Increased sperm chromatin decondensation in selected nonapoptotic spermatozoa of patients with male infertility

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Objective: To evaluate the sperm chromatin decondensation (SCD) rates of the annexin-negative (nonapoptotic) sperm fraction of patients with infertility using hamster intracytoplasmic sperm injection (H-ICSI). In healthy donors, the depletion of apoptotic sperm using annexin V-based magnetic-activated cell separation (MACS) enhances hamster oocyte sperm penetration but does not increase SCD rates following H-ICSI.
Design: A prospective-controlled study.
Setting: Male infertility clinic, European Academy of Andrology Center Leipzig.
Patient(s): Twenty-one male infertility patients with subnormal spermiogram.
Intervention(s): Spermatozoa were separated by Annexin V-MACS.
Main Outcome Measure(s): Apoptosis signaling (disruption of transmembrane mitochondrial potential, transmembrane mitochondrial potential [TMP], and activation of caspases-3 [CP3]) and SCD rates of human spermatozoa after hamster intracytoplasmic sperm injection.
Result(s): Infertility patients showed high levels of sperm with active CP3 and disrupted TMP, which correlated negatively with SCD rates. Annexin V-MACS resulted in a significant enrichment of spermatozoa with inactive CP3 and intact TMP in the annexin-negative fraction. Similarly, annexin-negative sperm had the highest SCD rates following H-ICSI compared with controls and annexin-positive sperm.
Conclusion(s): These results suggest that nonapoptotic spermatozoa prepared by annexin V-MACS display higher early fertilization potential following ICSI. The technique should be evaluated in a clinical setting for its impact on ICSI outcomes in patients diagnosed with infertility. (Fertil Steril® 2009;92:572–7. ©2009 by American Society for Reproductive Medicine.)

Key Words: Hamster oocyte, ICSI, human sperm, apoptosis, annexin V

Several apoptosis signaling pathways that were established in somatic cells have also been documented in human spermatozoa. Although there is a consensus on the implication of apoptosis in male infertility, the exact mechanisms of its involvement remain to be elucidated (1, 2). Decrease in fertilization potential of apoptotic sperm may be a contributing factor. In support, indices of activated apoptosis including caspase-3 (CP3) activation and disruption of the transmembrane mitochondrial potential (TMP) correlate well with the sperm fertilizing potential measured by sperm penetration assay (3).

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Another apoptosis-related event demonstrated in human spermatozoa is the externalization of the phospholipid phosphatidylserine (PS), which has been inversely correlated with routine sperm parameters (4, 5). The covalent binding of annexin V, a phospholipid binding protein that has high affinity for PS and lacks the ability to pass through an intact sperm membrane, can be used to label sperm with externalized PS (6). Subsequently, annexin V-conjugated superparamagnetic microbeads can be used effectively to separate nonapoptotic spermatozoa from those with deteriorated plasma membranes based on the externalization of PS using magnetic-activated cell separation (MACS). The separation of sperm yields two fractions: annexin V-negative (intact membranes, nonapoptotic) and annexin V-positive (externalized PS, apoptotic) (7, 8).

In semen samples from healthy donors, the integration of annexin V-MACS in standard sperm preparation protocols yields a sperm population with higher motility, viability, intact TMP, inactive CP3, DNA integrity, and oocyte penetration rates following the sperm penetration assay (SPA) (9, 10). However compared with conventional density gradient centrifugation, the selected nonapoptotic sperm fraction did

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not show higher sperm chromatin decondensation rates following hamster oocyte intracytoplasmic sperm injection (H-ICSI). Two animal models for assisted reproductive procedures were used in this study: the standardized hamster oocyte penetration assay simulating in vitro fertilization (IVF) and the H-ICSI model evaluating early sperm fertilization potential and the potential impact on ICSI results. Although the potential impact of annexin V-MACS in IVF protocols was demonstrated by the SPA results, the impact on ICSI procedures remained unclear.

Multiple studies have established that spermatozoa in patients diagnosed with male infertility display a higher incidence of apoptotic features (11–15). Therefore, sperm preparation protocols based on the selection of nonapoptotic cells should be evaluated in this unique population. The goal of our study was to determine whether annexin V-MACS can be used to separate spermatozoa with inactivated apoptosis signaling from sperm samples with subnormal parameters. In addition, we aimed to clarify the impact of annexin V-MACS on sperm chromatin decondensation (SCD) rates in subfertile patients using the H-ICSI model.

MATERIALS AND METHODS

Experimental Design

This study was approved by the institution review board of the University of Leipzig. Semen samples were obtained from 21 infertility patients following a period of 3 to 5 days of sexual abstinence. Semen analysis was performed according to the World Health Organization guidelines (16) and revealed oligoasthenoteratozoospermia and asthenoteratozoospermia in 17 of 21 and 4 of 21 of patients, respectively.

Semen samples were prepared by double-density gradient centrifugation (DGC, SupraSperm gradient, MediCult, Jyllinge, Denmark). Samples 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 an additional 7 minutes and resuspended in 0.5 mL human tubal fluid media (Irvine Scientific, Santa Ana, CA).

One aliquot of the sperm suspension (0.1 mL) served as the control, while the other aliquot (0.4 mL) was subjected to annexin V-MACS. Activated CP3 levels and TMP integrity were assessed as markers of apoptosis in the annexin V-negative and -positive aliquots following MACS as well as in the control aliquot. The extent of SCD following H-ICSI was used to assess early sperm fertilization potential.

Isolation of Spermatozoa with Deteriorated Membranes by MACS

Following washing in 2.0 mL annexin V-binding buffer for 4 minutes at 300 × *g* sperm pellets were incubated with annexin V-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at room temperature. One hundred microliters of microbeads was used for each 10 million separated cells. The sperm/microbead suspension was loaded in a separation column containing iron globes, which was fitted in a magnet (MiniMACS; Miltenyi Biotec). The annexin V microbead-labeled fraction composed of apoptotic spermatozoa was retained in the separation column and labeled as annexin V positive; the fraction with intact membranes that was eluted through the column was labeled as annexin V negative. To ensure the elution of the annexin V-negative sperm fraction the column was rinsed three times with 0.5 mL annexin V-binding buffer. The power of the magnetic field was measured as 0.5 Tesla between the poles of the magnet and up to 1.5 Tesla within the iron globes of the column. After the column was removed from the magnetic field, the retained annexin V-positive fraction was eluted using annexin V-binding buffer (Miltenyi Biotec).

Detection of Activated CP3

Activated CP3 levels were detected in spermatozoa using fluorescein-labeled inhibitor of caspase (FLICA), which is cell permeable, noncytotoxic, and binds covalently to active CP3 (17). The inhibitor was used with the appropriate controls according to the kit instructions provided by the manufacturer (Carboxyfluorescein FLICA, Immunochemistry Technologies, Bloomington, MN). A 150-fold stock solution of the inhibitor was prepared in dimethyl sulfoxide and 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 twice with the rinse buffer.

Evaluation of Mitochondrial Membrane Potential

A lipophilic cationic dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine chloride) was used to detect intact TMP in spermatozoa (ApoAlert Mitosensor KitTM, Clontech Laboratories, Mountain View, CA). Spermatozoa with intact mitochondria excite an intense red fluorescence because of the formation of the dye aggregates, whereas the monomer dye fluoresces green in the presence of sperm with a disrupted mitochondrial membrane. The kit was used according to the instructions of the manufacturer. Briefly, all aliquots were incubated at 37°C for 20 minutes in 1 μg of the lipophilic cation diluted in 1 mL PBS. Negative controls were processed identically for each fraction except that the stain was replaced with 10 μL PBS.

Flow Cytometry Analysis

The extent of activated CP3, intact MMP, and the externalized PS were evaluated by flow cytometric analyses. All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (Becton Dickinson, San Jose, CA). A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/sec. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm