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# Effect of Bovine Oviductal Estrus-Associated Protein on the Ability of Sperm to Capacitate and Fertilize Oocytes

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**ABSTRACT**: At estrus, an oviduct-specific, estrus-associated glycoprotein (EAP) of molecular weight 85–95,000, is secreted by the oviductal epithelium and found in cannula-derived bovine oviductal fluid (ODF). The objectives of these studies were to determine if bovine sperm were capacitated by EAP *in vitro*, whether this effect differed for EAP derived from ODF versus conditioned medium from oviduct ampullar explants, and to determine if sperm capacitated *in vitro* with EAP-fertilized bovine eggs. Sperm were incubated for up to 6 hours with partially purified EAP derived from ODF and assessed for capacitation by their ability to undergo the acrosome reaction following exposure to lysophosphatidylcholine. At 4 hours of incubation, the number of capacitated sperm in treatments containing 50% ODF or EAP plus bovine serum albumin (BSA) was similar, and it was significantly greater than the number of capacitated sperm in treatments containing antibodies to EAP. Using purified EAP derived from ampullar explant-conditioned medium, twice the number of sperm were capacitated after 4 hours with EAP from conditioned medium or with ODF than with treatments containing anti-EAP. The fertilizing ability of sperm incubated with EAP was significantly greater than that for sperm incubated without EAP or with anti-EAP. We conclude that bovine EAP, derived from both ODF and *in vitro* cultures, promotes sperm fertilizing ability.

Key words: Oviduct culture, protein secretion, oviductal fluid.

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The oviduct provides the environment for sperm capacitation, fertilization, and early embryo cleavage in mammals. Prior to fertilization, inseminated sperm acquire the ability to undergo the acrosome reaction (AR) and fertilize ova by a process termed capacitation (Austin, 1951; Chang, 1951). The oviductal isthmus is believed to be a primary site for sperm capacitation (Harper, 1973; Hunter and Hall, 1973; Hunter and Nichol, 1988). Capacitation is characterized by changes in sperm membrane lipid and protein constituents that occur within hours of deposition into the female reproductive tract and that confer upon sperm the ability to acrosome react and penetrate an egg. During capacitation, changes in sperm membrane constituents occur as a consequence of exposure to the oviductal environment. The components in oviductal fluid (ODF) that are present at the time of estrus would be expected to influence the process of sperm capacitation.

ODF contains serum transudate and substances secreted by the oviductal epithelium. Oviductal cell cultures from rabbits synthesize more proteins when primed with estrogen (Oliphant et al, 1984). Oviductal tissue cultures from baboons synthesize different proteins during different estrous cycle stages, and ampullar secretion of three glycoproteins is increased by estrogen administration (Verhage and Fazleabas, 1988).

Oviduct-specific proteins, secreted at estrus, have been reported for rabbits (Urzua et al, 1970), monkeys (Mastroianni et al, 1970), sheep (Sutton et al, 1984; Murray, 1993), mice (Kapur and Johnson, 1985), rats (Wang and Brooks, 1986), hamsters (Leveille et al, 1987; Robitaille et al, 1988), pigs (Brown and Cheng, 1986), baboons (Fazleabas and Verhage, 1986), women (Lippes et al, 1981; Verhage et al, 1988), and cows (Malayer et al, 1988; Gerena and Killian, 1990). Some oviductal proteins bind to zonae pellucidae of hamsters (Leveille et al, 1987; Kan et al, 1988; Oikawa et al, 1988) and cows (Wegner and Killian, 1991). Oviductal proteins have been detected on the zonae pellucidae and in the perivitelline space of early embryos in mice (Kapur and Johnson, 1986, 1988), sheep (Gandolfi et al, 1989), and baboons (Boice et al, 1990a). Oviductal proteins also bind to sperm of sheep (Voglmayr and Sawyer, 1986), humans (Lippes and Wagh, 1989), and bulls (McNutt et al, 1992).

An oviduct-specific, estrus-associated glycoprotein (EAP) with an approximate molecular weight of 95,000 is secreted by the oviductal epithelium in cows (Boice et al, 1990b; Wegner and Killian, 1992). The concentration

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of EAP is highest at estrus and the following 3 days, but it then decreases significantly during the luteal phase (Boice et al, 1990b; Gerena and Killian, 1990). Bovine EAP binds to zonae pellucidae (Wegner and Killian, 1991), and five bovine ODF proteins, including a 90-kDa EAP, bind to bull sperm (McNutt et al, 1992). Although the interaction between the oviductal epithelium, ODF constituents, and the gametes is suspected to promote gamete fertilizing ability, the role of EAP or other oviductal proteins in modulating gamete function or fertilization has not been defined.

The purpose of this study was to assess whether bovine EAP in ODF and conditioned media from oviductal explant cultures was biologically active. The specific objectives were to determine whether EAP capacitated sperm, whether the biological activity of EAP from both *in vitro* and *in vivo* sources was similar, and to determine whether bovine sperm capacitated *in vitro* with EAP were able to fertilize intact bovine eggs.

## Materials and Methods

### ODF Collection and Isolation of EAP

Daily samples of ODF were collected via indwelling oviductal cannulae (Kavanaugh and Killian, 1988). Based on serum progesterone concentration, non-luteal ODF (progesterone < 1.5 ng/ml), which included the days of estrus and ovulation, was pooled from three cows. The protein concentration of ODF was 10–12 mg/ml, with EAP comprising 7–10% of that total. Using 30–35 ml of ODF, EAP was enriched, from an initial concentration of 7–10% to approximately 40%, by use of ammonium sulfate precipitation to 70% saturation. This was followed by sequential reverse ammonium sulfate extractions from 65 to 35% saturation at 5% increments. The pellet was added to 10 ml of ammonium sulfate solution, stirred on ice, and then centrifuged at 12,000 × *g* for 20 minutes for each successive extraction. The protein was further purified by anion exchange column chromatography, using ecteola cellulose (Sigma Chemical Company, St. Louis, Missouri), to yield about 80% EAP and 20% albumin, based on densitometry of one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples were then assayed for total protein (Bradford, 1976) and stored at −20°C until used.

### Electrophoresis and Western Blotting

One-dimensional SDS-PAGE was used to separate ODF proteins and assess protein purity (Laemmli, 1970). Resolving gels were cast using 10% acrylamide, and stacking gels contained 4% acrylamide. To visualize protein bands after electrophoresis, gels were stained for 2 hours with an aqueous solution of Coomassie R-250 (0.125%) in 10% acetic acid with 40% methanol. Gels were destained with an aqueous solution of 10% acetic acid with 25% methanol.

Western blots were performed by transfer of proteins from SDS-PAGE gels to nitrocellulose paper (NCP) using a Milliblot semi-dry transfer system (Millipore Corporation, Bedford, Massachusetts) at 10–15 V (about 150 mA) for 30 minutes. Blots were blocked for 30 minutes with phosphate-buffered saline (PBS) containing 0.5% Tween-20 and 5% normal goat serum (NGS). All additional washes and incubations were performed in PBS plus 0.5% Tween-20 with 1% NGS, except for incubation with chromagen, which was performed in 0.01 M sodium citrate, pH 5.2. Western blots were probed with antibody to EAP (polyclonal IgG purified from the serum of New Zealand white rabbits immunized with EAP), followed by peroxidase-conjugated secondary antibody and diaminobenzidene as chromagen (Towbin et al, 1979).

### Characterization of Polyclonal Anti-EAP

Polyclonal antibody to purified bovine EAP (anti-EAP) was prepared by inoculation of New Zealand white rabbits. Bands containing EAP were excised from SDS-PAGE gels of ODF and ground into fine pieces using a small pestle. Approximately 100 μg of protein was added to 100 μl sterile saline. This was combined with 100 μl of Freund's complete adjuvant. After thorough mixing, antigen was injected subcutaneously (s.c.) at 10–12 sites in the scapular region of the rabbit. Two weeks after the initial injection, 100 μg EAP in 100 μl sterile saline was mixed with 100 μl of Freund's incomplete adjuvant and administered by s.c. injection. Antigen mixed with Freund's incomplete adjuvant was administered by s.c. injection as described above, 10 days after the previous s.c. injections and every 4–6 weeks thereafter to boost antibody production.

Antiserum was collected from a marginal ear vein and the IgG fraction isolated by ammonium sulfate precipitation and diethylaminoethyl (DEAE) cellulose column chromatography (Dunbar and Schwoebel, 1990). Total protein concentration was assayed, and aliquots were frozen.

Specificity of anti-EAP was determined by Western blotting of whole ODF, using anti-EAP as primary antibody. No cross-reactivity was observed with albumin or other ODF proteins.

### Sperm Incubation with In Vivo-Derived EAP

Semen was collected from three dairy bulls of known reproductive history using an artificial vagina. Sperm were recovered from seminal plasma by centrifugation (1,000 × *g* for 20 minutes) and washed twice in protein-free modified Tyrode's medium (MTM). To evaluate the effect of EAP on capacitation, $5 \times 10^7$ sperm/ml were incubated in MTM containing: 1) 50% ODF (5 mg protein/ml); 2) 20 mM Tris, pH 8.2; 3) 10% EAP (0.5 mg/ml); and 4) 10% EAP (0.5 mg/ml) plus 35% bovine serum albumin (BSA) (1.9 mg/ml) plus anti-EAP (1 mg/ml). Based on densitometry, we estimated the relative amounts of albumin and EAP found in cannula-derived estrous ODF to be 35% and 10%, respectively, of the total protein. All treatments were incubated for 6 hours at 39°C with 5% $CO_2$ in air to capacitate the sperm. Sperm incubated in MTM plus 20 mM Tris, pH 8.2, served as a negative control, and sperm incubated in MTM plus 50% ODF served as a positive control.

Lysophosphatidylcholine (LPC) was used to induce the acrosome reaction (AR) in capacitated sperm without affecting the population of non-capacitated sperm (Parrish et al, 1988). A 100-μl aliquot of sperm was incubated with 12.5 μl BSA (50 mg/

ml) and 16.2 μl (60 μg/ml) LPC for 10 minutes at 39°C. A 5-μl aliquot was then mixed with 5 μl of Fast Green FCF-Eosin B stain (Aalseth and Saacke, 1986) on the end of a glass slide, drawn into a smear, and dried immediately. Smears were coverslipped and evaluated by differential interference contrast microscopy. One hundred sperm were evaluated per slide and classified as acrosome-reacted live (ARL), acrosome-reacted dead (ARD), acrosome-intact live (IL) or acrosome-intact dead (ID). Because sperm must be live, motile, and capacitated to acrosome react and penetrate an egg, only ARL sperm were considered to be functionally capacitated.

Studies involving incubation of sperm with *in vivo*-derived EAP were performed following a repeated measures design. Four time points (0, 2, 4, and 6 hours) were observed for sperm from three different fertile bulls. The experiment was replicated on three different days. Significance of means for each bull from each treatment at each time point was determined by analysis of variance. Differences between bulls and treatments were determined by least square means and Bonferroni means comparisons (SAS Institute Incorporated, 1985). The significance level was $P < 0.05$ for all tests.

### Determination of LPC Concentration for AR Induction

A titration of LPC was performed to determine the lowest concentration of LPC needed to induce the AR without causing excessive ARD or ID sperm. A constant number of sperm ($5 \times 10^7$), pooled from three bulls, was capacitated *in vitro* by incubating in MTM plus 50% ODF (5 mg protein/ml) at 39°C for 4 hours. A 100-μl aliquot of sperm from each bull was incubated for 10 minutes with BSA (50 mg/ml) alone or BSA plus 15, 30, 60, or 120 μg/ml LPC to induce the AR. Smears were prepared and evaluated for percentages of ARL, ARD, IL, and ID sperm as described above. This experiment was repeated on four different days.

### Preparation of Conditioned Medium (CM) Containing EAP

Ampullar oviducts were obtained from three cows slaughtered on the day of behavioral estrus. Ampullae from each cow were cut into pieces approximately 1 mm square. Tissue pieces from each cow were placed in individual culture dishes containing 5 ml of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) supplemented with D-glucose and L-glutamine (cat. no. D5648; Sigma Chemical Company, St. Louis, Missouri) plus RPMI-1640 medium with L-glutamine (cat. no. R7634; Sigma). CM were collected after 48 hours of culture of the explant tissues. The CM from three different cows, designated CM1, CM2, and CM3, were dialyzed, lyophilized, then individually assayed for total protein (Bradford, 1976), and frozen until used. EAP was purified by electroeluting the excised 90-kDa band from 10% SDS-PAGE gels of pooled CM, and it was frozen until used. Electroeluted EAP was considered pure if it migrated as a single band on SDS-PAGE gels.

### In Vitro Sperm Incubation with CM Containing EAP

Ejaculated sperm from fertile dairy bulls were prepared as described above. After washing, $1.67 \times 10^7$ sperm/ml from each bull were combined, and $5 \times 10^7$ sperm/ml were added to MTM containing: 1) 50% ODF (5 mg protein/ml), 2) anti-EAP (1 mg/ml), 3) CM1 (1 mg/ml), 4) CM2 (1 mg/ml), 5) CM3 (1 mg/ml), and 6) pooled CM (1 mg/ml) plus anti-EAP (1 mg/ml). Pooled CM contained equal amounts of protein from each of the three explant cultures. Treatments were incubated for 4 hours at 39°C with 5% $CO_2$ in air to capacitate the sperm. Sperm incubated in MTM plus anti-EAP served as a negative control and sperm incubated in MTM plus 50% ODF served as a positive control.

To induce the AR, a 100-μl aliquot of sperm suspension was incubated with 12.5 μl of BSA solution (50 mg/ml), 8.1 μl MTM, and 8.1 μl LPC (30 μg/ml) for 10 minutes at 39°C. Stained smears were made and evaluated as described above.

Data from sperm incubations with conditioned medium containing EAP were analyzed in a $3 \times 6$ factorial. Sperm from three different bulls were pooled, and the experiment was replicated three times on three different days. Analysis of variance was performed using means from each treatment. Differences between treatments were determined by least square means and Bonferroni means comparisons (SAS Institute Incorporated, 1985). The significance level was $P < 0.05$ for all tests.

### In Vitro Sperm Capacitation Conditions to Assess Sperm Fertilizing Ability

To evaluate the role of bovine EAP in fertilization, a preliminary experiment was conducted to compare the fertilizing ability of sperm capacitated by *in vitro* incubation with EAP to sperm incubated with EAP plus anti-EAP. Ejaculates were collected from three fertile bulls. After sperm were washed twice in protein-free MTM, $5 \times 10^7$ sperm/ml were added to MTM containing: 1) 50% ODF (5 mg protein/ml), 2) anti-EAP (1 mg/ml), 3) EAP (1 mg/ml), and 4) EAP (1 mg/ml) plus anti-EAP (1 mg/ml). Sperm were capacitated by incubating 4 hours at 39°C with 5% $CO_2$ (Parrish et al, 1988). A 100-μl aliquot from each treatment was incubated with BSA (62.5 mg/ml) and LPC (30 μg/ml) for 10 minutes at 39°C to induce the AR. Sperm smears were prepared and stained with Fast Green FCF-Eosin B (Aalseth and Saacke, 1986), and dried immediately. Coverslips were bonded to slides using xylene:Permount (3:1), and smears were evaluated using differential interference microscopy. A minimum of 100 sperm per slide were classified as either ARL, ARD, IL, or ID to assess capacitation status. Only ARL sperm were considered to be functionally capacitated.

Based on the results of this experiment, a more in depth study was undertaken, and experimental conditions were modified to maximize differences between positive and negative controls. In subsequent experiments, semen was collected from three fertile bulls. Each bull was used twice as a donor to replicate the experiment on two different days. Twice-washed sperm ($5 \times 10^7$/ml) were added to 1) MTM alone and MTM containing 2) 50% ODF (5 mg protein/ml), 3) EAP (1 mg/ml), 4) anti-EAP (1 mg/ml), and 5) EAP (1 mg/ml) plus anti-EAP (1 mg/ml). Sperm were capacitated by incubating 4 hours at 39°C in 5% $CO_2$ in air. Sperm incubated in MTM alone served as a negative control, and sperm incubated in MTM plus 50% ODF served as a positive control. The AR was induced and capacitation status evaluated on stained sperm smears as described above.

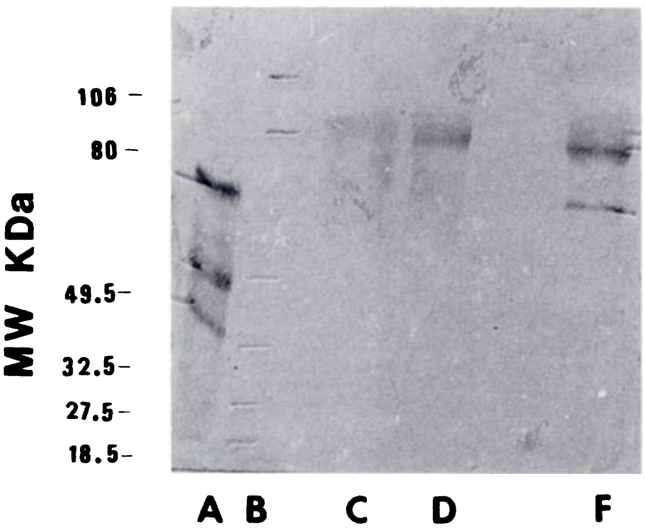



**FIG. 1.** Western blot of 20-μg protein of BSA (lane A), molecular weight standards (lane B), cannula-derived non-luteal ODF (lane C) and CM from cultured oviductal explants (lanes D and F) separated on one-dimensional SDS-PAGE. To establish protein purity, the blot was probed twice. The blot was first probed using polyclonal IgG to BSA as primary antibody, then probed with polyclonal IgG to bovine EAP. Lane F shows an impure sample containing both EAP and BSA.

### *In Vitro Fertilization (IVF)*

Ovaries were collected from slaughtered cows, and procedures previously reported by our laboratory for IVF of bovine oocytes were followed (McNutt and Killian, 1991). Briefly, small ovarian follicles (<5 mm) were aspirated and allowed to sediment at 39°C for 30 minutes. Oocytes with intact cumulus cells were washed and matured for 22–24 hours at 39°C with 5% $CO_2$ in air in TCM-199 supplemented with 2 mM $NaHCO_3$, 10% heat-treated fetal calf serum (GIBCO, Grand Island, New York), 0.25 mM pyruvate, 50 μg/ml gentamicin (cat. no. G1246; Sigma), luteinizing hormone (5 μg/ml, USDA-bLH-B-5; USDA Animal Hormone Program), follicle-stimulating hormone (0.5 μg/ml, NIH-P-1-Porcine; National Institute of Arthritis, Metabolism and Digestive Diseases), and β-estradiol (1 μg/ml, cat. no. E2758; Sigma) using the method of Sirard et al (1988). Mature eggs were recovered, and the remaining cumulus cells were dislodged by vortexing for 2 minutes. Eggs were washed and placed under paraffin oil in microdrops of 100 μl fertilization medium (Bavister et al, 1983).

In the preliminary experiment, oocytes with intact cumulus cells were washed and matured for 22–24 hours. Each microdrop containing 15 eggs was inseminated with $10^3$ sperm per drop ($10^4$ sperm/ml) and incubated at 39°C. After 22–24 hours of co-incubation, eggs were removed from each microdrop, washed, placed on a glass slide, and coverslipped. Slides were dehydrated in ethanol:acetic acid (3:1) for at least 24 hours, then stained with 1% aceto-orcein. Eggs were evaluated by light microscopy and considered fertilized if two polar bodies and/or two pronuclei were observed.

In subsequent experiments, washed mature eggs, in 100-μl microdrops (15 eggs per drop), were inseminated with $5 \times 10^4$ sperm per drop ($5 \times 10^5$ sperm/ml) and co-incubated for 16–18 hours at 39°C in 5% $CO_2$ in air.

A study using rabbit IgG prepared against a non-bovine antigen was performed to assess the effect of non-specific IgG on sperm capacitation. The IgG used in this study was prepared as stated above and was specific for bacterial histidase (anti-hist). Sperm ($5 \times 10^7$) were pooled from three bulls and capacitated *in vitro* by incubating in 1) MTM alone or in MTM containing 2) $EAP_{CM}$ (1 mg/ml), 3 anti-hist (1 mg/ml), 4) $EAP_{CM}$ (1 mg/ml) plus anti-hist (1 mg/ml), 5) $EAP_{CM}$ (1 mg/ml) plus anti-EAP (1 mg/ml), 6) $EAP_{ODF}$ (1 mg/ml), and 7) $EAP_{ODF}$ (1 mg/ml) plus anti-EAP (1 mg/ml) at 39°C for 4 hours. Sperm capacitation and the ability to fertilize oocytes *in vitro* were evaluated as stated above.

In the preliminary experiment involving IVF, sperm from each of three different bulls were incubated in each of the four treatments. These were statistically evaluated for capacitation using a 3 × 4 factorial. Sperm fertilizing ability in each of the four treatments was assessed in three microdrops, containing 12–15 mature eggs per drop. Microdrops served as replicates for each treatment. The range of eggs evaluated for each treatment and bull was 36 to 45, and the total number of eggs evaluated was 449.

In subsequent experiments involving IVF, sperm from each of the three bulls were incubated in each of five treatments. For this 3 × 5 factorial, each bull was replicated on two different days. Fertilizing ability of sperm from each of the five treatments was assessed by inseminating three microdrops, each containing 15 mature eggs. Analysis was performed as a 3 × 5 factorial, where microdrops served as replicates for each treatment. The number of eggs evaluated for each of the five treatments was 321, 329, 307, 334, and 331, for each treatment respectively, and totaled 1,622. Analysis of variance (SAS Institute Incorporated, 1985) was used to assess sperm capacitation prior to insemination. Using weighted means, both least square and Bonferroni means comparisons were used to assess IVF fertilization data. Significance level for all analyses was $P < 0.05$.

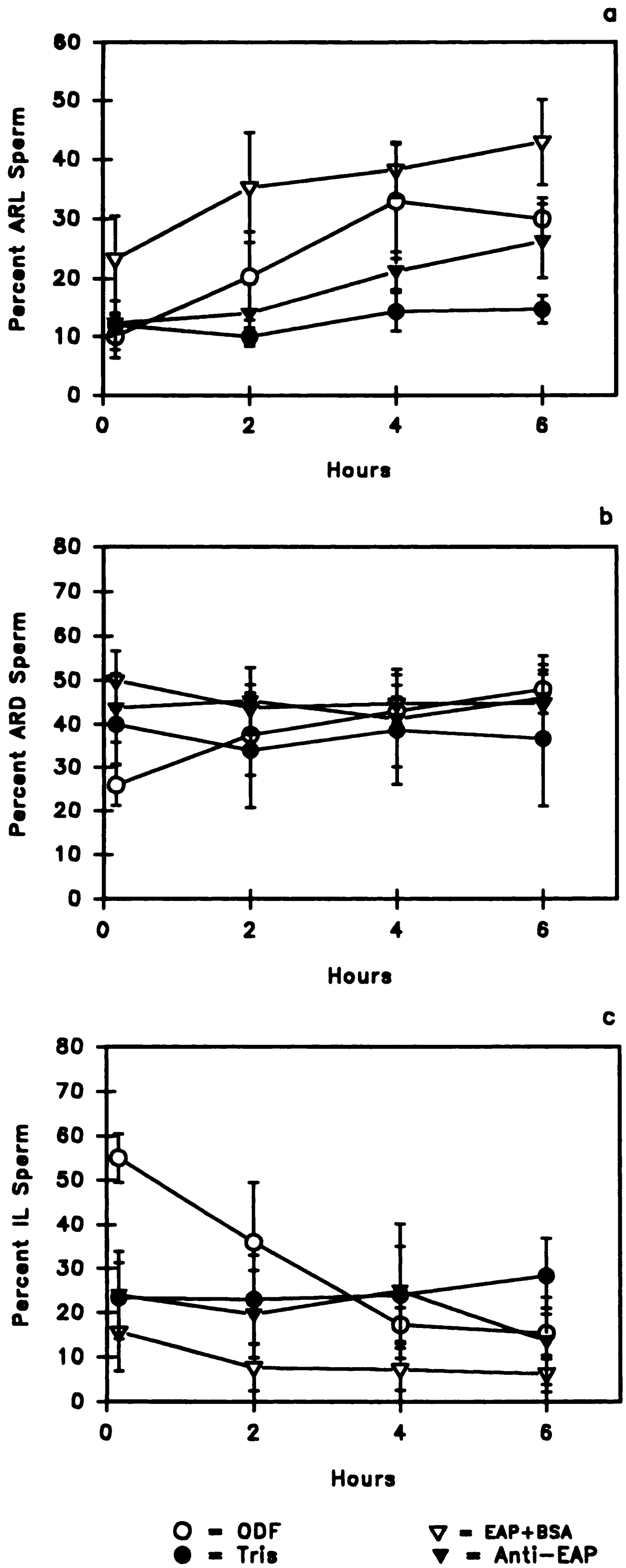
## Results

### *Electrophoresis and Western Blotting*

Purity of EAP isolated from ODF or CM from oviductal explants was assessed by SDS-PAGE and Western blotting. Although EAP in medium from cultured explants was immunologically similar to EAP from ODF on Western blots (Fig. 1), it had a slightly lower molecular weight than cannula-derived EAP, presumably due to less glycosylation. Because the amount of glycosylation often affects bioactivity and biological half life, it was important to determine if EAP from *in vitro* cultures was functionally similar to EAP from ODF.

### *Studies with In Vivo-Derived EAP*

There were no differences among bulls in the ability of sperm to become capacitated in response to a treatment. Treatment significantly affected ($P < 0.05$) ARL and IL,




but not ARD and ID. Percent ARL sperm (mean ± SE) increased over time in treatments containing EAP (50% ODF, 10% EAP, or 10% EAP plus 35% BSA), indicating that sperm became capacitated. Percent ARL sperm in the treatment without EAP (20 mM Tris) did not increase during the 6-hour incubation, and the percent ARL sperm in 10% EAP plus 35% BSA plus anti-EAP was suppressed (Fig. 2a), indicating minimal capacitation of sperm.

Percent ARD sperm was equivalent for all treatments by 2 hours, indicating that all treatments were equally affected by LPC and incubation conditions (Fig. 2b). Percent IL sperm decreased over time in treatments containing EAP (50% ODF, 10% EAP, or 10% EAP plus 35% BSA), corresponding to the increase in percent ARL. No decrease in IL sperm occurred in the treatment without EAP (20 mM Tris), and a slight decrease occurred at 6 hours in the treatment containing 10% EAP plus 35% BSA plus anti-EAP, corresponding to the slight rise in ARL at 6 hours (Fig. 2c).

The percentages of ARD sperm did not differ among treatments or incubation times, indicating that incubation conditions were not detrimental to the sperm. However, because the average percentage of ARD sperm for all treatments was 30–40%, a titration of LPC was performed to determine the lowest concentration of LPC needed to induce the AR without causing excessive ARD or ID sperm. No differences were found among bulls or treatments in ARL and ID sperm. Significant differences were found among treatments in ARD (Fig. 3a) and IL (Fig. 3b) sperm.

An increase in the percentage of ARD sperm was correlated to an increased concentration of LPC. After 4 hours of incubation, 30 μg/ml of LPC induced the highest percentage of AR (39.8% ARL sperm), with 20.4% ARD sperm and 39.8% IL sperm. The percentage of ARD sperm at 30 μg/ml LPC (20.4%) was significantly less than that for 60 μg/ml (35.3%) and 120 μg/ml (54.1%), but not different from that for 15 μg/ml (19.1%) or BSA alone (15.9%).

### *Studies with CM containing EAP*

Treatment had a significant effect on ARL and IL, but not on ARD and ID. Percent ARL sperm in treatments containing EAP without antibody (50% ODF, CM1, CM2, or CM3) was at least twice that of treatments either with-

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**FIG. 2.** Sperm were capacitated in MTM supplemented with non-luteal ODF (5 mg/ml), 20 mM Tris, EAP (0.5 mg/ml) plus BSA (1.9 mg/ml), or EAP (0.5 mg/ml) plus BSA (1.9 mg/ml) plus anti-EAP (1 mg/ml). Data are expressed as the percent of ARL sperm, representing functionally capacitated sperm (a); percent ARD sperm, representing cells which lost membrane stability (b); or percent IL, representing live uncapacitated sperm (c).

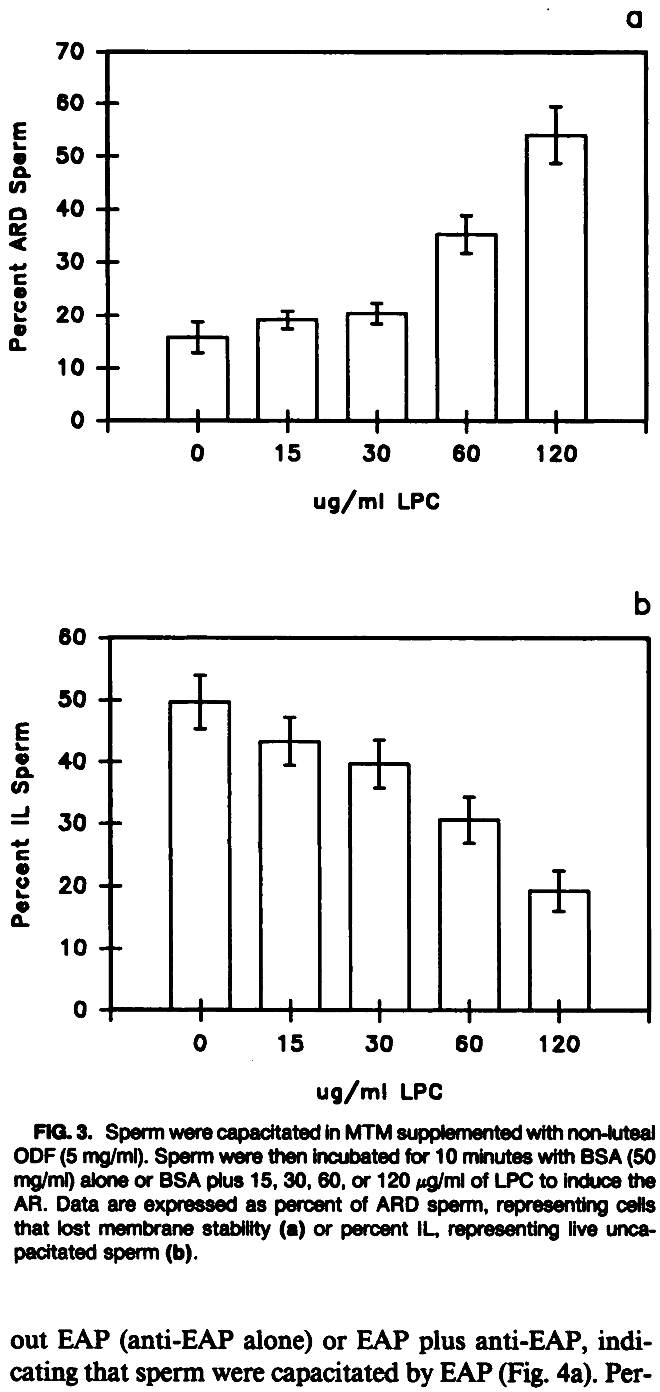


FIG. 3. Sperm were capacitated in MTM supplemented with non-luteal ODF (5 mg/ml). Sperm were then incubated for 10 minutes with BSA (50 mg/ml) alone or BSA plus 15, 30, 60, or 120 μg/ml of LPC to induce the AR. Data are expressed as percent of ARD sperm, representing cells that lost membrane stability (a) or percent IL, representing live uncapacitated sperm (b).

out EAP (anti-EAP alone) or EAP plus anti-EAP, indicating that sperm were capacitated by EAP (Fig. 4a). Percent ARD was lowest for treatments containing anti-EAP and CM plus anti-EAP, though not significantly. Therefore, sperm in all treatment groups were equally affected by LPC and incubation conditions (Fig. 4b). Significantly more IL sperm were present in treatments with anti-EAP and with CM plus anti-EAP, indicating that sperm were alive but not capacitated (Fig. 4c).

### *Effect of Bovine EAP on Sperm Fertilizing Ability*

In the preliminary experiment no differences among bulls were detected by analysis of variance. Treatment means (±SE) for ARL sperm (Fig. 5) were: 50% ODF (33.3 ± 2.7), anti-EAP (14.3 ± 2.2), EAP (32.7 ± 3.4), and EAP plus anti-EAP (18.0 ± 0.0). Percent ARL for sperm incubated in treatments containing EAP (ODF and EAP) were at least twice that ($P < 0.05$) of sperm from treatments containing anti-EAP (anti-EAP and EAP plus anti-EAP). No differences were observed among treatments in ARD, IL, and ID sperm.

Treatment means ± SE for percentages of eggs fertilized (Fig. 6) were: 50% ODF (57.4 ± 3.2), anti-EAP (39.1 ± 2.1), EAP (55.8 ± 4.1), and EAP plus anti-EAP (41.2 ± 4.5). Percentage of eggs fertilized was higher for sperm incubated in treatments containing EAP (ODF and EAP).

Based on the results of the preliminary study, experimental conditions were modified to obtain more information about the effect of EAP on sperm fertilizing ability. The number of sperm used to inseminate the microdrops containing eggs was titrated to determine the number of sperm needed to maximize differences between sperm incubated in MTM alone (negative control) and sperm incubated in MTM plus 50% ODF (positive control). Results of this trial indicated that the percentage of eggs fertilized using $\leq 2 \times 10^4$ sperm was about 20% for negative controls and about 40% for positive controls. The percentage of eggs fertilized by $4–8 \times 10^4$ sperm was <15% for negative controls and >50% for positive controls. Therefore, the number of sperm used for insemination in subsequent studies was increased to $5 \times 10^4$.

In subsequent studies, no differences were detected among bulls using analysis of variance. Treatment means (± SE) for ARL sperm (Fig. 7) were: MTM (14.5 ± 1.7), 50% ODF (25.6 ± 3.2), EAP (30.2 ± 3.0), anti-EAP (16.0 ± 2.1), and EAP plus anti-EAP (10.9 ± 1.2). Percent ARL for sperm incubated in treatments containing EAP without anti-EAP was higher ($P < 0.05$) than for sperm incubated without EAP or with anti-EAP. No differences were detected among treatments in ARD, IL, and ID sperm.

Treatment means (± SE) for percent eggs fertilized (Fig. 8) were: MTM (10.7 ± 2.6), 50% ODF (26.5 ± 3.4), EAP (27.1 ± 3.3), anti-EAP (12.8 ± 2.9), and EAP plus anti-EAP (10.7 ± 2.4). The percentage of eggs fertilized by sperm incubated in EAP without anti-EAP was greater than twice that of sperm incubated without EAP, or with anti-EAP ($P < 0.05$). Evaluation of sperm capacitation status after incubation of sperm for 18–20 hours without eggs, in microdrops of fertilization medium, revealed an increase in ARL sperm in all treatments, with a higher percentage of ARL sperm from treatments capacitated with EAP. Treatment means for ARL sperm at 18–20 hours were: 32, 66, 80, 32, and 28 for MTM, 50% ODF, EAP, anti-EAP, and EAP plus anti-EAP, respectively. Few ARD and near 0% ID sperm were observed in all treatment groups.

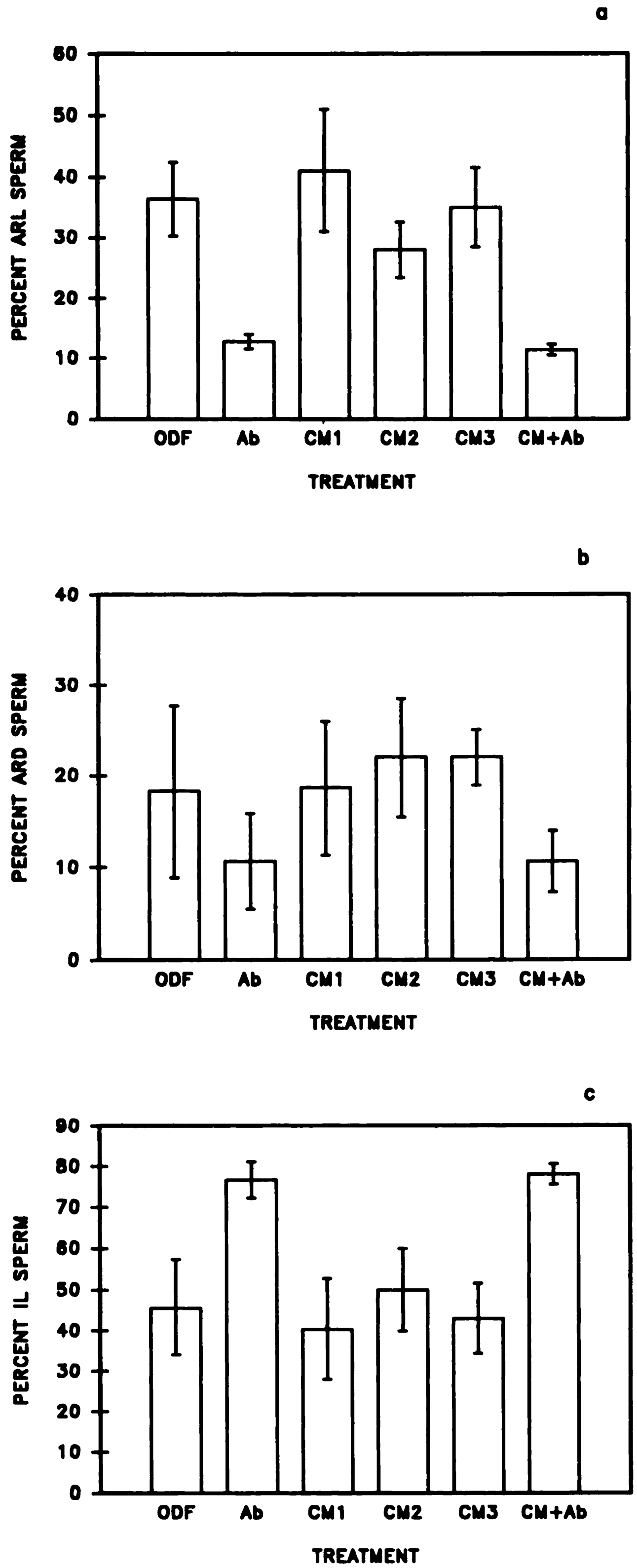


**FIG. 4.** Sperm were capacitated in MTM supplemented with non-luteal ODF (5 mg/ml), anti-EAP IgG (1 mg/ml), protein secreted by explant 1 (1 mg/ml), protein secreted by explant 2 (1 mg/ml), protein secreted by explant 3 (1 mg/ml), or pooled explant proteins (1 mg/ml) plus anti-EAP ← IgG (1 mg/ml). Data are expressed as the percent of ARL, representing functionally capacitated sperm (a), percent ARD sperm, representing cells that lost membrane stability (b), or IL, representing live uncapacitated sperm (c).

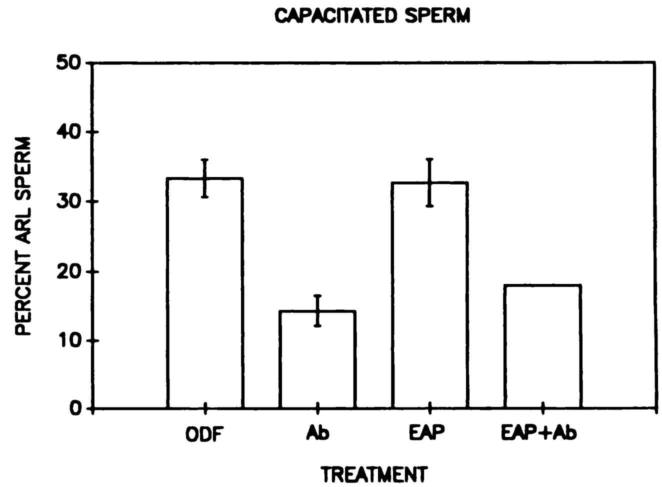


**FIG. 5.** Sperm were incubated in MTM supplemented with non-luteal ODF (5 mg/ml), anti-EAP (1 mg/ml), EAP (1 mg/ml), or EAP (1 mg/ml) plus anti-EAP (1 mg/ml). Data are percentages (mean ± SE) of ARL sperm, representing functionally capacitated sperm.

To address the question whether rabbit IgG, not specific for EAP, inhibited capacitation or fertilization, an additional study was undertaken. The rabbit IgG used was specific for bacterial histidase (anti-hist). After 4 hours of incubation at 39°C, the percentage of ARL sperm was: MTM (12 ± 6.0), $EAP_{CM}$ (35 ± 15.0), anti-hist (15.5 ± 0.5), $EAP_{CM}$ plus anti-hist (43 ± 11), $EAP_{CM}$ plus anti-EAP (16.5 ± 5.5), $EAP_{ODF}$ (45 ± 9.0), and $EAP_{ODF}$ plus anti-EAP (20 ± 5.0). The percentage of eggs fertilized after 16 hours of co-incubation was: MTM (13 ± 2.3), $EAP_{CM}$ (52 ± 4.0), anti-hist (10 ± 3.6), $EAP_{CM}$ plus anti-hist (33 ± 3.6), $EAP_{CM}$ plus anti-EAP (5 ± 2.0), $EAP_{ODF}$ (41 ± 2.0), and $EAP_{ODF}$ plus anti-EAP (8 ± 1.7). Sperm incubated with anti-hist alone did not become capacitated and fertilized a minimal percentage of eggs, whereas sperm incubated with the combination of anti-hist plus EAP became capacitated and fertilized a significant percentage of eggs.

## Discussion

### *Studies with In Vivo-Derived EAP*

The staining method used in this study permitted evaluation of the acrosomal membrane and live/dead status of individual sperm, and collectively these assessments defined the sperm population. Because the AR is induced by LPC only in capacitated sperm (Parrish et al, 1988),

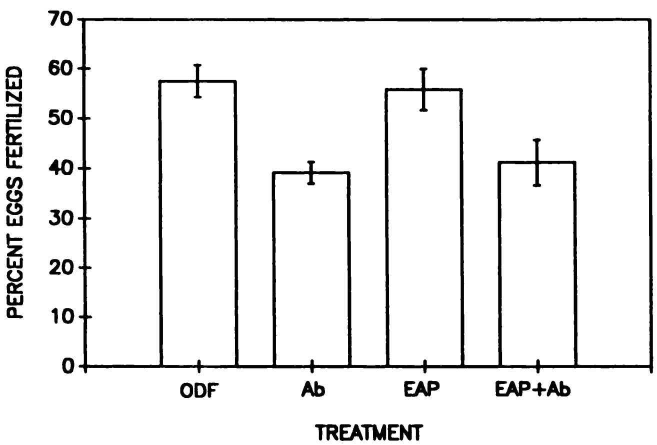


**FIG. 6.** Eggs were inseminated with sperm incubated in MTM supplemented with non-luteal ODF (5 mg/ml), anti-EAP (1 mg/ml), EAP (1 mg/ml), or EAP (1 mg/ml) plus anti-EAP (1 mg/ml). Data are percentages (mean ± SE) of eggs fertilized.

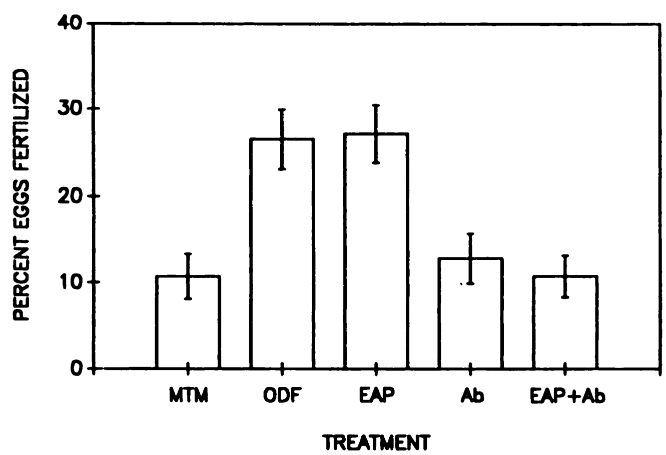


**FIG. 8.** Eggs were inseminated with sperm incubated in MTM alone or MTM supplemented with non-luteal ODF (5 mg/ml), EAP (1 mg/ml), anti-EAP (1 mg/ml), or EAP (1 mg/ml) plus anti-EAP (1 mg/ml). Data are percentages (mean ± SE) of eggs fertilized.

and only live sperm are able to fertilize an egg, the population of ARL sperm was considered to represent functionally capacitated cells. The acrosomal membrane may be lost after cell death, regardless of capacitation status. Although it could not be determined if ARD sperm were functionally capacitated prior to cell death, the ARD population did not increase during incubation. This suggests that sperm entering ARL status did not typically progress to ARD during the period of incubation.

The present studies were designed to evaluate physiological effects of EAP on sperm. Sperm were incubated with a concentration of EAP (10%) that corresponded to the physiological concentration in estrous ODF. Treatments containing EAP clearly had higher percentages of ARL sperm than treatments without EAP or with anti-EAP, suggesting that EAP facilitates sperm capacitation.

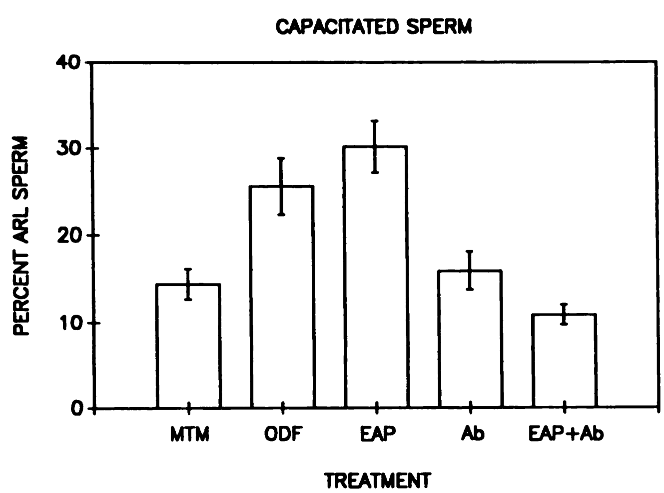


**FIG. 7.** Sperm were incubated in MTM alone or MTM supplemented with non-luteal ODF (5 mg/ml), EAP (1 mg/ml), anti-EAP (1 mg/ml), or EAP (1 mg/ml) plus anti-EAP (1 mg/ml). Data are percentages (mean ± SE) of ARL sperm, representing functionally capacitated sperm.

Incubation time also had a significant effect on ARL and IL sperm populations in treatments containing EAP, and it corresponded to sperm becoming capacitated. Functionally, capacitated sperm shifted from the IL to the ARL population after induction of the AR.

It has been shown that glycosaminoglycans (GAGs) promote fertilizing ability of sperm (Handrow et al, 1982; Lenz et al, 1982). Heparin, the GAG with highest sulfate content, had the greatest ability to stimulate *in vitro* sperm capacitation and induce the AR (Handrow et al, 1982; Miller and Ax, 1989). The incubation time needed to capacitate >30% of the sperm population *in vitro* with heparin was determined to be at least 4 hours (Parrish et al, 1988). It is of interest to note that 35% of the sperm population incubated with EAP became capacitated by 2 hours, whereas <20% incubated with heparin for 2 hours became capacitated, indicating that the potency and/or mechanism of action to capacitate sperm with EAP is different from that for heparin.

Addition of LPC has been shown to induce the AR in capacitated sperm *in vitro* without affecting uncapacitated sperm (Parrish et al, 1988). In the experiments using ODF-derived EAP, 60 $\mu$g/ml of LPC were added to induce the AR. Although the number of sperm in each treatment was the same, the protein concentration in each treatment differed. Based on the results of the LPC titration experiments, which specifically tested the sperm number and protein concentration used in this experiment, the LPC concentration needed to induce the AR with minimal ARD and ID sperm was 30 $\mu$g/ml.

The LPC dose used to induce the AR in sperm capacitated with heparin (60 μg/ml) was associated with a larger number of ARD sperm capacitated with EAP. However, the ARL results indicated that whereas EAP capacitated sperm, it did not induce the AR, because sperm generally did not undergo the AR without LPC addition. Use of a consistent protein concentration among treatments also allowed for titration of an LPC dose with bull sperm to validate experimental conditions, because the response of sperm to LPC is affected by the protein concentration and number of sperm.

### *Studies with CM containing EAP*

The biological activity of EAP from explant cultures appears to be similar to that of EAP derived from ODF. At 4 hours of incubation, percentages of ARL sperm for treatments containing culture-derived EAP versus ODF-derived EAP were comparable, despite the 5-kDa difference in molecular weight between the *in vivo*-synthesized and *in-vitro*-synthesized molecules.

In the experiment with CM containing EAP, the protein concentrations were kept constant in the treatments. This was more important than maintaining physiological percentages of protein in estrous ODF that were used in the previous experiment to determine if EAP could capacitate sperm at concentrations found in ODF.

Treatments containing EAP in CM had at least twice as many ARL sperm as treatments without EAP or with anti-EAP, clearly indicating that EAP from *in vitro* cultures facilitates sperm capacitation. The low ARL and high IL sperm observed in the treatment containing EAP plus anti-EAP suggests that the antibody suppressed capacitation to minimal levels without adversely affecting sperm viability.

The percentages of ARD sperm for treatments containing culture-derived EAP were not different from those containing anti-EAP; however, the percentages of IL sperm were significantly higher in treatments containing anti-EAP. The high percentages of IL sperm in treatments containing anti-EAP corresponds to the low percentages of ARL sperm in these treatments, indicating that sperm were not capacitated in the presence of anti-EAP. The lower percentages of ARD sperm in this experiment were apparently due to the lower concentration of LPC used to induce the AR (30 μg/ml), indicating that 60 μg/ml LPC produced conditions under which sperm membrane integrity was lost.

### *Effect of Bovine EAP on IVF*

IVF assays have been used to document fertilizing ability and assay the relative fertility of different populations of sperm. When zona-intact eggs are used in IVF assays, sperm bind to species-specific receptors on the egg surface, to be induced to acrosome react by zona pellucida constituents and penetrate the egg investments to achieve fertilization (Florman and First, 1988). These sperm–egg interaction events likely mimic those occurring *in vivo.* Although oviduct-specific EAP occur in many species, and appear to bind to both sperm and eggs, no biological function of EAP has been reported.

The observation that EAP capacitated sperm *in vitro* establishes a physiological role of this protein in preparing sperm for fertilization. EAP apparently induces membrane changes during sperm capacitation that serve to initiate a sequence of events that enable the sperm to acrosome react in response to the zona pellucida and penetrate the egg.

Binding of bovine sperm to the zona is mediated by ZP3, which also induces the AR in capacitated sperm (Florman and First, 1988). Based on evidence in mice, it is believed that the sperm head binds to ZP2 after the AR occurs (Bleil and Wassarman, 1986). Penetration of the ZP and perivitelline space can then occur, after which the sperm head binds to the oolemma of the egg, resulting in fertilization and formation of pronuclei within the egg.

Studies of the fertilizing ability of sperm capacitated with EAP, a naturally occurring constituent present during *in vivo* capacitation, provide evidence that sperm capacitated with EAP also are functionally competent to penetrate oocytes. Sperm must be functionally capacitated before they are able to acrosome react, bind to the ZP, and penetrate an egg (Yanagimachi, 1988; Florman and Babcock, 1991; Storey, 1991). Bovine EAP does not induce the AR, but does promote sperm fertilizing ability.

Although the initial study of capacitation of sperm with EAP demonstrated that they were capable of penetrating oocytes, the results also suggested the number of sperm used to inseminate eggs and length of time sperm were incubated with eggs could be modified to maximize differences between positive and negative controls. Because syngamy occurs by about 24 hours, the incubation time was decreased to facilitate evaluation of pronuclei in stained eggs. In addition, prolonged incubation with BSA, a constituent of fertilization medium, can induce capacitation in bull sperm *in vitro.* Cholesterol efflux from the sperm membrane is believed to be an important part of the capacitation process (Langlais et al, 1981). Several studies have shown that addition of cholesterol to incubation medium can decapacitate sperm *in vitro* (Davis, 1976; Davis and Hungund, 1976) and inhibit the AR and IVF (Davis, 1980). Inhibition of capacitation by cholesterol also has been observed in studies with bovine sperm (Ehrenwald et al, 1988a,b). Albumin, the most abundant protein in follicular fluid and ODF, can act as a sterol acceptor (Go and Wolf, 1985) and therefore, may mediate capacitation. By decreasing the co-incubation time of eggs with sperm from 22–24 hours to 16 hours, capacitation due to prolonged exposure to BSA was minimal. Therefore, to maximize differences among treatments, the number of sperm used for insemination was increased and the co-incubation time was decreased.

The results from these studies on EAP indicate that EAP enhances sperm capacitation and, therefore, fertilizing ability. Our assessment of fertilizing ability does not distinguish the specific site of EAP action. However, EAP appears to alter the sperm membrane and enable AR induction. In the absence of EAP, or its inhibition by antibody, this membrane alteration may not occur, which would effectively block signaling by the ZP to induce the AR. Bovine EAP also may be involved in sperm binding to the oolemma of the egg or in events after egg penetration, such as signaling egg activation and release of cortical granules, or pronuclear/DNA events. These potential effects remain to be demonstrated and are under investigation.

## Conclusions

These experiments suggest that bovine EAP facilitates sperm capacitation and that EAP from cannula-derived ODF and oviductal cultures is biologically active. These findings are important, not only because this is the first paper to demonstrate a function of EAP, but because EAP is a naturally occurring constituent of the environment in which fertilization occurs. Further study of the mechanism involved in EAP function should provide insight into the conditions surrounding gamete preparation for *in vivo* fertilization.

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