OS) observed in the seminal fluid of infertile patients (Jones et al., 1979; Agarwal et al., 2006), as evidenced by their lower total antioxidant capacity (Sharma et al., 1999).

The absence of a general consensus concerning the inclusion of ROS measurement as part of routine diagnostics of infertile men is ascribed to two main factors: (i) lack of standardization of what constitutes normal or pathological levels of ROS in semen; (ii) paucity of literature examining patterns of ROS generation in semen/sperm samples from infertile men who exhibit normal and abnormal sperm characteristics (Agarwal et al., 2006).

In a recent report (Donà et al., 2010), we showed that human sperm endogenous ROS production is a determinant for correct capacitation, evaluated as the percentage of acrosome-reacted cells (ARC). ROS content peaks in 45 min of incubation in capacitating conditions (CCs), then declines, and later slowly re-peaks, starting from the second hour of incubation. Concomitantly, sperm Tyr-P, which occurs only in the midpiece in basal conditions, expands all over the cell, starting from the head to the flagellum, in 45 min of incubation, declines in the following period to label only the midpiece, and then re-expands all over the cells as sperm incubation progresses. AR only occurs in the presence of both Tyr-P of the sperm head and peak ROS production.

In this work, we examined the sperm of 58 sub-fertile individuals by comparing their ROS production levels, Tyr-P patterns and percentages of ARC with those of five fertile men. We also investigated the source of endogenous ROS production by adding apocynin (APO), an NADPH oxidase inhibitor (Miraglia et al., 2009; Yang et al., 2010) or oligomycin (Oligo), an ATP synthase inhibitor which blocks the mitochondrial respiratory chain and inhibits ROS production (Aronis et al., 2003; Watabe and Nakaki, 2007), to the capacitating medium.

Materials and Methods

Semen collection and analysis

Healthy male donors (n = 5) of proven fertility (with 1–3 children) (age range, 30–35 years; average age, 32.5) already analysed in a previous study (Donà et al., 2010) were enrolled together with patients (n = 58) (age range, 28–38 years; average age, 34.7 years), from couples who had failed to conceive after at least 1 year of regular unprotected intercourse. The female partners of the men underwent gynaecologic evaluations, and all results were normal.

All recruited donors gave their informed consent and provided detailed lifestyle histories.

After 3 days of abstinence, semen samples were collected by masturbation in a sterile container and then assessed for sperm parameters. All sperm samples enrolled in this study had following characteristics: volume >2 ml, sperm count >60 x 10^6 cells/ml; motility >60%; (grades 1 and 2) and morphologically normal cells >40%. Fructose levels and pH values were in accordance with the criteria of World Health Organization (1999). All samples presenting any kind of contamination were discarded.

Chemicals

Anti-P-Tyr mouse monoclonal and goat anti-mouse immunoglobulin G (IgG)-fluorochrome fluorescein isothiocyanate (FITC) conjugate antibodies were purchased from Upstate (Becton Dickinson Italia SpA., Milan, Italy) and Santa Cruz Biotechnology (Heidelberg, Germany), respectively. Pure Sperm Wash (PSW) was obtained from Nidacocon International AB (Goteborg, Sweden), and ISolate density gradient was purchased from Irvine Scientific (Santa Ana, CA, USA), respectively. 12-myristate-13-acetate phorbol ester (PMA) and APO were purchased from Calbiochem, (Nottingham, UK); all other reagents were from Sigma-Aldrich (Milan, Italy).

Sample preparation

After semen analysis, ejaculated samples were laid on a discontinuous ISolate density gradient (40/90%) and centrifuged at 750g for 20 min at room temperature. The seminal plasma and sperm from the 40% ISolate interface were discarded, and the sperm from the bottom pellet (90% ISolate) were collected. Cells were washed with PSW, re-analysed for concentration, motility, viability and morphology, and the concentration was adjusted to 80 x 10^6 sperm cells/ml in PSW. If the total cells pelletted after gradient separation ranged from 100 to 120 x 10^6, the sample was divided and incubated for up to 180 min in CCs in the absence or presence of APO 100 μM or Oligo 100 μM, from stock solutions of 100 mM dissolved in dimethylsulphoxide (DMSO) and ethanol, respectively.

Aliquots from each sample, incubated for up to 180 min in a humid chamber at 37°C, were taken to be analysed for ROS production and, at different incubation times, also for Tyr-P labelling and AR.

ROS enhanced chemiluminescence

Production of ROS was measured by the chemiluminescence assay method with luminol (5-amino-2,3-dihydro-1,4-phthalalizinedione) as the probe (Saleh and Agarwal, 2002; Donà et al., 2010). Briefly, 2 μl of 25 mM luminol and 4 μl of 10 mg/ml horse-radish peroxidase, both prepared in DMSO, were added to 200 μl of a sperm suspension at a concentration of 10 x 10^6 cells/ml. ROS levels were determined by a luminometer (Fluoroscan Ascent FL, Labsystems, Helsinki, Finland) in the integrated mode for 180 min at 37°C. Results are expressed as Relative Luminescence Units (RLU) per 2 x 10^6 sperm.

Lastly, 2 μl of a 10 mM N-formylmethionyl-leucyl-phenylalanine (FMLP) stock was added and, after a further 10 min of incubation, 4 μl of a 1 mM stock solution of PMA was added, to exclude leucocyte contamination (Aitken et al., 1992). Only samples with negative responses to FMLP and PMA were considered and then processed.

Comparative values for fluorescence were obtained in the same conditions by analysing increasing concentrations of H2O2 in 200 μl of water at 37°C. Values are expressed as nmoles of H2O2/200 μl.
Anti-P-Tyr evaluation at confocal microscopy

Sperm aliquots (15 × 10^6 cells) were centrifuged for 5 min at 2300g, washed with phosphate-buffered saline (PBS)—containing vanadate I mM and protease inhibitor cocktail, fixed with 2% (w/v) paraformaldehyde in PBS and incubated overnight at 4°C on slides pre-coated with poly-L-lysine (Mortimer et al., 1987).

Slides were rinsed twice with PBS, treated with 0.2% (v/v) Triton X-100 in PBS for 15 min at 4°C, and incubated with anti-P-Tyr (diluted 1:20 in 1% bovine serum albumin (BSA) in PBS) for 1 h at 37°C in a humid chamber.

Slides were washed three times with PBS and then stained with antimouse IgG-FITC conjugate (diluted 1:100 in 1% BSA in PBS) for 1 h at 37°C in a humid chamber. Staining without primary antibody was used as negative control.

Evaluation of AR and cell viability

Acrosome status was monitored with acrosome-specific FITC-labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA) in conjunction with DNA-specific fluorescent propidium iodide (PI) as a viability test (Lukoseviciute et al., 2004). Briefly, AR was induced by incubating aliquots (15 × 10^6 cells) of each sample at different incubation times, for a further 30 min at 37°C, in the presence of Ca²⁺ ionophore A23187 from a 10 mM stock in DMSO, to a final concentration of 10 μM (Larson and Miller, 1999). Control tubes contained DMSO, but no ionophore.

Sperm aliquots were centrifuged for 5 min at 600g, resuspended in PBS, and exposed to PI (final concentration 12 μM) for 10 min at room temperature to evaluate cell viability. Sperm were washed with PBS, centrifuged, fixed with 2% (w/v) paraformaldehyde in PBS and incubated overnight at 4°C on poly-L-lysine-treated slides.

Slides were rinsed twice with PBS, permeabilized as described above, washed three times and stained with 1 mg FITC-PNA/ml for 15 min at 37°C in the dark, in a humid chamber (Mortimer et al., 1987). They were then washed three more times with PBS and mounted.

At least 200 cells were evaluated for each sample and fluorescence was detected with the UltraView LCI confocal system (Perkin Elmer, Waltham, MA, USA) equipped with a fluorescence filter set for excitation at 488 nm. Only sperm showing evenly distributed fluorescence over the acrosomal region were considered acrosome-intact.

Statistical analysis

Results are expressed as means ± SD. One-way analysis of variance (ANOVA) for independent samples was applied for multiple comparisons; Tukey's HSD (honestly significant difference) post hoc test (Tukey, 1977) or Student's t-test were used for comparisons between two classes of data. A P-value < 0.05 was considered statistically significant. Comparisons between parameters measured at different times for each group of patients were made by two-tailed Student's t-test for paired data.

To assess the ability of the measured parameters to discriminate among the four groups of patients divided according to ROS production, discriminant analysis was used. This is an analytical technique that uses discriminant functions which are linear combinations of the original variables, allowing the data set to be distinguished into a number of pre-defined groups. Analysis was performed with StatistXiXL statistical software, version 1.8 (StatistXiXL, Broadway, Nedlands, Western Australia).

Results

Cells from each sample were analysed for ROS generation in a luminometer for over 180 min, with luminol as the luminescence source. Samples were subdivided into four different groups according to the RLU expressed during incubation: low ROS production (LRP), range about 0.0–0.05 RLU (corresponding to the fluorescence recorded for 0–223 nmoles H₂O₂); normal ROS production (NRP), expressed by healthy control samples, range 0.05–0.1 RLU (or 223–607 nmoles of H₂O₂); high ROS production (HRP), range about 0.1–0.4 RLU (or 607–1357 nmoles H₂O₂); very high ROS production (VHRP), range 0.4–2.0 RLU (or 1357–5357 nmoles H₂O₂). Some samples, with high numbers of cells, were also incubated in the presence of APO or Oligo, to selectively inhibit ROS production from either the NADPH oxidase system (Miraglia et al., 2009) or mitochondrial respiratory chain (Aronis et al., 2003), respectively. Figure 1 shows the ROS production curves of the various groups, in the absence or presence of the inhibitors.

In LRP and NRP samples, ROS production was completely inhibited by the addition of APO, which brought ROS to the level expressed by the buffer (Fig. 1, inset). Instead, Oligo did not seem to induce any alteration in sperm ROS production, at least in the first 60 min; later, the ROS production curve rose steeply, reaching 0.4–0.6 units in the LRP (Fig. 1A) and NRP (Fig. 1B) groups. In the HRP and VHRP groups (Fig. 1C and Fig. 1D, respectively), Oligo addition did not make any detectable difference compared with their own basal curves (red compared with blue lines), whereas APO diminished ROS production by ~75 and 90%, respectively.

Aliquots of sperm suspensions were analysed for their Tyr-P content. Cells were counted and the values are listed in Table I. The percentage of cells presenting partial or total Tyr-P at all times of incubation was significantly different in each group of patients, in comparison with the NRP group (P < 0.001), the latter including seven samples from patients who showed the same ROS production range of the healthy controls.

We also analysed the Tyr-P pattern of phosphorylated sperm (Fig. 2) to determine which part of the cell was involved in the capacitation process. At the four different phases of incubation, except for APO- and Oligo-treated sperm, which were analysed after only 180 min of incubation, percentages of Tyr-phosphorylated head, flagellum or the whole, together with those of ARC and non-viable cells (NVC) were counted (Table II). Samples belonging to the NRP group showed almost homogeneous Tyr-P of the whole cell (Fig. 2B) and underwent AR after both 45 min and 180 min of incubation (63.9 ± 5.8 and 62.5 ± 5.4%, respectively). Incubation in the presence of APO or Oligo significantly (P < 0.0001) reduced the number of ARC and significantly (P < 0.0001) augmented that of NVC (Table II).

Interestingly, in the LRP group, the Tyr-P process never expanded over the whole cell as in the NRP group, but remained in the midpiece region and flagellum (Fig. 2A compared with 2B), with a time-dependent decrease at 180 min. Tyr-P labelling also involved a small number of cells, never exceeding 50% of the total (Table I), with a time-dependent increase in PI positivity (NVC, 49.8 ± 2.3 at 180 min) (Table II). At 45 min, AR characterized only 14.8 ± 2.3% of the cells (Table II), which is in the range of spontaneously occurring reactions obtained in the absence of A23187 (data not shown), with a time-dependent decrease during incubation (8.5 ± 2.6 at 180 min). The presence of APO did not significantly alter the ROS production curve (Fig. 1), which remained at buffer level, but considerably diminished the percentages of Tyr-P labeled cells (25.4 ± 3.5 versus 42.8 ± 2.5, P < 0.001,
Table I) and ACR (2.5 ± 1.4 versus 8.5 ± 2.6, P < 0.001) and significantly (P < 0.0001) augmented that of NVC (68.4 ± 3.2 versus 49.8 ± 2.3) after 180 min of incubation (Table II).

In the HRP group, Tyr-P labelling at 45 min (Fig. 2C, Table I) was lower than in NRP (39.5 ± 2.7 versus 82.5 ± 4.5, P < 0.001) and decreased to only 34.0 ± 3.3% after 180 min of incubation, compared with 76.9 ± 3.2 of NRP (P < 0.001) and involved both midpiece region and tail. ARCs were present at 45 min of incubation time, but in a very small percentage (12.4 ± 1.4) as in LRP, and declined as incubation time was prolonged. In addition, cell viability was seriously compromised throughout the incubation period (Table II).

In the VHRP group, almost all the cells were PI positive (percentage of NVC ranging from 91.0 ± 3.6 to 97.9 ± 1.6 during incubation), with Tyr-P labeling mainly occurring in the midpiece region and flagellum (Fig. 1D) and a very low ability to undergo AR (4.4 ± 0.6% at 45 min, and 1.1 ± 0.7% at 180 min) (Table II). The addition of APO or Oligo did not significantly affect either HRP or VHRP ARC and NVC percentages (Table II), although APO decreased ROS production by 75 and 90%, respectively.

Figure 3 shows the main characteristics observed for the cells incubated for 45 and 180 min. In particular, head Tyr-P, ARC and NVC values (expressed as percentage) were compared among the four
different patient groups presenting different ROS production levels. In comparison with the NRP group, considered as the normal reference, all the other three groups presented significantly different values in the three parameters evaluated ($P < 0.0001$).

As Fig. 4 shows, a close relationship was observed among the percentages of cells presenting head Tyr-P, ARC and NVC. At both 45 and 180 min, the NRP group was clearly distinct from the other three groups, which also showed differences.

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**Table I** Percentage of cells presenting Tyr-P, determined at different incubation times, according to patient group.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Incubation times (min)</th>
<th>$T_0$</th>
<th>45</th>
<th>75</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRP (0–0.05)</td>
<td>$n = 20$</td>
<td>32.8 ± 3.7**</td>
<td>44.6 ± 1.8**</td>
<td>24.3 ± 1.9**</td>
<td>42.8 ± 2.5**</td>
</tr>
<tr>
<td>APO ($n = 4$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.4 ± 3.5‡</td>
</tr>
<tr>
<td>Oligo ($n = 4$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.2 ± 2.3‡</td>
</tr>
<tr>
<td>NRP (0.05–0.1)</td>
<td>$n = 12$</td>
<td>75.2 ± 1.7</td>
<td>82.5 ± 4.5</td>
<td>69.4 ± 3.2</td>
<td>76.9 ± 3.2</td>
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<tr>
<td>APO ($n = 3$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.7 ± 2.6‡</td>
</tr>
<tr>
<td>Oligo ($n = 3$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.2 ± 7.5‡</td>
</tr>
<tr>
<td>HRP (0.1–0.4)</td>
<td>$n = 20$</td>
<td>45.5 ± 4.8**</td>
<td>39.5 ± 2.7**</td>
<td>35.8 ± 4.1**</td>
<td>34.0 ± 3.3‡</td>
</tr>
<tr>
<td>APO ($n = 3$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.7 ± 4.5‡</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>11.6 ± 3.7‡</td>
</tr>
<tr>
<td>VHRP (0.4–2)</td>
<td>$n = 11$</td>
<td>38.2 ± 3.9**</td>
<td>32.4 ± 1.6**</td>
<td>29.5 ± 1.4**</td>
<td>25.1 ± 2.7‡</td>
</tr>
<tr>
<td>APO ($n = 2$)</td>
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<td></td>
<td></td>
<td></td>
<td>9.8 ± 1.6‡</td>
</tr>
<tr>
<td>Oligo ($n = 2$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.5 ± 0.5‡</td>
</tr>
</tbody>
</table>

Data are the mean ± SD.

ROS production (RP) groups: low (L), normal (N), high (H) and very high (VH).

$**P < 0.001$, Tukey’s HSD post hoc test, following a significant ANOVA among the four patient groups; each group was compared versus NRP reference group for each respective time.

$‡P < 0.001$, Student’s t-test, comparing values at 180 min, against APO and Oligo treatments.

**Figure 2** Tyr-P pattern after 180 min of incubation in CCs of sperm from patients divided into four ROS content-dependent groups. Tyr-P detection by immunofluorescence cytochemistry of sperm from LRP (0.0–0.05 RLU) (A), NRP (0.05–0.08 RLU) (B), HRP (0.08–0.4 RLU) (C) and VHRP (0.4–1.0 RLU) (D). Images are combinations of immunofluorescence and phase contrast photos. Bar: unit of measurement.
Table II Parameters for cells, determined at different incubation times, according to patient group. Data are the mean ± SD.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Incubation times (min)</th>
<th>Tyr-P</th>
<th>ARC %</th>
<th>NVC %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Head %</td>
<td>Tail %</td>
<td>Midpiece %</td>
</tr>
<tr>
<td>LRP (0–0.05)</td>
<td>T₀</td>
<td>0.6 ± 0.3</td>
<td>2.6 ± 1.2</td>
<td>96.3 ± 2.9</td>
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<td></td>
<td>45</td>
<td>2.9 ± 0.8</td>
<td>49.3 ± 2.6</td>
<td>51.7 ± 3.5</td>
</tr>
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<td></td>
<td>75</td>
<td>1.3 ± 0.9</td>
<td>17.8 ± 2.4</td>
<td>87.8 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>2.4 ± 1.1</td>
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<td>16.1 ± 1.8</td>
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<tr>
<td>APO n = 4</td>
<td>180</td>
<td>1.7 ± 0.9 ns</td>
<td>76.9 ± 2.2</td>
<td>23.1 ± 1.5 †</td>
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<td>Oligo n = 4</td>
<td>180</td>
<td>1.6 ± 0.7 ns</td>
<td>75.7 ± 3.5 †</td>
<td>26.3 ± 1.9 †</td>
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<tr>
<td>NRP (0.05–0.1)</td>
<td>T₀</td>
<td>11.2 ± 1.4</td>
<td>3.7 ± 1.9</td>
<td>94.6 ± 2.3</td>
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<td>45</td>
<td>91.9 ± 2.8</td>
<td>61.3 ± 3.2</td>
<td>91.8 ± 2.7</td>
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<td></td>
<td>75</td>
<td>17.8 ± 2.3</td>
<td>28.4 ± 2.5</td>
<td>89.6 ± 3.2</td>
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<td>180</td>
<td>87.9 ± 4.3</td>
<td>71.3 ± 2.8</td>
<td>92.4 ± 2.2</td>
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<tr>
<td>APO n = 3</td>
<td>180</td>
<td>28.4 ± 2.8 †</td>
<td>35.3 ± 2.6 †</td>
<td>61.5 ± 5.3 †</td>
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<tr>
<td>Oligo n = 3</td>
<td>180</td>
<td>10.3 ± 6.7 †</td>
<td>18.6 ± 3.6 †</td>
<td>11.9 ± 3.4 †</td>
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<tr>
<td>HRP (0.1–0.4)</td>
<td>T₀</td>
<td>3.2 ± 0.9</td>
<td>57.2 ± 2.8</td>
<td>84.7 ± 3.4</td>
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<td>45</td>
<td>18.3 ± 1.5</td>
<td>31.8 ± 2.1</td>
<td>63.2 ± 2.6</td>
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<td>75</td>
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<td>26.5 ± 1.6</td>
<td>71.8 ± 3.3</td>
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<td>3.1 ± 0.7</td>
<td>74.1 ± 3.4</td>
<td>30.5 ± 2.1</td>
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<td>81.1 ± 2.9</td>
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<td>82.6 ± 4.2</td>
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<td>93.1 ± 4.5</td>
<td>4.9 ± 1.3</td>
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<td>APO n = 2</td>
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<td>3.3 ± 0.6 ns</td>
<td>89.3 ± 3.6 ns</td>
<td>6.5 ± 1.9 ns</td>
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<tr>
<td>Oligo n = 2</td>
<td>180</td>
<td>4.5 ± 1.8 ns</td>
<td>91.8 ± 3.2 ns</td>
<td>3.7 ± 2.1 ns</td>
</tr>
</tbody>
</table>

T₀ indicates basal condition, before capacitation start. ns, not significant; ROS production (RP) groups: low (L), normal (N), high (H) and very high (VH).
Tyr-P, tyrosine phosphorylation; ARC, acrosome reacted cells; NVC, non-viable cells.
Tyr-P-head-, Tyr-P-tail- and Tyr-P-midpiece indicate % of cells phosphorylated on head, flagellum or midpiece region, respectively.
†P < 0.0001.
‡P < 0.001.
*P < 0.05 at Student’s t-test, comparing values at 180 min, against APO and Oligo treatments.

For statistical evaluation of the ability of the parameters to indicate a membership to the four different groups of patients, divided according to ROS generation ability, linear discriminant analysis was used (Fisher, 1936). This mathematical approach calculates discriminant functions which are linear combinations of the original variables, allowing the data set to be separated into a number of pre-defined groups.

Table III lists the classification function coefficients (Fn), together with the constant values, obtained with the procedure, at both 45 and 180 min. According to the highest score obtained by the four functions applied to the data for each patient, the most probable group membership was estimated (see Supplementary data, Tables SI and SII). Discriminant analysis provided a 100% ability to distinguish all cases, according to the classification function coefficients obtained, at both times considered.

Discussion

We evaluated endogenous sperm ROS production in 58 individuals (with ROS expressed by the five healthy fertile volunteers also analysed in a previous study; Donà et al., 2010), over a period of 3 h in CCs. ROS values were lower or higher than those of the five fertile controls, with the exception of seven samples who shared ROS production with the healthy controls, values ranging between 0.05 and 0.1 RLU. By choosing this range as the cut-off value for normal sperm ROS production, we classified patients according to their particular capacity to produce ROS, and analysed both Tyr-P patterns and ARC as parameters defining the capacitation process (Larson and Miller, 1999; Whan et al., 2006; Carli et al., 2007; Donà et al., 2010). Sperm from LRP patients were unable to undergo Tyr-P involving the head region, resulting in a low percentage of ARC and a large percentage of NVC. Also in the HRP and VHRP groups, sperm showed very little ability to accomplish correct Tyr-P of the head and AR, as almost all cells lost viability as incubation progressed. Conversely, the seven samples included in the NRP group showed the same characteristics as healthy controls, also for Tyr-P patterns and percentages of ARC and NVC, thus confirming that the ROS production curve may predict correct/incorrect sperm functioning.
In idiopathic infertility (Baker and Aitken, 2005; Aitken, 2006; Saxena et al., 2008), ROS content in both sperm and seminal fluid is the most appropriate parameter able to partially predict the fertilizing power of cells (Agarwal et al., 2003, 2006; Moustafa et al., 2004; Donà et al., 2010). Although the positive role of ROS was ruled out early (O’Flaherty et al., 2006), much evidence has been accumulating on the role of ROS in causing sperm pathologies when they overwhelm the antioxidant defences of the cell. ROS-induced OS may cause damage to DNA (Moustafa et al., 2004) as well as general impairment of normal cell functions (Makker et al., 2009). For the first time, we demonstrate here the correlation between endogenous sperm ROS production and Tyr-P pattern and determine the threshold of a correct content of endogenous ROS for achieving higher percentages of both sperm viability and capacitation. In these conditions, the percentage of cells able to undergo AR (more than 60%) is clearly higher than that previously reported for sperm incubations in the presence of both oxidizing and anti-oxidizing agents (Leclerc et al., 1997), where the percentage of ARC never exceeded 25% and Tyr-P pattern
never expanded to the head. This is in accordance with the fact that, lacking any additional control of the endogenous ROS content, sperm could be alternatively classified in LRP or HRP/VRHRP, depending on the incubating conditions. In addition, the correctness of the proposed classification of sperm samples in the four groups, according to their own ability to produce ROS, was assessed by the highest score obtained by the discriminant analyses carried out by using the three main variables measured (head Tyr-P, ARC and NVC): the four functions applied to the data for each patient provided a 100% ability to distinguish all cases, at both times considered.

Therefore, ROS measurement and classification of sperm give a simple, rapid and not expensive system able to describe the probable percentage of NVC present in the sample, and to predict the probability that the cells undergo a correct capacitation (expressed as percentage of head Tyr-P and ARC).

Sperm may generate ROS through the NADPH-oxidase system(s) in the plasma membrane (Armstrong et al., 2002; Miraglia et al., 2009) and/or the mitochondrial NADH-dependent oxidoreductase (diphorase) complex (Gavella and Lipovac, 1992), through the oxidative phosphorylation generating ATP.

In this study, we confirmed the negative impact of high ROS content in sperm physiological parameters and evaluated the effect of APO (100 µmol/l) on sperm capacitation. APO, a NADPH oxidase (NOX) inhibitor (Miraglia et al., 2009), was used to investigate the contribution of NAD(P)H oxidase to H$_2$O$_2$ production by sperm suspensions. In all analysed samples, APO decreased ROS production to a considerable extent. In HRP and VHRP, high ROS production was not completely abolished by APO, which in any case affected almost 75–90% of ROS generation. In these conditions, APO further decreased ARC and augmented NVC percentages but with poor significance, due to the basically deteriorated condition of the samples (NVC %). In LRP and NRP, in which ROS production was below 0.1 RLU, APO completely abolished ROS production, causing a net decrease in ARC percentage and an increase in NVC. This is consistent with the idea that, when the threshold of ROS production is not achieved, sperm can no longer ensure correct cell functioning, probably due to an impairment in the metabolic pathway. For partial confirmation of this interpretation, ascorbic acid, which acts as a ROS scavenger rather than a ROS preventer like APO, induced a sort of resting condition, maintaining cells in an un-capacitated condition but ready to undergo capacitation (Donà et al., 2010). Instead, APO-induced prevention of ROS production caused cell death.

Further confirmation of the involvement of the NOX system in the correct sperm capacitation was obtained by incubating normal sperm in conditions expressing NOX activity modulation (Supplementary data, Fig. S1). By adding increasing concentrations of NADPH, we induced NOX activation, which was partially or totally inhibited by the addition of 10 µM APO. ROS evaluation confirmed both the activation and the gradual inactivation of the enzyme. The respective evaluation of ARC percentages varied accordingly.

Interestingly, the addition of Oligo did not seem to affect the ROS curves, at least for the first 60 min of incubation. Later, in the NRP group, ROS increased considerably and, after 180 min, were four to five times higher than those produced by cells in the absence of Oligo, with net impairment of cell survival. Also in the LRP, Oligo addition induced a very similar effect, although the ROS curve peaked at a lower extent after 180 min of incubation. In HRP and VHRP, no significant alterations were evident after Oligo addition, probably due to the starting anomalous condition of cells. Oligo, an ATP synthase inhibitor, has previously been reported to inhibit oxidative phosphorylation-related ROS production in mitochondria (Aronis et al., 2003; Watabe and Nakaki, 2007). Our results confirm that the contribution of mitochondrial ROS generation in human sperm is very low (Koppers et al., 2008), mainly when compared with an APO-sensitive NOX-dependent system. The latter is related to correct ROS-dependent functioning of normal sperm during capacitation and, probably, also accounts for the abnormal OS evidenced in HRP and VHRP. In fact, the addition of APO, but not of Oligo, succeeded in decreasing ROS generation in these groups. The Oligo-induced late ROS peaks observed in the LRP and NRP groups may be explained by the energetic collapse of the mitochondrial membrane, leading to ROS release into the cytosol. Increased intracellular levels of ROS (OS) facilitate apoptosis, while further increases have been reported to oxidize elements of the apoptotic pathway and to promote necrotic cell death (Samali et al., 1999).
Table III  Discriminant analysis results conducted using the three main variables determined (head Tyr-P, ARC and NVC) at 45 min and at 180 min, respectively.

<table>
<thead>
<tr>
<th>Variable</th>
<th>45 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fn (LRP)</td>
<td>Fn (NRP)</td>
</tr>
<tr>
<td>Head Tyr-P%</td>
<td>0.216</td>
<td>34.879</td>
</tr>
<tr>
<td>ARC%</td>
<td>2.067</td>
<td>5.353</td>
</tr>
<tr>
<td>NVC%</td>
<td>8.471</td>
<td>−0.237</td>
</tr>
<tr>
<td>Constant</td>
<td>−179.791</td>
<td>−1774.408</td>
</tr>
</tbody>
</table>

This study emphasizes the sensitivity of sperm to endogenous ROS production and, for the first time, the threshold for correct ROS content is established, together with a ROS-related classification of sperm, which is a rapid and inexpensive system able to predict the probability of the cells undergoing a correct capacitation (expressed as the percentage of head Tyr-P and the highest percentage of ARC). These results not only provided valuable information regarding sperm quality, but, if adopted in routine analysis, ROS content classification can also prove to be clinically significant for the correct treatment strategy to adopt in the idiopathic infertility and in vitro fecundation techniques.

A close relation between normal ROS production and Tyr-P labeling of the sperm head is demonstrated as a prerequisite for the AR. In addition, APO-sensitive, NADPH oxidase ROS generation has been evidenced as the major source of human sperm ROS, responsible for correct cell functioning.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles

G.D. and C.F. took part in the acquisition, analysis and interpretation of data and in the drafting of the manuscript, giving final approval of the version to be published. A.A., A.B. and E.R. took part in the acquisition and elaboration of data, and in the drafting of the manuscript, giving final approval of the version to be published. D.A. and G.C. gave their contribution to the interpretation of data, and drafted and revised manuscript critically for important intellectual content, giving final approval of the version to be published. L.B. gave substantial contributions to conception and design, acquisition, analysis and interpretation of data and drafted manuscript, giving final approval of the version to be published.

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References


Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 1999;14:2801–2807.


Yang J, Lane PH, Pollock JS, Carmines PK. Protein kinase C-dependent NAD(P)H oxidase activation induced by type 1 diabetes in renal medullary thick ascending limb. Hypertension 2010;55:468–473.