

# Effect of In Vitro Incubation on Spontaneous Acrosome Reaction in Fresh and Cryopreserved Human Spermatozoa

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**ABSTRACT: Objectives**-Capacitation and acrosome reaction are prerequisites for fertilization. However, in vitro capacitation is not necessary for an agonist-induced acrosome reaction. We studied whether in vitro capacitation is important in spontaneous acrosome reaction and analyzed how capacitation before cryopreservation influences the acrosomal status of thawed spermatozoa. **Methods**-Semen specimens from normal donors (n = 15) were processed by the swim-up technique and divided into two aliquots. One aliquot was capacitated (capacitation induced) for three hours by incubation in a modified-BWW medium with 3% HSA at 37°C under 5% carbon dioxide. The other aliquot did not receive any treatment. Both aliquots were analyzed by CASA to assess the capacitation status of the spermatozoa and then cryopreserved. Spontaneous acrosome reaction was assessed by FITC-PNA lectin before and after cryopreservation. Sperm viability was measured using Hoechst-33258 stain. **Results**-Before freezing, the frequency of spontaneous acrosome reaction was higher in the capacitation-induced sperm preparation (median, 20.5% [interquartile range, 17.2-37.8]) than in swim-up-induced specimens (median, 10.6% [range, 4.8-23.2]; P <.001). The percentage of viable cells showing acrosome reaction increased after cryopreservation in both swim-up-induced specimens (median, 241.4% [interquartile range, 37.1-678.6]; P <.001) and capacitation-induced specimens (median, 48.2% [range, 6.1-63.3]; P = 0.002). Although this increase was higher in the swim-up-induced specimens (P = 0.002), frequency of postthaw spontaneous acrosome reaction was similar in both groups (P = 0.18). **Conclusions**-We conclude that sperm capacitation significantly optimizes the acrosome reaction. However, a small proportion of normal spermatozoa do not require capacitation to undergo spontaneous acrosome reaction in vitro. After cryopreservation, the percentage of spermatozoa that had intact acrosomes was similar in both groups, despite the fact that one aliquot underwent prefreeze capacitation. These findings suggest that the acrosome reaction may involve complex mechanism(s) rather than a physiological change induced by capacitation. Int J Fertil 43(5):235-242, 1998

**KEY WORDS:** sperm activation, FITC-PNA labeling, cryopreservation, Hoechst-33258 stain

## INTRODUCTION

Under normal physiological conditions, ejaculated spermatozoa need to be washed free of seminal plasma to undergo capacitation, before they can undergo the acrosome reaction, which is needed for penetrating the zona pellucida [1]. Seminal plasma is removed as the spermatozoa transverse the cervical mucus, and capacitation occurs as they are transported across the cervix, uterus; or fallopian tubes [2]. Capacitation essentially comprises a series of cellular changes that permit an influx of calcium ions, an early component of the acrosome reaction. Under laboratory conditions, capacitation is simulated by the removal of the seminal plasma through centrifugation and incubation of the spermatozoa in a medium containing albumin or heat-treated serum.

Previous *in vivo* and *in vitro* studies on capacitation and acrosome reaction have unequivocally found that spermatozoa have to be capacitated before they can undergo the acrosome reaction and that an influx of external calcium is required for this process [3,4]. However, recent *in vitro* studies have shown that neither calcium influx nor preincubation under capacitating conditions is required for an agonist-induced acrosome reaction to occur, although these conditions can optimize the acrosome reaction in response to zona proteins [5,6].

This study was designed [1] to verify whether capacitation under *in vitro* conditions is an important prerequisite for a spontaneous acrosome reaction and [2] to study the influence of capacitation before cryopreservation on the acrosome reaction in cryopreserved spermatozoa.

## **MATERIALS AND METHODS**

### **Chemicals**

TEST yolk-buffer freezing medium, modified Biggers-Whitten-Whittingam medium (BWW) and human serum albumin (HSA) were purchased from Irvine Scientific (Santa Ana, CA). Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and bis-benzimide (Hoechst-33258) were obtained from Sigma (Sigma Chemical Co., St. Louis, MO).

### **Semen Collection and Assessment of Quality**

Semen samples were obtained from 15 normal, healthy volunteers. All subjects were asked to abstain from ejaculation for at least 48 hours before their appointments. Semen was collected at the appointment by masturbation into sterile specimen cups. The ejaculates were allowed to liquefy for 30 minutes at 37°C. All subjects had normal semen volume, sperm count, and motility as defined by the World Health Organization criteria [7]. A small aliquot was removed from each liquefied whole-semen specimen and was analyzed within one hour of collection on a computer-assisted semen analyzer (CASA; Motion Analysis, Cell-Trak, model VP 110, Santa Rosa, CA) to assess sperm concentration and motility and motion characteristics.

### **Sperm Preparation, Capacitation and Cryopreservation**

After the initial semen analysis, the highly motile sperm population from each specimen was isolated by the swim-up method [8]. In brief, the specimens after liquefaction were washed with BWW medium twice by resuspending and centrifuging at 250 g for seven minutes to remove the seminal plasma. The final pellet was reconstituted in BWW medium with 0.3 % HSA. Two to three round-bottom tubes containing 0.7 mL BWW medium were placed at an angle of 30°, and 0.2 mL of the reconstituted pellet was carefully layered at the bottom of each tube. Tubes were incubated for one hour at 37°C in a 5% carbon dioxide atmosphere. At the end of the incubation period, 0.5 mL of the supernatant from each tube was aspirated, combined and centrifuged for seven minutes. The pellet was resuspended in 1 mL of BWW medium, and each reconstituted specimen was subsequently divided into two equal aliquots. The first aliquot (swim-up-induced) received no subsequent treatment and was immediately cryopreserved. The second aliquot was capacitated (capacitation-induced) for three hours by incubation in a BWW medium with 3% human serum albumin (HSA) at 37°C, under 5% carbon dioxide, and then cryopreserved [4,6]. To assess the capacitation status of the spermatozoa, both aliquots were analyzed by CASA before freezing, for percent motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), and amplitude of lateral head displacement (ALH).

TEST-yolk buffer with glycerol was used as a freezing agent for cryopreservation. In brief, a 5-mL vial of the medium was thawed by incubation at 37°C. An aliquot of the medium equal to 25% of the original specimen volume was then added to the specimen. The specimen was gently mixed for five minutes using the Hema-Tek aliquot mixer (Miles, Elkhart, IN). This process was repeated to give a final volume of freezing medium to ejaculate of 1:1. Cryovials were placed at -20°C for eight minutes and submerged in liquid nitrogen at -79°C for two hours. The vials were then immersed in liquid nitrogen at -196°C for long-term storage. After at least 48 hours in liquid nitrogen, the vials were incubated at 37°C for 20 minutes, washed twice in BWW to remove the cryomedia, and reconstituted in 0.5 mL BWW medium with 0.3% HSA [9].

### **Calibration of Motion Analyzer**

The CASA calibration set-up was as follows: 2-well, 20  $\mu\text{m}$ , duration of data capture (frames): 15 (raw) and 30 (washed); minimum motile speed ( $\mu\text{m}/\text{sec}$ ): 600 (raw) and 800 (washed); distance scale factor ( $\mu\text{m}/\text{pixel}$ ): 0.9457; centroid cell size minimum (pixels): 2; centroid cell size maximum (pixels): 8; number of cells to find per well: 200; minimum number of fields per sample: 3. A high degree of correlation seen between the CASA and manual sperm counts ( $r^2 = 1$ , slope 1) and motility ( $r^2 = 0.97$ , slope 0.97) established the accuracy of CASA measurements. Reproducibility of the analyzer was determined by using a calibration videotape recording. Rejection criteria for results were values greater than 2 SD. For raw

specimens, the sperm count was: 38.3 to 42.5 x 10<sup>6</sup>/mL (30 frame/sec) and for washed specimens 33.1 to 34.7 x 10<sup>6</sup>/mL (60 frame/sec); sperm. motility: 60.6 to 75.0% (30 frame/sec) and for washed specimens 78.1 to 81.5% (60 frame/sec). Both frame rates were used at an identical threshold value.

### **Assessment of Acrosome Reaction**

The frequency of spontaneous acrosome reaction of swim-up-induced and capacitation-induced specimens was evaluated before and after freezing. Assessing the acrosome reaction involved the use of fluorescein isothiocyanate-conjugated peanut agglutinin in conjunction with a nuclear stain, bis-benzimide (Hoechst-33258) as a viability test [10-12]. Simultaneous assessment of viability by Hoechst-33258 and acrosome status by FITC-PNA was done by mixing 100  $\mu$ L of semen specimen with 100  $\mu$ L of 2  $\mu$ g/mL Hoechst-33258 solution and incubating for 10 minutes in the dark. Spermatozoa were then washed in phosphate-buffered saline (PBS) solution by centrifugation at 300 g for five minutes to remove excess stain, and the pellet was resuspended in 100  $\mu$ L of BWB. A 20- $\mu$ L aliquot of this solution was subsequently smeared on a microscope slide and allowed to air dry. Smears were prepared in triplicate, immersed in ice-cold methanol for 30 seconds to render the sperm membranes permeable, and allowed to air dry. The fixed smears were immersed in a 40- $\mu$ g/mL FITC-PNA solution, incubated at room temperature for 20 minutes in a foil-covered Coplin jar, and washed gently in PBS to remove the excess label. Scoring was completed within 48 hours of staining.

A Leitz Orthoplan fluorescence microscope (Leitz, Dialux-2, Germany) equipped with a Ploemopak epi-illumination module and a mercury ultraviolet source was used to examine the slides at 1000x magnification in the presence of an anti-quenching agent (Cargille immersion oil, type DF, Fisher Scientific, Pittsburgh, PA) to minimize the loss of fluorescence. Filter cube 1.2 was used for FITC-PNA, which fluoresces "apple-green," and cube A.2 for Hoechst-33258, which fluoresces a bright medium blue. Hoechst-33258 stains the nuclei of damaged cells (dead spermatozoa), which show a bright blue-white fluorescence; it is excluded from viable cells (live spermatozoa), which show a pale blue fluorescence [10]. In each case, the same slide was examined for FITC-PNA labeling and for Hoechst-33258 staining by interchanging the two filters.

### **Categorization of Staining Patterns**

Acrosome staining on FITC-PNA labeling was classified as follows. In an intact acrosome, the acrosomal region of the sperm head exhibited a uniform apple green fluorescence. In a reacted acrosome, only the equatorial segment of the acrosome was stained.

Viability staining with Hoechst-33258 was classified as follows. In viable spermatozoa, the sperm head showed a pale-blue fluorescence, and in a dead spermatozoon, a bright blue-white fluorescence. A total of 200 spermatozoa per, sample were scored. The acrosome reaction scores were based on viable cells only.

### **Reproducibility of Acrosomal Evaluations**

To evaluate the between-observer reproducibility of the acrosomal results, one slide from each donor (n = 15) was evaluated blindly by two observers. To determine the within-observer reproducibility of the acrosomal results, the same 15 smears were reevaluated blindly by one observer (SCE). Five slides were reevaluated a total of 5 times to determine the correlation coefficient of the method.

### **Statistical Analysis**

Repeated-measures analysis of variance (ANOVA) was used to test for statistical differences in sperm motion characteristics in whole semen, and swim up-induced and capacitation-induced specimens before cryopreservation. Paired Student's t test was used to analyze statistical differences in viability scores between swim-up-induced and capacitation-induced samples before and after cryopreservation. A P value of <.05 was considered to be significant. For multiple pairwise comparisons, the Bonferroni correction factor was used and a P value of  $\leq$ .02 was considered to be significant.

The Wilcoxon rank-sum test was used for pairwise comparison of the frequency of spontaneous acrosome reaction between groups, since the data were not normally distributed. An alpha level of  $\leq$ .02 (with Bonferroni correction) was considered statistically significant. In addition, a Wilcoxon signed-rank test was used to determine the magnitude of acrosome reaction change in both swim-up-induced and capacitation-induced groups after cryopreservation. An alpha level of <.05 was considered significant. Statistical analysis was performed using the SAS statistical software package (Cary, NC).

**TABLE I**  
**Characteristics of whole semen, and swim-up-induced and capacitation -induced sperm before cryopreservation.**

Sperm Characteristics	Whole Semen	Swim-up-Induced (P*)	Capacitation-Induced (P†)	P‡
Motility (%)	75.8 ± 11.6	91.0 ± 5.1 (<.001)	90.2 ± 4.0 (<.001)	0.49
VCL (µm/s)	37.2 ± 7.6	97.4 ± 18.9 (<.001)	102.9 ± 21.1 (<.001)	0.34
VSL (µm/s)	16.2 ± 4.0	39.3 ± 5.2 (<.001)	37.5 ± 8.7 (<.001)	0.42
VAP (µm/s)	24.5 ± 5.4	62.1 ± 10.2 (<.001)	66.2 ± 10.3 (<.001)	0.2
LIN(%)	42.2 ± 5.5	42.9 ± 7.2 0.09	38.4 ± 8.8 0.09	0.09
ALH (µm)	2.4 ± 0.4	3.2 ± 0.7 0.02	3.3 ± 0.7 (<.001)	0.52

Values are mean ± SD.

\* Comparison between whole and swim-up-induced groups; P ≤.02 was considered significant.

† Comparison between whole and capacitation-induced groups; P ≤.02 was considered significant.

‡ Comparison between swim-up-induced and capacitation-induced groups; P ≤.02 was considered significant.

VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity; ALH = amplitude of lateral head displacement.

## RESULTS

Sperm characteristics in the whole-semen and swim-up-induced and capacitation-induced specimens before freezing are compared in Table I. There was a significant increase in percent motility and in all motion characteristics, except linearity, in both swim-up-induced and capacitation-induced groups, compared to the whole-semen specimens. Motion characteristics in the capacitation-induced group were comparable to those of the swim-up-induced group, although the VCL, VAP, and ALH values were higher in the former.

Sperm viability assessed by Hoechst-33258 stain decreased significantly after cryopreservation in both swim-up-induced and capacitation-induced groups (P <.001; Table II). The percent change in viability (from prefreeze to postthaw) was not different between groups. In addition, postthaw viability results were similar in both swim-up-induced and capacitation-induced groups.

**TABLE II**  
**Sperm viability before and after cryopreservation in swim-up-induced and capacitation-induced groups, as measured by the Hoechst-33258 stain.**

Treatment	Sperm Viability (%)		P*
	Swim-up-Induced	Capacitation-Induced	
Pre-freeze	88.1 ± 5.8	88.7 ± 6.5	0.2
Post-thaw	29.1 ± 6.0	27.8 ± 6.2	0.57
% Change	-67.1 ± 5.2	-68.5 ± 6.5	0.43
P†	<0.001	<0.001	

Values are mean ± SD; n = 15.

\* Comparison between swim-up-induced and capacitation-induced groups; P <.05 was considered significant.

† Comparison between pre-freeze and post-thaw viability results; P <.05 was considered significant.

**TABLE III****Frequency of spontaneous acrosome reaction in whole semen, and swim-up-induced and capacitation -induced groups before cryopreservation.**

Treatment	Acrosome Reaction (%)						P <sup>‡</sup>
	Whole Semen		Swim-up-Induced (P <sup>*</sup> )		Capacitation-Induced (Pt)		
	Median	25 %-75 %	Median	25 %-75 %	Median	25 %-75 %	
	11.2	5.9-15.2	10.6	4.8-23.2	28.6	17.2-37.8	<.0008
	0.71			0.0008			

Values are median and 25 %-75 % interquartile range.  
\* Comparison between whole semen and swim-up-induced groups; P <.02 was considered significant.  
† Comparison between whole semen and capacitation-induced groups.  
‡ Comparison between swim-up-induced and capacitation-induced groups.

**TABLE IV****Frequency of spontaneous acrosome reaction in swim-up-induced and capacitation-induced groups before and after cryopreservation.**

Treatment	Acrosome Reaction (%)				P <sup>‡</sup>
	Swim-up-Induced		Capacitation-Induced		
	Median	25 %-75 %	Median	25 %-75 %	
Pre-freeze	10.6	4.8-23.2	20.5	17.2-37.8	<.0008
Post-thaw	32.4	26.9-40.0	28.6	25.0-45.9	0.18
Change	241.4	37.1-678.6	48.2	6.1-63.3	0
P <sup>†</sup>	0.001		0.002		

Values are median and 25 %-75 % interquartile range.  
\* Comparison between swim-up-induced and capacitation-induced groups; P <.05 was considered significant.  
† Comparison between pre-freeze and post-thaw acrosome reaction results; P <.05 was considered significant.

Before cryopreservation, the frequency of spermatozoa exhibiting a spontaneous acrosome reaction in the whole-semen specimen was comparable to that in the swim-up-induced acrosome reaction. However, the capacitation-induced acrosome reaction was significantly more frequent than in the swim-up induced acrosome reaction (Table III). After cryopreservation, the percentage of viable cells showing swim-up-induced (P <.001) and capacitation-induced (P = 0.002) acrosome reaction was significantly increased in comparison with the prefreeze values. This increase was higher in the swim-up-induced group than in the capacitation induced group (P = 0.002). However, the frequency of spontaneous acrosome reaction observed after cryopreservation was similar between groups (Table IV).

The mean difference in the measured frequency of spontaneous acrosome reaction between the first and second observer was  $1.31 \pm 9.53\%$  (P = 0.60), and the average coefficient of variation was 6.5%. The intraclass correlation coefficient between the observers was 0.807, with a 95 % confidence interval of 0.62-0.91. The mean difference for the duplicate evaluation of the same 15 slides by one observer was  $-0.29 \pm 2.41\%$  (P = 0.65), and the coefficient of variation was 1.6%. The intraclass correlation coefficient (ICC) between the two readings by the same observer was 0.98, with a 95 % confidence interval of 0.95-0.99. The average coefficient of variation of the five slides (on which the frequency of spontaneous acrosome reaction was evaluated 5 times each) was 1.4%, and of the ICC, 0.89, with a 95 % confidence interval of 0.76-0.96.

## DISCUSSION

It is well established that capacitation and subsequent acrosome reaction can be observed by incubating the semen specimen in a medium containing a high concentration of serum albumin in an atmosphere of 5 % carbon dioxide for a period of three hours. In the present study, the percentage of acrosome-reacted sperm was significantly higher in the capacitation -induced samples than in the swim-up-induced samples; one may conclude that the incubation process facilitates the acrosome reaction. On the other hand, neither the swim-up technique nor the washing procedure appears to increase the acrosome reaction. These results are supported by the present study, where the percentage of acrosome reaction was similar in the

whole semen and processed semen samples. This can be explained by the fact that sperm incubation for one hour in a medium supplemented with 0.3 % HSA during swim-up does not mimic the optimum conditions necessary to permit capacitation and the acrosome reaction.

In the present study, the small percentage of sperm undergoing the acrosome reaction (<5 %) observed in the swim-up-induced specimens occurred independently of capacitation. This study supports the idea that *in vitro* capacitation can no longer be defined as a series of biochemical changes that lead to the acrosome reaction, as proposed by others [5,6,13]. However, capacitation significantly optimizes the rate of spontaneous acrosome reaction before cryopreservation.

Abnormally high rates of spontaneous acrosome reaction (acrosome reaction prematurity) are seen in men with unexplained infertility [14]. The cause of the premature acrosome reaction is not known, but the premature (stimulus-independent) initiation of acrosomal exocytosis may be related to a perturbation of the plasma membrane. The exocytosis process decreases membrane stability, thereby increasing the chances of membrane fusion. In this situation, the acrosome reaction may not involve a premature activation of the receptor-mediated process, but rather reflect an inherent fragility of the sperm membrane, leading to a receptor-independent loss of acrosome.

Cryopreservation of human semen is now an established procedure worldwide, and it has many important applications in the treatment of infertility. Despite the availability of advanced techniques of assisted reproduction, cryosurvival rates remain poor [15]. Cryopreservation directly damages the sperm membrane, with resulting increased membrane permeability and subsequent cell death [16,17]. Cryopreservation can also cause permanent functional damage (sublethal damage), reducing the fertilizing ability of human sperm [18,19]. This reduction in fertility can be explained partially by the reduction in the percentage of normal, intact acrosomes and in total acrosin activity [20]. Damage to the acrosome after cryopreservation may also be secondary to cell death [16]. Nonetheless, an increase in the proportion of viable acrosome-reacted spermatozoa after cryopreservation has been reported by others [19,21].

In the present study, cryopreservation-induced acrosome reaction was significantly increased in viable spermatozoa, whether or not they had undergone capacitation. This increase in acrosome reaction was lower in the capacitation -induced group; however, this group had a higher frequency of spontaneous acrosome reaction (baseline) before freezing compared to the swim-up-induced group, which explains the differences in the results. Furthermore, the frequency of spontaneous acrosome reaction was similar in both sperm populations (capacitation-induced and swim-up-induced) after cryopreservation. This indicates that the sperm population with intact acrosomes (the population that potentially can undergo further acrosome reaction) remained similar in both groups despite the fact that in one group the spermatozoa had undergone capacitation. Our results support the idea that capacitation and acrosome reaction are independent events: the occurrence of the former does not necessarily lead to the latter.

Capacitation before cryopreservation does not appear to affect postthaw acrosomal status. Cryopreservation increases the incidence of viable, reacted sperm, as reported by others [19]. However, one cannot evaluate this evidence by analyzing the total sperm population. Only viable, acrosome-intact sperm were considered for the acrosome reaction, since they alone are able to fertilize the egg. The population of viable spermatozoa was considered as the total sperm population in our study. If we take into account only viable uncapacitated sperm prior to cryopreservation, 88% of the sperm population was potentially able to fertilize the egg. After cryopreservation, the number of viable sperm decreased, and only 29% of the preefreeze sperm survived. This sperm population represents a subgroup of the preefreeze population, and for this reason absolute values (total sperm count) cannot be used for comparison. Thirty-two percent of the spermatozoa underwent the acrosome reaction after freezing. A significant decrease in the live, acrosome-intact population was seen in fresh, acrosome-intact sperm (from 88 % to 68 % ). This population of spermatozoa can potentially fertilize the egg. Since cryopreservation was the main step between the two acrosome assessments, we believe that cryopreservation alone may be responsible for the change.

We speculate (1) that the deleterious effects of cryopreservation on the human sperm acrosome involve a complex mechanism that can be partially explained by sublethal damage, and (2) that a normal physiological change, such as capacitation, does not play an important role in this process.

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## REFERENCES

1. Henkel R, Muller C, Miska W, et al: Determination of the acrosome reaction in human spermatozoa is predictive of fertilization in vitro. *Hum Reprod* 8:2128-2132, 1993.
2. Mortimer D: From the sperm to oocyte: the long route in vivo and in vitro short cut, in Testart J, Frydman R (eds): *Human In-Vitro Fertilization. Actual Problems and Prospects*. Amsterdam, Elsevier Science Publishers, p 93, 1985.
3. Zaneveld LJ, De Jonge CJ, Anderson RA, et al: Human sperm capacitation and the acrosome reaction. *Hum Reprod* 6:1265-1274, 1991.
4. Wang C, Lee GS, Leung A, et al: Human sperm hyperactivation and acrosome reaction and their relationships to human in vitro fertilization. *Fertil Steril* 59:1221-1227, 1993.
5. Anderson RA, Feathergill KA, De Jonge CJ, et al: Facilitative effect of pulsed-addition of dibutyl cAMP on the acrosome reaction of noncapacitated human spermatozoa. *J Androl* 3:398-408, 1992.
6. Bielfeld P, Anderson RA, Mack SR, et al: Is capacitation or calcium ion influx required for the human sperm acrosome reaction? *Fertil Steril* 62:1255-1261, 1994.
7. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions, 3rd ed, New York, Cambridge University Press, 1992.
8. Berger T, Marrs RP, Moyler DL: Comparison of techniques for selection of motile spermatozoa. *Fertil Steril* 43:268-273, 1985.
9. Agarwal A, Tolentino MV, Jr, Sidhu RS, et al: Effect of cryopreservation on semen quality in patients with testicular cancer. *Urology* 46:382-389, 1995.
10. Cross NL, Morales P, Overstreet JW, et al: Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res* 15:213-226, 1986.
11. Mortimer D, Curtis EF, Camenzind AR: Combined use of fluorescent peanut agglutinin lectin and Hoechst-33258 to monitor the acrosomal status and vitality of human spermatozoa. *Hum Reprod* 5:99-103, 1990.
12. Aitken RJ, Buckingham DW, Fang HG: Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J Androl* 14:132-141, 1993.
13. Anderson RA, Feathergill KA, Drisdell RC, et al: Atrial natriuretic peptide (ANP) as a stimulus of the human sperm acrosome reaction and a component of ovarian follicular fluid: correlation of follicular ANP content with in vitro fertilization outcome. *J Androl* 15:61-70, 1993.
14. Tesarik J, Mendoza C: Alleviation of acrosome reaction prematurity by sperm treatment with egg yolk. *Fertil Steril* 63:153-157, 1995.
15. Polanski FF, Lamb EJ: Do the results of semen analysis predict future fertility? A survival analysis study. *Fertil Steril* 49:1059-1065, 1988.
16. Cross NL, Hanks SE: Effects of cryopreservation on human sperm acrosomes. *Hum Reprod* 6:1279-1283, 1991.
17. Watson PF, Kunze E, Cramer P, et al: A comparison of critical osmolarity and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. *J Androl* 13:131-138, 1992.
18. Barthelemy C, Royere D, Hammah S, et al: Ultrastructural changes in membranes and acrosome of human sperm during cryopreservation. *Arch Androl* 25:29-40, 1990.
19. McLaughlin EA, Ford WC, Hull MG: Motility characteristics and membrane integrity of cryopreserved human spermatozoa. *J Reprod Fertil* 95:527-534, 1992.
20. Mack SR, Zaneveld LJ: Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res* 18:375-383, 1987.
21. Centola GM, Mattox JH, Burde S, et al: Assessment of the viability and acrosome status of fresh and frozen-thawed human spermatozoa using single wavelength fluorescence microscopy. *Mol Reprod Dev* 27:130-135, 1990.