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In Vitro and Xenogenous Capacitation-Like Changes of Fresh, Cooled, and Cryopreserved Stallion Sperm as Assessed by a Chlortetracycline Stain

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ABSTRACT: Like the human female, the mare experiences reproductive tract pathology that may sometimes be circumvented by the use of assisted reproductive technologies (ARTs). One such technology, gamete intrafallopian transfer (GIFT), may be used in mares that exhibit ovulatory, oviductal, or uterine abnormalities that limit the use of common ARTs, such as embryo transfer. Homologous GIFT has been successfully performed in the horse; however, the logistics, costs, and associated risks of surgically transferring gametes to the oviducts of a recipient mare are considerably high. Use of a less costly species in a heterologous or xenogenous procedure would therefore be beneficial. This study represents the preliminary investigation into the use of sheep as recipients for xenogenous GIFT procedures using equine gametes. We investigated the capacitation response of fresh, cooled, or frozen stallion sperm after 1) in vivo incubation in the reproductive tract of estrous and anestrous ewes as well as 2) in vitro incubation in a modified Krebs/Ringer extender at 37°C with and without the addition of heparin at 10 IU/mL for up to 8 hours. A chlortetracycline (CTC) fluorescent stain was used to assess the capacitation response of sperm. Findings indicated that oviductal fluid samples recovered from estrous ewes had significantly higher numbers of sperm exhibiting capacitation-like staining patterns when compared to samples recovered from anestrous ewes ($P < .05$). Fresh semen yielded higher capacitation-like staining patterns after in vivo incubation than did frozen-thawed or cooled samples. A transition from majority CTC unreacted sperm to majority CTC non–acrosome intact sperm was demonstrated for both in vivo and in vitro studies. In vitro incubation of stallion sperm with heparin did not result in an increased capacitation-like staining response over time when compared with nonheparinized samples. Results from this study suggest that xenogenous capacitation of stallion sperm may occur in the estrous ewe.

Key words: Capacitation, estrous, ewe, equine, heparin.

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Ejaculated sperm from mice (Fraser, 1984), rabbits (Chang, 1957; Bedford, 1967a,b), hamsters (Yanagimachi, 1981), boars (Hunter and Hall, 1974), bulls (Ellington et al, 1993a), tomcats (Hamner et al, 1970), and humans (Lambert et al, 1985) have all been shown to require a period of residence within the female reproductive tract, within the fluids obtained from the latter, or within specific incubation media prior to their interaction and fusion with a homologous female gamete. During this incubation period, which appears to be species dependent, physiological modifications of the sperm plasma membrane occur that confer upon sperm the ability to fertilize ova. Such modifications are thought to arise from extracellular, transmembrane, and intracellular events that involve membrane cholesterol/phospholipid depletion, lectin binding, surface molecule alterations, intracellular pH changes, and calcium, potassium, and bicarbonate fluxes (Koehler, 1978; Babcock et al, 1979; Davis, 1981; Yanagimachi, 1981, 1994; Rufo et al, 1984; Visconte et al, 1998). Biophysical and biochemical changes also occur that influence sperm motility in some species (Yanagimachi, 1994; Suarez, 1996).

Capacitation is neither organ specific nor species specific. Numerous studies have indicated that a homologous tract is not a requirement for sperm capacitation (Saling and Bedford, 1981) and that successful fertilization is possible in a nonhomologous reproductive tract (xenogenous fertilization). Successful fertilization of goat (Rao et al, 1984), squirrel monkey, hamster (DeMayo et al, 1980), and bovine and porcine (Hirst et al, 1981) ova in rabbit oviducts has been demonstrated, thereby suggesting the occurrence of successful xenogenous capacitation. The existence of numerous equine, small ruminant, and bovine hybrid crosses also supports the nonspecificity of the capacitation process. Efforts to capacitate equine sperm in the reproductive tract of the rabbit have met with only minimal success (McKinnon et al, 1988). To date, neither the ovine reproductive tract nor its fluids has been

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reported to facilitate xenogenous capacitation of equine sperm.

Successful in vitro capacitation of equine sperm has been previously demonstrated (Samper et al, 1989; Blue et al, 1989; Ellington et al, 1993b,c; Varner et al, 1993). The methods used to induce capacitation included incubation of sperm in media that contained heparin, calcium ionophore A23187, caffeine, or lysophospholipids and coculturing of sperm with oviductal cells. Data from these studies suggest a capacitation time of 4.5 hours for equine sperm.

Limited work has been performed that documents the influence of stage of reproductive cycle on capacitation. The fact that capacitation naturally occurs around the time of estrus and ovulation suggests that the hormonal environment may play a role. This has been demonstrated for the rabbit and bull (Soupart, 1967; McNutt and Killian, 1991; King et al, 1994). In both species, estrus was associated with an increased sperm capacitation response when it was compared with diestrus. Glycosaminoglycans (heparin) have been noted in luminal fluid from the reproductive tract of numerous species. In mares, concentrations of such compounds are elevated during the follicular phase, compared with the nonfollicular phase, of the estrous cycle (Varner et al, 1991).

The objectives of the present study were 1) to determine if equine sperm would undergo capacitation-like changes in the reproductive tract of the ewe, as indicated by chlortetracycline (CTC) staining pattern, 2) to determine if the estrous status of the ewe influenced any observed capacitation response, 3) to compare the response of fresh, cooled, and frozen-thawed sperm, and 4) to determine whether in vitro incubation with heparin produced similar staining patterns. We suggest that the information obtained may aid in assessing the feasibility of xenogenous gamete intrafallopian transfer (GIFT) procedures (ie, transfer of equine gametes to the reproductive tract of a nonhomologous species such as the ewe) in promoting successful sperm capacitation and fertilization in vivo and may thus serve as a treatment option for certain forms of equine infertility.

Materials and Methods

Part A—In Vivo Capacitation of Equine Sperm

Ejaculated sperm from one 16-year-old Dutch Warmblood stallion of proven fertility was utilized for this study. This animal was stabled locally and had exhibited a pregnancy rate for the previous breeding season of 100%, over an average of 1.6 mare cycles. Prior to being used for the study, sperm from this stallion were collected daily for 4 days and the ejaculates discarded. This was done in an attempt to equilibrate sperm reserves, to decrease variation between samples, and to reduce the chance of obtaining an aged sample. Subsequently, ejaculates were collected daily for 2 days (anestrous ewes) or on the day ewes were first recorded as being in standing heat and were collected again the day of insemination (estrous ewes). Semen collection was performed with the aid of a breeding phantom and a Missouri-type artificial vagina (AV) purchased from Hamilton Thorn (Beverly, Mass).

Ewes—Twenty-three sexually mature, crossbred ewes were utilized for this study. Ewes were Finn/Dorset or Suffolk/Dorset crosses, and they were all approximately 16 to 18 months of age. They were kept at pasture until they were used, and they had unlimited access to water and mineral blocks. Mean body weight was 70.4 kg.

This study was conducted in 2 parts, the first part being carried out during the months of June to August and the second part being carried out during the months of October to December. During the former period, weekly venous blood sampling was performed for determination of luteal function. Blood samples were centrifuged, serum harvested, and stored at −20°C until progesterone concentration was measured by radioimmunoassay (RIA). Commercially available RIA kits were purchased from Diagnostic Products Corporation (Los Angeles, Calif) and were used according to the manufacturer's instructions. Ewes (anestrous) with serial plasma progesterone concentrations of less than 1 ng/mL were surgically inseminated with stallion sperm.

During the months of October to December, ewes were observed for behavioral estrus twice daily—in the morning and evening—with the aid of 3 vasectomized teaser rams. Ewes were recorded as being in estrus if they exhibited mating behavior (including standing heat) with the teaser more than once. Other less-reliable signs of estrus, such as tail twitching and the presence of a cloudy vaginal discharge, were also noted. Ewes observed in estrus were immediately segregated and surgically inseminated with stallion sperm approximately 12 to 16 hours later.

Semen Preparation/Processing—Immediately after collection, the gel fraction of each ejaculate was separated using an in-line milk filter fitted into the AV. Gel-free volume was recorded, and sperm motility, morphology, and concentration were evaluated. Sperm motility was evaluated using the Hamilton Thorn HTM-S computerized motility analyzer, sperm concentration was assessed using the Animal Reproduction Systems model 534B Densimeter (Chino, Calif), and sperm morphology was evaluated using a vital stain (Hancock's stain) containing eosin and nigrosin, which was obtained from the Society for Theriogenology (Nashville, Tenn). In order to evaluate sperm morphology, equal volumes of sample and stain were mixed and smeared across a slide. Approximately 100 sperm cells were examined under oil immersion, and the percentage of morphologically normal cells was recorded. Mean ejaculate volume (gel free), sperm concentration, motility, and morphology for this stallion were 75.8 mL, 105×10^6 sperm/mL, 57%, and 83% normal cells, respectively. Only ejaculates with an average motility of greater than 50% and a total concentration of greater than 1×10^9 sperm were used for this study.

Using the formula [C] × %MOT × VOL × %N/500 × 10^6, where [C] is the average concentration of sperm per mL, %MOT is the average motility, VOL is the volume of gel-free semen

collected, and %N is the average percentage of normal sperm per ejaculate, each ejaculate was divided into aliquots containing approximately 500 million motile sperm. Semen aliquots were centrifuged at 300*g* for 10 minutes, the supernatant discarded, and the pellet resuspended in approximately 5 mL of fresh E-Z Mixin–OF semen extender, which was purchased from Animal Reproduction Systems. Aliquots for use as fresh samples were used immediately for insemination, and those for use as cooled samples were stored in an Equitainer, which was purchased from Hamilton Thorn Research, for 24 hours. Manufacturer's specifications for the Equitainer state that controlled cooling of samples to a minimum temperature of 4°C occurs, and this temperature is maintained for up to 60 hours.

Frozen sperm samples were thawed in a water bath at 37°C for approximately 1 minute prior to use. Straws from a single ejaculate from the same stallion had previously been frozen in 0.5-mL straws by a commercial laboratory. The freezing extender used was an egg-yolk/glycerol equine freezing extender, and straws were frozen in liquid nitrogen vapor prior to being submerged in liquid nitrogen for storage. Each straw contained approximately 500 million sperm. Upon thawing, sperm motility, morphology, and viability were assessed as before for each straw. Semen processing time for all samples was approximately 20 minutes.

Sperm Viability—The percentage of live and dead sperm per sample (sperm viability) was also assessed using the Hancock's stain. Slide preparation and examination was similar to that used for sperm morphology. Under oil-immersion microscopy, stained and unstained sperm were considered to have been dead and live, respectively, at the time of staining.

Surgical Insemination—Prior to surgery, ewes were held off feed and water for approximately 12 hours. Ewes were sedated with xylazine hydrochloride, which was administered intramuscularly at a dose of 0.1 mg/kg, and anesthesia was induced using ketamine hydrochloride, which was administered intravenously at 0.05 mg/kg. Animals were placed in dorsal recumbency, a ventral midline laparotomy was performed posterior to the umbilicus, and the uterus was identified and exteriorized. Processed sperm was deposited in the uterine body via a small puncture site and was directed into both uterine horns using an 18-gauge, 2-inch Teflon catheter that we obtained from Becton Dickinson Vascular Access (Sandy, Utah). The volume of inseminate for fresh and cooled samples was 5 mL, and the volume of inseminate for frozen samples was 0.5 mL. Ewes were surgically inseminated with approximately 500 million sperm during estrous (n = 11) or anestrous (n = 12), as determined by season and behavior. Of the estrous ewes, 4 were inseminated with fresh sperm, 4 were inseminated with frozen sperm, and 3 were inseminated with cooled sperm. Anestrous ewes were divided into 3 equal groups (n = 4), and each was inseminated with fresh, cooled, or frozen sperm.

Following 4 to 6 hours of incubation time, ewes were again anesthetized, in the manner previously described. Both oviducts were surgically exteriorized and flushed with 10 mL of the modified Krebs/Ringer bicarbonate solution (Varner et al, 1993). Flushing was performed in a normograde manner using a 3½-french, 5½-inch tomcat catheter that was carefully introduced approximately 1 to 2 cm into each oviduct. The effluent was collected with the aid of a 10-french Foley catheter that was seated just caudal to the uterotubal junction of each uterine horn. Oviductal washings were then centrifuged at 300*g* for 5 minutes. Supernatants were discarded and pellets resuspended in 1 mL of fresh solution. Sperm cell viability and CTC staining patterns of recovered samples were evaluated within 10 minutes of recovery.

Chlortetracycline fluorescent staining patterns of equine sperm

Staining Category*	Fluorescence Patterns
Unreacted	Fluorescence of the sperm head with a line of brighter fluorescence at the equatorial region.
Capacitated	Fluorescence of the acrosomal region with a band of no fluorescence at the equatorial region.
Nonacrosome intact	Lack of fluorescence of most or all of the acrosome or the entire sperm head.

* Sperm was categorized as being functionally unreacted, capacitated, or nonacrosome intact based on fluorescence staining patterns described above. Functional validation was based on previous studies that reported similar staining patterns for stallion (Varner et al, 1993) and mouse sperm (Ward and Storey, 1984).

CTC Stain—CTC staining patterns of sperm were assessed prior to and following in vivo or in vitro incubation. The CTC stain was prepared as described in previous studies (Varner et al, 1987). Briefly, CTC powder (500 μM) was dissolved in tromethamine buffer, and the pH was adjusted to 7.8. The tromethamine buffer consisted of Trisma base (20 μM), L-cysteine (5 μM), and NaCl (130 μM) dissolved in distilled water. Stain was made fresh every 48 hours and was stored at 4°C in a flask that was protected from light. All staining procedures were performed within 10 minutes of insemination or collection and in the absence of strong light. Approximately 100 μL of each sperm sample was mixed with an equal volume of stain and incubated at 38°C for 1 minute. Following this, 1.6 μL of a 12.5% glutaraldehyde solution was added, and the mixture was incubated for an additional 5 minutes. After incubation, a drop of the mixture was examined microscopically. Microscopic assessment was made using a Nikon Labophot scope equipped with epifluorescence capability. The excitation beam was passed through a B-2A fluorescence filter block with a double cube illuminator and collector lens. Excitation wavelength emission was between 450 and 490 nm.

Sperm were categorized into one of 3 categories based on the pattern of CTC fluorescence observed. Categories recognized in this study included unreacted sperm (UR), capacitated acrosome-intact sperm, and non–acrosome intact sperm (AR) (the Table). Scored sperm were required to have a minimum of bright fluorescence over the midpiece of the flagellum. This indicated adequate uptake of the CTC stain by sperm.

Part B—In Vitro Capacitation of Equine Sperm

Fresh, cooled, and frozen-thawed sperm samples from the same stallion were incubated with and without the addition of heparin. Fresh samples were processed as before, except that for resuspension of pelleted sperm after centrifugation, 5 mL of the pre-

viously described modified Krebs/Ringer extender was used in place of the E-Z Mixin–OF stallion semen extender. After 24 hours of storage in the Equitainer, cooled samples were centrifuged at 300*g* for 10 minutes, and the pellets were also similarly resuspended. Frozen 0.5-mL straws (as before) were thawed, extended to 5 mL with E-Z Mixin–OF stallion semen extender, centrifuged at 300*g* for 10 minutes, and pellets resuspended with 5 mL of the modified Krebs/Ringer extender.

A total of 48 sperm samples (5 mL), each of which contained approximately 500×10^6 sperm, were prepared (fresh = 16; cooled = 16; frozen-thawed = 16). Sodium heparin (10 IU/mL) was added to one half of the fresh, cooled, and frozen-thawed samples prior to incubation at 37°C. Following incubation times of 0, 4.5, 6, and 8 hours, heparinized and nonheparinized, fresh, cooled, and frozen-thawed samples were evaluated for viability and CTC staining pattern.

Statistical Analysis

The NPAR1WAY procedure of SAS (version 6.12; SAS Institute, Cary, NC) was used to perform the Kruskal-Wallis test for the main effect of type of semen processing (fresh, cooled, frozen thawed), and the Wilcoxon test was used to determine the main effect of estrous status. When semen-processing effect was significant, samples were compared using a Kruskal-Wallis–based multiple comparison (Hollander and Wolfe, 1973). Statistical significance was set at $P < .05$. A paired *t* test was used to determine significant variation in serial progesterone values in the anestrous group.

Results

Part A: In Vivo Capacitation of Equine Sperm

Of the 23 ewes utilized in the study, 7 were eliminated because of nonrecovery of sperm. All 4 anestrous ewes inseminated with fresh sperm and 3 estrous ewes inseminated with cooled sperm had 0 sperm recovery from the oviducts. Estrous and anestrous ewes therefore numbered 9 and 7, respectively.

Mean serum progesterone concentrations in anestrous ewes were 0.74 ± 0.39 ng/mL and 0.99 ± 1.43 ng/mL for successive samples obtained a week apart. Estrous ewes had a mean progesterone level of 0.19 ± 0.19 ng/mL.

Mean incubation time for the study, measured as interval from surgical insemination to oviductal flush, was 5.54 ± 0.38 hours. Mean incubation times for anestrous and estrous ewes were 5.16 ± 0.17 and 5.72 ± 0.11 hours, respectively. The incubation time averaged approximately 36 minutes longer for estrous ewes and was significantly different compared with that of anestrous ewes ($P = .01$). In order to determine whether or not incubation time confounded the effects of estrous status on capacitation response, a linear regression analysis was performed. There was no significant relationship between incubation time and capacitation response ($P = .8$).

Sperm motility varied significantly in preinseminated samples. Fresh sperm samples had consistently higher sperm motility than did cooled or frozen-thawed samples. Sperm motility of preinseminated samples was not different between ewe groups ($P > .05$). Mean motilities for anestrous and estrous ewes were 66.3 ± 12.9 and 68.0 ± 4.6%, respectively. Mean motility for all preinseminated samples was 67.3 ± 8.9%. Recovered samples exhibited minimal oscillatory patterns.

Percentage of live sperm present, or viability of preinseminated samples, was significantly different with respect to type of sperm. Mean viability was higher for fresh samples compared with cooled or frozen-thawed samples. For fresh, cooled, and frozen-thawed sperm, mean viability was 81.7 ± 2.5, 70 ± 1.4, and 69.5 ± 0.7%, respectively. When recovered samples were evaluated, no significant difference in viability was observed between sperm type ($P > .05$). The mean viability of recovered samples was 30.1 ± 9.1%.

As detected by the CTC stain, capacitation-like changes recorded were significantly different between ewe groups ($P = .01$). Increased capacitation-like changes were observed in sperm after incubation within the reproductive tract of the estrous ewes compared with the anestrous animals (Figure 1). More capacitated sperm were recovered from estrous ewes. Changes in UR and AR sperm were not different between estrous groups ($P > .05$). UR sperm numbers decreased, and AR sperm numbers increased after in vivo incubation in both estrous and anestrous ewes.

Sperm type significantly affected changes in CTC staining patterns ($P = .0001$). Ewes inseminated with fresh sperm had higher percentages of recovered sperm that exhibited capacitation-like staining when compared with ewes inseminated with cooled or frozen-thawed samples (Figure 2). Within the reproductive tract of the ewe, frozen-thawed sperm had a greater capacitation-like response when compared with cooled sperm ($P = .004$). Cooled sperm exhibited an unexpected increase in numbers that displayed the unreacted CTC pattern after in vivo incubation. Changes in AR sperm for fresh, cooled, or frozen-thawed sperm were not significantly different.

Part B—In Vitro Capacitation of Equine Sperm

The greatest change in capacitation-like staining pattern of sperm occurred between 0 and 4.5 hours in heparinized and nonheparinized samples (Figure 3). Nonheparinized samples tended to exhibit greater capacitation-like changes, but significance was not established. At 4.5 hours, heparinized samples had a mean capacitation-like response of 7.67 ± 4.26%, compared with 17.67 ± 10.71% in nonheparinized samples. No significant influence of heparin on sperm exhibiting capacitation-like CTC staining patterns was observed over time ($P > .05$). Over the

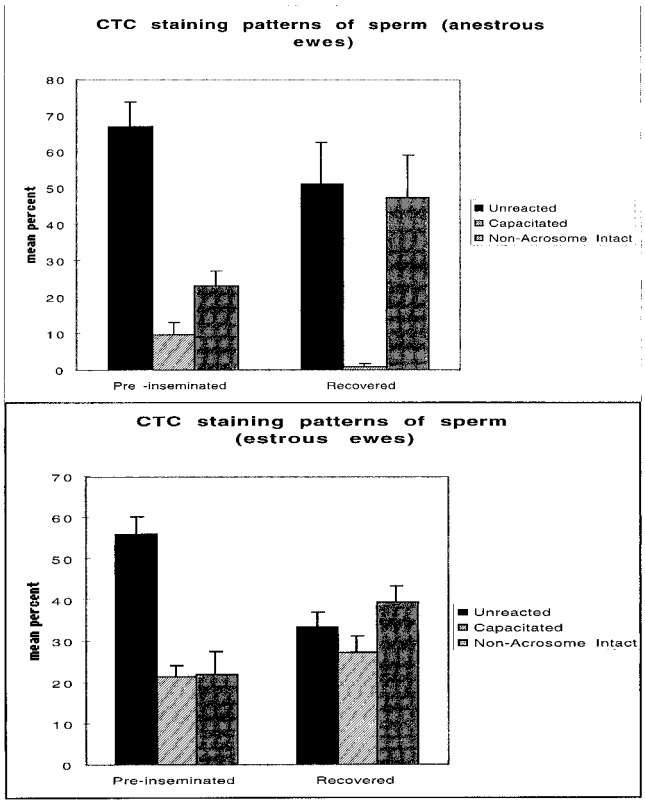

Figure 1. Effect of xenogenous in vivo incubation on stallion sperm. Sperm were deposited in the reproductive tract of anestrous (top graph) and estrous (bottom graph) ewes. Following an incubation period of approximately 6 hours, sperm were recovered from the oviducts of sheep via normograde flushing. Prior to insemination and after recovery, sperm samples were stained with a chlortetracycline (CTC) fluorescent stain to assess functional status (ie, unreacted, capacitated, or non–acrosome intact [acrosome reacted]). Data presented represent the means ± SEM of sperm CTC staining patterns. Capacitation-like changes were significantly different ($P = .01$) for equine sperm incubated in the reproductive tract of estrous ewes compared with sperm incubated in anestrous ewes. Unreacted and non–acrosome intact sperm changes did not differ between estrous and anestrous ewes.

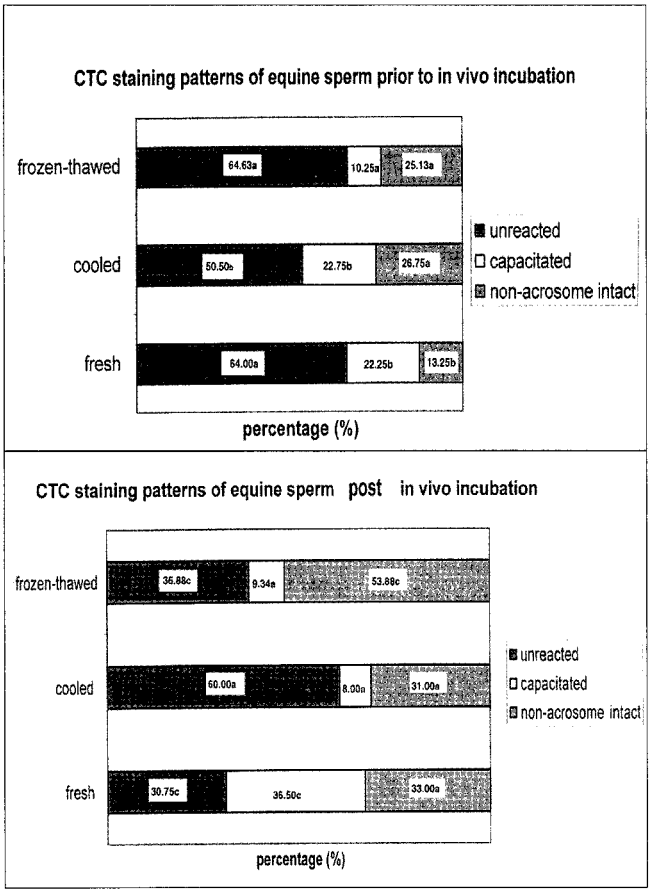

Figure 2. Chlortetracycline (CTC) staining patterns of fresh, cooled, and frozen-thawed stallion sperm before and after in vivo incubation. Sixteen ewes were inseminated with either fresh (n = 4), cooled (n = 4), or frozen-thawed (n = 8) stallion sperm. Insemination dose was approximately 500 million sperm. Prior to and after in vivo incubation for approximately 6 hours, sperm samples were stained with a CTC stain to assess functional status. Data presented represent mean values for unreacted, capacitated, and non–acrosome intact sperm. Means with dissimilar letters are statistically different ($P < .05$). For fresh and cooled samples, the percentage of sperm displaying CTC patterns suggestive of capacitation significantly increased and decreased, respectively, after in vivo incubation. After incubation, unreacted sperm numbers decreased for fresh and frozen-thawed samples. For cooled samples, the number of unreacted sperm increased postincubation. The number of non–acrosome intact sperm increased following incubation for all samples.

8-hour time period, the percentage of sperm displaying patterns indicative of UR and AR sperm decreased and increased, respectively, in both heparinized and nonheparinized samples. There were no significant CTC staining differences between fresh, cooled, nor frozen-thawed sperm samples over time ($P > .05$).

Sperm motility and viability decreased over time. At 8 hours, the majority of sperm incubated at 37°C were nonviable. In nonheparinized samples, mean motility decreased from 61.67 ± 1.67% at 0 hour to 0.67 ± 0.33% at 8 hours. Motility of heparinized and nonheparinized samples did not differ significantly. Mean viability of sperm at 8 hours was greater for heparinized samples than for nonheparinized samples. At 0 and 8 hours, mean viability changed from 74.67 ± 2.91 and 75 ± 3.21% to 15 ± 6.11 and 8.67 ± 4.48% in heparinized and nonheparinized samples, respectively.

Discussion

Mammalian sperm undergoing capacitation reportedly exhibit a characteristic CTC staining pattern. This has been demonstrated in mouse (Ward and Storey, 1984; Fraser and Herod, 1990), stallion (Varner et al, 1987, 1993; Ellington et al, 1993b,c), human (Lee et al, 1987; Kholkute et al, 1992), and boar (Mattioli et al, 1996) sperm. Using transmission electron microscopy (Varner et al, 1987, 1993), zona binding assays (Ellington et al, 1993c), and induction of zona-induced acrosome reaction (Ward and Story, 1984; Mattioli et al, 1996), the functional status of CTC-stained sperm has been previously validated. The

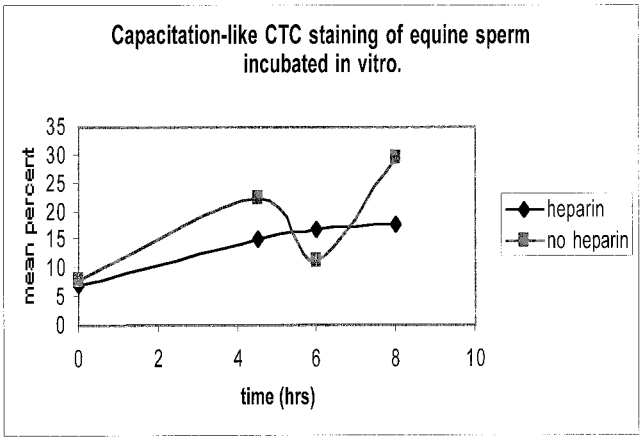

Figure 3. Chlortetracycline (CTC) patterns of in vitro–incubated stallion sperm. Fresh, cooled, and frozen-thawed sperm were incubated at 37°C for up to 8 hours with and without the addition of heparin at 10 IU/mL. Data presented represent mean sperm capacitation responses at 0, 4.5, 6, and 8 hours. There was no significant difference between heparinized and nonheparinized sperm samples over time.

reader is cautioned that the functional status of recovered sperm was not investigated in this study but was assumed based upon staining characteristics.

Ewes utilized in this study were endocrinologically in either the anestrous or estrous phase of the reproductive cycle, as documented by assessment of serum progesterone levels. Levels reported for estrous ewes are reported to be less than 0.2 ng/mL at time of estrus, and for anestrous ewes sampled at weekly intervals, levels of less than 1 ng/mL are reported. In this study, serum progesterone concentrations of estrous and anestrous ewes were within the above ranges.

The results of this study suggest that stallion sperm may undergo capacitation-like changes, as observed with the CTC stain, within the reproductive tract of the estrous ewe (ie, xenogenous capacitation). Duration of incubation within the reproductive tract of the ewe was approximately 6 hours, and adequate fluorescence was observed with the CTC technique employed. The mechanism of action of CTC staining is not clear; however, it may involve binding of CTC to a calcium affinity substance located on the sperm membrane after uptake of calcium by the sperm head. CTC chelates divalent cations, such as calcium, and emits increased fluorescence upon interaction (Caswell and Hutcheson, 1971). During capacitation, this affinity substance is lost, which results in the characteristic staining patterns. Sperm patterns exhibited in this study were consistent with those previously observed for the stallion, mouse, bull, and human.

Oviductal flushing enabled sampling of the crypt and luminal sperm populations and, to a lesser extent, of those sperm loosely attached to the oviductal epithelium. This technique was selected because experimental animals would not be sacrificed and because it was thought that flushing at or around the time of capacitation would increase the concentration of sperm in the luminal pool.

Increased percentages of sperm exhibited CTC staining patterns that were indicative of capacitation when preinseminated samples were compared with samples recovered from the oviducts of estrous sheep. The mean percentage of capacitated sperm within preinseminated samples was higher than previously recorded for fresh and cooled samples; however, this observation might have been a result of the semen extender. The extender used in this study was milk based and therefore may have contained moderate amounts of ionized calcium. Calcium has been shown to promote capacitation and the acrosome reaction in stallion sperm (Varner et al, 1993).

The influence of ovarian activity on sperm capacitation is not clear. In a somewhat similar experiment that evaluated bull sperm recovered from the reproductive tract of estrous and diestrous cows, investigators observed that fewer sperm underwent a true acrosome reaction in diestrous cows (Didion and Graves, 1986). The fact that capacitation occurs relatively near the time of ovulation and that the latter occurs during estrus suggests that the hormonal status of the female may also be involved in the mediation of capacitation. Estrus has been positively associated with sperm capacitation in the rabbit (Bedford, 1967b; Soupart 1967) and bull (King et al, 1994).

Freshly ejaculated semen should have very few capacitated or acrosome-reacted sperm. Numbers should increase as sperm become capacitated within the female reproductive tract. Percentages of sperm displaying the CTC UR and AR patterns changed, as expected, in this study. Prior to insemination, the percentage of sperm exhibiting the CTC UR pattern was higher than that exhibiting the AR pattern. This relationship was reversed in samples recovered from the oviducts and suggests sperm transition through to the acrosome reaction.

Fresh sperm samples gave the best results in this study, and this was expected, despite the low numbers of animals used. Frozen-thawed samples had more sperm transitioning to the AR stage than did cooled samples, a fact that might be explained by the capacitation-promoting properties of cryopreservation observed by some investigators (Cormier et al, 1997). Interestingly, however, preinseminated frozen-thawed samples had lower numbers of capacitated sperm than did fresh and cooled samples. It should be noted here that frozen samples were prepared in a previous year and may reflect differences in ejaculate quality. UR sperm numbers decreased in all but cooled samples. The unexpected increase in UR sperm staining when preinseminated and recovered samples were compared with cooled samples may be the result of low sperm recovery.

A review of the literature suggests that it is difficult to

reliably and repeatedly capacitate equine sperm in vitro. The reason can only be speculated upon, but it may involve the inhibition of heparin-induced capacitation by glucose, a component of the modified Krebs/Ringer extender (Varner et al, 1993). Heparin was not observed to have any effect on the numbers of sperm displaying capacitation-like changes in the second part of this study. Heparin had been previously shown to induce capacitation-like changes in bull (Parrish et al, 1988) and stallion (Varner et al, 1993) sperm. However, other investigators have not been as successful in duplicating the effect of heparin on stallion sperm (Blue et al, 1989; Ellington et al, 1993b; Hochi et al, 1996). We had hoped to document an increased CTC capacitation-like response of sperm after 4.5 to 6 hours of incubation with heparin, thereby justifying our incubation time for the in vivo experiment. That was not the case; however, we did document an increase in sperm that exhibited the AR pattern after 4.5 hours.

Our results support the theory that the process of capacitation is not species dependent. Xenogenous capacitation of equine sperm implies the possibility of xenogenous fertilization of equine gametes. The latter has recently been accomplished in our laboratory (Wirtu, unpublished data) and holds much promise for the use of assisted reproductive technologies, such as xenogenous gamete intrafallopian transfer (X-GIFT), for treatment of certain forms of infertility in the mare.

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References

Babcock DF, Singh JP, Lardy HA. Alteration of membrane permeability to calcium ions during maturation of bovine spermatozoa. *Dev Biol.* 1979;69:85–93.

Bedford JM. The importance of capacitation for establishing contact between eggs and sperm in the rabbit. *J Reprod Fertil.* 1967a;13:365–367.

Bedford JM. Experimental requirement for capacitation and observations on ultrastructural changes in the rabbit spermatozoa during fertilization. *J Reprod Fertil.* 1967b;2(suppl):35–48.

Blue BJ, McKinnon AO, Squires EL, Seidel GE, Muscari KT. Capacitation of stallion spermatozoa and fertilization of equine oocytes in vitro. *Equine Vet J.* 1989;8:111–116.

Caswell AH, Hutcheson JD. Visualization of membrane bounded cations by a fluorescent technique. *Biochem Biophys Res Commun.* 1971;42:43–49.

Chang MC. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. *Nature (Lond).* 1957;179:258–259.

Cormier N, Sirard M, Bailey J. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *J Androl.* 1997;18:461–468.

Davis BK. Timing fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. *Proc Natl Acad Sci USA.* 1981;78:7560.

DeMayo FJ, Mizogushi H, Dukelow WR. Fertilization of squirrel monkey and hamster ova in the rabbit oviduct (xenogenous fertilization). *Science.* 1980;208:1468–1469.

Didion BA, Graves CN. In vivo capacitation and acrosome reaction of bovine sperm in estrous and diestrous cows. *J Anim Sci.* 1986;62:1029–1033.

Ellington JE, Ball BA, Blue BJ, Wilker CE. Ability of epididymal or ejaculated bull spermatozoa to bind to cow zona pellucida and undergo the acrosome reaction. *J Reprod Fertil.* 1993a;48:851–856.

Ellington JE, Ball BA, Blue BJ, Wilker CE. Capacitation-like membrane changes and prolonged viability in vitro of equine sperm cultures with uterine tube epithelial cells. *Am J Vet Res.* 1993b;54(9):1505–1510.

Ellington JE, Ball BA, Yang X. Binding of stallion sperm to the equine zona pellucida after co-culture with oviductal epithelial cells. *J Reprod Fertil.* 1993c;98:203–208.

Fraser LR. Mouse sperm capacitation in vitro involves loss of a surface-associated inhibitory compound. *J Reprod Fertil.* 1984;72:373–384.

Fraser LR, Herod JE. Expression of capacitation-dependent changes in chlortetracycline fluorescence patterns in mouse sperm requires a suitable glycolysable substrate. *J Reprod Fertil.* 1990;88:611–621.

Hamner CE, Jennings LL, Sojka NJ. Cat spermatozoa require capacitation. *J Reprod Fertil.* 1970;23:477–480.

Hirst PJ, DeMayo FJ, Dukelow WR. Xenogenous fertilization of laboratory and domestic animals in the oviduct of the pseudopregnant rabbit. *Theriogenology.* 1981;15:67–75.

Hochi S, Korosue K, Choi YH, Oguri N. In vitro capacitation of stallion spermatozoa assessed by the lysophosphatidylcholine-induced acrosome reaction and the penetration rate into in vitro matured, zona-free mare oocytes. *J Equine Vet Sci.* 1996;16(6):244–248.

Hollander M, Wolfe DG. *Non-Parametric Statistical Methods.* New York, NY: Wiley; 1973.

Hunter RHF, Hall JP. Capacitation of boar spermatozoa. Synergism between uterine and tubal environments. *J Exp Zool.* 1974;188:203–214.

Kholkute SD, Meherji P, Puri CP. Capacitation and the acrosome reaction in sperm from men with various semen profiles monitored by a chlortetracycline fluorescence assay. *Int J Androl.* 1992;15:43–53.

King RS, Anderson SH, Killian GJ. Effect of bovine oviductal estrus-associated protein on the ability of sperm to capacitate and fertilize oocytes. *J Androl.* 1994;15(5):468–478.

Koehler JK. The mammalian sperm surface: studies with specific labeling techniques. *Int Rev Cytol.* 1978;54:73–108.

Lambert H, Overstreet JW, Morales P, Hanson FW, Yanagimachi R. Sperm capacitation in the female reproductive tract. *Fertil Steril.* 1985;43(2):325–328.

Lee MA, Trucco GS, Bechtol KB, Wummer N, Kopf GS, Blasco L, Storey BT. Capacitation and acrosome reaction in human sperm monitored by a chlortetracycline fluorescence assay. *Fertil Steril.* 1987;48(4):649–658.

Mattioli M, Barboni B, Lucidi P, Seren E. Identification of capacitation in boar sperm by chlortetracycline staining. *Theriogenology.* 1996;45:373–381.

McKinnon AO, Carnevale EM, Squires EL, Voss JL, Seidel GE. Heterogenous and xenogenous fertilization of in vivo matured equine oocytes. *Equine Vet Sci.* 1988;8(2):143–147.

McNutt TL, Killian GJ. Influence of bovine follicular and oviductal fluids on sperm capacitation in vitro. *J Androl.* 1991;12(4):244–251.

Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. *Biol Reprod.* 1988;38:1171–1180.

Rao VH, Sarmah BC, Bhattacharyya NK. Xenogenous fertilization of goat ova in the rabbit oviduct. *J Reprod Fertil.* 1984;71:377–379.

Rufo GA, Scholf PK, Lardy HA. Regulation of calcium content in bovine spermatozoa. *J Biol Chem.* 1984;259:2547–2552.

Saling PM, Bedford JM. Absence of species specificity for mammalian sperm capacitation in vitro. *J Reprod Fertil.* 1981;63:119–123.

Samper JC, Behnke EJ, Byers AP, Hunter AG, Crabo BG. In vitro capacitation of stallion spermatozoa in calcium free Tyrode's medium and penetration of zona free hamster eggs. *Theriogenology.* 1989; 31(4):875–883.

Soupart P. Studies on the hormonal control of rabbit sperm capacitation. *J Reprod Fertil.* 1967;2(suppl):49–60.

Suarez SS. Hyperactivated motility in sperm. *J Androl.* 1996;17:331–335.

Varner DD, Bowen JA, Johnson L. Effect of heparin on capacitation/acrosome reaction of equine sperm. *Arch Androl.* 1993;31:199–207.

Varner DD, Forrest DW, Fuentes F, Taylor TS, Hooper RN, Brinsko SP, Blanchard TL. Measurement of glycosaminoglycans in follicular, oviductal, and uterine fluid of mares. *J Reprod Fertil.* 1991;44(suppl): 297–306.

Varner DD, Ward CR, Storey BT, Kenney RM. Induction and characterization of acrosome reaction of equine spermatozoa. *Am J Vet Res.* 1987;48(9):1383–1389.

Visconte PE, Galantino-Homer H, Moore GD, Bailey JL, Ning X, Fornes M, Kopf GS. The molecular basis of sperm capacitation. *J Androl.* 1998;19(2):242–248.

Ward CR, Storey BT. Determination of the time course of capacitation in mouse sperm using a chlortetracycline fluorescence assay. *Dev Biol.* 1984;104:287–296.

Yanagimachi R. Mechanisms of fertilization in mammals. In: Mastroianni L, Biggers JB, eds. *Fertilization and Embryonic Development In Vitro.* New York, NY: Plenum; 1981:81–182.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* New York, NY: Raven Press; 1994: 189–317.