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Comparison of Fresh and Cryopreserved Human Sperm Attachment to Bovine Oviduct (Uterine Tube) Epithelial Cells *in Vitro*

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ABSTRACT: Formation of a prefertilization sperm reservoir in mammals is thought to occur via sperm cell attachment to fallopian tube or oviduct epithelial cells (OEC). Recent data suggests that such an interaction also occurs for human sperm in the fallopian tube. We have previously validated an *in vitro* sperm–OEC coculture system utilizing bovine OEC monolayers to study postejaculatory human sperm physiology. This study was done to evaluate aspects of human sperm attachment to OEC in coculture and to determine if such attachment and subsequent sperm survival differ between fresh and cryopreserved human sperm. In experiment 1, aliquots of fresh (n = 4) or cryopreserved sperm (n = 3) from normospermic donors were placed into coculture with OEC monolayers at dilutions ranging from 2×10^5 to 15×10^6 sperm per well. Numbers of each type of sperm attaching to OEC at each concentration were determined. In experiment 2, fresh and cryopreserved sperm from the same donors (n = 4) were put into OEC coculture to observe numbers attaching and subsequent survival time for each sperm type. Sperm attachment to OEC occurred in a linear, dose-dependent manner for fresh and cryopreserved sperm in experiment 1, both as a function of total sperm numbers and as a function of numbers of motile sperm applied ($R^2 \geq 0.79$). However, cryopreserved sperm attached to the OEC at a slower rate than fresh (as a function of the average increase in the number of sperm attaching per unit increase in the number of sperm applied; $P < 0.05$), with an overall lower percentage of the total and motile sperm applied attaching to OEC ($P < 0.01$) for cryopreserved versus fresh sperm. Fewer cryopreserved sperm also attached to the OEC, as compared with fresh sperm, in experiment 2 ($P < 0.05$), even after correcting for motility differences between the sperm types. Sperm survival time in coculture was also decreased for cryopreserved sperm as compared with fresh sperm ($P = 0.005$). Understanding the kinetics of sperm and OEC interactions may be useful for developing improved cryopreservation protocols or bioassays of sperm function.

Key words: Sperm coculture, sperm storage, artificial insemination, uterine tube, cryodamage.

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Cryopreservation of human sperm generally reduces subsequent fertility outcomes when compared directly with freshly ejaculated sperm (Woolley and Richardson, 1978; Cohen et al, 1981; Keel et al, 1987). Nonetheless, cryopreservation is an integral part of the assisted-reproduction industry, primarily for artificial insemination of women with commercially prepared donor sperm or by therapeutic donor insemination (TDI). In fact, it is one of the most common treatment interventions for subfertile couples because of its widespread availability and relatively lower costs as compared with other forms of intervention (Critser, 1998).

Understanding the differences in physiology and function between freshly ejaculated and cryopreserved sperm should promote the development of superior protocols for their use with the goal of improving overall fertility outcome. Previously, it has been established that cryopreservation of human sperm adversely effects sperm function, including postthaw motility, membrane function, and cervical mucus penetration (Ulstein, 1973; Keel and Black, 1980; Zavos and Cohen, 1980; Esteves et al, 1996). In addition, ultrastructure of the sperm cell is significantly altered during cryopreservation (Mahadevan and Trounson, 1984), and changes in chromatin structure and integrity can occur (Royere et al, 1988; Thompson et al, 1994; Ellington et al, 1998a).

Some studies have seen reductions in fertility for cryopreserved sperm that are more severe than can be accounted for by routine sperm function assay outcomes, such as postthaw motility or cervical mucus penetration (Salisbury, 1965; Ulstein, 1973; Cohen et al, 1981). Such studies suggest that sublethal damage to the sperm may have occurred that could affect postejaculatory, prefertil-

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ization sperm physiology (Ulstein, 1973; Check and Check, 1991; Coddington et al, 1991). Cryopreservation of sperm could also affect the formation of a sperm reservoir in women.

A postcoital reservoir of fertile sperm is maintained in women; the probability of conception from a single coitus, 5 or more days prior to ovulation, reportedly ranges between 10–13% (Barrett and Marshall, 1969; France et al, 1984; Wilcox et al, 1995). The site of this sperm storage in the woman remains under study. However, evidence suggests that storage in the fallopian tube may play a role similar to that seen in other mammals, with freshly ejaculated motile sperm recovered from the tubes of women days to weeks after insemination or reported coitus (Rubenstein et al, 1951; Sumi, 1982; Mansour et al, 1993). Also, intimate contact between sperm and tubal epithelial cells, suggestive of attachment between the two, has been observed during *in vitro* studies (Vigil et al, 1992; Pacey et al, 1995; Baillie et al, 1997; Ellington et al, 1998b). To date, it has not been possible to quantify or fully evaluate the physiology of sperm storage in the tubes of normal women because of ethical and logistical constraints.

The mechanisms of sperm storage are particularly relevant for cryopreserved sperm, which are thought to be more short lived in the woman's body as compared with fresh sperm, thus requiring intensive and accurate timing of insemination with ovulation (Hogerzeil et al, 1991; Deary et al, 1997; Matilsky et al, 1998). In spite of much work in this area, it has been difficult to identify an optimal protocol for TDI because of differences between studies in the numbers and quality of sperm used and in the recipient management techniques (Marshburn et al, 1992; Huang et al, 1996; Berg et al, 1997). Further, a lack of understanding of sperm function and physiology in the fallopian tube has slowed adoption of cryopreservation protocols that could enhance such physiologically relevant outcomes as sperm storage potential.

One way to advance the study of postejaculatory sperm physiology has been through the use of an *in vitro* sperm and oviduct (tubal) epithelial cell (OEC) coculture system, which has been recently adapted for human tissue studies (Pacey et al, 1995; Baillie et al, 1997; Ellington et al, 1998b). In this system, human sperm attach to polarized OEC monolayers, and their survival is prolonged *in vitro* as compared with that seen in media alone (Ellington et al, 1998b). Sperm attachment to OEC *in vivo* and *in vitro* is thought to be mediated by lectin binding for the species studied (DeMott et al, 1995; Dobrinski et al, 1996; Lefebvre et al, 1997; Suarez et al, 1998). Although species differences exist with regard to the specific oligosaccharides involved in sperm to OEC attachment, we have previously reported that numbers of human sperm attaching to either human or bovine OEC in coculture do not differ (Ellington et al, 1998b). This has allowed us to utilize frozen–thawed pools of bovine OEC from tubal pathology-free animals in the follicular phase of their cycle for a consistent coculture system across samples and replicates in an experiment.

In all species studied to date, sperm behavior in the OEC coculture system has mimicked sperm behavior observed in the oviduct *in vivo*, suggesting physiologic relevance of the system (Ellington et al, 1991; Thomas et al, 1994; Pacey et al, 1995). Additionally, the ability of sperm to attach to the OEC in coculture and subsequently survive appears to correlate with fertility outcomes of the male in animal model studies (Ellington et al, 1999b). Previous work has shown that cryopreservation of stallion sperm decreases the number of sperm attaching to OEC in coculture, even after controlling for the decreased sperm motility and membrane integrity observed in cryopreserved sperm (Dobrinski et al, 1995). It is hypothesized that a reduced ability of cryopreserved sperm to form a reservoir in the female may be, in part, responsible for the decreased fertility observed for cryopreserved sperm.

The present study was done to quantify aspects of human sperm attachment to OEC, specifically to determine if numbers of sperm attaching to OEC increased in relationship to the numbers of sperm applied to coculture wells. Additionally, we evaluated whether sperm attachment and subsequent survival in coculture differed for fresh and cryopreserved human sperm.

Methods

OEC Recovery and Culture

Oviducts were collected at slaughter from young, disease-free heifers in the follicular phase of their cycle, as determined visually (e.g., ≥6-mm follicle and no corpus luteum). To retrieve oviduct epithelial cells (OEC), the isthmic portion of the oviduct from each animal was lavaged with phosphate-buffered saline (PBS) + 10% fetal calf serum (FCS) + 2% antibiotic/antimycotic premix. The OEC from five or more animals were pooled together to diminish individual female effects on culture outcome (Ellington et al, 1993). The recovered OEC were then washed twice by centrifugation, and the OEC pellet was resuspended in a basal culture medium of 50:50 DMEM:Ham's F12 + 5 μg/ml insulin + 5 μg/ml transferrin + 5 ng/ml selenium + 10 ng/ml EGF (epidermal growth factor) + 1% antibiotic/antimycotic premix and 10% FCS.

Suspensions of OEC were then placed into 2-cm^2 tissue culture wells and were cultured until the OEC were confluent. At that time, the OEC were stained to confirm >90% epithelial cell content in the pools with a fluorescein isothiocyanate immunocytokeratin stain (Sigma, St. Louis, Missouri; Carney et al, 1990). Successful pools were then cryopreserved in basal medium with 50% FCS and 10% DMSO (dimethylsulfoxide) and

were stored in liquid nitrogen. A single pool of OEC was used in this study.

For these coculture studies, OEC were thawed and cultured in 100-mm^2 tissue culture plates until confluent, and 2×10^5 cells/well were passaged into 24-well plates that had been manually coated with 60 μl Matrigel (Becton Dickinson Lab, Bedford, Massachusetts). Culture of OEC on the Matrigel extracellular matrix is required to promote human sperm attachment to OEC monolayers *in vitro* (Ellington et al, 1998b). Unattached OEC were removed from the culture wells 18–24 hours after plating. The OEC were confluent monolayers in 2–3 days and were used in the following studies at that time.

Experiment 1—Dose-Dependent Study of Fresh and Cryopreserved Sperm Attachment to OEC Monolayers

Freshly ejaculated sperm from four normospermic donors with children and cryopreserved sperm from three commercial donors were used (one ejaculate per donor). Both types of sperm were washed by centrifugation through 35 ml of human tubal fluid (HTF) + 5% human serum albumin (HSA) (HTF+; Irvine Scientific, Santa Ana, California) at 300 × *g* for 10 minutes. The sperm pellets were then resuspended in HTF+. The concentration of sperm/ml in the initial sample was determined by Makler chamber count (Shiran et al, 1995), and the percentage of progressively motile sperm was observed subjectively. Aliquots of sperm from each sample containing specific total numbers of sperm were then applied to either a control, HTF+ solution (no OEC or Matrigel) or to wells with confluent OEC monolayers from a single pool of frozen–thawed OEC. Sample tubes containing the initial sperm suspensions were mixed gently with a vortex throughout the following experimental setup. Specifically, for each sample, eight different sperm concentrations, ranging from a calculated dose of 2×10^5 to 15×10^6 per well, were placed into one well each of control or coculture wells concomitantly. All samples were then incubated at 37°C in 5% CO_2 and air.

After 2 hours of culture, sperm numbers attaching to the OEC at each concentration were estimated. First, a most accurate determination of the number of sperm actually applied to the wells at each concentration was estimated by counting the sperm numbers in suspension in paired, control media-only wells (versus just relying on the initial sperm concentration in the sample as a whole). At each concentration, the sperm in coculture that had not attached to the OEC were then removed by vigorous mixing and rinsing of the wells as reported elsewhere (Ellington et al, 1998b). Presumptive numbers of attached sperm in coculture were calculated by subtracting the number of sperm removed from coculture wells (e.g., not attached to OEC) from the numbers of sperm in the control suspensions (e.g., applied). Five aliquots from each concentration, treatment, and donor were processed in this manner to determine the mean number of attached sperm for each donor at each concentration.

Experiment 2—Comparison of Fresh and Cryopreserved Sperm Function in Coculture

Freshly ejaculated sperm from four normospermic donors with children (≤2 years of age) were used in this experiment. Aliquots of fresh sperm were used immediately, and portions of the ejaculates were cryopreserved by standard methods with a Tris–egg yolk buffer (Irvine Scientific, La Jolla, California; Prins and Weidel, 1986) for later analysis. The same pools of frozen–thawed OEC were utilized across all replicates and treatments. Sperm and OEC were prepared as above. An initial sperm analysis was done on all samples to determine the percentage of progressively motile sperm, the percentage of viable sperm (eosin–nigrosin staining), and the percentage of sperm with functionally intact membranes (the hypoosmotic swell test [HOS]; Oosterhuis et al, 1996).

In order to compare attachment of both fresh and cryopreserved sperm from all four donors, a single concentration of approximately 3×10^6 sperm per well of each sperm type (in 500 μl of media) was added to at least three wells each of either media alone (controls) or OEC coculture wells. Sperm in suspension from either treatment were then pooled for an average estimation of sperm numbers in control media or in OEC coculture for each sample. The experiment was replicated with different ejaculates, twice for three donors and three times for one donor. Numbers of sperm attaching to the OEC were calculated as above.

In addition, the survival time for both types of sperm (fresh or cryopreserved) in either OEC coculture or media alone was determined. This was done by applying fresh HTF+ to the OEC coculture wells with attached sperm after unattached sperm were removed for counting at 2 hours. The cocultures were then placed back into the incubator. Control suspensions of sperm were also replenished with 100 μl of fresh HTF+ and continued in culture. Sperm in both treatments subsequently received a 100 μl HTF+ media exchange every 24 hours throughout the remainder of culture. Survival time for sperm in both treatments was evaluated at 12-hour intervals until <5% of the sperm in a treatment showed flagellar activity (coculture treatments) or progressive motility (controls).

Statistical Analysis

In experiment 1, linear regression studies were done to determine the relationship between sperm numbers applied for each donor and the numbers of sperm attaching. The slope of the line formed by this relationship was calculated separately for fresh and cryopreserved sperm, and these were compared to determine whether attachment rates differed for the fresh and cryopreserved sperm. Because the mean percentage of motile sperm differed between the two sperm types (fresh and cryopreserved), a corrected analysis was done to determine the relationship between the number of motile sperm applied and the number of sperm attaching. Specifically, the actual number of motile sperm applied to the OEC was estimated for each sperm sample, and the rate of average increase observed in the number of sperm attaching per unit increase in the number of motile sperm applied was determined. The percent of motile sperm attaching (out of those applied) was also calculated for each concentration, and the overall difference between fresh and cryopreserved sperm was evaluated by the Mann-Whitney test.

In experiment 2, comparisons of the mean numbers and percentages of sperm attaching to OEC for fresh and cryopreserved sperm from the same donor were evaluated as a function of both the total numbers and the numbers of motile sperm applied in

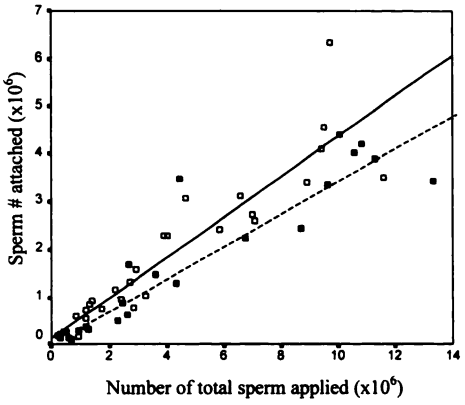

FIG. 1. The number of sperm attaching to OEC in 2-cm² coculture wells versus total number of sperm applied over a range of concentrations for both fresh and cryopreserved human sperm.

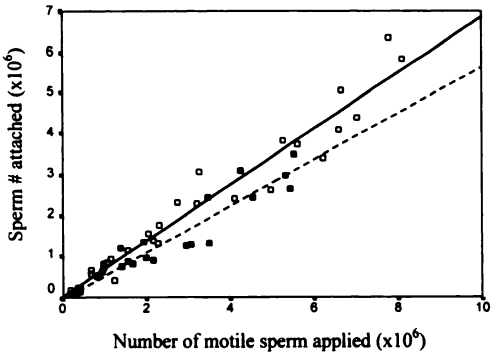

FIG. 2. The number of sperm attaching to OEC in 2-cm² coculture wells versus the number of motile sperm applied for both fresh and cryopreserved human sperm.

each sample. Further analysis was done by an ANOVA model to determine whether there were any donor-by-sperm type (fresh or cryopreserved) interactions and to compare the survival time of both sperm types in the two culture treatments (media only vs. OEC coculture). The relationship between sperm function measurements (percent motile, viable, and intact) and sperm numbers attaching was evaluated by linear regression studies.

Results

Experiment 1—Sperm Attachment to OEC Is Dose Dependent

The initial mean percentage of motile sperm differed between the fresh and cryopreserved groups (74 ± 2% and 43 ± 3%; $P = 0.001$), although the percentage of sperm with normal morphology did not differ between groups and exceeded 50% for all samples. The actual numbers of sperm evaluated across this dose-dependent study ranged from 2.2×10^5–13.7×10^6.

Sperm attachment to the OEC for the concentrations studied here did occur in a dose-dependent fashion for both fresh sperm ($R^2 = 0.87$; sperm numbers applied versus those attached) and cryopreserved sperm ($R^2 = 0.79$; sperm numbers applied versus those attached). Data for the actual counts of sperm applied versus the counts of sperm attaching are shown in Figure 1, and the corrected counts of motile sperm applied versus sperm attaching are shown in Figure 2.

Qualitatively, sperm attachment to OEC in coculture was similar to that previously reported (Ellington et al, 1998b). Specifically, the head of the sperm were in contact ("attached") to the OEC, with tails maintaining vigorous flagellar activity. Initially, most, if not all, sperm attached to the OEC had flagellar motion in both the fresh and cryopreserved sperm groups. There were no qualitative differences in the sperm attached to the OEC for sperm from the fresh or cryopreserved groups, other than the fewer numbers of sperm attaching in the cryopreserved group. In general, sperm tended to attach to OEC in distinct focal areas, rather than uniformly across the well. The majority of sperm remained attached to the OEC at a given location during observation, although a subpopulation of sperm did release, progress forward, and reattach to OEC in another area. Sperm in the control wells were easily resuspended into a uniform suspension by pipet mixing of the well and thus did not "attach" to the plastic control wells.

Overall, the mean percentage of sperm attaching out of the number of motile sperm applied was lower for cryopreserved sperm than for fresh sperm (59 ± 3% vs. 72 ± 6%; $P = 0.01$). Also, the rate of sperm attachment as determined by the slope of the line differed ($P < 0.05$) between fresh and cryopreserved sperm, suggesting that cryopreserved sperm were attaching to OEC at a slower rate (as a function of the average increase in the number of sperm attaching per unit increase in the number of sperm applied), as well as by fewer overall numbers and a lower percentage.

Individual donors within a sperm type (fresh or cryopreserved) had a significant effect on sperm attachment rates ($P = 0.02$). Conversely, the initial percentage of motile sperm in a sample had only a limited effect on the numbers of sperm attaching to OEC ($R^2 = 0.25$).

Table 1. *The mean (±SEM) number of sperm applied and attaching to OEC in coculture wells for fresh and cryopreserved sperm from the same donors (n = 4)*

	Fresh sperm	Cryopreserved sperm
Total sperm in well ($\times 10^6$)	2.9 ± 0.3	3.1 ± 0.4
Total sperm attaching to OEC ($\times 10^6$)*	1.7 ± 0.2 (56%)	0.9 ± 0.2 (30%)
% Motile sperm attaching*	82 ± 3	70 ± 4

OEC indicates oviduct epithelial cells.
* Values differ at $P < 0.05$.

Experiment 2—Cryopreservation of Sperm Alters Subsequent Attachment to and Function in OEC Coculture

Sperm quality differed for fresh and cryopreserved samples from the same donors ($P < 0.001$). Fresh and cryopreserved sperm samples had a respective percent motility of 73 ± 3% and 41 ± 2%; percent viable of 68 ± 5% and 43 ± 6%, and percent with intact membranes (HOS) of 62 ± 3% and 48 ± 3%.

The mean percentage of sperm that attached to OEC differed for fresh and cryopreserved sperm from the same donors (Table 1), both as a function of total sperm numbers and as a function of motile sperm numbers applied to the wells ($P = 0.001$). The percentage of motile sperm that attached to the OEC wells differed: for fresh sperm, 82 ± 3% and for cryopreserved sperm, 70 ± 4%; $P = 0.01$. No interaction between donor and sperm type occurred (Fig. 3). In general, sperm from donors that attached to OEC at a relatively higher or lower rate did so in both their fresh or cryopreserved sperm samples.

Much of the variability observed in numbers of sperm attaching to OEC was explained by a linear regression of the following independent variables: sperm numbers applied, individual donor effect, and the sperm type (fresh or cryopreserved; $R^2 = 0.748$). Individually, the percent motile sperm, the percent of sperm with intact membranes (HOS), and the percent viable sperm in the initial sperm sample had little association with the numbers of sperm attaching to the OEC ($R^2 = 0.26$). Taken together, these routine sperm analysis parameters had a more predictive relationship of the numbers of sperm attaching ($R^2 = 0.57$ for number of sperm attaching = percentage motile + percentage intact HOS + percentage viable ± error).

Survival time was significantly shorter for sperm in control media as compared with sperm in OEC coculture ($P = 0.001$). However, in control media, fresh sperm lived longer than did the cryopreserved sperm ($P = 0.005$; 108 ± 5 hours vs. 75 ± 7 hours; Fig. 4). Likewise, in coculture, the fresh sperm also lived longer than did the cryopreserved sperm (141 ± 7 hours vs. 108 ± 4 hours; $P = 0.005$). Only a limited relationship was seen between numbers of sperm attaching to OEC in coculture and their subsequent survival time ($R^2 = 0.37$; $P = 0.07$).

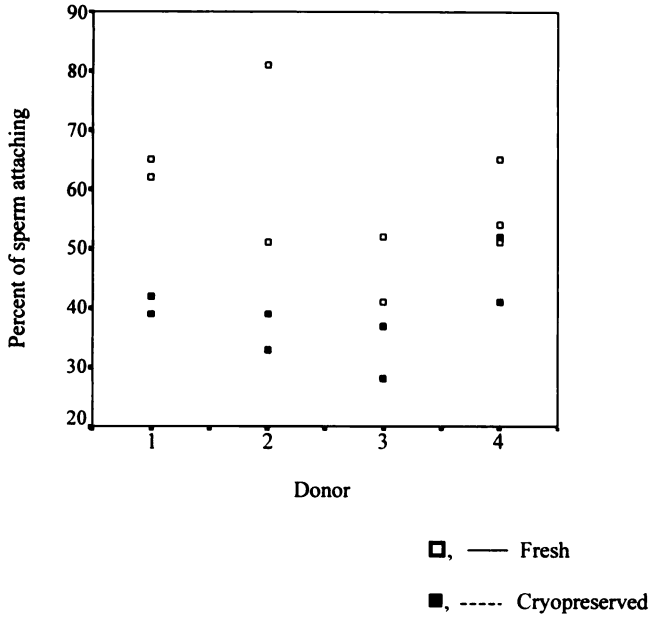

FIG. 3. The percentage of sperm attaching to OEC in coculture (out of the motile sperm applied) for fresh or cryopreserved sperm from the same donor ($n = 4$).

Discussion

These studies suggest that human sperm attachment to OEC in coculture occurs in a dose-dependent fashion at the concentrations studied here. Specifically, the relation-

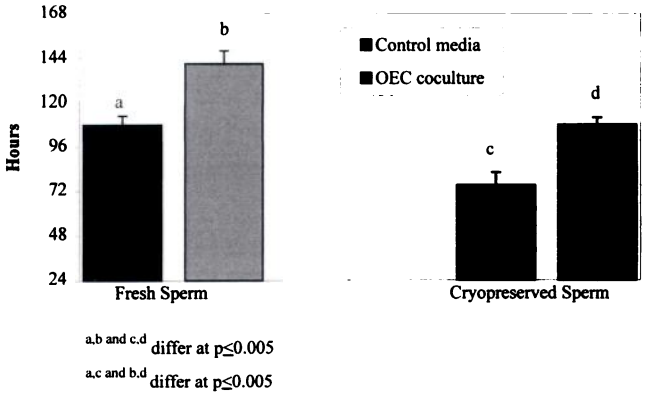

FIG. 4. The hours (mean ± SEM) of sperm survival in control media culture or OEC coculture for fresh or cryopreserved human sperm.

ship between sperm numbers applied and numbers attaching in experiment 1 was linear and well correlated ($r \geq 0.81$). However, a lower percentage of cryopreserved sperm attached to the OEC and at a slower rate (e.g., less of an average increase in the number of sperm attaching per unit than increase in the number applied) than that observed for fresh sperm. This decreased rate of cryopreserved sperm attachment to OEC in coculture was observed even when differences in sperm motility and membrane integrity between the fresh and cryopreserved sperm were taken into account. Subsequent survival of the cryopreserved sperm attached to OEC in coculture was also shorter than that seen for the freshly ejaculated sperm. These findings suggest that cryopreserved human sperm may be less fertile, in part, because of decreased storage and survival in the fallopian tube prior to ovulation.

In this study, we utilized equal numbers of total sperm from each treatment group (fresh or cryopreserved) in the coculture studies rather than equal numbers of motile sperm in the initial inseminate. This was done on the basis of previous work showing a lack of correlation between motility in a sample and subsequent attachment to OEC for stallion sperm in coculture (Dobrinski et al, 1995; Ellington et al, 1999b). Taken together, these studies plus our current data suggest that while almost all sperm that attach to OEC in coculture are motile (e.g., have flagellar activity), not all motile sperm attach to OEC. This may be because attachment of sperm to OEC in coculture appears to selectively involve the higher quality sperm in a sample, including those with the higher quality chromatin (DNA; Ellington et al, 1999a), and damage to sperm chromatin can occur or be present without a concomitant decrease in sperm motility (Twigg et al, 1998; Evenson et al, 1999).

In general, cryopreservation induces many sublethal cellular changes in sperm, including the following: partial capacitation, loss of chromatin integrity, ultrastructural damage, and reactive oxygen species accumulation (Mahadevan and Trounson, 1984; Alvarez and Storey, 1992; Cormier et al, 1997; Ellington et al, 1998a). Many of these cellular changes (such as capacitation) have been shown to decrease sperm attachment to OEC both *in vivo* and *in vitro* (Smith and Yanagimachi, 1991; Thomas et al, 1994; Dobrinski et al, 1997). A decreased ability of cryopreserved sperm to bind to the zona pellucida has also been observed (Coddington et al, 1991; Dobrinski et al, 1995), as well as inferior embryonic development following fertilization *in vitro* with cryopreserved sperm (as compared directly with fresh sperm; Janny and Menezo, 1994). Development of future methods for cryopreserving sperm should mitigate cellular changes that negatively impact physiologic function of sperm (such as storage or fertilization) in order to improve outcomes, rather than focusing on poorly predictive variables like postthaw motility.

Survival of the fresh and cryopreserved sperm in this study, particularly in OEC coculture, surpassed the hypothesized lifespan of sperm in the woman's body (e.g., ±48 hours and <24 hours, respectively; reviewed by Eddy and Pauerstein, 1980). It is currently unknown when motile sperm may lose their ability to fertilize an oocyte; however, conceptions resulting from coitus 5 or more days prior to ovulation do occur (Barrett and Marshall, 1969; Wilcox et al, 1995). The mechanisms by which OEC promote sperm survival *in vitro* and *in vivo* likely include stabilization against capacitation and prevention of reactive oxygen species damage and chromatin breakdown (Dobrinski et al, 1997; Murray and Smith, 1997; Ellington et al, 1998a). Exploitation of such factors to stabilize sperm function during *in vitro* handling, such as cryopreservation, may be useful.

It was an unexpected finding of the current work that the numbers of both fresh and cryopreserved sperm attaching to OEC continued to increase linearly at the concentrations studied here. We had hoped to saturate the system with the high-end concentrations (approximately 15 million sperm per milliliter). Temporally, we have found that attachment of human sperm to OEC in our coculture system continues to occur over 24 hours, along with an increasing tenacity of attachment (e.g., less "transient migration" of sperm across the OEC surface; Ellington et al, unpublished). However, a subpopulation of previously attached sperm begins to be shed from the OEC starting around 4 hours of coculture, so that the highest overall percentage of sperm in a sample are attached between 2 and 4 hours. Thus, this time was chosen for comparison of attachment between treatments in the current study.

Although the numbers of sperm utilized in this study were likely much higher than those reaching the fallopian tube after coitus or *in vivo* insemination (reviewed by Eddy and Pauerstein, 1980), these data suggest that numbers of motile sperm delivered to the woman may influence sperm numbers participating in a fallopian tube sperm reservoir. These results are in agreement with an earlier study (Settlage et al, 1973) that found that the numbers of sperm found in the tube (*in vivo*) after insemination with fresh, whole ejaculates were directly related to sperm numbers in the inseminate.

Increasing the number of sperm inseminated into the female increases the number of sperm available to form the sperm reservoir and thus participate in fertilization up to a threshold level (Huang et al, 1996; Berg et al, 1997). However, the threshold number of sperm required to optimize human fertilization both naturally and for TDI remains a topic of debate (Marshburn et al, 1992; Berg et al, 1997; Bonde et al, 1998).

In general, the use of intrauterine insemination and double insemination techniques likely act to deliver more sperm closer to the site and time of ovulation, thus increasing the available sperm reservoir (Matorras et al, 1996; Deary et al, 1997; Ford et al, 1997; Matilsky et al, 1998). Even with such modifications, average single-cycle pregnancy rates using cryopreserved human sperm remain low at around 10% per cycle (although some clinics support better outcomes). Additionally, current protocols for TDI are not able to optimize fertility outcome without superovulation of the recipient, which results in an increased incidence of multiple-order pregnancies that can lead to increased spontaneous abortion rates and increased medical costs (Matorras et al, 1996; Pittrof et al, 1996; Deary et al, 1997). Increasing our understanding of the unique requirements and physiology of cryopreserved sperm may allow us to develop systems for TDI that optimize fertility outcomes without relying on these more invasive and expensive protocols.

The numbers of sperm attaching to OEC in this study were indicative of trends, rather than quantitatively accurate, given the constraints of Makler chamber counts at the lower concentrations of sperm studied here. However, accuracy of the Makler chamber down to 5×10^6 sperm per milliliter has been reported (Shiran et al, 1995), and we have found this method more repeatable than Coulter counts, computerized semen analyzer counts, or hemocytometer counts for studies of the OEC coculture system.

A significant donor effect on sperm attachment to the OEC was also observed in this study. A significant relationship between field fertility for stallions bred to 20 or more mares and the number of sperm attaching to OEC or their subsequent survival in coculture has been reported (Ellington et al, 1999b). We are currently evaluating differences in sperm numbers attaching for a larger group of men with a variety of sperm quality. It is possible that the coculture system could serve as a bioassay for sperm function of both fresh and cryopreserved human sperm.

In summary, human sperm attach to bovine OEC *in vitro* in a dose-dependent manner. Attachment is decreased for cryopreserved sperm versus fresh sperm in a manner separate from any difference in sperm motility between the two. Sperm survival in coculture is also decreased for cryopreserved sperm. Donor differences in these sperm and OEC interactions were observed. The physiologic relevance of these observations requires further evaluation. However, further understanding of sperm and OEC interactions may be useful for optimizing donor insemination protocols or for evaluating donor differences in sperm function.

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