Apoptosis and necrosis in human ejaculated spermatozoa

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BACKGROUND: Features of both apoptosis and necrosis have been reported in ejaculated human spermatozoa. This study examines the relative contribution of these two modes of cell death to the demise of these terminally differentiated cells. METHODS: Sperm fractions were prepared from aliquots of semen samples from young normozoospermic donors by simple washing from seminal plasma, by discontinuous density gradient centrifugation or by swim-up technique. They were subsequently incubated in vitro at 37°C for 24 h. Sperm motility, viability, and the presence of two apoptotic markers, phosphatidylserine externalization (annexin-V binding) and DNA fragmentation (TUNEL), were examined before incubation and again after 4 and 24 h of incubation. RESULTS: The swim-up technique was the most efficient in terms of the recovery of viable, motile and non-apoptotic spermatozoa, followed by density gradient centrifugation and finally simple washing. No changes in the parameters tested were observed after 4 h of incubation, but a significant decrease in sperm motility and viability was detected after 24 h irrespective of the sperm preparation technique employed. However, these changes were not accompanied by any increase in the incidence of spermatozoa showing markers of an active apoptotic process. CONCLUSIONS: Healthy human ejaculated spermatozoa appear incapable of initiating apoptosis, at least under in vitro conditions.

Key words: apoptosis/cell death/DNA fragmentation/phosphatidylserine externalization/spermatozoa

Introduction

Mature sperm cells have been reported to present distinct signs of apoptosis-related cell damage (Gorczyca et al., 1993; Baccetti et al., 1996; Aravindan et al., 1997; Sun et al., 1997; Sakkas et al., 1999b; Barroso et al., 2000; Gandini et al., 2000; Irvine et al., 2000; Muratori et al., 2000; Oosterhuis et al., 2000; Shen et al., 2002), although they lack transcripational activity and have a very small amount of cytoplasm (Weil et al., 1998). It is not clear whether the apoptotic markers detected in spermatozoa are residues of an abortive apoptotic process started before ejaculation (Sakkas et al., 1999a,b; Tesarik et al., 2002) or whether they result from apoptosis initiated in the post-ejaculation period.

This study was undertaken to examine the relative contribution of apoptosis and necrosis to the demise of ejaculated spermatozoa.

Materials and methods

Sperm source and preparation

Semen samples were obtained after 72 h of sexual abstinence from seven men, aged between 20 and 30 years, participating in our sperm donation programme. Basic semen parameters, including sperm concentration, motility, viability and morphology, were assessed according to the World Health Organization (WHO) guidelines (World Health Organization, 1999). All samples used in this study were normal according to the WHO criteria (World Health Organization, 1999).

Each sample was divided into three aliquots. One aliquot was diluted (1:10) in phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 220 g for 10 min. The seminal plasma supernatant was discarded and the resulting pellet was resuspended in 1 ml of Menez B2 medium (BioMérieux, France). A small sample (100 µl) of this cell suspension was removed immediately after this step for the evaluation of sperm motility, viability, and DNA fragmentation and DNA integrity (see below). The rest of this aliquot was divided into two equal parts which were incubated in B2 medium equilibrated with 5% CO₂ at 37°C for 4 and 24 h, respectively.

The second aliquot was layered on to a two-layer discontinuous (40–80%) Percoll isotonic gradient and centrifuged at 200 g for 20 min. The medium used to dilute the Percoll was modified Ham’s F10 medium (Sigma, St Louis, MO) supplemented with 1% bovine serum albumin and 25 mM HEPES pH 8.0 (both purchased from Sigma). The 40% fraction was discarded, and the higher density layer was resuspended in 10 ml of PBS and centrifuged again at 600 g for 10 min. The resulting pellet was resuspended in 1 ml of B2 medium. As in the former group, an aliquot of 100 µl was removed for the evaluation of sperm motility, viability, and DNA fragmentation and DNA integrity (see below), and the rest of the aliquot was divided into two equal parts which were incubated in vitro for 4 and 24 h, respectively.

The third aliquot was prepared using a simple wash in PBS and centrifugation at 220 g for 10 min. After the removal of the seminal
plasma, 1 ml of B2 medium was gently layered onto the sperm pellet followed by incubation (vertically) for 1 h at 37°C under 5% CO2 in air. At the end of the incubation, 500 μl of the overlying medium was separated to select the motile sperm fraction. As previously, an aliquot of 100 μl of the resulting cell suspension was removed for sperm analysis, and the resting cell suspension was subjected to in vitro incubation.

**Motility analysis**

The proportions of sperm that were motile and immotile were evaluated with the use of a Makler chamber. A drop of sperm sample was placed into the chamber and directly observed under a 40× objective. The operation was repeated twice and the mean of the two evaluations was taken into account. Each time, at least 100 spermatozoa were counted.

**Supravital eosin staining**

To evaluate sperm viability, sperm samples were centrifuged at 220 g for 10 min. The supernatant was discarded, and the pellet resuspended in 100 μl of PBS heated to 37°C. To assay sperm viability, 10 μl of the resulting cell suspension was mixed with 10 μl of the vital stain eosin–nigrosin and incubated for 30 s. The specimens were then smeared on a glass slide, air dried and observed with a 100× oil-immersion objective. For each smear, at least 200 spermatozoa were examined and the percentage of unstained spermatozoa was recorded as the percentage of viable spermatozoa.

**Evaluation of annexin-V binding and supravital propidium iodide staining**

Externalized phosphatidylserine was visualized by incubating sperm samples at 37°C with annexin-V–fluorescein labelling reagent and 20 μl of propidium iodide in 1 ml of HEPES buffer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. The sperm cell suspensions were then smeared on glass slides and air dried. Slides were observed under a fluorescent microscope with a 100× oil-immersion objective. For each smear, 500 spermatozoa were examined. Spermatozoa stained either by propidium iodide alone or by both propidium iodide and annexin-V were considered as necrotic, whereas those stained with annexin-V only were considered as apoptotic. Unstained cells were considered alive.

**Evaluation of DNA fragmentation**

The presence of apoptosis-related DNA strand breaks in spermatozoa was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), using the Apoptosis Detection System, Fluorescein (Boehringer Mannheim, Mannheim, Germany). Washed sperm samples were smeared on microscope slides, fixed with 4% paraformaldehyde in PBS at 4°C for 25 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The rest of the procedure was carried out according to the manufacturer’s instructions. Briefly, the specimens were washed twice in PBS, equilibrated with the equilibration buffer at room temperature for 15 min and incubated at 37°C for 1 h in TUNEL incubation buffer containing nucleotide mix and the enzyme terminal transferase. After stopping the enzyme reaction, the slides were washed three times in PBS for a total time of 20–30 min to ensure complete removal of non-specific fluorescence. Slides were observed in a fluorescent microscope with a 100× oil-immersion objective. For each slide, 500 spermatozoa were evaluated. The negative and the positive control were performed, respectively, by omitting the enzyme terminal transferase and by pre-incubating fixed and permeabilized sperm samples with DNase I (1 mg/ml) for 20 min at room temperature.

**Statistical analysis**

The significance of differences in the percentage of immotile, eosin-positive, propidium-positive, annexin-V-positive and TUNEL-positive spermatozoa in individual sample groups was evaluated by χ2 test using the StatView II statistical package (Abacus Concepts, Berkeley, CA).

**Results**

**Sperm samples prepared by simple washing from seminal plasma**

When whole sperm samples were simply washed from seminal plasma and incubated in vitro at 37°C, sperm motility did not change during the first 4 h of incubation, but an increased percentage of immotile spermatozoa was found after 24 h of incubation (Table I). Similarly, the percentage of non-viable spermatozoa (determined by supravital eosin and propidium iodide staining) did not change during the first 4 h but increased after 24 h of incubation (Table I).

On the other hand, the percentage of spermatozoa with detectable markers of apoptosis (phosphatidylserine externalization visualized by annexin-V binding and DNA fragmentation visualized by TUNEL) did not change throughout the 24 h in vitro incubation period (Table I).

**Sperm samples prepared by discontinuous density gradient centrifugation**

In samples prepared by discontinuous density gradient centrifugation (Table II), the percentage of immotile and non-viable (eosin-positive and propidium-positive) spermatozoa was lower than in crude sperm samples prepared by simple washing (Table I). However, similar to the crude sperm samples, the percentage of immotile and non-viable spermatozoa remained unchanged during the first 4 h of in vitro incubation but increased after 24 h of incubation (Table II).

As in the crude sperm samples, the percentage of spermatozoa with detectable markers of apoptosis (phosphatidylserine externalization visualized by annexin-V binding and DNA fragmentation visualized by TUNEL) did not change throughout the 24 h in vitro incubation period (Table II).

**Sperm samples prepared by washing followed by swim-up**

Of the three sperm preparation methods used in this study, sperm washing followed by swim-up selection resulted in the isolation of sperm populations with the lowest percentages of immotile and non-viable (eosin-positive and propidium-positive) spermatozoa (Table III). However, the pattern of change for sperm motility, viability and apoptosis during subsequent in vitro incubation was similar to that of the other sperm preparation methods (Table III).

**Discussion**

Out of the three sperm preparation methods used in this study, the highest enrichment in motile, viable and non-apoptotic spermatozoa was observed for the swim-up procedure, followed by discontinuous density gradient centrifugation and simple sperm washing from the seminal plasma. These data are
in agreement with previous studies demonstrating a high efficacy of the swim-up technique in terms of elimination of non-viable and apoptotic spermatozoa (Aitken and Clarkson, 1988; Ng et al., 1992; Zini et al., 2000; Sakkas et al., 2000; Younglai et al., 2001). The percentage of spermatozoa stained supravitally with eosin tended to be higher than that of spermatozoa stained with propidium iodide in all groups. This observation may be explained by the fact that the former stain is cytoplasmic and the latter nuclear. Supravital staining with cytoplasmic stains only requires permeability of the plasma membrane for the stain, whereas a nuclear stain needs to traverse the nuclear envelope. Thus, spermatozoa may stain supravitally with eosin at an early stage of necrosis when the propidium iodide still cannot gain access to the nucleus.

Irrespective of the preparation technique used, the percentage of motile and viable spermatozoa remained unchanged during the first 4 h of in vitro incubation, followed by a significant increase in the percentage of immotile and non-viable spermatozoa observed in all groups after 24 h of incubation. This increase was roughly proportional to the initial incidence of immotile and non-viable spermatozoa in individual groups. However, the overall increase in the percentage of immotile and non-viable spermatozoa was not accompanied by any detectable increase in the population of spermatozoa showing phosphatidyserine externalization and DNA fragmentation, two distinctive signs of an active apoptotic process. Thus, these data suggest that sperm cell death during incubation at 37 °C occurs by necrosis rather than by apoptosis.

Several authors have described the presence of apoptotic markers (phosphatidyserine externalization, DNA fragmentation and Fas expression) in spermatozoa from men with normal and deranged spermatogenesis (Sakkas et al., 1999a; Gandini et al., 2000; Oosterhuis et al., 2000; Tesarik et al., 2001; Ricci et al., 2002). However, it is not clear whether ejaculated spermatozoa still retain the ability to activate the apoptotic signalling cascade or whether the apoptotic markers detected in ejaculated spermatozoa are merely relics of an abortive apoptotic process started before ejaculation (Sakkas et al., 1999a). The abortive outcome of the apoptotic process may be related to a premature release of spermatids at early stages of apoptosis from Sertoli cells which normally should actively participate in the elimination of such cells. The present data strongly suggest that the presence of apoptotic markers in human ejaculated spermatozoa is a consequence of processes.

### Table I.

<table>
<thead>
<tr>
<th>Time of incubation (h)</th>
<th>% Sperm cells</th>
<th>Immotile</th>
<th>Eosin-positive</th>
<th>Propidium-positive</th>
<th>Annexin-positive</th>
<th>TUNEL positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>37.6 ± 3.8a</td>
<td>34.7 ± 3.0a</td>
<td>21.6 ± 3.0a</td>
<td>11.6 ± 1.8a</td>
<td>12.5 ± 2.2a</td>
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<td></td>
<td>41.2 ± 4.2ab</td>
<td>38.3 ± 4.0ab</td>
<td>26.7 ± 3.2ab</td>
<td>10.8 ± 1.9a</td>
<td>11.7 ± 2.0a</td>
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<tr>
<td>24</td>
<td></td>
<td>51.8 ± 4.0b</td>
<td>44.3 ± 3.3b</td>
<td>35.0 ± 3.7b</td>
<td>8.0 ± 1.7a</td>
<td>8.6 ± 1.7a</td>
</tr>
</tbody>
</table>

abValues which do not share a common superscript within each column are significantly different from each other (P < 0.05).

### Table II.

<table>
<thead>
<tr>
<th>Time of incubation (h)</th>
<th>% Sperm cells</th>
<th>Immotile</th>
<th>Eosin-positive</th>
<th>Propidium-positive</th>
<th>Annexin-positive</th>
<th>TUNEL-positive</th>
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<tr>
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<td>14.4 ± 2.2b</td>
<td>18.3 ± 1.8a</td>
<td>12.1 ± 2.0a</td>
<td>7.2 ± 1.2a</td>
<td>7.6 ± 1.1a</td>
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<tr>
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<td></td>
<td>19.4 ± 2.9ab</td>
<td>20.8 ± 2.2ab</td>
<td>13.8 ± 2.0a</td>
<td>7.1 ± 1.3a</td>
<td>7.6 ± 1.2b</td>
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<tr>
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<td></td>
<td>35.1 ± 3.0b</td>
<td>28.5 ± 2.9b</td>
<td>24.2 ± 2.8b</td>
<td>4.6 ± 1.5a</td>
<td>5.9 ± 1.6o</td>
</tr>
</tbody>
</table>

abValues which do not share a common superscript within each column are significantly different from each other (P < 0.05).

### Table III.

<table>
<thead>
<tr>
<th>Time of incubation (h)</th>
<th>% Sperm cells</th>
<th>Immotile</th>
<th>Eosin-positive</th>
<th>Propidium-positive</th>
<th>Annexin-positive</th>
<th>TUNEL-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>4.6 ± 1.7a</td>
<td>10.8 ± 1.9a</td>
<td>7.8 ± 1.8a</td>
<td>1.2 ± 0.6a</td>
<td>1.7 ± 0.8a</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>8.1 ± 1.8a</td>
<td>12.1 ± 2.0a</td>
<td>11.4 ± 1.7a</td>
<td>0.8 ± 0.7a</td>
<td>1.2 ± 0.7a</td>
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<tr>
<td>24</td>
<td></td>
<td>24.5 ± 2.7b</td>
<td>23.3 ± 2.8b</td>
<td>21.6 ± 2.6b</td>
<td>0.7 ± 0.6a</td>
<td>0.9 ± 0.7a</td>
</tr>
</tbody>
</table>

abValues with different superscripts within each column are significantly different from each other (P < 0.05).
started before ejaculation and that the death of healthy ejaculated spermatozoa occurs by necrosis rather than by apoptosis.

In fact, if the increase in the percentage of spermatozoa supravitally stained with propidium iodide and eosin, observed after 24 h of in vitro incubation, resulted from an apoptotic process started in healthy ejaculated spermatozoa at the beginning of incubation, an increase in the percentage of spermatozoa with externalized phosphatidylserine would be expected to be detectable at least at the 4 h time point. In agreement with the present findings, the presence of active caspases, which are mediators of the classical apoptotic process in most cell types, was only rarely detected in human post-meiotic germ cells, and the presence of caspase activity did not correlate with DNA fragmentation (Tesarik et al., 2002).

The association of apoptotic markers with the death of human spermatozoa, observed by different authors (Sakkas et al., 1999a,b; Blanc-Layrac et al., 2000; Ramos and Wetzel's, 2001), may be explained by an increased sensitivity of those spermatozoa whose apoptosis has been started in a late phase of spermatogenesis and interrupted after spermatization to the effects of external damaging agents. This may be due to membrane or mitochondrial damage caused by the incipient apoptotic process. In this study, however, all sperm samples came from sperm donors with normal basic sperm parameters in whom the occurrence of aborted apoptosis in ejaculated spermatozoa can be expected to be low. This may explain why simple necrosis, rather than atypical apoptosis, was responsible for cell death of most of the spermatozoa that did not survive during the 24 h incubation period.

In conclusion, these data strongly suggest that ejaculated spermatozoa lack the capacity of initiating the apoptotic pathway of cell death, and their demise occurs mainly by necrosis. Because sperm cell necrosis is accompanied by the loss of motility, there appears to be no significant increase in the risk of mistaking dying spermatozoa for viable ones after prolonged in vitro incubation periods. Consequently, sperm in vitro incubation for up to 24 h is unlikely to compromise outcomes of assisted reproduction using ICSI, at least when normozoospermic sperm samples are used. The death patterns of ejaculated spermatozoa from men with deranged spermatogenesis remain to be evaluated.

References


Submitted on August 26, 2003; resubmitted on October 20, 2003; accepted on November 18, 2003