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# Membrane Contact with Oviductal Epithelium Modulates the Intracellular Calcium Concentration of Equine Spermatozoa In Vitro[1]

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## ABSTRACT

**Interaction of equine spermatozoa with oviductal epithelial cells (OEC) prolongs sperm viability and maintains low intracellular calcium concentration ($[Ca^{2+}]_i$) in spermatozoa. Experiments were designed to investigate 1) whether release of spermatozoa from OEC in vitro is associated with elevated $[Ca^{2+}]_i$ and 2) whether soluble products from OEC or direct membrane contact between spermatozoa and OEC mediates the effects of OEC on sperm $[Ca^{2+}]_i$. In the first experiment, changes in $[Ca^{2+}]_i$ in spermatozoa loaded with indo-1 acetoxymethylester were determined in motile spermatozoa released from OEC monolayers after 4 h of culture compared to $[Ca^{2+}]_i$ in spermatozoa still attached to OEC. In addition, $[Ca^{2+}]_i$ was determined in spermatozoa incubated with OEC-conditioned medium for 6 h compared to that in spermatozoa incubated in control medium. $[Ca^{2+}]_i$ was higher in motile spermatozoa released from OEC than in spermatozoa still attached to OEC after 4 h of incubation. Incubation in OEC-conditioned medium resulted in lower sperm $[Ca^{2+}]_i$ only at 4 h of incubation, but not at 0.5, 2, or 6 h of incubation. In the second experiment, a suspension of apical plasma membrane vesicles (AMV) isolated from isthmic oviductal epithelium was used to study the specific effect of sperm contact with OEC membranes on sperm viability, capacitation, and $[Ca^{2+}]_i$. Direct membrane contact between spermatozoa and AMV prolonged sperm viability, delayed capacitation, and maintained low $[Ca^{2+}]_i$ in spermatozoa. These results indicated that membrane contact between equine spermatozoa and OEC is required to maintain low $[Ca^{2+}]_i$, delay capacitation, and prolong viability of spermatozoa in vitro. Modulation of capacitation rate for spermatozoa stored in the isthmic sperm reservoir might ensure the availability of a competent sperm population at the time of fertilization.**

## INTRODUCTION

The mammalian oviduct is the site of final gamete maturation, fertilization, and early embryonic development. After insemination, spermatozoa from several mammalian species are stored in the isthmus of the oviduct (rabbit [1]; pig [2]; sheep [3]; mouse [4]; cattle [5]; hamster [6]). Within the isthmic reservoir, spermatozoa attach to oviductal epithelial cells (OEC) by their rostral plasma membrane [7, 8]. Contact of spermatozoa with OEC serves to maintain viability of spermatozoa stored in the oviduct [8]. This may be particularly important in the mare, where fertilization can occur several days after breeding [9]. In the horse, the existence of sperm storage in the oviductal isthmus is supported by evidence obtained from explants of oviductal epithelium in vitro [10].

Because the life span of capacitated spermatozoa is relatively short [11, 12], regulation of the rate of capacitation of spermatozoa stored in the oviductal isthmus could represent an important mechanism for ensuring availability of spermatozoa at the time of fertilization. Capacitated spermatozoa lose their ability to attach to OEC in vivo and in vitro [6, 13, 14], and spermatozoa released from OEC in vitro are capacitated and will bind to the zona pellucida [15]. Capacitation of spermatozoa in the female reproductive tract involves changes in the sperm plasma membrane and an influx of calcium ions [16–18]. In equine spermatozoa attached to OEC in vitro, the intracellular calcium concentration ($[Ca^{2+}]_i$) is maintained at basal levels [19]. High $[Ca^{2+}]_i$ is associated with impending cell death [20], and maintenance of low $[Ca^{2+}]_i$ might therefore represent a mechanism for extending sperm viability during storage in the oviduct. From previous experiments, it could not be determined whether modulation of $[Ca^{2+}]_i$ in spermatozoa attached to OEC was mediated by direct cell contact or by OEC secretory products. Secreted products from OEC may preserve sperm longevity by stabilizing sperm membranes and delaying capacitation [21, 22] and have been shown to influence sperm motility [23–25]. Conversely, incubation of rabbit spermatozoa with apical plasma membrane vesicles from oviductal epithelium was shown to lengthen the life span of the spermatozoa, suggesting the possibility that direct membrane contact between spermatozoa and OEC also plays a role in maintaining sperm viability during storage [26].

The objective of the present study was to further investigate the role of OEC in modulating $[Ca^{2+}]_i$ in equine spermatozoa. Specifically, experiments were designed to determine 1) whether release of spermatozoa from OEC is associated with elevated $[Ca^{2+}]_i$ in spermatozoa and 2) whether the effect of OEC on $[Ca^{2+}]_i$ in spermatozoa is mediated by soluble products from OEC or by direct membrane contact between spermatozoa and epithelial cells. For examining the effects of membrane contact alone, apical plasma membrane vesicles (AMV) were prepared, using a method originated by Kinne-Saffran and Kinne [27] adapted for oviductal epithelium [26].

## MATERIALS AND METHODS

### *Experimental Design*

For all experiments, ejaculated equine spermatozoa were washed and loaded with the fluorescent calcium indicator indo-1 acetoxymethylester (AM) unless stated otherwise. $[Ca^{2+}]_i$ was determined by ratio image analysis of images captured at 405- and 490-nm emission as described below. In experiment 1, $[Ca^{2+}]_i$ was measured in spermatozoa re-

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leased from OEC compared with spermatozoa attached to OEC after 4 h of incubation. Progressive motility, membrane integrity, and acrosomal status were assessed before coculture and in sperm released from OEC after 4 h of culture. In experiment 2, $[Ca^{2+}]_i$ was measured in spermatozoa incubated in media conditioned by OEC monolayers alone or by sperm-OEC cocultures in comparison with spermatozoa in control media at 0.5, 2, 4, and 6 h of incubation. In experiment 3, spermatozoa were incubated with AMV derived from either equine isthmic oviductal epithelium or kidney, or without AMV. The $[Ca^{2+}]_i$ was measured at 0.5, 3, and 6 h of incubation. Sperm viability and capacitation were evaluated in spermatozoa that had not been loaded with indo-1 AM prior to and at 6 and 24 h of incubation. In experiment 4, spermatozoa were incubated with or without isthmic oviductal AMV in the presence or absence of a polyclonal antibody previously shown to inhibit sperm attachment to OEC in vitro [28], or in the presence of control antibody. The $[Ca^{2+}]_i$, sperm viability, and capacitation were assessed as in experiment 3.

All experiments were replicated using 2 ejaculates from each of 3 stallions. Epithelial cells harvested from different mares were used in experiments 1 and 2, and OEC from a single mare were used within each experiment.

### General Methods

*Preparation of OEC monolayers.* Unless otherwise mentioned, all reagents were supplied by Sigma Chemical Co. (St. Louis, MO). Monolayers of OEC were derived from the oviductal isthmi of two mares in the preovulatory stage of the cycle [29]. Briefly, oviducts were opened longitudinally, and epithelial cells were mechanically dissociated from the underlying stroma. Epithelial cells were cultured in 50% Dulbecco's Modified Eagle Medium and 50% Ham's F-12 (50:50 DMEM:F-12) with 10% fetal bovine serum (FBS; Hyclone, Langhorne, PA), 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Monolayers were grown to confluence, trypsinized, and frozen in 10% dimethylsulfoxide (DMSO) and 50% FBS [30]. Cells were thawed and cultured to confluence in slide chamber wells (Lab-Tek 4-chamber slides; Nunc Inc., Naperville, IL; experiment 1) or in 24-well culture plates (Sarsted Inc., Newton, NC; experiment 2) coated with 50 μl of basement membrane extract (Matrigel; Collaborative Research Inc., Bedford, MA) at a concentration of 2 mg/ml. All cultures were incubated at 38.5°C in a humidified atmosphere of 5% $CO_2$ in air. Monolayers of OEC were used at confluence five days after initiation.

*Preparation of oviductal AMV.* AMV were derived from oviductal isthmi of 8 mares in the preovulatory stage of the cycle and from the kidney of a single horse as described previously [26]. The final AMV fraction was resuspended in protein-free modified Tyrode's solution (TALP; [31]). Protein content was determined colorimetrically [32]. Specific activity of the apical plasma membrane marker γ-glutamyl transpeptidase [33, 34] was determined using the method of Naftalin et al. [35]. The degree of enrichment was expressed as fold increase in γ-glutamyl transpeptidase activity in the final AMV fraction compared to the initial homogenate.

*Semen collection and processing.* Ejaculated semen was collected with an artificial vagina and diluted to $100 \times 10^6$ spermatozoa/ml in TALP. Spermatozoa were washed by centrifugation ($300 \times g$, 6 min), resuspended to $50 \times 10^6$/ml in TALP, and incubated with 5 μM indo-1 AM (Molecular Probes Inc., Eugene, OR) for 40 min at 38.5°C [36]. Extracellular dye was removed by centrifugation ($300 \times g$, 6 min), and spermatozoa were resuspended in 50:50 DMEM:F-12 (experiment 1), in OEC-conditioned medium (experiment 2) to $10 \times 10^6$/ml, or in TALP to $70 \times 10^6$/ml (experiments 3 and 4). Before $[Ca^{2+}]_i$ was measured, spermatozoa were incubated for 20 min to allow de-esterification of indo-1 AM to its calcium-sensitive form indo-1 [36]. In experiments involving OEC (experiments 1 and 2), 50:50 DMEM:F12 was used as incubation medium to achieve optimal culture conditions for OEC. For experiments not involving OEC (experiments 3 and 4), spermatozoa were incubated in TALP as a defined medium commonly used for incubation of spermatozoa.

*Determination of $[Ca^{2+}]_i$ in spermatozoa.* Fluorescent images were acquired and processed as described previously [20]. Briefly, the fluorescence ratio (R) was calculated as F2/F1, where F2 is from the image taken at 405 nm ($Ca^{2+}$-bound indo-1) and F1 is from the image taken at 490 nm ($Ca^{2+}$-free indo-1). The average fluorescence ratio of five image pairs was determined for each of 25 motile spermatozoa for each treatment group. The fluorescence ratio was determined for each individual spermatozoon, whereas the other variables used to calculate $[Ca^{2+}]_i$ were determined for the experimental conditions by adding bromo-A23187 (10 μM for 1 min) to spermatozoa in solutions of known $[Ca^{2+}]$ [36]. Only motile spermatozoa were included, as determined by the presence of a flagellar beat in sperm attached to OEC and by progressive motility in free-swimming spermatozoa.

### Experiment 1

Experiment 1 was designed to investigate the effect on sperm $[Ca^{2+}]_i$ of attachment to, or release from, OEC monolayers. Cocultures of spermatozoa and OEC were established by addition of $5 \times 10^6$ spermatozoa loaded with indo-1 AM to each culture well in a total of 500 μl medium. Cocultures were incubated for 4 h. After 0.5 and 4 h of incubation, the medium overlying the monolayer was aspirated to remove any unbound spermatozoa. Cocultures were then rinsed once with 500 μl of medium, and 500 μl of fresh medium was introduced into each well. Cocultures were incubated for an additional 15 min, and the overlying medium containing spermatozoa released during the 15-min incubation was collected. The cocultures were rinsed again as above. The chamber well gasket was then removed, and a coverslip was placed over the OEC on the slide to allow observation of the spermatozoa still attached to OEC. The $[Ca^{2+}]_i$ was measured in spermatozoa released from OEC by placing 5 μl sperm suspension on slides and covering it with 22-$mm^2$ coverslips. $[Ca^{2+}]_i$ was determined at 4 h and 15 min of incubation in 25 motile spermatozoa still attached to OEC and in 25 free-swimming spermatozoa that had been attached to OEC at 4 h of incubation but were subsequently released from OEC.

Progressive motility, membrane integrity, and acrosomal status were determined before establishment of coculture and in spermatozoa released from OEC after 4 h and 15 min of incubation. Progressive motility was evaluated subjectively by phase-contrast microscopy (×200 magnification) of wet-mounted preparations at 37°C. Membrane integrity and acrosomal status were determined as described previously [37]. This method allows for the simultaneous assessment of membrane integrity, by exclusion of the su-

pravital nuclear dye Hoechst 33258, and of acrosomal integrity, by labeling with fluoresceinated *Pisum sativum* agglutinin. Preliminary experiments determined that indo-1 fluorescence was only faintly visible under the conditions used to evaluate membrane integrity (350-nm exciter filter, 400-nm dichroic mirror, 420-nm barrier filter) and could easily be distinguished from the more intense Hoechst 33258 fluorescence. On the basis of the observed fluorescence pattern, each spermatozoon was assigned to one of four categories: 1) viable/acrosome-intact, 2) viable/acrosome-missing, 3) dead/acrosome-intact, or 4) dead/acrosome-missing. A total of 100 spermatozoa were examined per sample.

*Experiment 2*

Experiment 2 was designed to investigate the effects of soluble products from OEC monolayers on sperm $[Ca^{2+}]_i$. In experiment 2a, OEC-conditioned medium was harvested from confluent OEC monolayers after 24 h of incubation, pooled, and centrifuged at 2000 × *g* for 10 min. The supernatant was filtered through 0.45-μm syringe filters, aliquoted, and frozen at −20°C. Aliquots of the same batch of medium not exposed to OEC were frozen as control medium.

Experiment 2b was designed to account for changes in secretory products from OEC induced by coculture with spermatozoa [38]. Spermatozoa ($1 \times 10^6$/well) were allowed to attach to OEC monolayers, unbound spermatozoa were removed after 30 min of incubation, medium was replaced, and incubation was continued for another 4 h. Sperm/OEC-conditioned medium was harvested after 4 h of sperm/OEC coculture, pooled, centrifuged, filtered, and frozen as in experiment 2a. For sperm-conditioned control medium, spermatozoa ($2 \times 10^6$/ml) were incubated in the same batch of culture medium for 4.5 h, and control medium was processed and frozen as described for OEC-conditioned medium.

Spermatozoa were loaded with indo-1 AM and resuspended in OEC-conditioned medium or control medium (experiment 2a) or in sperm/OEC-conditioned medium or sperm-conditioned medium (experiment 2b) to $10 \times 10^6$/ml and were incubated for 6 h. $[Ca^{2+}]_i$ was measured in 25 motile, free-swimming spermatozoa per treatment at 0.5, 2, 4, and 6 h of incubation as described for experiment 1.

*Experiment 3*

Experiment 3 was designed to investigate the effect of direct membrane contact between spermatozoa and OEC in the absence of intact OEC or soluble products from OEC. This was accomplished by incubating spermatozoa with AMV isolated from isthmic OEC or from kidney tubule epithelial cells as controls. Semen was collected and processed as for experiments 1 and 2, except that spermatozoa were not loaded with indo-1 AM for experiment 3a. In experiment 3a, washed spermatozoa ($35 \times 10^6$/ml) were incubated with isthmic or kidney AMV or without AMV for 24 h at 38.5°C in 5% $CO_2$ in air. Total protein concentration was adjusted to 3.1 mg/ml by addition of BSA to the control incubation.

Prior to and at 6 and 24 h of incubation, sperm viability and capacitation status were determined by epifluorescence microscopy of spermatozoa stained with propidium iodide (PI) and carboxyfluorescein diacetate (CFDA), or by chlortetracycline, respectively. For assessment of sperm viability, 10 μl of sperm suspension were mixed with 10 μl of TALP containing 10 μg/ml PI and 20 μg/ml CFDA for 15 min at 38.5°C in 5% $CO_2$ in air [39]. TALP containing PI/CFDA was freshly prepared from stocks (0.5 mg/ml PI in water, 0.5 mg/ml CFDA in DMSO) immediately before staining. Spermatozoa were examined by epifluorescence microscopy using filters for fluorescein or rhodamine, respectively. Spermatozoa fluorescing green were considered to be viable; spermatozoa fluorescing red were considered to be dead. A total of 100 spermatozoa were examined per sample.

Capacitation status of spermatozoa was assessed as described previously [40]. On the basis of membrane fluorescence patterns, spermatozoa were classified as either uncapacitated, capacitated, or acrosome missing. A total of 100 spermatozoa were examined per sample.

To examine the interaction of spermatozoa with AMV by transmission electron microscopy, an aliquot of spermatozoa incubated with isthmic or kidney AMV for 1 h was fixed in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate and postfixed in 2% osmiumtetroxide in 0.1 M sodium-cacodylate. Samples were embedded in 2% agar, stained with 2% uranyl acetate, sequentially dehydrated, embedded in epon/araldite (Electron Microscopy Sciences, Fort Washington, PA), sectioned, and processed for transmission electron microscopy.

In experiment 3b, spermatozoa loaded with indo-1 AM ($35 \times 10^6$/ml) were incubated with isthmic or kidney AMV or without AMV for 6 h. At 0.5, 3, and 6 h of incubation, $[Ca^{2+}]_i$ was measured in 25 motile spermatozoa per treatment as described for experiments 1 and 2.

*Experiment 4*

Experiment 4 was designed to determine whether AMV binding to the sperm plasma membrane is required to elicit the observed effects on sperm viability, capacitation, and $[Ca^{2+}]_i$. If AMV binding is necessary, then inhibition of AMV binding to the sperm plasma membrane should abolish the effect of AMV on sperm viability, capacitation, and $[Ca^{2+}]_i$. Antigen-binding fragments (Fab) from a polyclonal antibody raised against the periacrosomal plasma membrane of equine spermatozoa, which had been shown to inhibit sperm binding to OEC monolayers and explants [28], were used to inhibit attachment of AMV to equine spermatozoa. Semen was collected and processed as in experiments 1 and 2, except that spermatozoa were not loaded with indo-1 AM for experiment 4a. In experiment 4a, washed spermatozoa ($35 \times 10^6$/ml) were incubated for 24 h at 38.5°C in 5% $CO_2$ in air with the following 5 treatments:

1) To provide a medium control, spermatozoa were incubated in TALP, with the protein concentration adjusted to equivalency to the protein concentration of AMV suspensions (1.7 mg/ml) with BSA. 2) To determine the effect, if any, of Fab on sperm function, spermatozoa were incubated with 0.5 mg Fab/ml. This had been shown previously to significantly reduce sperm-binding to OEC monolayers. 3) To provide reference values for effects of AMV on sperm function as in experiment 3a, spermatozoa were incubated with isthmic AMV. 4) To control for nonspecific effects of Fabs on sperm-AMV interaction or sperm function, spermatozoa were incubated with cFab (Fab derived from nonimmune rabbit serum, 0.5 mg/ml) and AMV. 5) To test whether inhibition of sperm-binding to AMV reduces the effects of AMV on sperm function, spermatozoa were incubated with Fab as in 2) above and AMV.

TABLE 1. Motility, viability, and acrosomal integrity of indo-1 AM-loaded equine spermatozoa prior to coculture and after release from OEC following 4.25 h of coculture as determined by phase-contrast microscopy, simultaneous exclusion of Hoechst 33258, and acrosomal staining with *Pisum sativum* agglutinin (experiment 1; means ± SD).

| Sperm population | % Progressively motile | % Viable, acrosome intact | % Viable, acrosome missing | % Membrane damaged, acrosome intact | % Membrane damaged, acrosome missing |
|---|---|---|---|---|---|
| Prior to coculture | 54.2 ± 6.6[a] | 64.5 ± 4.8[a] | 7.8 ± 0.8 | 10.2 ± 4.3 | 17.5 ± 3.1[a] |
| Released from OEC | 24.2 ± 12.8[b] | 44.3 ± 12.7[b] | 8.0 ± 3.6 | 16.5 ± 11.1 | 31.5 ± 6.0[b] |

[a,b] Means with different letters within columns are significantly different ($p < 0.05$).

The main comparison is between treatments 3 (sperm and AMV) and 5 (sperm, Fab, and AMV). Treatments 1, 2, and 4 served as controls. The total protein concentration in all treatments was 1.7 mg/ml in TALP. Prior to and at 6 and 24 h of incubation, sperm viability and capacitation status were determined as in experiment 3a. Aliquots of spermatozoa incubated with Fab and AMV (treatment 5) and of spermatozoa incubated with AMV only (treatment 3) were fixed and processed for transmission electron microscopy as described above. Spermatozoa with intact plasma membranes (200/treatment) were evaluated for the presence of AMV bound to their periacrosomal plasma membrane.

In experiment 4b, spermatozoa loaded with indo-1 AM ($35 \times 10^6$/ml) were incubated for 6 h with Fab (0.5 mg/ml), isthmic AMV, or Fab and AMV, corresponding to treatments 2, 3, and 5 above. At 0.5, 3, and 6 h of incubation, $[Ca^{2+}]_i$ was measured in 25 motile spermatozoa per treatment as described for experiments 1 and 2.

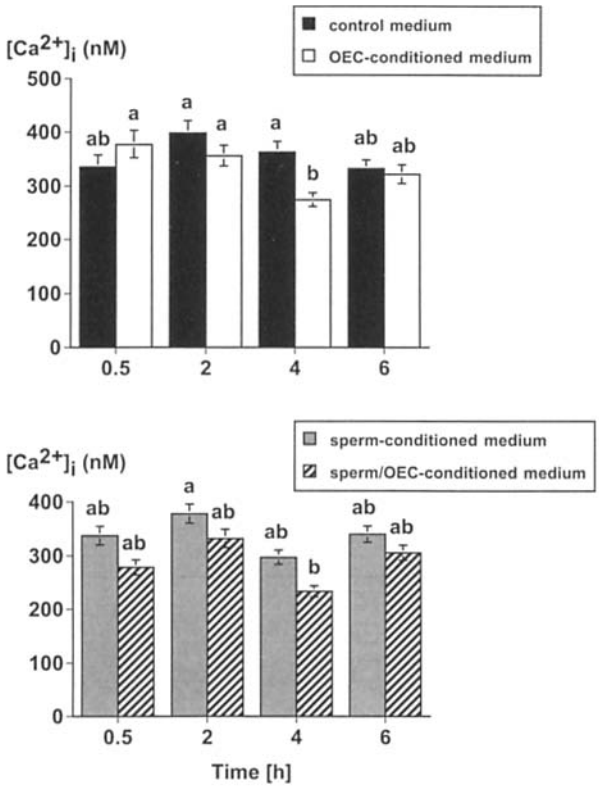


FIG. 1. $[Ca^{2+}]_i$ in equine spermatozoa incubated in OEC-conditioned medium vs. control medium (top), and in OEC/sperm-conditioned medium vs. sperm-conditioned medium (bottom) (experiment 2). Least-squares means ± SEM, n = 6 ejaculates. Means with different letters are significantly different ($p < 0.05$).

### *Statistical Analysis*

In all experiments, a randomized block design was employed, with treatments applied within ejaculate and ejaculates blocked by stallion. Calcium concentration data were transformed 1/sqrt(x) to achieve equality of variance in experiments 1, 7, and 8, and were log-transformed in experiment 2 to achieve a Gaussian distribution. Percentage data were transformed by arcsin(sqrt[x]). Data were analyzed by ANOVA using Linear Models of Statistix (Analytical Software, Tallahassee, FL). The dependent variable was $[Ca^{2+}]_i$, percentage of viable, or percentage of capacitated spermatozoa, and data were examined for effect of treatment, time, and interactions. Pairwise comparisons of means were made using Tukey's studentized range test (HSD). A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

### *Experiment 1*

$[Ca^{2+}]_i$ was higher ($p < 0.001$) in spermatozoa released from OEC after 4 h of coculture than in spermatozoa still attached to OEC (344.6 ± 17.5 nM vs. 110.3 ± 4.7 nM, mean ± SEM).

Sperm motility, viability, and acrosomal status before initiation of coculture and in spermatozoa released from OEC after 4.25 h of coculture are shown in Table 1. The percentage of progressively motile spermatozoa and of viable, acrosome-intact spermatozoa was lower ($p < 0.05$) in spermatozoa released from OEC after 4.25 h of coculture than in spermatozoa before coculture.

### *Experiment 2*

$[Ca^{2+}]_i$ was not different in spermatozoa incubated in conditioned medium and in spermatozoa incubated in control medium, except at 4 h of incubation in experiment 2a (Fig. 1).

ANOVA revealed an overall effect of treatment ($p < 0.01$) in experiment 2b, but $[Ca^{2+}]_i$ was not significantly lower in spermatozoa incubated with sperm/OEC-conditioned medium than in spermatozoa incubated with sperm-conditioned medium at any observation time (Fig. 1).

### *Experiment 3*

Specific activity of the marker enzyme γ-glutamyl transpeptidase was increased 28-fold in the final fraction compared to the initial tissue homogenate for isthmic AMV, and 11-fold for kidney AMV. These data suggest that the final fraction was markedly enriched with apical plasma membrane.

Transmission electron microscopy of spermatozoa incubated with isthmic AMV showed vesicle material attached

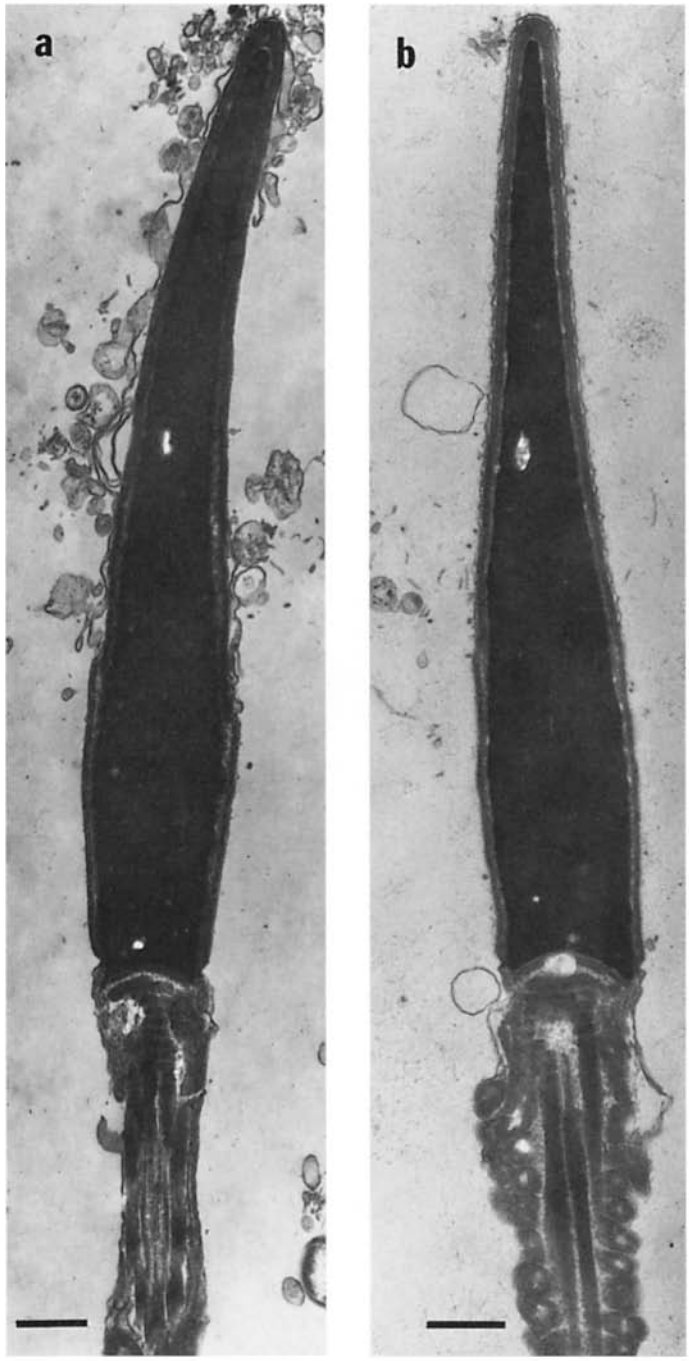


FIG. 2. Transmission electron micrographs of equine sperm heads **a**) incubated with isthmic AMV (experiment 3a) and **b**) incubated with isthmic AMV in the presence of Fab (experiment 4a). Bar represents 400 nm.

only to the periacrosomal plasma membrane of the sperm head (Fig. 2a), and AMV did not attach to the postacrosomal plasma membrane, midpiece, or tail. Kidney AMV attached to spermatozoa in a similar fashion, with the vesicle material appearing more heterogeneous in size.

Viability was higher ($p < 0.01$) at 6 and 24 h of incubation in spermatozoa incubated with isthmic AMV than in spermatozoa incubated with kidney AMV or in medium alone. Kidney AMV had an effect on sperm viability that was intermediate between isthmic AMV and medium alone (Fig. 3). Fewer ($p < 0.01$) spermatozoa incubated with isthmic AMV exhibited a capacitated staining pattern at 6 and

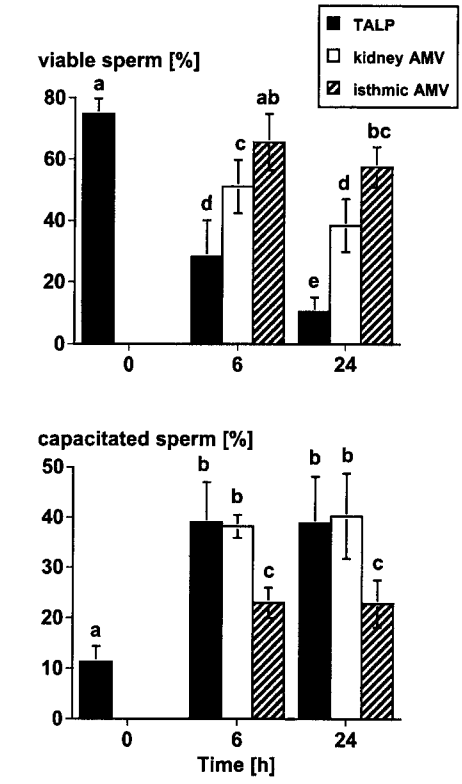


FIG. 3. Percentage of viable and capacitated equine spermatozoa incubated with kidney AMV, with isthmic AMV, or without AMV (TALP). Means ± SD, n = 6 ejaculates. Means with different letters are significantly different ($p < 0.05$).

24 h of incubation than did spermatozoa incubated with kidney AMV or in medium alone (Fig. 3).

$[Ca^{2+}]_i$ was lower ($p < 0.05$) in motile spermatozoa incubated with isthmic AMV than in spermatozoa incubated with kidney AMV or without AMV at 0.5, 3, and 6 h of incubation (Fig. 4).

*Experiment 4*

Transmission electron micrographs revealed that few AMV bound to the sperm plasma membrane when sper-

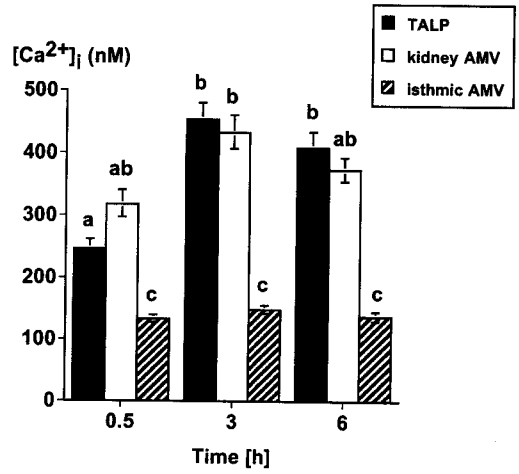


FIG. 4. $[Ca^{2+}]_i$ in equine spermatozoa incubated with kidney AMV, with isthmic AMV, or without AMV (TALP). Least-squares means ± SEM, n = 6 ejaculates. Means with different letters are significantly different ($p < 0.05$).

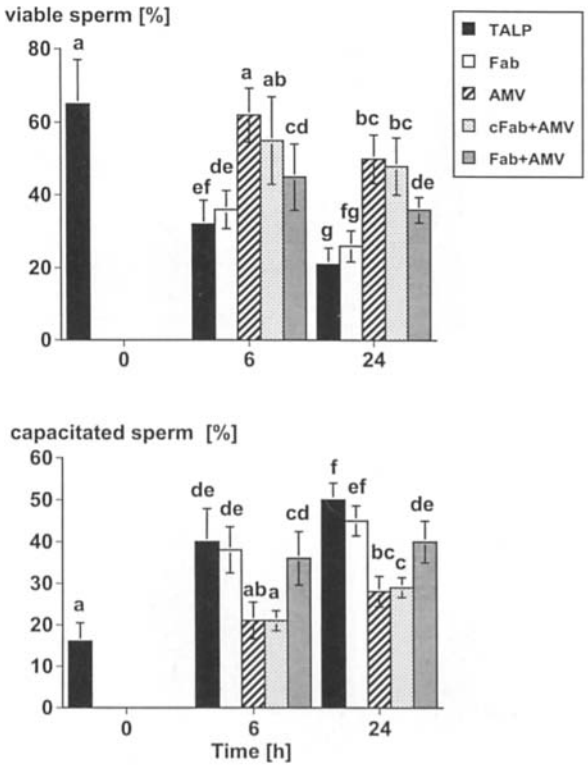


FIG. 5. Percentage of viable and capacitated equine spermatozoa incubated with or without isthmic AMV in the presence or absence of Fab directed against equine periacrosomal sperm plasma membrane or Fab from nonimmunized rabbit serum (cFab). Means ± SD, n = 6 ejaculates. Means with different letters are significantly different ($p < 0.05$).

matozoa and AMV were incubated in the presence of Fab (Fig. 2b). After incubation in the presence of Fab (treatment 5), only 34% of spermatozoa with intact plasma membranes had AMV bound to their periacrosomal plasma membranes, compared to 82% of spermatozoa after incubation with AMV only (treatment 3); however, this reduction in AMV binding was not statistically analyzed because of small sample size.

Viability was higher ($p < 0.05$) at 6 and 24 h of incubation in spermatozoa incubated with AMV or with AMV and control-Fab (treatments 3 and 4) than in spermatozoa incubated without AMV in the presence or absence of Fab (treatments 1 and 2). In spermatozoa incubated with AMV in the presence of Fab (treatment 5), the percentage of viable cells was significantly higher than in spermatozoa incubated without AMV (treatment 1), but significantly lower than in spermatozoa incubated with AMV or with AMV and control-Fab (treatments 3 and 4) at 6 and 24 h of incubation ($p < 0.05$; Fig. 5).

Fewer ($p < 0.05$) spermatozoa were capacitated at 6 and 24 h of incubation when incubated with AMV or with AMV and control-Fab (treatments 3 and 4) than when incubated in medium alone in either the presence or absence of Fab (treatments 1 and 2). In spermatozoa incubated with AMV in the presence of Fab (treatment 5), the percentage of capacitated cells was lower than in spermatozoa incubated without AMV (treatment 1) at 24 h of incubation, but higher than in spermatozoa incubated with AMV or AMV and control-Fab (treatments 3 and 4) at 6 and 24 h of incubation ($p < 0.05$; Fig. 5).

$[Ca^{2+}]_i$ was lower ($p < 0.05$) in motile spermatozoa incubated with AMV than in spermatozoa incubated with Fab

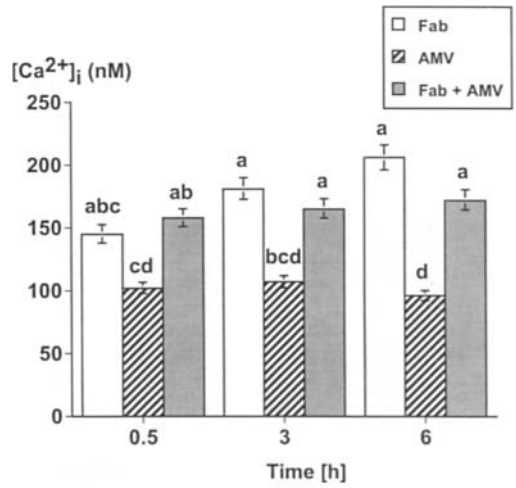


FIG. 6. $[Ca^{2+}]_i$ in equine spermatozoa incubated with isthmic AMV, Fab only, or AMV and Fab. Least-squares means ± SEM, n = 6 ejaculates. Means with different letters are significantly different ($p < 0.05$).

or with Fab and AMV at 0.5, 3, and 6 h of incubation (Fig. 6).

## DISCUSSION

A series of experiments was performed to investigate modulation of $[Ca^{2+}]_i$ in equine spermatozoa by OEC in vitro. Capacitation of spermatozoa involves an increase in $[Ca^{2+}]_i$ [16] and subsequently leads to acrosomal exocytosis and the death of the spermatozoon if fertilization does not occur [11, 41]. The prevention of premature capacitation in spermatozoa stored in the isthmic sperm reservoir by modulation of $[Ca^{2+}]_i$ might represent a mechanism to ensure availability of competent spermatozoa at the time of ovulation. This may be particularly important in species like the horse, in which insemination can precede ovulation by several days [9].

We demonstrated previously that equine spermatozoa attached to OEC maintain low $[Ca^{2+}]_i$ compared to spermatozoa incubated without OEC [19]. In the present study, equine spermatozoa released from OEC after 4 h of coculture had elevated $[Ca^{2+}]_i$ compared to spermatozoa that remained attached to OEC (experiment 1). This is in agreement with reports that equine spermatozoa released from OEC monolayers are capacitated and can bind to the zona pellucida [42]. Capacitated spermatozoa do not appear to attach to OEC [6, 13, 14]. This implies that changes in sperm surface characteristics associated with capacitation trigger the release of spermatozoa from the oviductal epithelium [6]. Furthermore, it has been suggested that hyperactivated motility may aid in the release of mouse sperm from the oviductal lining [43], and hyperactivation was described previously in equine spermatozoa released from OEC monolayers [42]. Hyperactivated motility in hamster sperm was associated with elevated $[Ca^{2+}]_i$ in the sperm head and the flagellum [36]. In our study, motility patterns of released sperm were not assessed; therefore the role of hyperactivation in release of equine sperm from OEC in vitro remains to be investigated.

Although no direct relationship between elevated $[Ca^{2+}]_i$ in spermatozoa and release from OEC could be established in the present study, it can be speculated that a subpopulation of spermatozoa attached to OEC undergoes changes in membrane properties associated with capacitation and subsequently are released from OEC, allowing further in-

flux of calcium. Conversely, a spontaneous increase in $[Ca^{2+}]_i$ could occur in a subpopulation of spermatozoa bound to OEC, leading to the subsequent release of spermatozoa from OEC. About 50% of spermatozoa released from OEC had intact plasma membranes and acrosomes, with only about half of those exhibiting progressive motility. This is consistent with earlier observations [44]. In the present study, $[Ca^{2+}]_i$ was determined only in motile spermatozoa, which presumably represent a functionally competent subpopulation of spermatozoa.

Interaction of equine spermatozoa with OEC serves to maintain basal $[Ca^{2+}]_i$ in spermatozoa [19]. However, it was not known whether soluble products from OEC present in the culture medium or direct cell contact between spermatozoa and OEC mediated the observed effect. A role for OEC secretory products has been proposed because OEC-conditioned medium can maintain motility and viability of bovine and human spermatozoa [21, 45]. In addition, viability-promoting effects have been reported that were dependent on the region of the oviduct from which the secretions were collected [24]. Bovine oviduct-specific glycoproteins secreted during the follicular phase of the cycle associated with spermatozoa and maintained sperm motility and viability [25]. In the present study, only a small reduction in sperm $[Ca^{2+}]_i$ was observed with OEC-conditioned medium (experiment 2). This is in agreement with previous studies reporting that OEC-conditioned medium promoted capacitation rather than delaying it [24, 42, 46]. Thus, it appears that modulation of $[Ca^{2+}]_i$ in equine spermatozoa attached to OEC is not mediated by soluble products present in OEC-conditioned media.

To test the hypothesis that the maintenance of low $[Ca^{2+}]_i$ in spermatozoa that occurs during incubation with OEC is attributable to direct membrane contact between OEC and spermatozoa, we incubated spermatozoa in a suspension of isolated AMV derived from isthmic oviductal epithelium [26]. This approach provides the advantage that direct membrane effects can be studied independently from other effects introduced by intact oviductal epithelial cells in the culture system. With use of differential precipitation, an apical plasma membrane fraction was obtained from isthmic oviductal epithelium. A 28-fold increase in the specific activity of the apical plasma membrane marker enzyme γ-glutamyl transpeptidase indicates that the AMV fraction used in this study was highly enriched in apical membranes. This is comparable to the enrichment values reported for AMV generated from rabbit oviductal epithelium [26]. Similar to rabbit oviductal AMV [26], AMV from equine isthmic epithelium attached to the rostral plasma membrane of the sperm head, which is the area of the sperm plasma membrane involved in binding of spermatozoa to OEC [4, 7, 8, 10]. Taken together, these observations support the use of oviductal epithelial AMV to study the direct effects of membrane contact between sperm and OEC.

Unlike that in the rabbit, binding of equine AMV to the periacrosomal area of the sperm membrane did not result in significant agglutination of spermatozoa during incubation with AMV. As previously described [26], incubation with AMV maintained sperm viability for significantly longer periods of time than did incubation in culture medium alone (experiment 3a). Progressive motility of spermatozoa incubated with AMV was not evaluated in this experiment. In the present study, kidney AMV were found to have a moderately beneficial effect on sperm viability. This is in contrast to the report by Smith and Nothnick [26], who observed no significant effect of kidney AMV on rabbit sperm viability. Incubation of equine spermatozoa with isthmic AMV delayed capacitation (experiment 3a) and maintained low $[Ca^{2+}]_i$ (experiment 3b) in these spermatozoa. These effects appeared to be specific for oviductal tissue in the present study. These results indicate that direct membrane contact between equine spermatozoa and the apical plasma membrane of the OEC is sufficient to promote sperm viability, delay capacitation, and maintain low sperm $[Ca^{2+}]_i$.

To ensure that the effects we observed were attributable to the direct contact between spermatozoa and AMV and not to the mere presence of AMV in the incubation medium, spermatozoa and AMV were incubated in the presence of antigen binding fragments (Fab) directed against the periacrosomal sperm plasma membrane. The presence of Fab in the incubation medium largely abolished the effects of AMV on the prolongation of sperm viability, delay of capacitation, and modulation of sperm $[Ca^{2+}]_i$ (experiment 4). The polyclonal antibody used in the present experiments previously has been shown to reduce equine sperm binding to OEC monolayers and explants by about 70% at the same Fab concentration [28]. In the present study, incubation of spermatozoa and AMV in the presence of Fab resulted in a 60% reduction in the number of spermatozoa with AMV bound to their periacrosomal plasma membrane compared to spermatozoa incubated with AMV only. Antigen binding fragments alone did not affect sperm function, and Fab generated from nonimmunized rabbit serum did not interfere with the effects of AMV on spermatozoa. Taken together, these results confirmed that direct contact between the sperm plasma membrane and the apical plasma membrane of OEC appears to mediate the effects on sperm viability, capacitation, and $[Ca^{2+}]_i$.

The mechanism by which membrane contact between spermatozoa and OEC modulates $[Ca^{2+}]_i$ and prolongs sperm viability is unknown. Intimate association between sperm and OEC membranes could serve to stabilize the sperm plasma membrane and prevent membrane damage [47]. This could explain the positive effect of kidney AMV on sperm viability. Alternatively, recognition of glycoproteins expressed on the surface of OEC by a lectin-like receptor on the sperm surface has been postulated in different species, including the horse [48–50]. This putative receptor-ligand interaction could trigger events within the spermatozoa that act to prevent an increase of $[Ca^{2+}]_i$. Millimolar concentrations of calcium ions have been detected in bovine isthmic oviductal fluid [51]. In order to maintain low $[Ca^{2+}]_i$ in the presence of high concentrations of extracellular calcium, spermatozoa would need to prevent calcium influx and/or actively extrude calcium from their cytoplasm. Stabilization of the sperm plasma membrane could aid in preventing passive influx of calcium ions due to increased membrane permeability. Spermatozoa of several species possess calmodulin-regulated $Ca^{2+}$ ATPase (cattle [52, 53]; guinea pig [54]; mouse [55]; pig [56]; human [57]) and $Na^+/Ca^{2+}$ antiporters [58] in their plasma membranes. Recently it has been reported that a decapacitation factor from seminal plasma [59] maintains low $[Ca^{2+}]_i$ and prevents capacitation in mouse spermatozoa by activation of a calmodulin-sensitive $Ca^{2+}$ ATPase [60]. Calmodulin is lost from bovine spermatozoa during capacitation [61], which could decrease the activity of $Ca^{2+}$ ATPase, allowing for a capacitation-related increase in $[Ca^{2+}]_i$. Furthermore, the bovine seminal plasma protein caltrin [62], which associates with spermatozoa at ejaculation, has been shown to

initially inhibit the $Na^+/Ca^{2+}$ exchanger [58], thereby preventing calcium influx into bovine spermatozoa in which $Ca^{2+}$ ATPase might not be important [58, 63]. It remains to be seen whether any of these mechanisms could be involved in modulation of $[Ca^{2+}]_i$ in equine spermatozoa attached to OEC. It is possible that binding of a putative sperm receptor to its respective ligand on the epithelium in the oviductal isthmus could stimulate calcium extrusion from the sperm cell, thereby keeping $[Ca^{2+}]_i$ low in the presence of high extracellular $[Ca^{2+}]$ in the preovulatory isthmic fluid. Maintenance of low $[Ca^{2+}]_i$ and delay of capacitation could extend the functional life span of spermatozoa stored in the oviductal reservoir. Membrane changes associated with capacitation might occur in a subpopulation of cells at any given time [11] or in association with ovulation [64], causing release of spermatozoa from the OEC and allowing a concomitant increase in $[Ca^{2+}]_i$.

In conclusion, maintenance of low $[Ca^{2+}]_i$, delay of capacitation, and prolonged viability in equine spermatozoa attached to OEC in vitro appears to be mediated by membrane contact between spermatozoa and OEC. The release of spermatozoa from OEC is associated with an increase in $[Ca^{2+}]_i$ in spermatozoa. Modulation of capacitation in spermatozoa stored in the isthmic sperm reservoir would ensure the availability of functionally competent spermatozoa at the time of fertilization.

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