—Full Paper—

Relationship between Caspase Activity and Apoptotic Markers in Human Sperm in Response to Hydrogen Peroxide and Progesterone

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Abstract. Apoptosis plays an essential role in normal spermatogenesis, but deregulations of this biological process, which is closely associated with male infertility, have been found. Whereas calcium homeostasis is a key regulator of cell survival, sustained elevation of intracellular calcium plays a role in apoptosis. The aim of this research was to determine the role of two different calcium mobilizing agents, hydrogen peroxide (H$_2$O$_2$) and the physiological agonist progesterone, on the apoptosis process of human ejaculated spermatozoa. Translocation of membrane phosphatidylserine was examined with an annexin V binding assay, DNA damage was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL assay) and caspase-3 activity was assessed using a fluorometric assay. After incubation of spermatozoa for 1 h with either 10 μM H$_2$O$_2$ or 20 μM of progesterone, there was a significant increase in both caspase-3 activity and the percentage of annexin V-positive cells. Similarly, the TUNEL results were significantly higher 1 h after incubation with either 10 μM H$_2$O$_2$ or 20 μM of progesterone. In fact, progesterone-treated cells showed a three-fold increase (from 17.6 to 52.9%) of TUNEL-positive cells compared to untreated cells, while H$_2$O$_2$-treated cells exhibited a two-fold increase (from 17.6 to 37.9%). In sum, our results suggest that spermatozoa treated with calcium mobilizing agents, such as H$_2$O$_2$ and progesterone, seem to undergo an apoptosis process that is dependent on caspase-3 activation.

Key words: Apoptosis, Caspases, Phosphatidylserine exposure, Spermatozoa, TUNEL

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by TUNEL assay) has been correlated with oxidative stress (endogenous generation or exogenous stimulus) and with impaired sperm functional competence, including poor fertilization rates using in vitro fertilization [23–26].

Finally, it is well-known that one of the earliest and most consistently observed features of apoptosis is the activation of a series of cytosolic cysteine proteases called caspases [27], which cleave multiple protein substrates en masse, leading to loss of cellular structure and function and ultimately resulting in cell death [28]. In particular, caspase-3, caspase-8 and caspase-9 play relevant roles in apoptosis: caspase-9 in the mitochondrial pathway, caspase-8 in Fas/CD95 pathway, and caspase-3 an executioner caspase activated by multiple pathways more downstream [29]. On the other hand, it has been reported that calcium ion, a key regulator of cell survival, also plays an important role in cell death [30] since sustained elevation of intracellular calcium produces a calcium overload in mitochondria and may subsequently induce apoptosis by both stimulating release of apoptosis-promoting factors from the mitochondrial intermembrane space into the cytoplasm and impairing mitochondrial function [31]. Interestingly, it has been very recently reported that the agonist progesterone [32] and H2O2 induced an increase in the cytosolic free-calcium concentration ([Ca2+]c) by depletion of intracellular calcium stores and mitochondrial apoptosis in human spermatozoa, which was strongly dependent on caspase-3 and caspase-9 activation [33].

The aim of this study was to determine the role of two different calcium mobilizing agents, hydrogen peroxide (H2O2) and the physiological agonist progesterone, on the apoptosis process of human ejaculated spermatozoa as well as to analyze the relationship between caspase activity with both early (phosphatidylserine externalization) and late (DNA fragmentation assessed by TUNEL assay) apoptotic markers in spermatozoa from infertile patients.

Materials and Methods

Reagents

Progesterone, H2O2, CHAPS, N-acetyl-Asp-Glu-Val-Asp-7-amido-4-ethylcoumarin (AC-DEVD-AMC), dithiothreitol (DTT) and RPMI-1640 medium were obtained from Sigma (Spain). N-acetyl-Leu-Glu-His-Asp-7-amido-4-fluoromethylketone (z-LEHD-FMK) was obtained from Bachem (Germany). An in situ cell death detection kit, fluorescein, was obtained from Boehringer Mannheim (Indianapolis, IN, USA).

Subjects

Human semen was obtained from infertile patients at the Extremadura Centre of Assisted Reproduction (Badajoz, Spain), as approved by a local committee, the institutional review board of the University of Extremadura and the ethics committee of Infantile Hospital (Badajoz, Spain) in accordance with the Declaration of Helsinki.

Semen was obtained from forty-five men (20–50 years old) being counseled for infertility and who were undergoing evaluation at our andrology laboratory. The patients suffered from primary infertility of at least 1 year in duration. Each subject was ascertained to be in good health by means of their medical histories and a clinical examination including routine laboratory test and screening. The subjects were all non smokers, were not using any medication and abstained from alcohol. Informed consent was obtained from all the participants.

Sperm preparation

Samples were collected by masturbation after 4–5 days of sexual abstinence and allowed to liquefy for 30 min at room temperature. Routine seminal parameters were evaluated according to the World Health Organization criteria [34]. The variables taken into consideration were ejaculated volume; spermatozoa concentration > 2 × 107 cells/ml, in order to be able to perform multiple assays in the same sample (patients with total concentrations of motile sperm < 2 × 107 cells/ml in a liquefied sample were excluded from the study); total spermatozoa number > 4 × 107 cells; motility (grades a+b, where a and b indicate rapid and slow progressively motile spermatozoa, respectively), which was analyzed using a computer-assisted semen analysis (CASA) system; round cells (ratio of spermatozoa to leukocytes > 100:1); vitality; and morphology based on strict criteria (normal morphology > 15%) (see Table 1).

Semen was washed twice in RPMI medium (250 × g, 10 min), the supernatant was discarded and the pellet was resuspended in Na-HEPES solution containing 140 mM NaCl, 4.7 mM KCl, 1.1 mM CaCl2, 10 mM glucose and 10 mM HEPES, (pH adjusted to 7.4 with NaOH).

The samples were divided in two fractions. The first fraction of sperm was assayed for caspase activity after incubation with 10 μM H2O2 and 20 μM of progesterone for 60 min, and the second one

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patients’ values (mean ± SEM)</th>
<th>WHO criteria (range)</th>
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</thead>
<tbody>
<tr>
<td>Age</td>
<td>33.11 ± 6.15</td>
<td>–</td>
</tr>
<tr>
<td>Volume</td>
<td>2.93 ± 1.70</td>
<td>–</td>
</tr>
<tr>
<td>Concentration (cell/mL)</td>
<td>12.95 ± 8.69 × 10^7</td>
<td>5.75–16 × 10^7</td>
</tr>
<tr>
<td>Motility A (%)</td>
<td>36.70 ± 6.92</td>
<td>27–51</td>
</tr>
<tr>
<td>Motility B (%)</td>
<td>22.76 ± 9.28</td>
<td>8–40</td>
</tr>
<tr>
<td>Motility A+B (%)</td>
<td>60.23 ± 10.95</td>
<td>40–70</td>
</tr>
<tr>
<td>Round cells (%)</td>
<td>1.51 ± 0.50</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Vitality (%)</td>
<td>76.52 ± 9.48</td>
<td>60–88</td>
</tr>
<tr>
<td>Normal morphology range (%)</td>
<td>16.41 ± 2.09</td>
<td>14–21</td>
</tr>
</tbody>
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was evaluated using a TUNEL assay after the same treatments. These doses and incubation time were chosen because it has been previously demonstrated by our research group that they are able to induce calcium release from intracellular stores [32, 33] and apoptosis, which is due to mitochondria being subjected to an excessive calcium load [33].

**Caspase activity assay**

To determine caspase activity, stimulated and resting cells were sonicated, and cell lysates were incubated with 2 ml of substrate solution (20 mM HEPES, pH 7.4; 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 8.25 μM of caspase substrate) for 1 h at 37 C, as described in elsewhere [33]. Substrate clearance was measured by using a fluorescence spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preliminary experiments showed that caspase-3 substrate clearing was not detected in the presence of an inhibitor of caspase-3, DEVD-CMK. The activity of caspase-3 was calculated from the cleavage of its respective specific fluoregenic substrate, AC-DEVD-AMC. The data were calculated as fluorescence units per milligrams of protein and were presented as percentages above the control (untreated samples).

**Determination of phosphatidylserine externalization**

The phosphatidylserine externalization of resting and stimulated cells was determined according to a procedure published elsewhere [35]. Briefly, cells were stimulated in HEPES-buffered saline, and samples of cell suspensions (500 μl) were transferred to 500 μl of ice-cold 1% (wt/vol) glutaraldehyde in phosphate-buffered saline for 10 min. Cells were then incubated for 10 minutes with annexin V-fluorescein isothiocyanate conjugate (0.6 μg/ml) in phosphate-buffered saline that was supplemented with 0.5% (w/v) bovine serum albumin and 2 mM CaCl2.

After incubation, the cells were collected by centrifugation for 60 sec at 10,000 g and were resuspended in phosphate-buffered saline. Cell staining was measured by using a Hitachi spectrofluorimeter. Samples were excited at 488 nm, and emission was recorded at 516 nm. The data were calculated as fluorescence per milligram protein and presented as percentages above the control (untreated samples).

**TUNEL assay**

DNA damage can be detected by TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) assay, as previously described [36]. Thus, DNA fragmentation was measured using an *in situ* cell death detection kit, fluorescein, using fluorescein-dUTP as a label according to the manufacturer's instructions.

Semen samples were washed, the supernatant was then discarded and the cell pellet was finally resuspended in Na-HEPES solution. Each sample was divided into three fractions. One fraction, which contained untreated spermatozoa, was considered as the control slide, and the other two fractions, which were treated with 10 μM H2O2 and 20 μM of progesterone for 60 min, respectively, were considered the treated slides.

The three slides were air-dried for 24 h. Afterwards, the air-dried slides were washed in PBS. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were then incubated in the dark at 37°C for 1 h in TUNEL reaction mixture containing 50 μl of the mixture of terminal deoxynucleotidyl transferase and dUTP. At least 100 cells were randomly analyzed per slide in five fields. Spermatozoa were first identified in the field using phase contrast microscopy and then analyzed using fluorescence microscopy. Each cell was categorized as apoptotic (intense green nuclear fluorescence) or normal (no fluorescence).

**Statistical analysis**

Data were expressed as means ± SEM of the number of determinations. Analysis of statistical significance was performed by using the Student’s *t*-test. One-way analysis of variance followed by Tukey’s *t*-test was used for multiple comparisons. *P*<0.05 was considered a statistically significant difference.

**Results**

**H2O2- and progesterone-induced DNA damage**

It has been previously reported that the reactive oxygen species H2O2 and the physiological agonist progesterone induce calcium release from intracellular stores [32, 33] in human spermatozoa, which is related to the apoptotic process. The TUNEL assay is a well-established method for detection of DNA cleavage, a relatively late apoptotic marker [36]. In addition, this technique has been widely used to determine DNA damage in human sperm [37, 38]. As shown in Figs. 1A and 1B, treatment of spermatozoa from infertile men with both 10 μM H2O2 and 20 μM of progesterone for 60 min leads to DNA fragmentation, which could be observed as intense green fluorescence in the nuclear region, indicating that spermatozoa might be undergoing the late stages of apoptosis.

Furthermore, we found that a relatively high proportion of untreated spermatozoa (control cells) had DNA damage (17.6±1.4%; Fig. 2). However, when control cells were exposed to treatment with 10 μM H2O2, a significantly higher percentage of TUNEL-positive cells was found after 60 min of incubation (37.9±2.5% above the untreated cells; *P*<0.05), which represents a two-fold increase above the control (see Fig. 2). Similarly, when control cells were stimulated with 20 μM of progesterone, a three-fold increase of TUNEL-positive cells took place after 60 min of incubation (from 17.6 ± 1.4 to 52.9 ± 2.4%; *P*<0.05) (see Fig. 2). Interestingly, the percentage of TUNEL-positive cells induced by progesterone was significantly greater (*P*<0.05) than those induced by H2O2.

**Phosphatidylserine externalization and caspase-3 activation in human spermatozoa**

To further investigate the relationship of DNA fragmentation with the mechanism of apoptosis, we checked the plasma membrane translocation of phosphatidylserine residues, which reflects a relatively early apoptotic stage. As shown in Fig. 3, treatment of spermatozoa with 10 μM H2O2 for 60 min induced a significant increase in phosphatidylserine externalization (22.4 ± 4.2% above the control cells; *P*<0.05). Moreover, when spermatozoa from
infertile patients were treated with 20 μM of progesterone for 60 min, a statistically significant increase was found in the percentage of annexin V-positive cells (12.9 ± 3.7% above the control cells; P<0.05; see Fig. 3). Although the percentage of annexin V-positive cells was significantly higher (P<0.05) in the H2O2-treated spermatozoa than in the progesterone-treated spermatozoa, the results of annexin V staining demonstrated a similar proportion of cells depicting membrane phospholipid externalization in both spermatozoa subsets.

Likewise, to examine the effect of H2O2 on caspase-3 activation, human spermatozoa were treated with 10 μM H2O2 for 60 min. As shown in Fig. 4, the treatment produced significant activation of caspase-3 (20.0 ± 5.3% above the control cells; P<0.05). Similarly, when we incubated the cells with 20 μM of progesterone for 60 min, caspase-3 activation was also significantly increased (33.5 ± 7.2% above the control cells; P<0.05; Fig. 4). In this case, caspase-3 activation was significantly higher (P<0.05) in progesterone-treated spermatozoa than in H2O2-treated spermatozoa.

It is worth noting that the spontaneous (without adding any apoptotic inducer) phosphatidylserine externalization and caspase-3 activation after 60 min were 98.3 ± 4.1 and 93.5 ± 7.2% compared with the phosphatidylserine and caspase-3 activity at the beginning of incubation (time=0; data not shown). Additionally, it appears that there was a positive correlation between late apoptotic markers

Fig. 1. TUNEL assay results observed under fluorescence microscopy. Panels A and B show TUNEL-positive cells after stimulation with 10 μM H2O2 or 20 μM progesterone (PROG) for 60 min, respectively. DNA fragmentation assessed by TUNEL assay was determined as described in Materials and Methods. A spermatozoon with DNA fragmentation shows intense green fluorescence in the nuclear region, whereas normal cells showed no green fluorescence.

Fig. 2. H2O2 and progesterone (PROG) induce DNA fragmentation in human spermatozoa. Cells were stimulated for 60 min with 10 μM H2O2 or 20 μM progesterone, and then DNA fragmentation (assessed by TUNEL assay) was determined as described in Materials and Methods. Values are presented as means ± SEM of seven independent experiments and expressed as percentages of TUNEL-positive cells. *P<0.05 compared to control values. □P<0.05.

Fig. 3. Annexin-V binding assay results after the treatments with H2O2 and progesterone (PROG). Cells were stimulated for 60 minutes with 10 μM H2O2 or 20 μM progesterone, and then phosphatidylserine (PS) exposure was determined as described in Materials and Methods. Values are presented as means ± SEM of five to eight separate experiments and expressed as percentages above the control (untreated samples). *P<0.05 compared to control values. □P<0.05.
Moreover, it has been recently displayed that H2O2 and the physiologically-related apoptotic markers in both fertile donors and infertile patients [40]. Some somatic cell apoptosis stimuli and display some somatic cell apoptosis markers in ejaculated sperm, which leads to the apoptotic process owing to calcium overloading in mitochondria [33]. In other words, agents that are able to produce alterations in calcium homeostasis or simply sustain increases in [Ca2+]c may one of the mechanisms that lead to an apoptotic process in human spermatozoa.

Discussion

Apoptosis is an important process involved in normal spermatogenesis [39]. However, deregulations of this biological process involve abnormalities in the production of male gametes and male infertility. In recent years, much attention has been given to the role of apoptosis in ejaculated sperm. In fact, a previous report has shown that ejaculated sperm exhibit several apoptosis-related proteins, express caspase catalytic activity that could be elevated by some somatic cell apoptosis stimuli and display some somatic cell apoptosis markers in both fertile donors and infertile patients [40]. Moreover, it has been recently displayed that H2O2 and the physiologically-related apoptotic stimuli progesterone increase the cytosolic free-calcium concentration due to calcium release from intracellular stores in human spermatozoa, which leads to the apoptotic process owing to calcium overloading in mitochondria [33].

In the present study, we mainly analyzed the changes that H2O2 or progesterone caused in mature, ejaculated sperm cells from infertile men at the membrane and nuclear levels, and we then checked the relationship between such apoptosis markers and caspase-3 activity. Thus, treatment of ejaculated sperm with either H2O2 or progesterone for 60 min resulted in increased DNA fragmentation (as depicted by TUNEL), confirming the results of Lopes et al. [25] and Wang et al. [41], but in contrast to those presented by Hughes et al. [42]. Therefore, some of these spermatozoa might be undergoing late stages of apoptosis. Nevertheless, detection of fragmented DNA using a TUNEL assay was assessed by TUNEL was <4% in the majority of the samples [24]. Moreover, Oosterhuis et al. concluded that 20% of spermatozoa showed DNA fragmentation using TUNEL in ejaculated sperm from infertile men [17]. Similarly, Barroso et al. found that nuclear damage (TUNEL assay) was present in approximately 11% of the sperm cells from infertile patients [15]. The differences in the proportion of sperm with DNA damage among all these studies may be due to the sperm samples analyzed (donors versus different patient populations), the various sperm separation methods and the different methods used to detect DNA fragmentation.

On the other hand, spermatozoa have high levels of polyunsaturated fatty acids in their membranes, which are responsible for fluidity and, therefore, the motility of the sperm. The presence of free radicals causes changes in the distribution of the phosphatidylserine of the membrane, which can be measured with the Annexin V-binding assay [44]. In this way, we have established that treatment with either H2O2 or progesterone for 60 min also induced staining with annexin V in ejaculated sperm from infertile patients, confirming the results obtained in previous studies [37, 40]. Additionally, we previously demonstrated that translocation of phosphatidylserine induced by H2O2 or progesterone is an event dependent on caspase activation because H2O2- and progesterone-induced phosphatidylserine exposure was abolished by pretreatment with z-LEHD-FMK, a specific caspase-9 inhibitor [33]. Moreover, other investigators have shown evidence that phosphatidylserine exposure in human sperm is characteristic of apoptosis [45]. Therefore, it seems that some of these spermatozoa might be undergoing the early stages of apoptosis. Conversely, we cannot rule out the possibility that phosphatidylserine exposure is involved in capacitation of human spermatozoa and plays a physiological role in sperm, being necessary for acquisition of fertilizing ability.

Caspase-dependent apoptosis is a well-characterized and ubiquitous mechanism in eukaryotes for removing senescent, defective or unnecessary somatic cells, but the roles for caspases and apoptosis in ejaculated sperm are still in question. Nonetheless, we substantiate herein that both H2O2 and progesterone provoke caspase-3 activation, as assessed by a fluorometric assay. In this sense, Weng...
et al. showed unequivocal evidence for the presence of inactive and active caspases (including caspase-3) in ejaculated human sperm from both infertile patients and fertile donors by three distinctly different methods, including an in vitro fluorometric assay, immunoblot analysis and immunocytochemistry [11]. In spite of the fact that our findings are consistent with those obtained in previous reports showing caspase-3 activation in response to H$_2$O$_2$ and progesterone by fluorometric assay and immunoblotting [33, 40], there is still some doubts concerning the presence of inactive and active caspases in human sperm samples. In fact, de Vries et al. reported that both forms of caspase-3 are absent in mature sperm from fertile donors [46]. However, it seems that caspases (including caspase-3) are present in a restricted site for apoptosis (cytoplasmatic droplets) in spermatids and immature spermatozoa (cytoplasmatic droplets) in spermatids and immature spermatozoa [47, 48], which contribute to poor sperm quality in samples obtained from infertile men.

Furthermore, we previously found that H$_2$O$_2$- and progesterone-evoked caspase-3 activation is dependent on caspase-9 activation, as proved by pretreatment with a specific caspase-9 inhibitor, z-LEHD-FMK [33]. Curiously, we observed that treatments with both H$_2$O$_2$ and progesterone induced relatively low levels of both caspase enzyme activity and phosphatidylserine exposure compared with the untreated controls, which is consistent with the results reported in previous studies [40]. This might be because the caspase activity and membrane damage were already elevated in the infertile patients’ ejaculates and/or perhaps due to the fact that such sperm contain more defective machinery, making them less susceptible to the induction.

In summary, our findings demonstrate that two calcium mobilizing agents, H$_2$O$_2$ and the physiological agonist progesterone, stimulate two well-known somatic cell caspase-dependent events, annexin V-binding to phosphatidylserine on the outer leaflet of the membrane, a relatively early apoptotic event, and DNA fragmentation, a relatively late apoptotic event. Additionally, our results show that either H$_2$O$_2$ or progesterone also increase caspase-3 enzyme activity. However, the roles of caspase-dependent apoptosis mechanisms in post- ejaculated human sperm clearly require further analysis.

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References


