CHAPTER 9

Relationship between Sperm Apoptosis Signaling and Oocyte Penetration Capacity

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ABSTRACT

Human sperm have been documented to display apoptosis-like features such as externalization of phosphatidylserine (EPS), disruption of the transmembrane mitochondrial potential (MMP) and activation of caspases. Our aim was to evaluate possible association between activation of the apoptosis cascade in human sperm and its oocyte penetration capacity using the zona free hamster oocyte penetration assay (SPA). Semen specimens from 76 unselected donors were subjected to double density gradient centrifugation followed by incubation under capacitating conditions for 3 hours and SPA. Apoptosis signaling was monitored by assessment of EPS, disruption of MMP and activation of caspase-3 by flow cytometry. Semen samples with subnormal SPA values (<20% penetrated oocytes) contained significantly higher amounts of spermatozoa with EPS, disrupted MMP and activated caspase-3 compared to those samples with normal SPA values (>20 % penetrated oocytes, $p<0.01$). All three apoptosis markers showed a significantly negative correlation with the percentage of penetrated oocytes as well as to the numbers of sperm per penetrated oocyte ($p<0.001$). Apoptosis-related signaling appears to have a negative association with sperm-oocyte penetration. The exclusion of sperm presenting with those apoptosis-related features during assisted reproduction may improve success rates of procedures such as intrauterine insemination and in vitro fertilization.

Key words: annexin; caspase; magnetic-activated cell sorting; mitochondrial membrane potential; phosphatidylserine; sperm preparation; IUI, IVF

INTRODUCTION

Infertility affects approximately 15% of all couples trying to conceive and a male factor is the sole or contributing factor in almost half of the cases (Sharlip et al., 2002). Although assisted reproductive techniques (ART) have become the treatment of choice in many cases of male and female infertility (Gleicher et al., 2006), the current success rates of these procedures remain suboptimal (Gleicher et al., 2006). The quality of sperm samples is one of the factors determining the success of an ART cycle (Ombelet et al., 2003). The quality of spermatozoa can be evaluated by conventional semen analysis determining sperm concentration, motility, viability and morphology using light microscopy. Although the conventional analysis gives considerable information, it does not assess the presence of apoptosis signaling in spermatozoa, which may be partially responsible for the low fertilization and implantation rates seen with assisted reproductive techniques (Oehninger et al., 2003).

Human spermatozoa have been documented to display features of several apoptosis signal transduction pathways such as the externalization of phosphatidylserine (EPS), disruption of the transmembrane mitochondrial potential (MMP) and activation of caspases (Glander and Schaller, 1999; Oehninger et al., 2003; Paasch et al., 2004c). The apoptosis signaling cascade was found to be activated to a higher extent in spermatozoa that are immature and in those from infertility patients. In addition, the activation of apoptosis was documented following sperm conservation or preparation methods such as cryopreservation (Paasch et al., 2001; Paasch et al., 2004c; Said et al., 2004). Interestingly, a strong correlation of apoptosis markers with motility has been also shown (Paasch et al., 2004b). Therefore, apoptosis appears to have an important association with sperm parameters, which denotes that similarly it might affect the sperm fertilization potential.

The sperm penetration assay (SPA) using fresh or cryopreserved hamster oocytes is an established in-vitro model to analyze the sperm fertilization potential (Johnson et al., 1995). It gives functional information of sperm and can be used as a predictor of IVF outcome (Irvine and Aitken, 1986; Shy et al., 1988; Soffer et al., 1992). Our aim was to investigate the possible impact of activated apoptosis signaling cascade in human spermatozoa in relation to their oocyte penetration capacity using the zona free hamster oocyte penetration assay.

MATERIALS AND METHODS

Sample preparation

This study was approved by the Institution Review Boards of the Cleveland Clinic Foundation and the Faculty of Medicine, University of Leipzig.

Semen specimens (n=76) were collected from unselected donors following a period of 3 – 5 days of sexual abstinence. Semen parameters did not need to exceed the World Health

Organization reference ranges for the normal fertile population (World Health Organization, 1999). Semen samples contained 31.3 ± 15.3 million sperm/ mL (mean ± SD); 50.9 ± 26.9 % of the spermatozoa were motile. Specimens containing more than 1 million/ mL of leucocytes measured by routine peroxidase staining were not included in the study. Samples were prepared by double density gradient centrifugation (PureCeption®, SAGE BioPharma, Bedminster, NJ). Specimens were loaded onto a 40% and 80% discontinuous gradient and centrifuged at 300 g for 20 minutes at room temperature (25°C.). The resulting 80% pellet was washed by centrifugation for additional 7 minutes and re-suspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA). An aliquot from each semen sample was taken for evaluation of the apoptosis related parameters MMP, active caspase-3 and EPS at the time the SPA was performed.

Monitoring of transmembrane mitochondrial potential

A lipophilic cationic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine chloride) was used to detect intact transmembrane potential of mitochondria in spermatozoa (ApoAlert Mitosensor Kit ™, Clontech, CA, USA). Spermatozoa with intact mitochondria excite an intense red fluorescence due to the formation of the dye aggregates whereas the monomer dye fluoresces green in the presence of sperm with a disrupted mitochondrial membrane. Mitosensor ™ was used according to the instructions of the manufacturer. Briefly, all aliquots were incubated at 37 °C for 20 min in 1 µg of the lipophilic cation diluted in 1 mL PBS. Human neutrophils (5 x 10^6 cells) treated with 1 mM cycloheximide for 6 hours were used as positive controls for induction of apoptosis (Pozarowski et al., 2003). Negative controls were processed identically for each fraction except that the stain was replaced with 1mL PBS.

Detection and evaluation of activated caspase-3

Levels of activated caspases-3 were detected in viable spermatozoa using fluorescein labeled inhibitor of caspase (FLICA), which is cell permeable, non-cytotoxic and binds covalently to active caspase-3 (Ekert et al., 1999). The inhibitor was used with the appropriate controls according to the kit instructions provided by the manufacturers (Carboxyfluorescein FLICA, Immunochemistry Technologies, Bloomington, MN). A 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide (DMSO) and was further diluted in phosphate buffered saline (PBS) to yield a 30-fold working solution. All test aliquots and controls (with 100 µL PBS) were incubated at 37 °C for 1 hour with 10 µL of the working solution and subsequently washed with the rinse buffer. In concordance with the monitoring of transmembrane mitochondrial potential, human neutrophils (5 x 10^6 cells) treated with 1 mM cycloheximide for 6 hours were

used as positive controls for induction of apoptosis. The negative controls were processed identically for each fraction, except that the stain was replaced with 10 µL PBS.

Monitoring of externalization of phosphatidylserine

Externalization of phosphatidylserine was examined using a monoclonal mouse anti-human phosphatidylserine antibody, clone 1H6 (Upstate cell signaling solutions, Lake Placid, NY). Spermatozoa were incubated with the phosphatidylserine-antibody at a final concentration of 0.5 µg/mL in PBSB (phosphate buffered saline containing 2 % bovine serum albumin) for 20 minutes on ice, followed by addition of 150 µL PBSB and centrifugation 1600 rpm for 5 minutes at 20 °C. After discarding the supernatant each sperm pellet was incubated protected from light with 50 µL of secondary antibody (goat anti-mouse IgG [H+L], fluorescein conjugate, Upstate Cell Signalling Solutions, Lake Placid, NY) on ice for 20 minutes. A second washing step in PBSB (1600 rpm for 5 minutes at 20 °C) was performed to remove excess antibody that was not bound to the spermatozoal surface. For assessment by flow cytometry, sperm pellets were diluted in 400 µL PBSB. Human neutrophils (5 x 10^6 cells) treated with 1 mM cycloheximide for 6 hours served as positive controls for induction of apoptosis. The negative controls were processed identically for each fraction, except that the primary antibody was replaced with 200 µL PBS.

Flow cytometry analysis

All the fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (Becton Dickinson, USA). A minimum of 10,000 spermatozoa was examined for each assay at a flow rate of < 100 cells/s. The sperm population was gated using 90-degree and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. Green fluorescence (480-530 nm) was measured in the FHL1 channel and red fluorescence (580-630nm) in the FHL-2 channel. The percentage of positive cells and the mean fluorescence was calculated on a 1023 channel scale by software Expo32ADC (Coulter, Germany).

Zona-free hamster oocyte penetration assay

The assay was performed as described by Johnson et al. (1995) with slight modifications to mimic the human IVF procedure. Sperm aliquots used for the evaluation of sperm penetration assay were subjected to capacitating conditions (incubation in HTF media containing 3% bovine serum albumin for 3.5 hours at 37°C and 5% carbon dioxide). Frozen-thawed hamster oocytes (Embryotech, Wilmington, MA) were briefly exposed to acidified Tyrode's media (Irvine Scientific, Santa Ana, CA) to remove the zona pellucida and in turn their species specificity. The

zona-free hamster oocytes were placed in 50 µL sperm droplets (concentration adjusted to 3-5 X 10^6 sperm/mL), overlaid with mineral oil (Sigma, St Louis, MO) and incubated for 3 hours at 37°C, 5% CO_2. A total of 20 oocytes (5 oocytes per sperm droplet) were used for each of the 3 experiment aliquots.

Following incubation, oocytes were washed in PBS + 10% bovine serum albumin to remove the excess sperm attached to their surface and examined by 400X phase-contrast microscopy (Olympus, BH2-PC, Melville, NY). The presence of decondensed nucleus with an attached tail was taken to represent a positive penetration. The number of oocytes scored for each of the 3 experimental aliquots ranged from 15 to 20 depending on the number of oocytes damaged during the assay. Results were evaluated as the percentage of oocytes penetrated by sperm (SPA) and the average number of sperm penetrated per oocytes (sperm capacitation index (SCI).

Statistical analysis

Normal distribution was proven by Shapiro-Wilks Test. Pearson's correlation between sperm parameters were calculated and tested against zero. Student's *t*-test for independent samples was used to calculate the difference between samples. Pair-wise Pearson's correlation coefficients were used to study the association between different parameters. All tests were two-tailed, and significance is indicated by $p<0.05$. The statistical analysis was performed using Statistica 6.0 software (StatSoft; Tulsa, OK).

RESULTS

Semen samples were categorized into groups A and B according to their results in the zona-free hamster oocyte penetration assay (Johnson et al., 1995). In group A, 22 of the 76 collected semen samples presented with subnormal SPA values (<20 % penetrated oocytes), while in group B, the remaining 54 samples had normal SPA values (>20 % penetrated oocytes). The percentage of penetrated oocytes was significantly lower in group A vs. B (15.8 ± 0.8 vs. 34.1 ± 1.5; $p<0.01$). In concordance, the number of sperm per penetrated oocyte (SCI) differed significantly between both groups (group A: 1.2 ± 0.3 sperm vs. group B: 1.5 ± 0.5 sperm, $p<0.05$). Figure 1 displays an example of a penetrated hamster oocyte.

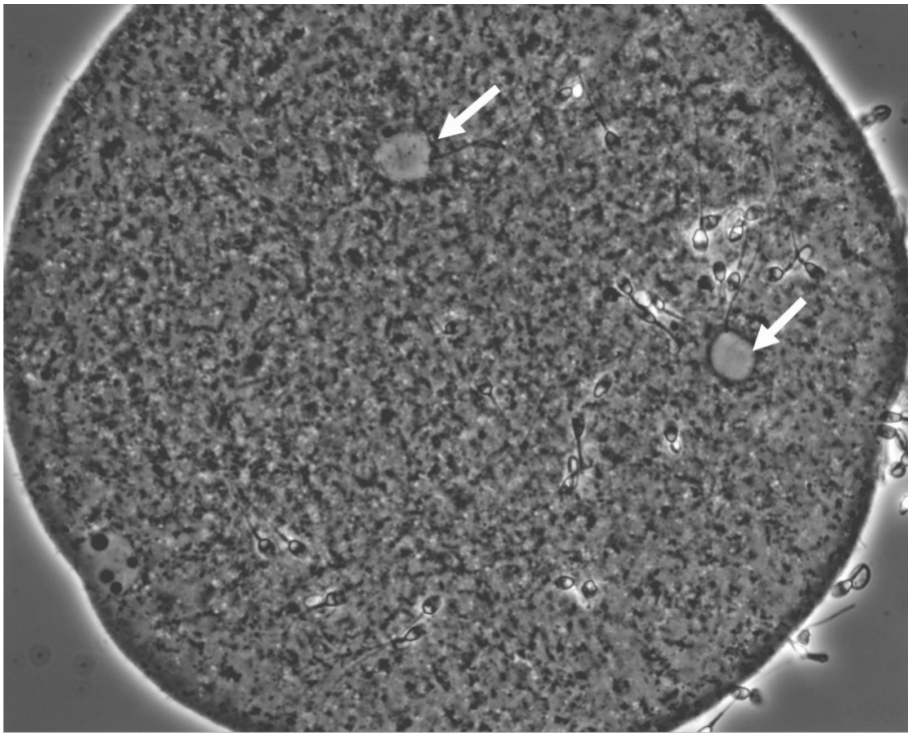

Figure 1. *Zona-free hamster oocyte following the sperm penetration assay. Positive penetration is demonstrated by completely decondensed sperm heads; the tail is still visible (white arrows). All other sperm are seen attached to the surface only and are considered negative. Magnification: X67.*

Furthermore, both groups were compared with regards to the activation of the apoptosis signaling cascade as measured by disrupted MMP, active caspase-3 and the EPS. Semen specimens with low fertilizing capacity (group A, abnormal SPA values) contained significant higher number of spermatozoa with activated apoptosis signaling. The mitochondrial transmembrane potential was disrupted in 67.1 ± 4.7 % of the spermatozoa, caspase-3 was activated in 41.4 ± 3.8 % and externalized phosphatidylserine was found on the surface of 37.1 ± 4.9 %. The most pronounced difference was found at the mitochondria. Semen samples with subnormal oocyte penetration values (group A) had 31.6 % more sperm with disrupted MMP compared to group B. This corresponds with the significant lower motility of semen samples in group A (30.2 ± 15.3% motile sperm, WHO A+B) when compared to group B. There was a strong correlation observed between mitochondrial integrity and motility (Pearson's correlation coefficient: $r = 0.87$, $p<0.01$). Semen samples of group B (normal SPA values) contained 59.3 ± 26.2 % motile sperm (WHO A+B), 35.5 ± 4.2 % sperm with disrupted MMP, 23.0 ± 3.1 % sperm with activated caspase-3 and 22.3 ± 3.6 % sperm with externalized phosphatidylserine (Table 1).

	Group A: SPA < 20% (n=22)	Group B: SPA > 20% (n=54)	p-value
SCI [sperm per penetrated oocyte]	1.2 ± 0.3	1.5 ± 0.5	<0.05
Motility [% motile spermatozoa]	30.2 ± 15.3	59.3 ± 26.2	0.001
Disrupted MMP [% spermatozoa]	67.1 ± 4.7	35.5 ± 4.2	<0.0001
Active caspase-3 [% spermatozoa]	41.4 ± 3.8	23.0 ± 3.1	0.001
EPS [% spermatozoa]	37.1 ± 4.9	22.3 ± 3.6	0.01

Table 1: *Comparison of SCI, motility and apoptosis markers in semen samples with normal and subnormal SPA values. Values are given as mean ± SEM. Statistical test: Student's t -tests for independent samples, p-values < 0.05 were considered significant. SCI= sperm capacitation index, MMP=mitochondrial membrane potential; EPS=externalized phosphatidylserine.*

All of the apoptosis markers were negatively correlated with the percentage of penetrated oocytes (Pearson's correlation coefficient: disrupted MMP: r = -0.67, activated caspase-3: r = -0.59 and EPS: r = -0.52, p<0.01, Figures 2 A, B, C). In addition, the apoptosis markers were negatively correlated to the number of sperm per penetrated oocyte (disrupted MMP: r = -0.49, activated caspase-3: r = -0.35, p<0.01). EPS showed the same trend, but failed to be significant (r = -0.23, p>0.05). The portion of motile sperm (WHO A+B) was significantly positive correlated to the SPA results (r = 0.70) as well as to the SCI values (r = 0.51, p<0.01). Serving as an internal control, there was a strong positive correlation between caspase-3 activation and the disruption of the mitochondrial transmembrane potential (r = 0.74, p < 0.001) and externalization of phosphatidylserine (r = 0.76, p < 0.01). Also, EPS and disrupted MMP correlated positively (r=0.71, p<0.01).

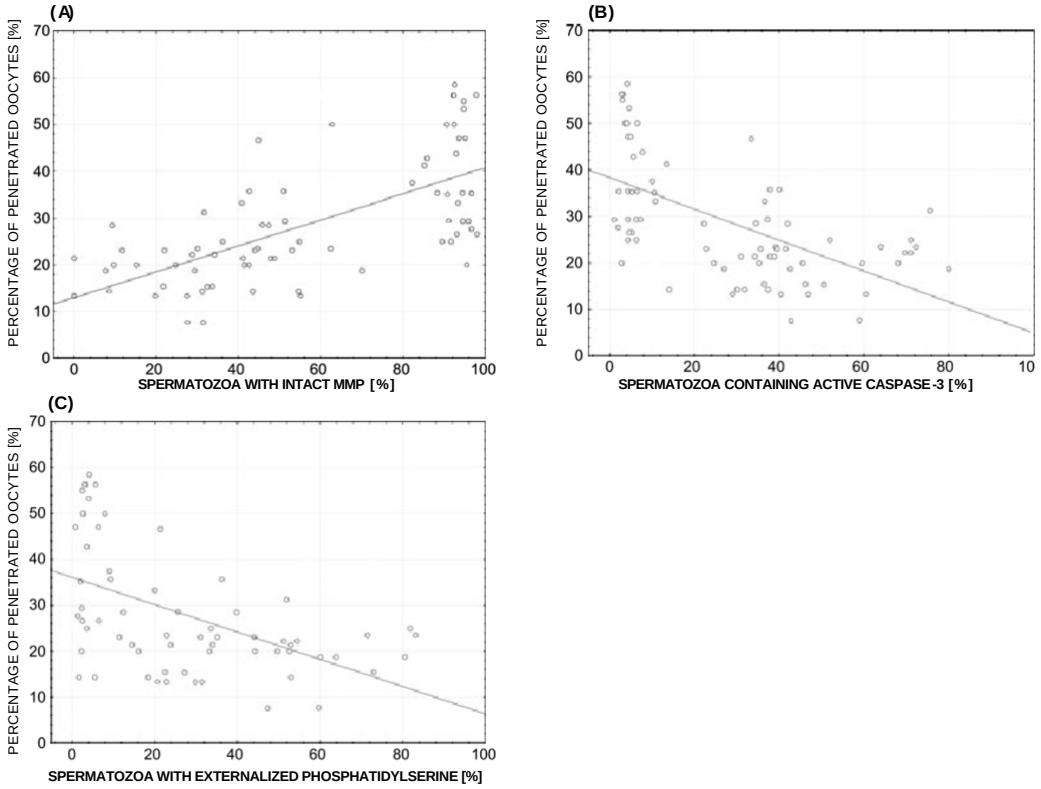

Figure 2. ***(A)*** *The integrity of the transmembrane mitochondrial potential is one of the key points in apoptosis signaling in human sperm. It shows a strong positive correlation with the sperm oocyte penetration capacity (Pearson's correlation coefficient r = 0.67, p<0.001).* ***(B)*** *Activation of the main apoptosis executor caspase-3 is significantly correlated with poor sperm oocyte penetration rates (Pearson's correlation coefficient: r = 0.59, p<0.001).* ***(C)*** *Externalization of phosphatidylserine to the outer leaflet of the sperm membrane is one of the earliest features of terminal apoptosis and correlates significantly negative with the sperm oocyte penetration capacity (Pearson's correlation coefficient r = 0.52, p<0.001).*

DISCUSSION

The zona-free hamster oocyte penetration assay examines the ability of human spermatozoa to capacitate, undergo the acrosome reaction, fuse with the vitelline membrane of the oocyte and initiate nuclear decondensation. Although it is an animal model with its own limitations, it gives insight in the functional status of the sperm and reflects the fertilizing potential of the sperm (Johnson et al., 1995). Clinically, results of the SPA correlate with the outcome of human IVF

and the achievement of spontaneous pregnancy in long-term prospective studies involving infertile patients (Irvine and Aitken, 1986; Shy et al., 1988; Soffer et al., 1992). At the molecular level, results of the SPA also correlate with apoptosis markers such as annexin V staining (Sion et al., 2004).

However, surface exposure of phosphatidylserine on sperm cell activation in vitro was found to be restricted to the apical area of the head plasma membrane and related to acrosome reaction too (Martin et al., 2005). On the other hand exposure of phosphatidylserine and its specific binding to Annexin-V has been used to characterize and to select apoptotic spermatozoa. Own data show that the subpopulation of EPS negative sperm has the same proportion of CD46 positive cells as compared to the neat semen (Grunewald et al., 2006).

The presence and functionality of apoptosis associated pathways known from somatic cells remains subject to controversy when it comes to human sperm (Agarwal and Said, 2005; Sakkas et al., 2003; Taylor et al., 2004). Extensive studies of the activation and localization of caspases in human sperm were performed previously. While caspase -9 was located almost exlusively to the mitochondria, caspase-3 was predominantly found in the cytoplasm (Paasch et al., 2004a).

Our findings indicate a strong negative correlation between the apoptosis related parameters: disruption of the MMP, activation of caspase-3 as well as EPS and the performance of the spermatozoa in the hamster oocyte penetration assay. Semen samples showing subnormal SPA values were characterized by significantly increased levels of disruption of the MMP, activation of caspase-3 and EPS, indicating an impact of apoptosis-related processes not only at the plasma membrane but also at the mitochondrial and cytoplasmic level on the spermatozoal capacity to penetrate oocytes.

Conclusions

The loss of the integrity of the mitochondrial transmembrane potential as well as caspase activation were previously demonstrated as processes seen in varying percentages of sperm in all semen samples. The amount of sperm showing those features was found to be increased not only in patients presenting with infertility, but also after cryopreservation and thawing. The association between those apoptosis markers in sperm and the oocyte penetration potential as demonstrated in our findings further supports the negative impact of apoptosis on in vivo and in vitro fertilization. In this context, the exclusion of sperm presenting with those apoptosis-like features from ART by techniques such as annexin-V-magnetic activated cell sorting may improve success rates.

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