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ORIGINAL ARTICLE

Sperm Biology

Induction of ultra-morphological features of apoptosis in mature and immature sperm

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There is a fundamental body of evidence suggesting that activated apoptosis signaling in ejaculated human sperm negatively influences their fertilization potential. However, it is still controversial whether this apoptotic signaling is a relic of an abortive apoptosis related to spermatogenesis or if it should be regarded as a functional preformed pathway in mature sperm leading to stereotypical morphological changes reflecting nuclear disassembly. To address this question, apoptosis was induced using betulinic acid in mature and immature ejaculated human sperm enriched by density gradient centrifugation. Execution of apoptosis was monitored by observing ultra-morphological changes via transmission electron microscopy. Typical morphological signs of apoptosis in somatic cells include plasma membrane blebbing with the formation of apoptotic bodies, impaired mitochondrial integrity, defects of the nuclear envelope, and nuclear fragmentation; these morphologies have also been observed in human sperm. In addition, these apoptotic characteristics were more frequent in immature sperm compared to mature sperm. Following betulinic acid treatment, apoptosis-related morphological changes were induced in mature sperm from healthy donors. This effect was much less pronounced in immature sperm. Moreover, in both fractions, the betulinic acid treatment increased the percentage of acrosome-reacted sperm. The results of our ultra-morphological study prove the functional competence of apoptosis in mature ejaculated human sperm. The theory of a sole abortive process may be valid only for immature sperm. The induction of the acrosome reaction by stimulating apoptosis might shed light on the biological relevance of sperm apoptosis.

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INTRODUCTION

Apoptosis is a directed series of cellular, morphological, and biochemical alterations that result in cell death without eliciting an inflammatory response. Critical events of the signaling cascade, including activation of caspases, disruption of the mitochondrial transmembrane potential, externalization of phosphatidylserine, and DNA fragmentation, have been detected in ejaculated human sperm.^{1–7} However, the relevance of these events is controversial due to the unique physiology of sperm.^{8–12}

The sperm cell is compartmentalized into the head (with the nucleus and only a narrow margin of cytoplasm), midpiece (containing densely packed mitochondria), and tail. The majority of the cytosolic proteins is attached to the cytoskeleton. Moreover, all mRNAs present in the sperm arise from transcription during spermatogenesis. Translation is not possible in the sperm cytoplasm due to the lack of ribosomes in this cellular compartment.^{13–15} As only a small number of mRNAs at the mitochondrial ribosomes (in polysome complexes) are translated during capacitation,¹⁶ we questioned whether apoptosis is possible under these conditions.

After an initial decline of apoptosis in human sperm,¹⁷ the theory of “abortive apoptosis” was introduced.¹⁰ This theory is based primarily on data of receptor-mediated apoptosis. Due to its low correlation to the sperm DNA fragmentation rate, activation of the apoptotic signaling cascade is regarded as an abortive process during spermatogenesis

that leads to the presence of such “immature” sperm in the ejaculate.¹⁸ Semen specimens of subfertile men contain higher levels of sperm with active apoptosis signaling.^{19,20} Until now, all trials attempting to induce receptor-mediated sperm apoptosis were unsuccessful. Neither incubation with Fas ligand/anti-CD95 specific antibody nor cryopreservation and thawing led to apoptosis induction.^{4,6,21}

However, results of numerous other studies do not conform to the theory of apoptosis as an abortive process during spermatogenesis. In contrast to the receptor-mediated pathway, mitochondria-mediated apoptosis is inducible in mature human sperm from healthy donors. A short incubation with the specific inducer betulinic acid leads to strong activation of the mitochondrial apoptosis pathway in sperm.^{6,22} Moreover, the same apoptosis signaling cascade, which includes disruption of the mitochondrial transmembrane potential, release of pro-apoptotic regulator proteins such as Bax and AIF, and activation of caspase-9 and caspase-3, is induced following cryopreservation and thawing.^{4,23–28}

In somatic cells, apoptosis signaling results in degradation of proteins via effector caspases-3 and -7. The resulting inactivation of poly(ADP) ribose-polymerase-1 (PARP-1) generates the typical DNA fragmentation leading to programmed cell death. However, this process is likely to be somewhat different in sperm. The DNA is densely packed on protamines, and although the DNA repair enzyme PARP is present in ejaculated sperm, the 24 kD fragment of PARP specific

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to apoptosis could not be detected until now.^{3,29,30} In addition, it has been reported that sperm DNA fragmentation can occur independent from apoptosis.³¹

Activation of apoptosis signaling has a major impact on the fertilization potential of sperm. Activation of the effector caspase-3 is not only associated with impaired sperm motility³² but also associated with the reduced ability of sperm to undergo capacitation, acrosome reaction, and oocyte penetration^{33,34} as well as reduced oocyte fertilization rates.³⁵

Our study aimed to investigate the consequences of specific activation of sperm apoptosis signaling on ultra-morphological level using transmission electron microscopy to improve the understanding of human sperm cell death.

MATERIALS AND METHODS

Experimental design

This study was approved by the Ethical Committee of the University of Leipzig, Saxony, Germany, (No. 136-10-31052010), and conducted in accordance with ethical standard guidelines of the University.

Semen samples were obtained from eight healthy donors following a period of 3–5 days of sexual abstinence. Before sample collection, written informed consent was obtained from all participants. Semen analysis was performed according to the World Health Organization guidelines.³⁶ Samples with $\geq 15 \times 10^6$ spermatozoa ml^{-1} and at least 50% progressive sperm motility were selected for the study. Five semen specimens were used for pretests to determine the optimal concentration of betulinic acid necessary as well as incubation times to induce sperm apoptosis.

The remaining three semen samples were prepared with a discontinuous density gradient centrifugation (Isolate[®], Irvine Scientific, Santa Ana, CA, USA) to separate mature and immature/defective sperm fractions. Samples were loaded onto a 47% and 90% discontinuous gradient and centrifuged at 600 $\times g$ for 20 min at room temperature (25°C). The resulting 90% and 47% pellets were washed by centrifugation for additional 5 min at 300 $\times g$ and resuspended in human tubal fluid (HTF) medium with Gentamicin (Irvine Scientific, Santa Ana, CA, USA).

The sperm suspensions were divided into two fractions: one served as control whereas the other was subjected to specific induction of mitochondria-mediated apoptosis with betulinic acid. Apoptosis induction was halted by washing the sperm twice in 500 μl HTF followed by centrifugation at 300 $\times g$ for 5 min. To allow for ultramorphologic changes, all of the aliquots were stored under physiological conditions (37°C, 5% CO_2) for 6 h before preparation of the sperm for transmission electron microscopy (modulation phase). Figure 1 provides an overview of the experimental design.

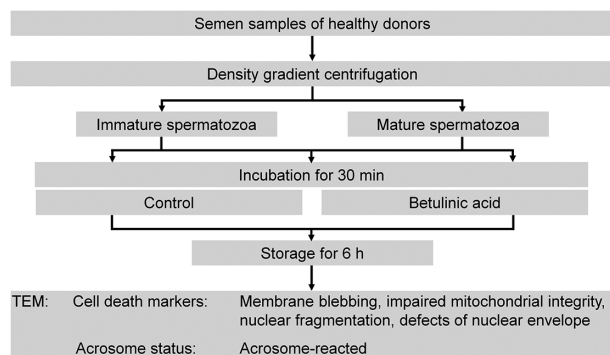


Figure 1: Experimental design overview.

Apoptosis induction

Betulinic acid (EnzoLifeScience, Lörrach, Germany), a pentacyclic triterpenoid, was used to induce mitochondria-derived apoptosis.^{6,37} Betulinic acid was dissolved in dimethylsulfoxide (DMSO) to 50 mmol l^{-1} as the stock solution. To determine the optimal concentration and incubation time to induce ultra-morphological changes, washed spermatozoa were treated with 10, 50, 100, 150, 200, or 500 $\mu\text{mol l}^{-1}$ betulinic acid at 37°C in the dark. After 10, 30, 60, or 120 min, the incubation was halted by washing the sperm twice in 500 μl HTF followed by centrifugation at 300 $\times g$ for 5 min.

To investigate the temporal requirements of ultra-morphological changes by spermatozoa, both untreated and betulinic acid-treated spermatozoa were stored under physiological conditions (37°C, 5% CO_2) for 0, 6, or 12 h before preparing the sperm for transmission electron microscopy.

Detection of activated caspase-3

Activated caspase-3 was detected in spermatozoa using the fluorescein-labeled inhibitor peptide FAM-DEVD-FMK of caspase-3 (FLICA), which is cell permeable, noncytotoxic and binds covalently to active caspase-3.³⁸ Once bound by an active caspase, the fluorescent signal is retained within the sperm. The inhibitor was used with the appropriate controls according to the kit instructions provided by the manufacturer (FAM FLICA[™] Caspase-3 and -7 Assay Kit, Immunochemistry Technologies, Bloomington, MN, USA). A 150-fold stock solution of the inhibitor was prepared in DMSO and further diluted in phosphate-buffered saline (PBS) to yield a 30-fold working solution. All test aliquots and controls (with 75 μl PBS) were incubated at 37°C for 1 h with 2.5 μl of the working solution followed by subsequent washing twice with the rinse buffer. Green FAM-FLICA[™] excites at approximately 490 nm and emits at 530 nm in a flow cytometer.

Evaluation of the acrosomal status

To evaluate the optimal concentrations of betulinic acid for ultra-morphological investigation of apoptosis, acrosome-reacted spermatozoa were detected in pretests using a monoclonal FITC-labeled mouse anti-human CD46 antibody (BioRad AbD Serotec Inc., Pulheim, Germany). After incubation with betulinic acid, sperm aliquots were washed in 1 ml PBS, centrifuged for 5 min at 300 $\times g$, and resuspended in 150 μl PBS containing 6 μg of CD46-FITC. Acrosome-reacted sperm is labeled as CD46-positive. After incubation for 30 min under light protection, the sperm was washed twice with 1 ml PBS followed by centrifugation for 5 min at 300 $\times g$. The pellets were finally resuspended in 100 μl PBS. CD46-FITC excites at approximately 490 nm and emits at 530 nm in a flow cytometer.

Flow cytometry

The percentage of CD46-positive sperm and sperm with activated caspase-3 was evaluated using the MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). A minimum of 10 000 spermatozoa were examined for each assay at a flow rate of <500 cells s^{-1} . The sperm population was gated using side and forward scatter to exclude debris, aggregates, and other cell types. The excitation wavelength (488 nm) was supplied by 30 mW diode-pumped solid-state (DPSS) laser. Green fluorescence (525/50 nm) was measured in the B1 channel. The percentage of positive cells and the mean fluorescence was calculated using the MACSQuantify software (Miltenyi Biotec, Bergisch Gladbach, Germany).

Electron microscopy

Spermatozoa from three donors were washed twice in HEPES buffer, fixed in 2% glutaraldehyde (pH 7.3 in 0.1 mol l^{-1} cacodylate

buffer) for 2 h, and centrifuged for 10 min at 800 \times g. The pellets were placed in low-melt agarose and postfixed in ice-cold 1% OsO₄ for 2 h. The samples were dehydrated through a graded series of acetone, en bloc contrasted with uranyl acetate, and phosphotungstic acid, further dehydrated through another graded series of acetone and embedded in Durcupan™ (Fluka AG, Buchs, Switzerland). Semi-thin sections were stained with toluidine blue to determine orientation, and ultrathin sections were cut, mounted on uncoated copper grids, and counterstained with Reynolds lead citrate. The ultrathin sections were observed under a Zeiss EM 900 electron microscope (Oberkochen, Germany). Digital photographs were acquired using a linked charge-coupled device (CCD) sensor. Within each sperm aliquot, fifty sperm cells and 200 mitochondria were investigated.

Statistical analysis

The descriptive statistical analysis was performed using Statistica 7.0 software (StatSoft, Tulsa, OK, USA). Values are given as mean or mean \pm standard deviation if applicable.

RESULTS

We first optimized the assay to determine the optimal concentration of betulinic acid and adequate incubation time to ensure induction of apoptosis in mature sperm. Sperm motility and vitality were used as sensitive markers.^{39–41} As expected, betulinic acid affected both sperm motility and vitality in a dose- and time-dependent manner (Figure 2a and 2b). To further evaluate the optimal concentration of betulinic acid, flow cytometric analysis of the apoptosis signaling cascade (i.e., active caspase-3 positive sperm) and acrosome reaction (i.e., CD46 positive sperm) was performed (Figure 2c and 2d). Based on the first pretests, treatment with 100 μ mol l⁻¹ of betulinic acid for 30 min was chosen to induce apoptosis. The third pretest investigated the time span until ultra-morphological changes occur. Two aliquots of the high-density sperm fractions of three semen samples were taken: the first was untreated as a control and the second aliquot was incubated with 100 μ mol l⁻¹ betulinic acid for 30 min. Fixing the sperm for electron microscopy was performed either immediately after betulinic acid incubation or after intervals of 6 h and 12 h at 37°C and 5% CO₂, respectively. In both aliquots, visible morphological changes resembling

apoptosis in somatic cells were present within 6 h after incubation with betulinic acid (Table 1).

Following protocol optimization (incubation in 100 μ mol l⁻¹ betulinic acid for 30 min followed by a modulation phase of 6 h at 37°C and 5% CO₂), ultra-morphological analysis of sperm apoptosis was performed in mature (i.e., the high-density sperm fraction after density gradient centrifugation) and immature sperm (i.e., the corresponding low-density sperm fraction) from three healthy donors with corresponding controls.

Examples of ultra-morphologic intact sperm are shown in Figure 3. All aliquots were screened for distinct morphological changes that are known to define apoptotic cell death in somatic cells: blebbing of the plasma membranes, formation of apoptotic bodies, impairment of the mitochondria, nuclear fragmentation, and defects of nuclear envelope.⁴² Chromatin condensation cannot be used as an apoptosis marker in sperm because sperm chromatin is physiologically condensed until fertilization of the oocyte. All of the aforementioned ultra-morphological signs of apoptosis could be detected in the sperm aliquots from all of the donors by transmission electron microscopy (Figure 4). However, the apoptotic percentages differed between mature and immature sperm and were influenced by betulinic acid (Table 2). Mature sperm showed signs of apoptosis less frequently compared to their immature counterparts. In particular, their mitochondria were more intact, the nucleus was minimally fragmented, and nuclear envelope defects were rarely observed (Figure 3). In contrast, the

Table 1: Time-dependent formation of ultra-morphological changes by in betulinic acid treated mature spermatozoa

Interval before temperature (h)	Control (h)			Betulinic acid (h)		
	0	6	12	0	6	12
Membrane blebbing and apoptotic bodies (% sperm)	4	10	34	14	36	32
Impaired mitochondrial integrity (% mitochondria)	0	5	27	46	90	95
Nuclear fragmentation (% sperm)	2	4	4	6	4	44
Defects of nuclear envelope (% sperm)	0	0	0	0	6	4
Loss of acrosome (% sperm)	6	16	20	28	80	84

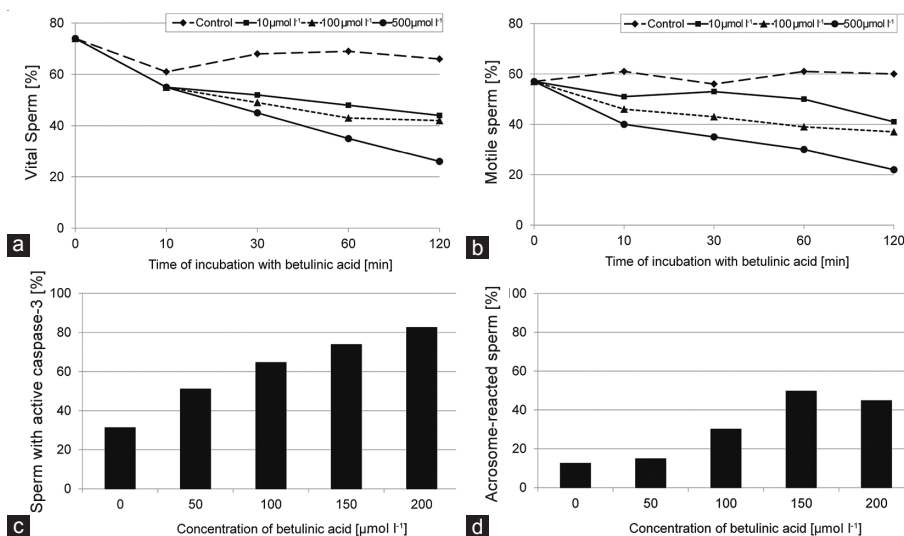


Figure 2: Optimization experiments to determine the ideal betulinic acid concentration and incubation time by measuring (a) sperm vitality, (b) progressive motility, (c) caspase-3 activation, and (d) acrosome reaction/CD46+.

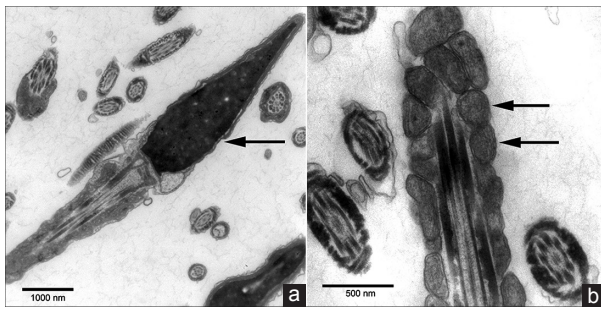


Figure 3: Transmission electron microscopy images of (a) an intact spermatozoon and (b) morphologically intact mitochondria.

immature sperm displayed all of the apoptotic characteristics at least twice as frequently as mature sperm. Only the frequency of membrane blebbing and formation of apoptotic bodies was similar in both sperm fractions.

The maturity status of the sperm influenced the effect of apoptosis stimulation. Whereas mature sperm presented with higher portions of impaired mitochondria, nuclear fragmentation, and defects of nuclear envelope, the immature sperm retained the same morphologies as those not treated with betulinic acid (**Table 2**). Notably, in both sperm fractions, incubation with betulinic acid induced an acrosome reaction (**Table 2**).

DISCUSSION

Stimulation of the sperm apoptosis signal cascade resulted in defined changes of sperm ultra-morphology. Within 6 h, signs of apoptosis as observed in somatic cells (namely plasma membrane blebbing with formation of apoptotic bodies, impaired mitochondrial integrity, defects of nuclear envelope, and nuclear fragmentation) were observed. All of these changes were already present in immature sperm (i.e., the low-density sperm fraction) without specific apoptosis induction. However, in the high-density sperm fraction, i.e., in mature human sperm, these apoptosis markers could be induced by treatment with betulinic acid. The results underline numerous other studies showing an activation of the apoptosis signaling cascade in sperm following a variety of triggers, e.g., cryopreservation and thawing.^{6,23,25,28}

This pilot study provides evidence of a healthy mature sperm's ability to undergo full apoptosis. The theory of a sole abortive apoptosis process¹² in human sperm appears to be invalid, particularly for mature sperm. It might play a role in immature sperm.

As novel protein expression in mature sperm is highly unlikely,¹⁴ it is possible that all components of the apoptosis pathway are produced during spermatogenesis. One theory is that they are present in mature sperm in an inactive form and become activated upon specific stimulation.⁴³ Although the study is limited by the low number of semen specimen ($n = 3$) which allows only a descriptive analysis, these pilot data are of high value for further analyses. The consistent observation of specific ultra-morphological signs of apoptosis in hundreds of sperm makes them applicable as sperm apoptosis markers.

Interestingly, following the induction of apoptosis, a substantial percentage of sperm underwent the acrosome reaction. This feature was discussed in an earlier study observing phosphatidylserine externalization following induction of the acrosome reaction⁴⁴ and required further investigation, which might elucidate the biological function of sperm apoptosis.

AUTHOR CONTRIBUTIONS

CS carried out the experiments and helped to draft the manuscript.

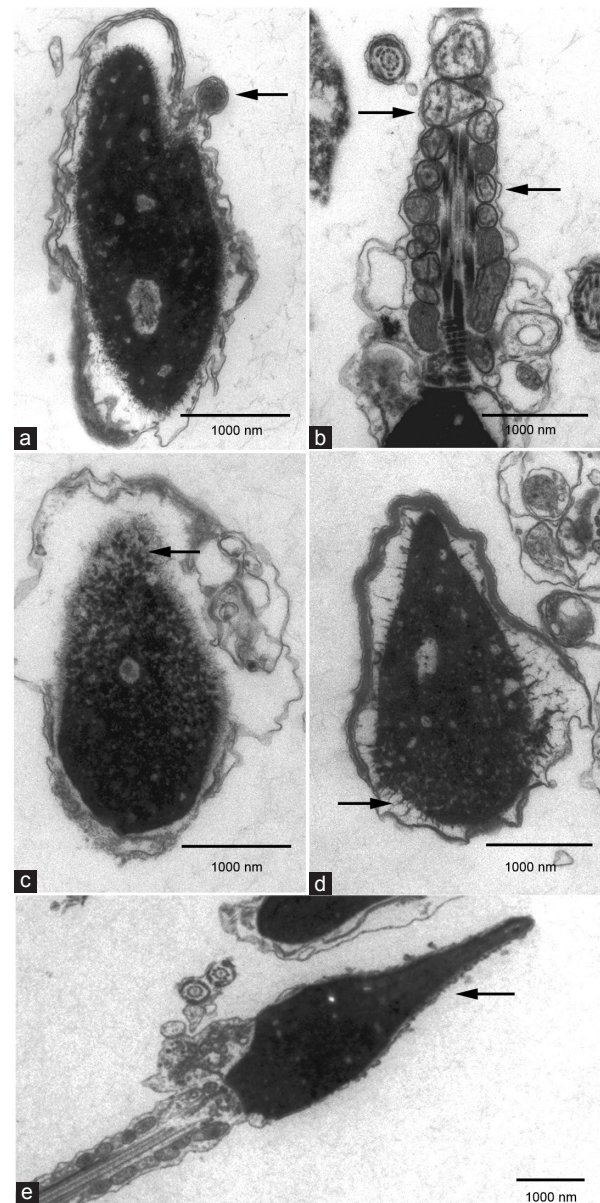


Figure 4: Transmission electron microscopy images of apoptosis-related morphologic changes in human mature sperm: (a) membrane blebbing and the formation of apoptotic bodies, (b) impaired mitochondrial integrity, (c) nuclear fragmentation, and (d) nuclear envelope defects. Induction of sperm apoptosis also resulted in loss of acrosome (e).

GF participated in the electron microscopy. SG designed the study, performed statistical analysis, and drafted the manuscript. All authors read and approved the final manuscript.

COMPETING INTEREST

The authors declared no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 2: Ultra-morphological changes in mature and immature spermatozoa following apoptosis induction by betulinic acid treatment

Sperm fraction	Mature sperm		Immature sperm	
	Control	Betulinic acid	Control	Betulinic acid
Incubation protocol				
Membrane blebbing and apoptotic bodies (% sperm)	34.67±7.02	38.67±21.39	31.33±11.55	32.00±9.17
Impaired mitochondrial integrity (% mitochondria)	21.27±14.91	67.98±5.88	55.9±12.70	56.95±36.74
Nuclear fragmentation (% sperm)	2.67±4.62	11.33±7.57	23.33±7.02	26.00±9.17
Defects of nuclear envelope (% sperm)	0.67±1.15	2.00±2.00	3.33±1.15	0.00±0.00
Loss of acrosome (% sperm)	12.67±11.72	47.33±28.59	46.00±7.21	61.33±24.44

Per aliquot, 50 sperm cells and 200 mitochondria were investigated 6 h after apoptosis induction by transmission electron microscopy

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