Influence of Salt Storage on Equine Zonae Pellucidae: Electrophoretic Properties and Interaction with Spermatozoa

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ABSTRACT
Successful spermatozoal penetration of the zona pellucida (ZP) occurs only when spermatozoa have undergone capacitation. Use of salt-stored oocytes in bioassays for detecting sperm maturation has been reported in several species. The objective of the present study was to compare oocytes obtained from frozen ovaries and oocytes stored in a concentrated salt solution in bioassays for monitoring capacitation of stallion spermatozoa. Salt storage did not alter the migration of ZP proteins separated by SDS-PAGE, and four identical glycoproteins (molecular masses of 90, 75, 60, and 50 kDa) were identified for salt-stored and frozen ZP. Ejaculated spermatozoa harvested from seminal plasma via a discontinuous Percoll gradient and suspended in Ham's F-10 required 120 min for binding and subsequent penetration of ZP of salt-stored oocytes. Moreover, when salt-stored and frozen oocytes were compared, each bound similar numbers of spermatozoa to their ZP following 120 min of culture. When spermatozoa were challenged after 30 and 120 min of culture with solubilized ZP of salt-stored and frozen oocytes, the percentages of live, acrosome-reacted spermatozoa were similar. These data suggest that salt-stored equine oocytes maintain spermatozoal receptors on the ZP and can be used to assess spermatozoal maturation and potential fertilizing ability.

INTRODUCTION
Assessments of spermatozoal motility, morphology, and concentration are used commonly to evaluate semen samples of many species. Although objective measures of semen and spermatozoal characteristics are correlated significantly with fertility [Amann, 1989], better predictions can be derived from their combination with in vitro tests based on the functional characteristics of spermatozoa [Bousquet & Brackett, 1982]. Recently, assays based on the spermatozoan's ability to bind and penetrate the zona pellucida (ZP) have been developed to predict fertilization potential in humans [Overstreet & Hembree, 1976; Yanagimachi et al., 1979; Burkman et al., 1988], cattle [Wheeler & Seidel, 1987; Fazeli et al., 1993], rabbits [Fayrer-Hosken & Brackett, 1987], hamsters [Boatman et al., 1988], and cats [Andrews et al., 1989].

In 1979, Yanagimachi et al. demonstrated that the ZPs of unfertilized hamster and human eggs stored in a highly concentrated neutral salt solution retained their biological functions. In these and other species evaluated (bovine [Chian et al., 1991], rabbit [Fayrer-Hosken & Brackett, 1987], cat [Andrews et al., 1989]), the only significant difference in sperm interaction between the zonae of a fresh, living egg and a salt-stored egg was that salt storage apparently destroyed the cortical granule-mediated block to polyspermy, thus allowing multiple penetration by spermatozoa [Yanagimachi et al., 1979]. This has made it possible to substitute salt-stored oocytes for fresh oocytes in evaluating the fertilizing ability of spermatozoa [Fayrer-Hosken et al., 1987; Boatman et al., 1988] and in assessing gamete interaction [Chian et al., 1991]. The use of salt-stored oocytes could be particularly helpful in evaluating stallion fertility because of the difficulty in obtaining a large number of fresh equine oocytes at one time. Therefore, our objective in this investigation was to compare sperm-oocyte interactions through use of salt-stored oocytes and oocytes obtained from frozen ovaries.

MATERIALS AND METHODS
Oocyte Collection and Preparation
Oocytes were collected from frozen/thawed equine ovaries that had been harvested at an abattoir (Central Nebraska Packing, Inc., North Platte, NE) and frozen within 12 h of collection. ZP-intact oocytes were used for ex-
periments II and III, whereas ZP ghosts were used for experiments I and IV. Palpable antral follicles were aspirated by means of a 3.81-cm, 18-gauge needle attached to a 12-ml syringe. Follicular fluid was deposited into petri dishes and allowed to settle for 10–30 min prior to being searched for oocytes (stereomicroscope, 10–20×). Isolated oocytes were transferred (micropipette, Wiretroll II; Drummond Scientific Company, Broomall, PA) into a 200-μl drop of Dulbecco’s PBS (Gibco Laboratories, Grand Island, NY) and treated with 0.3% hyaluronidase (testicular; Sigma Chemical Company, St. Louis, MO) for 20 min to remove cumulus cells. Denuded oocytes were rinsed four times in 200-μl drops of PBS before being stored in a concentrated salt solution or being used (within 12 h) in experiments.

The salt storage solution contained 0.75 M MgCl₂·6H₂O, 0.5 M (NH₄)₂SO₄, 0.2 mM ZnCl₂, 0.1 mg/ml polyvinyl alcohol, and 40 mM HEPES [Andrews et al., 1989]. All reagents were purchased from Sigma Chemical Company. The medium was adjusted to pH 7.2, sterilized by passage through a Sterivex-GS 0.22-μm filter unit (Millipore, Bedford, MA), and then stored at 4°C until used. After processing, oocytes to be stored were transferred into a 200-μl drop of concentrated salt solution. After a 30-min adaptation period, the intact oocytes, which settled to the bottom of the dish, were transferred into clean microcentrifugation tubes containing 0.5 ml of salt solution and stored at 4°C for up to 6 mo.

Prior to insemination (experiments II and III), stored oocytes were recovered from the salt solution, washed through five 200-μl drops of fresh PBS (20 min in each droplet), and transferred into 100-μl drops of Ham’s F-10 (SpermPrep; ZBL Laboratories, Lexington, KY). Washed oocytes were held at 4°C until placed in the incubator for warming (37°C) prior to experimental use. Freshly thawed oocytes that had been denuded and washed as described above also were placed in petri dishes in 100-μl drops of Ham’s F-10 at the same time as the salt-stored oocytes. For experiments involving intact oocytes, they were examined on an inverted microscope (Olympus, Tokyo, Japan; 40×, brightfield) for structural integrity prior to insemination.

Spermatozoal Collection and Preparation

For all experiments, ejaculates were collected from stallions by means of an artificial vagina. Gel-free fractions were evaluated initially for volume, spermatozoal concentration, and progressive motility. A portion of the sample then was extended 1:1 in Ham’s F-10. Extended semen was maintained at 37°C during transport to the laboratory, where spermatozoa were separated from seminal plasma through use of a commercially available, two-step discontinuous Percoll gradient (Perception; Fertility Technologies, Inc., Natick, MA) with slight modification of the manufacturer’s instructions. Briefly, 2.5 ml extended semen was placed gently onto the Percoll column containing 2.5 ml of upper (47%) and lower (90%) phases. The loaded column was centrifuged at 400 × g for 20 min at room temperature. After the supernatant was removed, the spermatozoal pellet was washed with 2.5 ml of Ham’s F-10 (400 × g, 10 min) and resuspended with 1 ml of Ham’s F-10 medium. Resuspended samples were evaluated for spermatozoal concentration and percentage of progressively motile spermatozoa. Unopette-WBC-determination kits (Becton, Dickinson, Rutherford, NJ) were used to dilute semen samples for determining concentration (hemocytometer).

Insemination and Evaluation Procedures

Spermatozoa were diluted to 1 × 10⁶/ml for experiments II and III, and 100 μl of the insemination dilution was added to each drop containing oocytes, bringing the final insemination dosage to 5 × 10⁵ spermatozoa/ml. After coincubation, oocytes were transferred to a fresh 50-μl drop of Ham’s F-10. To determine the number of loosely attached sperm, oocytes were washed by five passages through a pulled glass pipette that measured approximately 1.9 times the size of an average oocyte. To determine the number of bound or penetrated sperm, oocytes were passed five times through a pulled glass pipette that measured approximately 0.9 times the size of an average oocyte. Pipettes were kept consistent within experiments. Oocytes, in separate droplets of 3–5 μl, then were placed on a glass microscope slide, and associated sperm were fixed and permeated with the addition of 2 μl of 70% ethanol (v/v). After 5 min, spermatozoa were labeled with 2 μl Hoescht 33258 (1 μg/ml; Sigma). Slides were allowed to air dry in a darkened environment to fix oocytes onto them. Slides were rinsed gently in distilled water to remove excess unbound stain and again allowed to dry
in a darkened environment before being viewed on a fluorescent microscope for determination of sperm attachment, binding, and/or penetration.

Experiment I

In order to determine whether or not storage of equine ZP (eZP) in a highly concentrated salt solution affected ZP proteins, one-dimensional SDS-PAGE (Bio-Rad Modular Mini-Protean II System; Bio-Rad Chemical Division, Richmond, CA) was run to compare migration patterns of ZP proteins isolated from either salt-stored oocytes or frozen oocytes. Zona ghosts were created by rupturing the zona and manually removing the ooplasm. Zona ghosts of 50 frozen or 50 salt-stored equine oocytes, each in 10 µl PBS, were placed into an equal volume of double-strength treatment buffer (0.125 M Tris-Cl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8) and boiled for 2 min. The samples then were centrifuged at 10,000 rpm for 8 min. Samples were loaded onto a 12% SDS-PAGE gel at room temperature for 1.5 h at 100 V, 20 mA. Molecular mass markers were used in a separate lane to define the apparent molecular mass of the isolated proteins.

A silver stain method of protein detection was used to determine the number and location of the eZP protein families. The gel was washed in three sequential 20-min washes of 50% ethanol and distilled water. A final rinse contained distilled water and 5 µg/ml dithiothreitol. The gel was placed into a 0.2% silver nitrate solution for 45 min and then rinsed three times in fresh water. The color was developed by shaking the gel in a solution of 3% sodium carbonate and 0.5 ml/L 38% formaldehyde. Development was stopped with 10% acetic acid.

Experiment II

To investigate the kinetics of sperm-ZP binding and subsequent ZP penetration by spermatozoa, spermatozoa from two ejaculates of one stallion were cultured at 37°C under oil for 5, 30, 60, 120, 240, or 480 min with a total of 107 salt-stored oocytes. The numbers of spermatozoa loosely attached, tightly bound, or penetrating the ZP were determined at each time point.

Experiment III

Single ejaculates from three stallions were utilized to compare sperm attachment, binding, and penetration between salt-stored and frozen oocytes. Oocytes were cocultured with sperm for 30 or 120 min at 37°C in a humidified atmosphere. After the coculture, oocytes were washed, stained, and examined for attachment, binding, and penetration.

Experiment IV

Zona proteins were solubilized (ZP) and used to determine the ability of ZP of salt-stored (SZP) and frozen oocytes (FZP) to induce acrosome reactions in spermatozoa incubated for 5, 60, and 120 min prior to ZP challenge. After spermatozoal preparation, 100 µl of a sperm dilution containing 1 × 10^6 progressively motile spermatozoa was incubated for 15 min with 100 µl H33258 (Sigma) to label nonviable spermatozoa. Samples then were washed with 200 µl of Ham’s F-10 (8 min, 400 × g) to remove excess stain. After centrifugation, the supernatant was removed, and the sperm pellet was resuspended in 100 µl of Ham’s F-10. After 5, 60, and 120 min of culture, 5 µl of the sperm dilution was cocultured in microcentrifugation tubes at 37°C in a humidified atmosphere for 20 min with 5 µl of each of the following treatments: 1) BSA (5 mg/ml), 2) SZP (2 ZP/µl), or 3) FZP (2 ZP/µl). Solubilization was brought about by placing isolated zona ghosts (4/µl) in microcentrifugation tubes, which were covered with parafilm and held in a boiling water bath for 2 min. After the coculture period, 5 µl of the solution was added to 5 µl ethanol on a glass slide. After the specimen had dried, the slide was rinsed gently in distilled water and again allowed to air dry. To determine acrosomal status, medium containing 100 µg/ml fluorescein isothiocyanate (FITC)-labeled Pisum satium (PSA) lectin (Sigma) then was added, and the slide was refrigerated at 4°C for 30 min. In this study, spermatozoa were classified as acrosome-reacted or acrosome-intact based on PSA fluorescence patterns previously described by Casey et al. [1993].

Slides were evaluated by epifluorescence microscopy using filters appropriate to visualize bound FITC-PSA (acrosomal status) and ultraviolet filters to visualize H33258 (viability). Viability and acrosomal status of at least 100 sperm per slide were assessed. With dual fluorescent labeling, each spermatozoon was classified as viable/acrosome-intact; viable/acrosome-reacted; dead/acrosome-intact; or dead/acrosome-reacted.
FIG. 1. Silver stain analysis of equine ZP by one-dimensional PAGE. Protein was harvested from 50 salt-stored (lane A) and 50 frozen (lane B) oocytes. Molecular masses indicated are for standards. Four identical protein bands with molecular masses of 90, 75, 60, and 50 kDa were detected for each type of stored ZP.

Statistical Analyses

Data for experiment III were examined by analysis of variance procedures (PROC GLM), using Statistical Analysis Systems (SAS, 1993), as a split plot over time, as outlined by Milliken & Johnson (1992). Ejaculates were split to test treatments, which were further split to allow repeated measures over time. Storage was treated as a fixed effect, whereas all other effects were considered random. Therefore, the error term for testing the influence of storage on the other variables was the storage by stallion mean square error.

The model statement used was:

\[ y = \alpha + T_i + \beta_j + T\beta_{ij} + \gamma_k + T\gamma_{ik} + \varepsilon \]

where \( T = \) ith storage, \( \beta = \) jth stallion, \( T\beta = \) storage by stallion interaction (whole plot error term), \( \gamma = \) kth time, \( T\gamma = \) interaction between storage and time, and \( \varepsilon = \) residual.

Data for experiment IV were analyzed as a generalized linear model with a logistic distribution (PROC GENMOD) through use of Statistical Analysis System (SAS, 1993). This procedure most adequately analyzes binary data.

The model statement used was:

\[ \gamma_{ijk} = \alpha + \beta_i + \gamma_j + T_k + \beta\gamma_{ij} + \beta T_{ik} + \zeta + \epsilon \]

where \( \beta = \) ith treatment, \( \gamma = \) jth stallion, \( T = \) kth time, \( \beta\gamma = \) treatment by stallion interaction, \( \beta T = \) treatment by time interaction, \( \zeta = \) viability, and \( \epsilon = \) residual.

RESULTS

Experiment I

Zona protein from 50 salt-stored and 50 frozen oocytes subjected to SDS-PAGE revealed four identical proteins with molecular masses of 90, 75, 60, and 50 kDa (Fig. 1).
USE OF SALT-STORED OOCYTES

FIG. 2. The mean (± SEM) number of (A) loosely attached and tightly bound, and (B) penetrating spermatozoa (per salt-stored oocyte) during 4 h of culture. For each category the means represent an average of 5 oocytes per time point.

**Experiment II**

An increase in the number of spermatozoa loosely attached to salt-stored oocytes was observed through 240 min of coculture with a rapid decrease occurring after that time point (Fig. 2). An increase, though less dramatic, also occurred in the number of spermatozoa bound to oocytes through 120 min of coculture, followed by either a plateau or a gradual decline in the number of tightly bound spermatozoa. Penetration first occurred at 120 min. The number of spermatozoa that penetrated appeared to decline at 480 min; however, only four oocytes were evaluated at that time.

**Experiment III**

When salt-stored and frozen oocytes were compared, no difference was observed between them in the number of spermatozoa attached (p = 0.9), bound (p = 0.8), or penetrating (p = 0.7) the ZP after 120 min of culture (Fig. 3). However, a trend (p = 0.06) towards a time by treatment interaction was detected for total sperm attached per oocyte when analyzed within observation time. The number of sper-
Experiment IV

When spermatozoa were cultured alone or cocultured with SZP (2 zonae/μl), FZP (2 zonae/μl), or BSA, differences \( (p = 0.0005) \) were detected in the percentage of live acrosome-reacted (PAR) spermatozoa among treatments (Fig. 4). Spermatozoa challenged by both SZP and FZP had a higher \( (p = 0.003) \) PAR than the unchallenged spermatozoa. Additionally, PAR spermatozoa were increased \( (p = 0.02) \) by coculture with FZP as compared to BSA. However, no difference \( (p > 0.05) \) was observed in PAR spermatozoa between FZP and SZP. The FZP resulted in a 19% increase in acrosome-reacted sperm as compared to the control, whereas the SZP produced a 12% increase. No treatment by time or treatment by stallion interactions were detected \( (p > 0.05) \).

DISCUSSION

Experiments utilizing equine oocytes are challenging in that methods of oocyte isolation established for other species that yield large numbers of oocytes, such as mincing ovarian tissue [Dunbar et al., 1980], have not been successful. Equine oocytes normally are harvested by follicular aspiration [Vogelsang et al., 1987; Arns et al., 1991; Miller et al., 1992], which is a tedious process and is technician dependent [Vasquez et al., 1993]. The recovery rates for harvesting oocytes from frozen/thawed ovaries in the studies reported herein approached 80% on a per follicle basis. Oocyte recovery was not reported on a per ovary basis because of the tremendous variability in follicular activity. Tissue availability becomes a limiting factor in the design of experiments using equine oo-
cytes. The ability to develop reserves of oocytes thus would be beneficial for studies in which equine gamete physiology is to be investigated.

When equine oocytes were placed in concentrated salt solution, the cytoplasm shrunk, creating an enlarged perivitelline space, but no gross morphological change was observed in the ZP when viewed with a phase-contrast microscope. In a few instances, the vitellus shrunk to a dumbbell shape instead of retaining the spherical shape. This is in agreement with the original findings of Yanagimachi et al. [1979]. In some, but not all, instances, the cytoplasm re-expanded when stored oocytes were rinsed prior to coincubation. Yanagimachi et al. [1979] also noted this phenomenon in hamster oocytes, but only when using a storage solution containing MgCl₂ and polyvinylpyrrolidone. For the cytoplasm to remain shrunken is advantageous, because this allows for easier visualization of spermatozoa located in the perivitelline space.

In experiment III, the percentage of oocytes recovered (only oocytes with intact ZP were evaluated) from culture (final number of oocytes observed divided by the number initially placed into culture) tended to be lower (p = 0.1) for salt-stored oocytes than for frozen oocytes (82 vs. 91%, respectively). Boatman et al. [1988] observed a similar problem with salt-stored hamster eggs when using the original formula [Yanagimachi et al., 1979] containing dextran but eliminated the problem by substituting ZnCl₂ and polyvinyl alcohol for dextran. Throughout the current experiments, some of the salt-stored oocytes exhibited a stickiness that caused them to adhere to the petri dishes. Even though this stickiness was not characteristic of all oocytes, it occurred often enough to cause concern, because it intensifies handling; this, in turn, increased the possibility of oocyte damage. The storage solution used in this study has been used in the domestic [Andrews et al., 1989] and exotic cat [Howard et al., 1991] and is a modification of the solution used by Boatman et al. [1988]. The stickiness we observed was eliminated in later investigations in our laboratory by changing to noncoated petri dishes (Fisher Scientific, St. Louis, MO).

Proteins isolated from salt-stored and frozen oocytes exhibited similar patterns of protein migration with apparent molecular masses of 90, 75, 60, and 50 kDa. These molecular masses are in agreement with those reported by Miller et al., [1992], with the exception of the smallest, which was absent in their report. The appearance of this glycoprotein family may have been due to processing, because those authors were able to identify only the three larger molecular mass families by immunological techniques.

To monitor sperm-oocyte interaction, we used a combination of fluorescent and brightfield microscopy as described by Boatman et al. [1988]. Spermatozoa on the ZP could be distinguished through the use of brightfield microscopy; however, bound or penetrated sperm could be seen and counted only when they were labeled with the fluorochrome H33258. To substantiate our findings, we evaluated oocytes (data not shown) by transmission and scanning electron microscopy (SEM). Spermatozoa were seen clearly associated with the ZP, as well as being deeply embedded into it. Only one oocyte from each treatment was recovered and examined at each time point by SEM. Therefore, micrographs were not used for the purpose of drawing definitive conclusions regarding sperm-oocyte interactions between treatments. However, they did provide direct evidence that spermatozoa were bound to the ZP and were in different stages of ZP penetration.

The work presented here demonstrates that the ZPs of equine oocytes harvested from frozen ovaries and stored in concentrated salt solution were as penetrable by in vitro-capacitated spermatozoa as the ZPs of oocytes from frozen ovaries not stored in salt solution. Frozen ZP and salt-stored ZP were equal in their ability to stimulate attachment, binding, and penetration by equine sperm. The ability of equine spermatozoa to bind and subsequently penetrate the ZP of salt-stored oocytes is similar to that observed for spermatozoa of other species [Yanagimachi et al., 1979; Boatman et al., 1988; Andrews et al., 1989].

A day difference was detected (p < 0.03) in experiments III and IV. The day effect is probably a stallion effect but is confounded by day and therefore should be interpreted with caution. However, variability between sperm donors has been well documented [O’Rand & Fisher, 1987; Berger et al., 1989] and is in agreement with our observations. It is interesting that in addition to the stallion differences observed in sperm-oocyte interaction, an apparent difference occurred in the rate at which spermatozoa underwent spontaneous acrosome reactions (data not shown). The stallion that ejaculated spermatozoa showing the most rapid increase in spontaneous acrosome
reactions (within 15 min) had the lowest number of spermatozoa bound to the ZP at 120 min, whereas the stallion that ejaculated spermatozoa showing the slowest increase in spontaneous acrosome reactions (~60 min) had the highest number of spermatozoa bound. This suggests that stallion spermatozoa must be acrosome-intact prior to binding the ZP, a concept that is similar to results reported for the mouse [Saling & Storey, 1979]. However, future studies are necessary to distinguish which population of stallion spermatozoa (acrosome-intact or -reacted, or both) initiates binding to the ZP.

Capacitation events that allow for acrosome reaction and fertilization in situ require approximately 9 h in the mare [Enders et al., 1987]. Previous work from this laboratory [Buzby et al., 1993] suggested that equine spermatozoa separated from seminal plasma by a discontinuous Percoll gradient and re-exten­
ded in Ham's F-10 become capacitated within 30 min of in vitro culture. This is a more rapid occurrence than has been reported previously in the horse [Blue et al., 1989; Samper et al., 1989; Arns et al., 1991] and may be attributable to differences in processes used to stimulate capacitation. The ability of spermatozoa to successfully bind and penetrate the ZP is dependent upon their undergoing, first, capacitation and, subsequently, the acrosome reaction [Florman & Storey, 1982; Cherr et al., 1986; Cross et al., 1988; Florman & First, 1988]. The binding assays conducted in the present study support the hypothesis that capacitation had occurred first, thus allowing spermatozoa to undergo a physiological acrosome reaction followed by ZP penetration. Subsequent studies in this laboratory have substantiated this finding in the horse [Arns & Shepherd, 1994].

Solubilized ZPs from frozen and salt-stored oocytes were also similar in their ability to stimulate a receptor-mediated acrosome reaction in a population of in vitro-capacitated equine spermatozoa. Induction of the acrosome reaction by solubilized ZP has been reported in the mouse [Bleil & Wassarman, 1980], hamster [Cherr et al., 1986], rabbit [O’Rand & Fisher, 1987], and stallion [Arns et al., 1991]. The present study supports earlier work with solubilized eZP and its ability to induce the acrosome reaction in equine spermatozoa. The increased PAR in spermatozoa challenged with BSA was not expected. If spermatozoa were capacitated before incubation with BSA, as was suggested by experiments II and III, then additional serum albumin, a substance known to be necessary to stimulate sperm maturation in vitro [Andrews et al., 1989], might have intensified the destabilization of the acrosomal membranes, thus hastening spontaneous acrosome reactions [Florman & First, 1988]. Although a high incidence of spontaneous acrosome reactions existed, the spermatozoal response to solubilized ZP proteins was similar to findings reported earlier [Arns et al., 1991] in the horse.

These data provide evidence that salt-stored equine oocytes/zonae can be used in bioassays to monitor sperm-oocyte interaction and provide information regarding spermatozoal maturation and potential fertilizing ability.

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REFERENCES


