Detection of altered acrosomal physiology of cryopreserved human spermatozoa after sperm residence in the female reproductive tract

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At least some of the spermatozoa that remain motile following cryopreservation have sustained sublethal damage that reduces their functional capacity *in vivo*. Although it is believed that acrosomal damage is partly responsible for impaired sperm function *in vivo*, direct evidence for this hypothesis is lacking because spermatozoa have not been collected from the female reproductive tract for evaluation. In the study reported here, cervical mucus was collected from women 24 h after artificial insemination by cervical cup. For both cryopreserved and nonfrozen inseminates, spermatozoa within the cervical mucus and spermatozoa that migrated out of mucus into culture medium ($t = 1$ h) were viable and had intact acrosomes. However, although nonfrozen spermatozoa did not initially respond to induction of the acrosome reaction with follicular fluid, a significant proportion of cryopreserved spermatozoa did respond. These results demonstrate that cryopreservation increases the acrosomal lability of spermatozoa residing in the female reproductive tract. An *in vitro* test was developed to detect this form of cryodamage. Sperm-free mucus was collected before insemination and spermatozoa from the inseminate were allowed to swim into this column of mucus *in vitro*. Spermatozoa recovered from this mucus sample were compared with spermatozoa from the paired sample collected from the cervix 24 h later. This *in vitro* test could detect acrosomal lability in cryopreserved semen samples, and this approach may prove valuable for studying sublethal cryodamage to the acrosome.

Introduction

Extensive research on cryopreservation has been carried out with spermatozoa from many species over the last forty years. Nevertheless, conception rates following artificial insemination with cryopreserved spermatozoa are lower than those obtained using nonfrozen spermatozoa, even when equal numbers of motile spermatozoa are inseminated. The lower fertility of cryopreserved spermatozoa suggests that those that survive freezing and remain motile after thawing have impaired functional competence *in vivo*. It is possible that all of the spermatozoa have reduced function, or that the size of the functional population is reduced. In general, it is agreed that at least part of this impaired function is due to abnormal sperm transport in the female reproductive tract, including decreased longevity of spermatozoa following insemination (Saacke, 1982; Hawk, 1987). Several lines of evidence support this hypothesis (1) longevity during incubation *in vitro* is reduced for cryopreserved spermatozoa (Saacke and White, 1972; Keel and Black, 1980; Critser *et al.*, 1987b); (2) transport to the oviduct of motile, cryopreserved spermatozoa has been shown to be

impaired in some species (Mattner *et al.*, 1969; Lightfoot and Salamon 1970; Lineweaver *et al.*, 1970; Pursel *et al.*, 1978; Saacke, 1982); (3) longevity or retention of cryopreserved spermatozoa in the female reproductive tract is reduced (Mattner *et al.*, 1969; Pursel *et al.*, 1978; Saacke, 1982; Parrish and Foote, 1986); and (4) conception rates achieved with cryopreserved spermatozoa can approach those for nonfrozen spermatozoa if insemination is more carefully timed with respect to ovulation (Smith *et al.*, 1981; Parrish and Foote, 1986) or spermatozoa are inseminated closer to the oviduct (Lightfoot and Salamon, 1970; Byrd *et al.*, 1990).

The sublethal cryodamage that results in abnormal sperm function has proved difficult to detect *in vitro*. The lack of laboratory methodology for detecting sperm cryodamage has been an impediment to studies of sperm cryobiology. Without good experimental endpoints, it is difficult to determine the mechanisms underlying diminished sperm function, and it is difficult to develop improved techniques of sperm cryopreservation. Endpoints such as embryonic development and rate of cell division that are useful for studying cryobiology in other cellular systems are not applicable to spermatozoa. Assessment of viability alone is useful for monitoring cryodamage in many cells, but this approach cannot detect the sublethal changes that can disrupt the highly complex function of mammalian

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spermatozoa. At the present time, the only effective measurement of sperm function *in vivo* is the conception rate following insemination (Amann, 1989). With the exception of dairy cattle, this approach cannot be used for initial testing of cryopreservation methods. *In vitro* assays of sperm function are required for preliminary studies to identify methods for testing in clinical trials.

Cryopreserved spermatozoa have been shown to differ from nonfrozen spermatozoa by several laboratory criteria (references are examples for human spermatozoa), including the ultrastructure (Woolley and Richardson, 1978), percentage of motile spermatozoa (Critser *et al.*, 1987b), swimming velocity (Pilikian *et al.*, 1982), metabolism (Ackerman and Behrman, 1975), percentage of intact acrosomes (Pilikian and Guerin, 1986; Critser *et al.*, 1987a; Centola *et al.*, 1990), acrosomal enzymes (Mack and Zaneveld, 1987), acrosomal function (Cross and Hanks, 1991), longevity (Critser *et al.*, 1987b), penetration of cervical mucus (Ulstein, 1973), binding to the zona pellucida (Coddington *et al.*, 1991), and fusion with zona-free hamster oocytes (Critser *et al.*, 1987b). However, the relationship of these measures to sperm function in the female reproductive tract is not well understood. In human semen, distinct subpopulations of spermatozoa can be distinguished on the basis of physiological characteristics (e.g. Davis and Katz, 1988; Robertson *et al.*, 1988; Mortimer and Camenzind, 1989; Menkveld *et al.*, 1991). Measurements that provide mean values for the entire population of spermatozoa in semen may not detect differences in the subpopulations of spermatozoa that are biologically important. Assays that do not distinguish between motile and immotile spermatozoa may be particularly insensitive for detecting changes in spermatozoa that are functionally significant. *In vitro* tests for sperm cryodamage must be carefully chosen to approximate conditions encountered by the fertilizing spermatozoon *in vivo*. The standard assays of sperm longevity may detect an increased sensitivity of cryopreserved spermatozoa to conditions *in vitro* (e.g. pH, toxic components of seminal plasma), but these conditions may have little relevance to the environment of the female reproductive tract.

In the experiments reported here, we have studied the motility and acrosomal status of cryopreserved human spermatozoa following a period of residence in the female reproductive tract. Such spermatozoa can be sampled noninvasively from the cervical canal, and they represent a population that is similar to that evaluated clinically in the post-coital test (Hanson *et al.*, 1982). The physiology of nonfrozen spermatozoa recovered from cervical mucus has been studied in some detail. These cervical spermatozoa remain motile, acrosome intact, and capable of fertilizing oocytes for at least three days after insemination (Gould *et al.*, 1984; Zinaman *et al.*, 1989). These properties of cervical spermatozoa are consistent with prolonged functional integrity and with the hypothesis that the cervix is a reservoir for spermatozoa that can eventually participate in fertilization. In this communication, we report observations of abnormal acrosomal physiology of cryopreserved spermatozoa recovered from the cervix, and we describe an *in vitro* assay of sperm-cervical mucus interaction that may be useful for predicting this altered physiology of cryodamaged human spermatozoa.

Materials and Methods

Preparation of cryopreserved and fresh semen for insemination

The six research subjects who provided semen for this study were donors in the therapeutic artificial insemination programme. After approval was obtained from the University of California, Davis Institutional Review Board, informed consent was obtained to use these samples for this research. Each donor was screened to exclude genetic and health problems as recommended by the American Fertility Society (1990), and they were further screened for good semen quality and sperm cryosurvival. Cryopreserved semen from these donors was proven to be fertile in the artificial insemination programme.

The cryopreservative diluent was modified from the TEST-yolk medium of Bolanos *et al.* (1983). All reagents used were obtained from Sigma Chemical Company (St Louis, MO). The buffer solution contained 188.7 mmol Tes l^{-1} , 84.8 mmol tris base l^{-1} , 11.1 mmol glucose l^{-1} , 15 iu penicillin $G ml^{-1}$ (sodium salt), and 25 iu streptomycin sulfate ml^{-1} in HPLC-grade water. Complete diluent was prepared by combining 80% (v/v) buffer solution and 20% fresh egg yolk. The yolk granules were sedimented by centrifuging the diluent at 1000 g for 30 min. Approximately the upper two-thirds of the supernatant solution was collected, and the pH was adjusted to 7.40 with 1.0 mol NaOH l^{-1} . After filtering the diluent through a sterile, 0.45 μm pore-size filter, 10 ml aliquots of diluent were frozen in 15 ml centrifuge tubes (Corning Glass Works, NY) and stored at $-20^{\circ}C$. The diluent was thawed and warmed above $20^{\circ}C$ before use.

Semen was collected by masturbation and was allowed to liquify at room temperature. After liquefaction, the volume was measured and the concentration and percentage of motile spermatozoa in the semen were determined. For semen to be cryopreserved, the total number of motile spermatozoa in the ejaculate was calculated. Sperm 'freezability' was defined as the ratio of the percentage of motile spermatozoa after thawing to the percentage of motile spermatozoa in the fresh ejaculate. For each donor, the mean sperm freezability for the three previous ejaculates was used to estimate motility loss during cryopreservation, and the semen was diluted to produce a predicted after thawing insemination dose of 30×10^6 motile spermatozoa in 0.5 ml. If the motile sperm density was inadequate to dilute at least 1:1 with diluent, the specimen was not used. Glycerol was added in three aliquots over 30 min to a final 5% (v/v) concentration. After the final addition of glycerol, the semen was mixed thoroughly by gentle inversion, and 0.5 ml aliquots were pipetted into 1.2 ml, internal thread, screw-top cryovials (Corning Glass Works). Cryovials were attached to canes and loaded into a programmable freezer equipped with a cane rack (CryoMed 1010, CryoMed, New Baltimore, MI). The cooling programme produced an intra-vial cooling rate of $1^{\circ}C min^{-1}$ to $4^{\circ}C$ and $10^{\circ}C min^{-1}$ to $-100^{\circ}C$. Once the intra-vial temperature stabilized at $-100^{\circ}C$, the freezing chamber was opened and the canes were quickly transferred to liquid nitrogen. Cryopreserved semen was stored in liquid nitrogen for at least six months before use.

Semen was thawed by transferring individual cryovials rapidly to a thawing thermos (Continental Plastics, Baraboo, WI) containing water at $37^{\circ}C$. After 5 min, the cryovial was

transferred to room temperature and dried carefully before opening. Diluent, 0.5 ml, was added to produce 1 ml of cryopreserved semen for insemination.

Insemination and collection of cervical mucus

Informed consent to obtain mucus samples for this study was obtained from patients undergoing therapeutic donor insemination. Artificial insemination was performed using a cervical cup as described previously (Hanson and Overstreet, 1981). Twenty-four women were inseminated with cryopreserved spermatozoa and nine were inseminated with nonfrozen spermatozoa from the same donors. Each patient was studied in a single experiment. Patients were instructed to abstain from sexual intercourse for 2–3 days before insemination and 2–3 days after insemination. Ovulation was detected using a home testing kit for urinary LH (Ovuquick; Monoclonal Antibodies, Inc., Sunnyvale, CA). A single insemination was performed on the day that the LH rise was detected. Before placing the cervical cup, 25–50 µl of cervical mucus was collected from the cervical canal (Katz *et al.*, 1980) for use in the *in vitro* assay described below. Either liquified, nonfrozen semen or thawed, cryopreserved semen was drawn into a polyethylene catheter attached to a 3 ml syringe. A small aliquot (approximately 50 µl) of the semen was used for the *in vitro* assay described below. After placement of the cervical cup, at least 1.5 ml of nonfrozen semen or 1.0 ml of cryopreserved semen was used for insemination. The cup was left in place for 4–6 h and was removed by the patient. The day after insemination (16–24 h), 25–50 µl of mucus was collected as described previously and used for recovery of cervical spermatozoa.

Evaluation of the acrosomal status of cervical spermatozoa

The procedures for evaluation of spermatozoa in cervical mucus, recovery of spermatozoa from cervical mucus and evaluation of sperm acrosomal status have been described in detail by Zinaman *et al.* (1989). Briefly, a 10–20 µl aliquot of mucus was incubated in 1 µg Hoescht 33258 ml⁻¹ (H258; Sigma) in Dulbecco's phosphate-buffered saline (DPBS, pH 7.40) at 37°C for 10 min to evaluate the viability of cervical spermatozoa. The mucus was recovered with forceps, rinsed with 2 ml warm DPBS while supported on nylon mesh, and transferred to a microscope slide. A smear was then made and the slide was transferred to a slide jar containing 100% ethanol at -20°C. After at least 1 h, the slide was air-dried, and the mucus smear was treated with 100 µg fluoresceinated *Pisum sativum* lectin ml⁻¹ (FITC-PSA; Vector Laboratories, Burlingame, CA) in DPBS for 10 min. The slide was rinsed in deionized water and dehydrated in 100% ethanol for at least 1 h. Slides were mounted with a minimal volume of ethanol containing 100 mg 1,4-diazabicyclo [2.2.2] octane ml⁻¹ (DABCO; Sigma) and the slide preparation was sealed with nail enamel. These observations were made only on cryopreserved spermatozoa.

Spermatozoa were recovered from cervical mucus by emptying the remaining mucus from the catheter into 400 µl of BWW medium as modified by Overstreet *et al.* (1980). The mucus was observed under a stereomicroscope with the mirror adjusted to produce a dark background. If spermatozoa were present,

they could be seen as small, refractile points, migrating within the mucus. Only motile spermatozoa migrate out of the mucus (authors' unpublished observation), and spermatozoa have never been observed re-entering the mucus under these conditions. After incubation for 60 min (37°C, 5% CO₂ in water-saturated air), the mucus was removed and evaluated as described for the freshly collected mucus. The suspension containing spermatozoa that migrated out of mucus was evaluated for acrosomal status immediately (*t* = 1 h) and after a further 5 h incubation (*t* = 6 h). Spermatozoa were evaluated for spontaneous acrosome reactions and for acrosome reactions induced with human follicular fluid.

The human follicular fluid used in this study was collected from a single patient undergoing follicular aspiration before *in vitro* fertilization. After the oocytes were removed, follicular aspirates were frozen and stored at -20°C until use. This batch of follicular fluid was screened for bioactivity as described by Zinaman *et al.* (1989). Just before use, follicular fluid was thawed and microfuged for 60 s to sediment any particulate material. The supernatant was used immediately.

The sperm suspension recovered from cervical mucus was mixed gently before removing two 80 µl test samples. Twenty microlitres of human follicular fluid was added to the first sample to induce acrosome reactions, and 20 µl BWW was added to the second sample as a control treatment (spontaneous acrosome reactions). One microlitre of H258 (100 µg ml⁻¹ deionized water) was added after incubation for 5 min. After incubation for a total of 15 min, the spermatozoa were fixed by adding 100 µl of 2% paraformaldehyde in DPBS. Ten minutes later, the spermatozoa were collected on polycarbonate filters and stained with FITC-PSA. The filters were transferred to slides, then mounted and sealed as described for mucus.

In each sample, one hundred spermatozoa were examined with appropriate epifluorescence microscopy. Individual spermatozoa were considered viable if they excluded the DNA stain H258. They were considered acrosome reacted if they were viable and did not label with FITC-PSA over the acrosomal cap region (Cross *et al.*, 1986). The percentage of acrosome-reacted spermatozoa was calculated as the quotient of the number of acrosome-reacted spermatozoa and the total number of viable spermatozoa.

Selection of spermatozoa by penetration of cervical mucus in vitro

The methods used for recovery and evaluation of spermatozoa after penetration of cervical mucus *in vitro* were the same as those described by Zinaman *et al.* (1989), except that in these experiments the *in vitro* and *in vivo* experiments were paired. That is, the *in vitro* test was carried out with mucus that was obtained from the patient just before insemination and with a small aliquot of the inseminate. Of twenty-four patients inseminated with cryopreserved spermatozoa, paired samples were obtained for thirteen experiments. After the mucus was examined to ensure that it did not contain spermatozoa, both ends of the collection catheter were sealed with tube sealer (Seal-ease; Clay Adams, Parsippany, NJ) and the catheter was cut in half. The cut ends of the catheter were positioned such that the mucus was in contact with 50 µl of semen contained in a 1.5 ml cryovial. The cryovial and catheters were incubated in a 17 × 100 mm culture tube (Falcon) for 1 h. After incubation,

Table 1. Acrosomal response of nonfrozen and cryopreserved human spermatozoa to follicular fluid after recovery from cervical mucus and incubation for 1 h and 6 h *in vitro*

Sperm treatment	Number of experiments	Percentage of acrosome-reacted spermatozoa			
		1 h incubation		6 h incubation	
		BWW control	Follicular fluid	BWW control	Follicular fluid
Nonfrozen	9	1 ± 0	2 ± 1	4 ± 1	17 ± 3
Cryopreserved	24	3 ± 1	7* ± 1	8 ± 2	19 ± 3

Data are presented as means ± SEM where experiment is the experimental unit. One hundred spermatozoa were scored in each experiment. Spermatozoa were considered to be acrosome reacted if they excluded the supravital dye Hoescht 33258 and had no evidence of acrosomal cap staining with the fluoresceinated pea lectin FITC-PSA. BWW: BWW culture medium as modified by Overstreet *et al.* (1980).

*Cryopreserved spermatozoa were significantly different ($P < 0.0001$) from nonfrozen spermatozoa.

the catheters were removed from the semen, the ends were rinsed with 1–2 ml BWW, and 5 mm was cut from the rinsed ends of the catheter to remove any spermatozoa adhering to the mucus surface. The mucus in these catheters was then recovered and evaluated in the same manner as mucus collected after insemination.

Statistical analysis

Differences between nonfrozen and cryopreserved spermatozoa were detected using general linear model analysis of variance with sperm treatment as a fixed factor. Spermatozoa that penetrated mucus *in vitro* were compared with spermatozoa recovered from the cervical mucus after insemination by paired *t* test.

Results

Viability and acrosomal status of cryopreserved spermatozoa in cervical mucus

The viability of cryopreserved spermatozoa in the cervical mucus was high and the incidence of acrosome reactions was low. In the fourteen samples of cervical mucus collected on the day after insemination, the viability was $91 \pm 2\%$ and the percentage of viable, acrosome-reacted spermatozoa was $0.1 \pm 0.3\%$. These results are comparable to our previous observations on nonfrozen spermatozoa (Zinaman *et al.*, 1989). Similarly, $4 \pm 1\%$ of the spermatozoa remaining in the mucus after 1 h in BWW medium were acrosome reacted, which is also similar to our previous results for nonfrozen spermatozoa.

Response of cryopreserved spermatozoa recovered from cervical mucus to induction of the acrosome reaction

In nine experiments with nonfrozen spermatozoa, the acrosomal physiology of spermatozoa recovered from cervical mucus was similar to that observed in our previous study (Zinaman *et al.*, 1989). That is, very few spermatozoa were

acrosome reacted after migrating out of the mucus (Table 1), and, at this time ($t = 1$ h), nonfrozen spermatozoa did not acrosome react in response to challenge with follicular fluid. After further incubation ($t = 6$ h), the level of spontaneous acrosome reactions increased slightly, and a significant proportion of spermatozoa acrosome reacted in response to follicular fluid. Cryopreserved spermatozoa differed from this pattern in that a higher proportion of spermatozoa responded to follicular fluid immediately after migrating from the mucus (Table 1).

The response of cryopreserved spermatozoa to follicular fluid varied among experiments. For nonfrozen spermatozoa, the range of acrosome reactions in response to follicular fluid at $t = 1$ h was 0–5%. This was also the case in our previous experiment with nonfrozen spermatozoa. The distribution of samples for this variable was bimodal such that about half of the samples fell within the normal and half had more acrosome reactions than normal. When the experiments with cryopreserved spermatozoa having a normal acrosomal response were grouped separately from experiments with large numbers of acrosome reactions, the spermatozoa from the latter experiments not only had a greater acrosomal response to follicular fluid at $t = 1$ h, but also appeared to have more spontaneous acrosome reactions at this time (Table 2). The variation among donors in the response of cryopreserved spermatozoa to follicular fluid at $t = 1$ h was significant ($P < 0.03$; data not shown). However, each of the six donors for whom multiple samples were analysed had normal acrosomal response to follicular fluid at $t = 1$ h in some experiments (1–5% acrosome-reacted spermatozoa) and higher responses in other experiments.

Acrosomal response to follicular fluid of cryopreserved spermatozoa following mucus penetration *in vitro*

In the thirteen experiments for which paired data were collected, there was no significant difference in acrosomal status between spermatozoa that penetrated cervical mucus *in vitro* and spermatozoa that were recovered from cervical mucus 24 h after insemination (Table 3). In eleven of thirteen experiments, the $t = 1$ h acrosomal response of spermatozoa to follicular fluid after mucus penetration *in vitro* was predictive of whether

Table 2. Two groups of cryopreserved human spermatozoa having different responses to follicular fluid after recovery from cervical mucus and incubation for 1 h and 6 h *in vitro*

Acrosomal response	Number of experiments	Percentage of acrosome-reacted spermatozoa			
		1 h incubation		6 h incubation	
		BWW control	Follicular fluid	BWW control	Follicular fluid
Normal	13	2 ± 0	3 ± 1	6 ± 2	17 ± 5
Increased	11	4 ± 1	11 ± 1	10 ± 3	21 ± 4

Data are presented as means ± SEM where experiment is the experimental unit. One hundred spermatozoa were scored in each experiment. Spermatozoa were considered to be acrosome reacted if they excluded the supravital dye Hoescht 33258 and had no evidence of acrosomal cap staining with the fluoresceinated pea lectin FITC-PSA. BWW: BWW culture medium as modified by Overstreet *et al.* (1980). Spermatozoa in a given experiment were considered to have increased responsiveness to follicular fluid if the percentage of acrosome reactions was ≥ 6% after follicular fluid challenge at the 1 h time point.

Table 3. Acrosomal response of cryopreserved human spermatozoa to follicular fluid after penetration of cervical mucus *in vitro* or after 24 h in the female reproductive tract (*in vivo* assay)

Source of cervical mucus	Percentage of acrosome-reacted spermatozoa			
	1 h incubation		6 h incubation	
	BWW control	Follicular fluid	BWW control	Follicular fluid
<i>In vitro</i>	2 ± 1	6 ± 1	6 ± 2	27 ± 4
<i>In vivo</i>	3 ± 1	7 ± 1	9 ± 2	23 ± 4

Data are presented as means ± SEM for thirteen paired experiments. One hundred spermatozoa were scored for each treatment. There were no significant differences between *in vitro* and *in vivo* penetration. Spermatozoa were considered to be acrosome reacted if they excluded the supravital dye Hoescht 33258 and had no evidence of acrosomal cap staining with the fluoresceinated pea lectin FITC-PSA. BWW culture medium as modified by Overstreet *et al.* (1980). The *in vitro* assay was performed with a mucus sample collected just before insemination, and incubated with an aliquot of the semen used for the insemination. The *in vivo* assay was performed with mucus collected from the cervix 24 h after insemination.

the response for spermatozoa recovered from the cervix would be normal (≤ 5% acrosome reactions) or increased (≥ 6% acrosome reactions). In one case the response *in vitro* was normal (4%) and the response *in vivo* was increased (10%), whereas in a second case the response *in vitro* was increased (9%) and the response *in vivo* was normal (5%).

Discussion

In this study the physiology of a highly selected subpopulation of cryopreserved spermatozoa was evaluated. The semen samples used for insemination were obtained from men with high semen quality having spermatozoa relatively resistant to cryodamage. The spermatozoa were further selected for their ability to penetrate cervical mucus, remain viable in the cervix

and subsequently migrate out of mucus. Although there is no direct evidence that these spermatozoa are representative of the fertilizing population, there is indirect evidence in a number of species that the cervix functions as a reservoir for spermatozoa (reviewed in Drobnis and Overstreet, 1992). Human spermatozoa that remain motile following cryopreservation have reduced ability to penetrate cervical mucus (Ulstein, 1973). This decreased function is probably due in part to inadequate sperm motility, but changes in the sperm surface are also likely to be involved. In addition to penetrating the cervical mucus, the spermatozoa that we recovered for study had also remained viable within the cervix for many hours and could migrate out of the cervical mucus into culture medium *in vitro*. A very high proportion of both cryopreserved and nonfrozen spermatozoa in the cervical mucus were viable and motile. Cryopreserved human spermatozoa have reduced longevity *in vitro* (Critser *et al.*, 1987b), and they disappear from cervical mucus more rapidly than do nonfrozen spermatozoa (Overstreet and Drobnis, 1993), suggesting that nonmotile spermatozoa are preferentially lost from the cervix. The current model for sperm transport in the ruminant cervix (Mullins and Saacke, 1989) proposes that immotile spermatozoa are flushed from the cervix by mucus secretion, whereas motile spermatozoa are retained in the cervix because of their continued migration, which is oriented against the direction of mucus flow. If human sperm transport is consistent with this model, the cryopreserved spermatozoa retained in the cervix represent a subpopulation that maintain their motility during prolonged residence in the female reproductive tract. In light of the fact that the spermatozoa recovered from cervical mucus represent such a select subpopulation of cryopreserved spermatozoa, it is remarkable that they differed from nonfrozen spermatozoa in their physiology.

It has been suspected for more than twenty years that cryopreservation damages the sperm acrosome and that acrosomal damage results in decreased fertility. The proportion of spermatozoa in semen with intact acrosomes is well-correlated with conception rates after artificial insemination in cattle (Saacke and White, 1972), suggesting that motile spermatozoa in the insemination with damaged acrosomes have reduced functional capacity *in vivo*. However, these studies used techniques

involving sperm fixation that did not distinguish between motile and immotile spermatozoa. It is possible that the spermatozoa with abnormal acrosomes were dead, and that this form of lethal damage was merely coincident with other sublethal damage that was directly related to the abnormal function of the motile spermatozoa. This problem of interpretation applies generally to previous studies of cryopreservation damage to the acrosome. Our experiments which assess living, cryopreserved spermatozoa recovered from the human cervix provide the first direct evidence of abnormal acrosomal physiology in the population of highly motile, cryopreserved spermatozoa that remain viable in the female tract.

The increased incidence of acrosome reactions and the enhanced acrosomal response to follicular fluid are consistent with premature capacitation of cryopreserved spermatozoa. The acrosome reaction in the fertilizing spermatozoon is believed to occur in the vicinity of the oocyte (reviewed in Meizel, 1978, 1985; Yanagimachi, 1981, 1988; Talbot, 1985), and premature capacitation and acrosome reactions may be responsible for the decreased duration of normal function observed for cryopreserved spermatozoa. This hypothesis is supported by the observation that cryopreserved human spermatozoa have the ability to fuse with zona-free hamster oocytes immediately after separation from seminal plasma, and rapidly lose this ability during subsequent incubation (Critser *et al.*, 1987b). In contrast, nonfrozen spermatozoa require several hours of preincubation to complete capacitation *in vitro* and they retain the ability to fuse with oocytes for many hours. Not only do premature acrosome reactions reduce sperm longevity, but untimely completion of this cellular event may inhibit transport of spermatozoa to the site of fertilization and normal interaction with the oocyte investments. For example, acrosome-reacted hamster spermatozoa have reduced ability to penetrate the cumulus oophorus (Suarez *et al.*, 1984; Cherr *et al.*, 1986; Cummins and Yanagimachi, 1986). It is generally believed, although not proven, that the fertilizing spermatozoon completes the acrosome reaction during interaction with the oocyte investments, and that spermatozoa that undergo the acrosome reaction outside the vicinity of the oocyte cannot participate in fertilization.

The *in vitro* assay used in this study measures the acrosomal physiology of the subpopulation of spermatozoa that can migrate through cervical mucus. The acrosomal physiology of cryopreserved spermatozoa, as evaluated with this assay, was similar to that of spermatozoa recovered from the cervix after insemination. In paired comparisons, the assay could predict subsequent abnormal acrosomal physiology of cryopreserved spermatozoa *in vivo*. This technique can be used to study sublethal cryodamage to spermatozoa and to compare various cryopreservation methods for their ability to reduce this form of damage to the sperm acrosome.

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