

# 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

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  $300 \times g$  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  $300 \times g$  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  $\mu\text{L}$  PBS) were incubated at 37°C for 1 hour with 10  $\mu\text{L}$  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  $\mu\text{g}$  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  $\mu\text{L}$  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

supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FL-1 channel and red fluorescence (580–630 nm) in the FL-2 channel. The percentage of positive cells and the mean fluorescence were calculated on a 1,023-channel scale using the flow cytometer software Expo32 ADC (Beckman Coulter, Krefeld, Germany).

### Hamster Oocyte Intracytoplasmic Sperm Injection

Micromanipulation was performed using a 200×-inverted microscope (Nikon, Tokyo, Japan) using Hoffman optics. The microscope is equipped with two hydraulic Narishige micromanipulators (M0-102M) and two microinjectors (Eppendorf, Hamburg, Germany). Intracytoplasmic sperm injection was performed as described by Palermo et al. (18). In brief, 1 μL of sperm suspension was diluted with 4 μL of 7% polyvinyl pyrrolidone (Irvine Scientific) and placed in the center of injection dish. Each frozen–thawed hamster oocyte (Embryotech, Wilmington, MA) was placed in 5 μL of IVF medium (MediCult, Jyllinge, Denmark) surrounding the central drop containing the sperm suspension and covered with mineral oil. A total of 20 oocytes were injected for each of the three experiment aliquots, a total of 1,260 cryopreserved–thawed hamster oocytes (21 patients × 3 aliquots × 20 oocytes). The selected sperm was aspirated into the injection micropipette and introduced through the zona into the ooplasm. The micropipette was slowly withdrawn, and the injected oocytes were kept at 37°C, 6% CO<sub>2</sub> for 18 to 22 hours.

To evaluate the occurrence of SCD, oocytes were fixed by incubation in 2% formaldehyde + 0.02% Triton X (Merck Laboratories, Darmstadt, Germany) for 30 minutes at 37°C. Following incubation, oocytes were washed in PBS + 10% bovine serum albumin and stained with Hoechst 33258 (10 μg/mL; Sigma-Aldrich, St. Louis, MO) for 45 minutes at 37°C. Washing was repeated to remove the excess stain and oocytes were mounted on glass slide using Slow-Fade Gold anti-fade reagent (Invitrogen, Eugene, OR) (19). The number of oocytes scored for each of the three experimental aliquots

ranged from 10 to 20, depending on the number of oocytes damaged during the assay.

### Statistical Analysis

One-way analysis of variance with repeated measures was used to compare various parameters among the three experimental aliquots. Pairwise Pearson’s correlation coefficients were used to study the association between different parameters. The common correlation coefficient, which can be regarded as a measure of the average correlation across treatment groups, was calculated when necessary from a weighted average of the Fisher’s Z transformations of the aliquot-specific correlations. All tests were two tailed, and significance was indicated by  $P < .05$ . The statistical analysis was done using Statistica 6.0 software (StatSoft; Tulsa, OK).

## RESULTS

### Evaluation of Semen Samples

The mean sperm concentration and percentage progressive motility (WHO a + b) in the raw ejaculates were  $35.8 \pm 38.9 \times 10^6/\text{mL}$  and  $41.2 \pm 15.7\%$ , respectively. Separation on a double-density gradient resulted in an average sperm concentration of  $15.7 \pm 17.1 \times 10^6/\text{mL}$  and an average motility of  $31.7 \pm 24.8\%$ . After annexin V-MACS the average sperm concentration was  $3.1 \pm 3.7 \times 10^6/\text{mL}$  within the annexin V-negative fraction. Because of further centrifugation steps, motility was negatively affected by the annexin-V MACS procedure. Compared with the control aliquots, both annexin V-positive ( $P < .001$ ) and -negative sperm ( $P = .004$ ) displayed lower progressive motility. However, sperm motility values were significantly higher in the annexin V-negative fraction following MACS compared with the annexin V-positive fraction ( $P = .006$ , Table 1).

### Apoptosis Signaling in Spermatozoa

The annexin-negative spermatozoal fraction showed significantly lower activation of CP3 and higher intact TMP

**TABLE 1**

**Results of sperm apoptotic markers and sperm chromatin decondensation (SCD) following ICSI in Annexin V-negative and -positive sperm and controls of infertile patients.**

	Motility (% motile)	Caspase-3 (% active)	TMP (% intact)	SCD (% decondensed)
Annexin V-negative (A)	11.4 ± 14.2	26.8 ± 12.3	71.6 ± 1.5	44.2 ± 15.8
Annexin V-positive (B)	0.5 ± 2.3	58.4 ± 11.7	9.8 ± 12.0	18.3 ± 6.7
Control (C)	31.7 ± 5.4	43.5 ± 13.8	54.7 ± 23.2	31.3 ± 13.1
<i>P</i> value				
A vs. B	<.001	<.001	<.001	<.001
A vs. C	<.001	<.001	<.001	<.01
B vs. C	<.001	<.001	<.001	<.01

*Note:* TMP = transmembrane mitochondrial potential; ICSI = intracytoplasmic sperm injection. Results are expressed as mean ± standard deviation.  $P < .05$  was considered significant using one-way ANOVA with repeated measures.

Grunewald. Fertilizing capacity of nonapoptotic spermatozoa. *Fertil Steril* 2009.

compared with annexin-positive fraction and controls ( $P < .001$ , Table 1). Compared with controls, annexin V-MACS combined with DGC significantly reduced the percentage of CP3-positive sperm ( $-16.7\%$ ) as well as that of sperm with disrupted TMP ( $-16.9\%$ ) within the annexin V-negative fraction. In absolute counts, this means almost a bisection of sperm with activated apoptosis signaling compared with sperm prepared by DGC only (controls). Sperm with active CP3 and disrupted TMP were significantly increased within the annexin V-positive fraction ( $P < .001$ ) compared with annexin V-negative and control aliquots.

### Hamster Oocyte Intracytoplasmic Sperm Injection

Following H-ICSI, the percentage of SCD was higher in both annexin V-negative sperm and controls compared with annexin V-positive sperm ( $+25.9\%$  SCD,  $P < .001$  and  $+13.0\%$  SCD,  $P = .001$ , respectively). Furthermore, SCD rates in the annexin V-negative sperm fraction were significantly higher compared with those detected in controls ( $+12.9\%$  SCD,  $P = .005$ , Table 1).

In samples evaluated by H-ICSI ( $n = 21$ ), the SCD had a negative correlation with the extent of CP3-positive sperm ( $r = -0.45$ ,  $P < .001$ ) and a positive correlation with the percentage of TMP-intact sperm ( $r = 0.47$ ,  $P < .001$ ). No significant correlation was found between the percentage of motile sperm and the percentage of spermatozoa showing SCD following H-ICSI ( $r = 0.16$ ,  $P > .05$ , Table 2).

### DISCUSSION

The last 2 decades have witnessed a steady increase in the use of assisted reproductive techniques (ART) for the treatment of male and female infertility. Despite phenomenal advances in the ART field, success rates can still be improved. The identification and selection of the sperm with adequate fertilization potential is a strategy that could be used to improve ART success rates. Selection of human spermatozoa during ART currently is based on criteria such as viability, motility,

and morphology (20). These criteria clearly exclude sperm abnormalities at the molecular level, which may impact fertilization. Abnormalities such as DNA fragmentation and activated apoptosis signaling have been directly linked to failure of fertilization during ART (3, 21). Recently, a novel sperm preparation technique has been standardized for the separation of nonapoptotic spermatozoa. The protocol combines annexin V-MACS with density gradient centrifugation. Thus, spermatozoa are separated based on their molecular characteristics as well as physical properties (22).

Previously conducted studies using semen samples from healthy donors showed that annexin V-MACS allows the selection of nonapoptotic spermatozoa with optimal results obtained by combining it with DGC (7, 9, 23). In healthy donors, the selection of nonapoptotic spermatozoa resulted in a significantly improved oocyte penetration, but no benefits were observed in SCD using hamster oocytes ICSI as an animal model (10). The question was raised as to whether these results can be transferred to infertility patients. For this reason, we repeated our study (10) with semen from infertility patients using the same study design and methodology.

### Sperm Recovery Using the Combination of DGC and MACS

The low sperm concentration after DGC and annexin V-MACS is not likely to result from a massive sperm loss during the procedure. In fact, the rinsing procedure during the collection of annexin V-negative sperm dilutes the sample (1:3). More importantly, semen samples from infertility patients contain high amounts of sperm with activated apoptosis signaling which are predominantly separated in the annexin V-positive fraction (15). In a clinical setting it might be of advantage to skip the rinsing steps during the elution of annexin V-negative sperm.

### Impact of MACS on Apoptosis Markers

In this study, semen specimens from infertility patients contained significantly higher levels of sperm with activated

**TABLE 2**

**Sperm motility, markers of apoptosis, and sperm chromatin decondensation following ICSI showing the correlation with each other and the significance.**

n = 21	Motility (% motile)		Caspase-3 (% active)		TMP (% intact)		SCD (% decondensed)	
	r	P	r	P	r	P	r	P
Motility	1.00		-0.21	.10	0.34	<.01	0.16	n.s.
Caspase-3			1.00		-0.63	<.001	-0.45	<.001
TMP					1.00		0.47	<.001
SCD							1.00	

Note: TMP = transmembrane mitochondrial potential; SCD = sperm chromatin decondensation; n.s. = not statistically significant; ICSI = intracytoplasmic sperm injection. The average correlations are derived from the weighted averages of the Fisher's Z transformations of the group specific Pearson's correlations.  $P < .05$  is considered significantly different from zero using a Wald test.

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apoptosis signaling compared with results previously derived from healthy donors (10). In addition, the amount of CP3-positive and TMP-disrupted sperm after DGC (control aliquot) was higher in ejaculates from subfertile patients compared with control aliquots of healthy donors evaluated in a previous study (+28.3% and 23.3%, respectively) (10). Similarly, the comparison of the annexin V-negative fractions of healthy donors and infertility patients revealed significantly more annexin V-negative sperm with active CP3 (+18.0 %) and disrupted TMP (+17.0 %) in semen specimens from patients (10).

These findings are in accord with results from other studies indicating that semen samples from infertility patients contain higher levels of activated CP3 and disrupted TMP compared with healthy donors (12, 15, 24). In support of the study hypothesis, separation using annexin V-MACS resulted in a depletion of sperm with activated CP3 and disrupted TMP within the annexin V-negative fraction, whereas sperm having those features were enriched in the annexin V-positive fraction. Earlier studies demonstrated a strong interrelationship between the externalization of phosphatidylserine (annexin V binding) and caspase activation as well as the disruption of the TMP. Nevertheless, because of different time frames and induction pathways of externalized phosphatidylserine, activated CP3 and disrupted TMP the annexin V-negative sperm fraction is not completely free of sperm showing features of apoptosis signaling (14, 23).

### Impact of MACS on Sperm Early Fertilization Potential

In this study, an animal model for ART was applied to evaluate the sperm early fertilization potential for infertility patients. The investigation of human SCD following H-ICSI provides a method to test the sperm early fertilizing capacity and can be used as a measure for training and proficiency testing (25, 26). As mentioned above, the sperm cells of infertile patients contained higher levels of sperm with activated apoptosis signaling than did the sperm cells of healthy donors. The inclusion of annexin V-MACS in semen preparation resulted in significantly improved SCD in annexin V-negative sperm after H-ICSI compared with controls. This may be attributed to the significant reduction of sperm containing active CP3 (−16.7%) and disrupted TMP (−16.9%) in the annexin V-negative fraction after MACS. In addition, the negative correlation between activated sperm apoptosis signaling and SCD was stronger than previously reported in donors (10). This finding gives the first evidence of an association between sperm apoptosis signaling and sperm fertilization rate after ICSI. Because of the limitation of our animal model, assessment of embryonic development was not possible in the present study, but other studies have proven the correlation of DNA fragmentation with later stages of the fertilization process such as embryo development, blastocyst development rate, and clinical pregnancy rates (27–29). However, in patients with oligoasthenoteratozoospermia and asthenoteratozoospermia, motility was impaired after annexin V-MACS. This might be attributed

to the additional centrifugation steps during the sperm preparation. The effect was not observed previously in semen samples from healthy donors and indicates the higher susceptibility of samples with oligoasthenoteratozoospermia and asthenoteratozoospermia.

In healthy donors, no difference has been observed between annexin V-negative fraction and controls with regard to the SCD rate. Donor samples contained lower levels of sperm with activated apoptosis signaling in the control aliquots; the reduction of CP3-positive and TMP disrupted sperm by annexin V-MACS was lower than that observed in the current study (10). Therefore, it is evident that the benefits of using annexin V-MACS would be much greater in samples from infertile patients characterized by high levels of activated apoptosis signaling pathways. Furthermore, the annexin V-positive aliquot of healthy donor sperm with high levels of CP3-positive sperm and those with disrupted MMP displayed significantly reduced SCD rates compared with controls (10). This evidence further supports the negative effects of apoptosis on sperm fertilization potential. These results indicate that semen samples from infertility patients contain higher numbers of apoptotic sperm correlating to decreased SCD rates following H-ICSI. Subsequently, the inclusion of annexin V-based separation in sperm preparation protocols may enhance the outcome of ICSI.

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