

Caspase activation in human spermatozoa in response to physiological and pathological stimuli

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Objective: To investigate caspase activation in response to a variety of pathological and physiological stimuli in light of the fact that current research offers no clear consensus about caspase activation pathways in spermatozoa.

Design: A prospective, controlled study.

Setting: Male infertility clinic, Glickman Urological Institute, Cleveland Clinic Foundation, Cleveland, Ohio.

Patient(s): Fifteen healthy volunteers.

Intervention(s): Spermatozoa were exposed to [1] Fibroblast-associated (Fas) death receptor activation, [2] mitochondrial apoptosis induction using betulinic acid, [3] oxidative stress, and [4] prolonged incubation up to 3 hours without any external stimuli.

Main Outcome Measure(s): Active caspases-1, -3, -8, and -9 were examined in human spermatozoa by flow cytometry using carboxyfluorescein derivatives.

Result(s): Inducing Fas antibody did not result in any caspase activation. Conversely, betulinic acid significantly triggered caspase-9 and -3 activation. The application of oxidative stress and prolonged incubation (3 hours) failed to result in caspase activation.

Conclusion(s): These results suggest that Fas has no functional relevance in mediating caspase activation in human ejaculated spermatozoa. Although spermatozoal mitochondria are highly susceptible to specific agonists of apoptosis such as betulinic acid via caspase activation, oxidative stress-induced apoptosis appears to be caspase independent. (*Fertil Steril*® 2005;83(Suppl 1):1106–12. ©2005 by American Society for Reproductive Medicine.)

Key Words: Apoptosis pathways, caspase activation, ejaculated spermatozoa, oxidative stress

Apoptosis induces a series of cellular, morphological, and biochemical alterations that lead to cell death (1). Certain features are characteristic of cells undergoing apoptosis, including nuclear fragmentation, mitochondrial swelling, and externalization of phosphatidylserine at the plasma membrane. These features have been identified in ejaculated spermatozoa as well as intratesticular germ cells (2–4). Results from multiple animal (5–8) and human studies (9–13) indicate that apoptosis plays a major role in the pathogenesis of male factor infertility (14).

A central component of the apoptotic machinery involves a family of aspartic acid-directed cysteine proteases called caspases (cysteiny aspartate-specific proteinases) (15). Once activated, caspases transduce a signal to effector caspases, leading to the degradation of cellular substrates (16). Activation via membrane receptors (type I or extrinsic apoptosis) represents one of the major pathways for caspase activation

(17). Specific ligands are required for the transmission of the death signal (18).

Fibroblast-associated ligand (FasL) is a transmembrane protein. Upon engagement to Fas, an intrinsic program of apoptotic death is stimulated, which leads to the activation of caspase-8 (19). In turn, caspase-8 may initiate terminal apoptosis by directly activating caspase-3, the most efficient effector caspase. However, in type II or intrinsic apoptosis, the caspase-8-derived signals can also be amplified by mitochondrial signaling, resulting in activation of caspase-9. Caspase-3 becomes activated after forming the so-called apoptosome (20).

Because mitochondria are extremely susceptible to specific apoptotic agonists, researchers are beginning to use these organelles as novel targets for anticancer treatments (21). Betulinic acid, a proapoptotic signal-transducing molecule that acts on mitochondria, has been used to induce apoptosis in brain tumors and melanoma (22, 23). Another pathological phenomenon, oxidative stress, has been associated with the activation of caspase-9, disruption of the transmembrane mitochondrial potential, and the release of other regulating proteins (24, 25). Interestingly, reactive oxygen species (ROS)-mediated apoptosis induced by hydrogen peroxide in somatic cells is Fas independent and requires the

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release of mitochondria-derived ROS and the activation of nuclear factor-kappaB (NF- κ B) and caspase-3 (26).

Current research offers no clear consensus about what pathways are activated in ejaculated sperm and whether caspase can be autoactivated in the absence of specific stimuli (27–29). Because caspases interact with each other and connect different signaling systems, identifying the dynamics of caspase activation may lead to a better understanding of the many molecular aspects of male factor infertility. In addition, future modification(s) of caspase regulation may help direct the apoptotic machinery for therapeutic benefits.

The objective of this study was to investigate caspase activation in response to the following pathological and physiological stimuli: [1] Fas death receptor activation, [2] specific apoptosis induction at the mitochondrial level using betulinic acid, [3] oxidative stress resulting from hypochlorous acid, and [4] prolonged incubation without specific stimuli to monitor autoactivation of caspases.

MATERIALS AND METHODS

Selection Criteria of Semen Samples

After Institutional Review Board approval, a total of 15 ejaculates from 15 healthy donors were collected after a period of sexual abstinence of 2–3 days. After liquefaction of the ejaculates, an aliquot from each sample was examined for sperm concentration and motility according to the World Health Organization standard guidelines (30). To ensure that an adequate number of spermatozoa were available for all evaluations, only samples with a sperm concentration $>50 \times 10^6$ /mL were used in our study. Samples containing $>1 \times 10^6$ round cells/mL (verified by Endtz staining) were excluded to avoid a potential source of ROS generation by leukocytes.

Sperm Preparation and Exposure to Pathological and Physiological Stimuli

Unprocessed (neat) semen samples were washed in phosphate-buffered saline (PBS) by centrifugation at 400 *g* for 5 minutes. The supernatant was discarded, and the pellet was diluted in 1 mL PBS; aliquots were created for the following experiments:

Induction of Receptor-Mediated Apoptosis (Type I Apoptosis). Fibroblast-associated-receptor (CD95/Fas)–mediated (type I/extrinsic) apoptosis was induced using anti-Fas antibody (clone APO-1-3, Kamiya Biomedica, Seattle, WA) according to the manufacturer's instructions. Briefly, 5 μ g of the antibody diluted in 1 mL PBS was added to the sperm pellet, gently vortexed, and incubated for 1 hour at room temperature. In addition, an aliquot from each semen sample was diluted in 1 mL PBS and incubated for 1 hour at room temperature. This second aliquot served as a negative control. After incubation, the samples were centrifuged at 400 *g* for 5 minutes and resuspended in 100 μ L PBS to detect caspase activation.

Induction of Mitochondria-Derived Apoptosis (Type II Apoptosis). Betulinic acid (BA; Alexis, Gruenberg, Germany) was used to induce mitochondria-derived (type II/intrinsic) apoptosis. Previous studies from our group found that betulinic acid (60 μ g/mL) significantly reduces sperm motility (unpublished data). Spermatozoa were incubated with 1 mL BA solution (60 μ g/mL) for 10 minutes at room temperature. A semen sample diluted in 1 mL PBS and incubated under identical conditions served as a negative control. The samples were centrifuged at 400 *g* for 5 minutes, resuspended in 100 μ L PBS, and prepared for caspase detection.

Generation of Oxidative Stress by Hypochlorous Acid. Sodium hypochlorite (Sigma-Aldrich, St. Louis, MO) was dissolved in PBS to produce hypochlorous acid (HOCl). It was added to the designated sperm pellets at a concentration of 5×10^{-5} M, and the samples were incubated for 1 hour at room temperature. The reaction was terminated by adding 10 μ L taurine (Sigma-Aldrich) to remove all traces of HOCl (31). The aliquots incubated with PBS only served as the negative controls. All aliquots were centrifuged at 400 *g* for 5 minutes, resuspended in 100 μ L PBS, and examined for caspase activation.

Prolonged Incubation for Autoactivation of Caspases. Autoactivation of the caspase cascade without any external stimuli was examined by incubating all controls containing PBS for 10 minutes and 1 and 3 hours.

Detection and Evaluation of Activated Caspases

Levels of activated caspases-8, -9, -1, and -3 were detected in viable spermatozoa using fluorescein-labeled inhibitors of caspases (FLICA). These inhibitors are cell permeable and noncytotoxic and bind covalently to active caspases (32). The fluorogenic substrate becomes fluorescent upon cleavage by the caspases (33). The inhibitors were used with the appropriate controls according to the kit instructions provided by the manufacturers (CaspTag, Intergen Company, Purchase, NY; and FAMFLICA, Immunochemistry Technologies, Bloomington, MN).

A 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide (DMSO). It was further diluted in PBS to yield a 30-fold working solution. All test aliquots and controls (with 100 μ L PBS) were incubated at 37°C for 1 hour with 10 μ L of the working solution and subsequently washed with the rinse buffer.

Granulocytes separated from whole blood samples by density gradient centrifugation and incubated with betulinic acid (60 μ g/mL) for 10 minutes served as positive controls for caspase activation.

Flow Cytometric Analysis of Activated Caspases

Levels of activated caspase-8, -9, -1, and -3 were evaluated using flow cytometric analyses (fluorescence-activated cell

TABLE 1**Percentage of activated caspases in controls and after stimulation with anti-CD95.**

| | Control | Anti-CD95 stimulation |
|-----------|-------------|--------------------------|
| Caspase-8 | 37.8 ± 15.6 | 36.7 ± 17.3 ^a |
| Caspase-9 | 27.7 ± 6.4 | 36.7 ± 17.3 ^a |
| Caspase-1 | 36.8 ± 13.7 | 37.3 ± 15.5 ^a |
| Caspase-3 | 33.3 ± 15.0 | 35.4 ± 13.2 ^a |

Note: Values are expressed as mean ± SD; $P < .05$ was significant by Student's *t*-test compared with the controls.

^a Not significant.

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sorting, Becton Dickinson, San Jose, CA). A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. An argon laser at 15 mW was used, and green fluorescence (480–530 nm) was measured in the FHL1 channel. Both the percentage of positive cells and the mean fluorescence were calculated on a 1,023-channel scale using the flow cytometer software CellQuest (version 3.3, BD Biosciences, San Jose, CA).

Statistical Analyses

Evaluation of data in terms of differences was performed using parametric tests (Student's *t*-test and linear correlation) as appropriate for data type and distribution (investigated by the Shapiro-Wilk test). All calculations were performed using the computer program STATISTICA 6.0 (StatSoft, Tulsa, OK). $P < .05$ were considered statistically significant. All values are given as mean ± SD or as differences between the samples and controls.

RESULTS

Impact of CD95/Fas Induction on Caspase Activation

Anti-CD95 treatment did not result in type I apoptosis as no significant increase was seen in the activation of caspase-8 and caspase-3 compared with the control. Similarly, levels of activated caspase-9 and caspase-1 were not altered by CD95 (Fas induction) (Table 1).

Impact of Betulinic Acid on Caspase Activation

Type II apoptosis was induced by betulinic acid at a 60 µg/mL concentration as observed by a significant increase of the percentage of spermatozoa containing activated caspase-9 (43.6% ± 13.4% vs. 25.5% ± 10.7%; $P < .001$) and caspase-3 (45.3% ± 17.8% vs. 26.1% ± 8.2%; $P < .001$). After incubation with betulinic acid, a significant increase was observed in the num-

ber of caspase-8-positive spermatozoa (46.3% ± 16.6% vs. 34.2% ± 16.4%; $P < .05$). Caspase-1 was not significantly activated by betulinic acid (Table 2; Fig. 1).

Impact of Oxidative Stress on Caspase Activation

Oxidative stress induced by incubation with HOCl for 1 hour did not result in a significant increase in activation of the caspase cascade (Table 3). Neither caspase-8 (receptor-mediated) nor caspase-9, -3, and -1 (mitochondria-mediated) pathway were significantly altered.

Autoactivation of Caspase Cascade

A comparison of the varying incubation periods in the controls (10 minutes, 1 hour, and 3 hours) revealed no significant change in levels of caspase-8, -9, -1, and -3 activation. Similarly, no autoactivation of the caspase cascade was seen in the absence of capacitation and external stimuli after the samples were incubated at room temperature in PBS for up to 3 hours.

DISCUSSION

Caspases have been implicated in the pathogenesis of various andrological pathologies such as impaired spermatogenesis, decreased sperm motility, sperm DNA fragmentation, testicular torsion, varicocele, and immunological infertility (13, 28, 34–38). Caspase-dependant apoptosis is a well-characterized mechanism that not only removes senescent, defective cells, but also promotes the subsequent maturation of certain specialized cells such as those found in the lens and myofibroblasts. Therefore, it appears that caspase activation may occur under physiological as well as pathological conditions (39).

Fibroblast-associated receptors have been detected on the surface of intratesticular germ cells (17) as well as in subsets of ejaculated spermatozoa (9). However, their functional impact during spermatogenesis is unclear. Earlier studies

TABLE 2**Percentage of activated caspases in controls and after stimulation with betulinic acid.**

| | Control | Betulinic acid stimulation |
|-----------|-------------|----------------------------|
| Caspase-8 | 34.2 ± 16.4 | 46.3 ± 16.6 ^a |
| Caspase-9 | 25.5 ± 10.7 | 43.6 ± 13.4 ^a |
| Caspase-1 | 39.0 ± 13.5 | 45.0 ± 14.0 |
| Caspase-3 | 26.1 ± 8.2 | 45.3 ± 17.8 ^a |

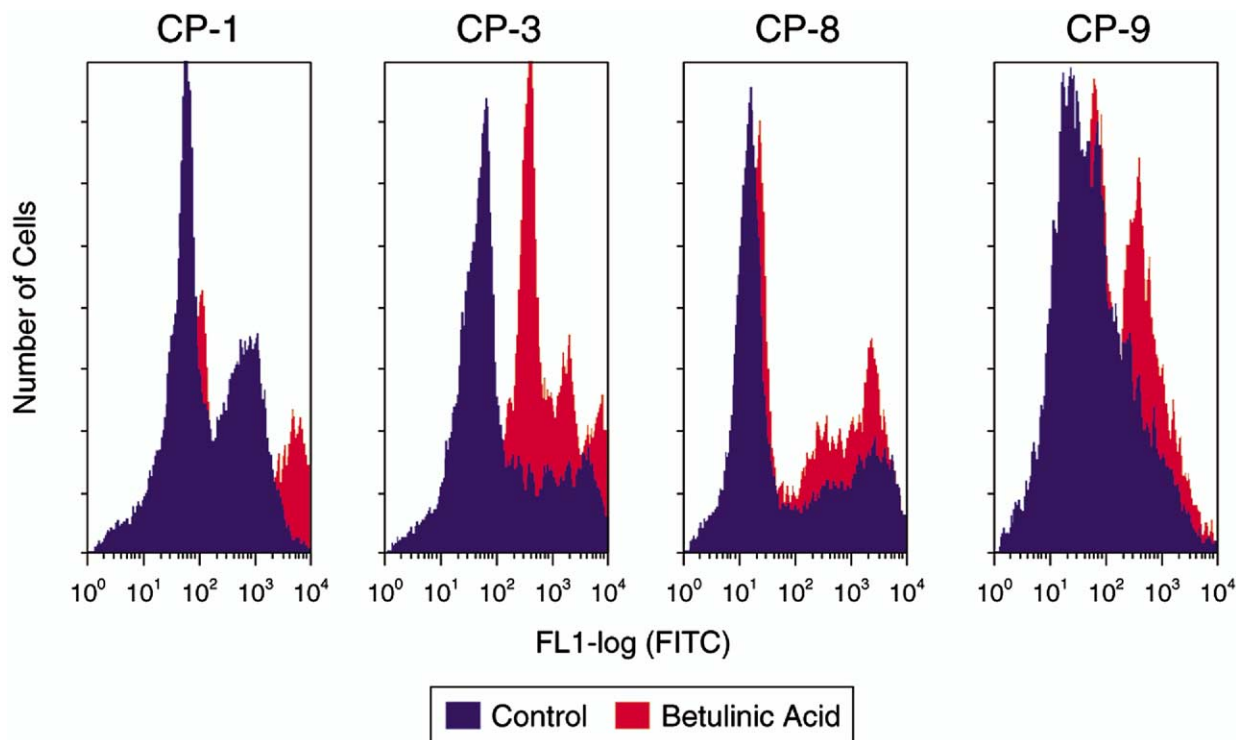
Note: Values are expressed as mean ± SD.

^a $P < .05$ was significant by Student's *t*-test compared with the controls.

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FIGURE 1

Sample histograms of spermatozoa after administration of 60 $\mu\text{g}/\text{mL}$ betulinic acid in comparison with controls stained with the carboxyfluorescein-labeled caspase inhibitors for activated caspases (CP)-1, -3, -8, and -9 (FLICA). The two clearly demarcated cell populations are the caspase-negative (left peak) and caspase-positive (right peak) cells. The X-axis of each histogram depicts the intensity of fluorescence in the green spectrum (FLH-1 channel) on a logarithmic scale. The Y-axis depicts the frequency in terms of the number of cells.



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suggested that the Fas-mediated pathway is a paracrine control mechanism of the Sertoli cell that helps regulate germ cell output during spermatogenesis by inducing type I apoptosis (40, 41). In one study (9), Fas was detected in fewer than 10% of spermatozoa obtained from healthy donors and in more than 10% of spermatozoa from donors with abnormal spermogram parameters. The fact that some ejaculated spermatozoa are Fas-positive indicates that in some men with abnormal semen parameters an “abortive apoptosis” has taken place (9). This in turn implies that some spermatozoa may have escaped a defective apoptotic process. In a recent study, we were able to demonstrate that the presence of Fas-positive spermatozoa increases after cryopreservation, particularly in sperm with altered membranes (42). However, it is important to note that the activation of the Fas/FasL does not exclusively mediate apoptosis (43–45).

In our present study, the initiation of type I apoptosis using an inducing Fas antibody did not significantly increase levels of caspase-3 and -8. Furthermore, it had no effect on caspase-9 and -1 activation. These results suggest that Fas may not have a functional relevance in terms of receptor-

mediated (type I) caspase activation in human ejaculated spermatozoa. Because the presence of Fas is restricted to specific subsets of spermatozoa (42), this finding supports the hypothesis that these cells might have escaped an initially desirable apoptotic process during spermatogenesis. However, since the role of the Fas/FasL system in the testis is unclear, further investigations are needed.

Mitochondria may be especially susceptible to various stimuli such as proapoptotic signals (bcl-2 proteins), cellular stress caused by cryopreservation, oxidative stress, and increased intracellular calcium levels due to the compartmentalization of the midpiece (28). In addition, certain evidence suggests that the classic mitochondria-derived apoptotic signaling cascade is activated in spermatozoa. This evidence includes the presence of caspase-9 and caspase-3 activation as well as the fact that the mitochondrial membrane depolarizes in response to the aforementioned stimuli (24, 25, 28).

In our current study, betulinic acid significantly triggered caspase-9 and caspase-3 activation. Caspase-8 may have been activated to a certain level as a result of caspase “cross

TABLE 3

Percentage of activated caspases in controls and after stimulation with hypochlorous acid (HOCl).

| | Control | HOCl stimulation |
|-----------|-------------|--------------------------|
| Caspase-8 | 37.8 ± 15.6 | 37.4 ± 15.8 ^a |
| Caspase-9 | 27.7 ± 6.4 | 25.2 ± 17.8 ^a |
| Caspase-1 | 36.8 ± 13.7 | 33.6 ± 13.0 ^a |
| Caspase-3 | 33.3 ± 15.0 | 38.8 ± 16.8 ^a |

Note: Values are expressed as mean ± SD; $P < .05$ was significant by Student's *t*-test compared with the controls.

^a Not significant.

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talk." These results suggest that spermatozoal mitochondria are extremely susceptible to specific agonists of apoptosis. Betulinic acid molecules are currently being used experimentally in anticancer treatments, and their side effects on the male reproductive system should be carefully considered.

Oxidative stress has been identified as a crucial factor leading to male factor infertility largely due to DNA fragmentation and the peroxidative damage it causes to the sperm cell membrane (37, 46, 47). Reactive oxygen species can be generated at the mitochondria level during apoptosis (48). Depending on ATP levels, signaling is induced toward apoptosis (high ATP levels) or necrosis (49). HOCl is one of the ROS that may have detrimental effects on spermatozoa. In our study, HOCl did not result in caspase-8 activation, which is also the case in somatic cells (26). In addition, none of the type II pathway caspases or the terminal caspase-3 was activated. Thus, it appears that oxidative stress-induced apoptosis is mainly caspase independent.

Endogenous generation of ROS is significantly and positively correlated with oxidative stress-induced damage to sperm, suggesting that additional mechanisms may be involved in cell damage (50). One of the proposed mechanisms would be the direct action of apoptosis inducing factor (AIF). Exposing mitochondria to ROS results in the release of AIF, which directly interacts with the DNA and leads to DNA fragmentation (50, 51).

Caspase-1 connects the calcium-calpain system via efficient and direct cleavage of its inhibitor calpastatin (52). The cross talk of the caspase-1 signaling system and the calcium-calpain pathway suggests that the caspase system might be involved in the physiological process of capacitation. This proposed interaction is supported by a recent study in which inhibition of calpain but not of caspases prevented collateral DNA damage after inducing experimental testicular torsion (53). Both calpain and caspase cleave a wide variety of cellular proteins in apoptotic and/or necrotic conditions (54). Therefore, inhibition of both signaling systems might be a

novel target of therapeutic interventions aimed at [1] preventing premature activation of spermatozoa by blocking physiological functions for contraceptive purposes or [2] inhibiting collateral damage due to pathologies. The validity of this hypothesis awaits further investigation.

A significant time-dependent increase in the externalization of phosphatidylserine residues has been detected in the spermatozoal membrane (55). However, externalization of phosphatidylserine in spermatozoa does not necessarily reflect apoptotic changes (27, 29).

Our current experiment was designed to evaluate the functional role of caspase activation in human spermatozoa and to study possible mechanisms of activation. Our study may be limited by the lack of positive controls, which was due to the lack of a proven protocol to induce caspase activation in human spermatozoa. Treatment with betulinic acid may possibly serve as a positive control in future experiments. Although our study included only 15 healthy donors, significant results were obtained even in this small sample size.

In conclusion, our results highlight the extreme susceptibility of spermatozoal mitochondria to specific agonists of apoptosis and downplay the functional relevance of the Fas/FasL receptor in mediating caspase activation in human ejaculated spermatozoa. Oxidative stress-induced apoptosis appears to be caspase independent. The importance of caspase activation might be seen in interactions between the caspase cascade and other proteinase systems such as the calpain-calpastatin system.

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