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Fertilizing Ability of Bovine Spermatozoa Cocultured with Oviduct Epithelial Cells[1]

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ABSTRACT

The effects of bovine oviduct epithelial cell monolayers (OECM), derived from the different segments of the oviduct and under various conditions, on oocyte penetration by bovine sperm were determined by in vitro cell culture techniques. The oocyte penetration rate by sperm treated with OECM+OECM-conditioned medium was 93% (155 of 166). Sperm penetration rates in OECM+fresh medium and in OECM-conditioned medium alone were 66% (115 of 173) and 52% (87 of 167), respectively, significantly lower ($p < 0.01$) than in OECM+OECM-conditioned medium. However, the percentage of penetrated oocytes in OECM-conditioned medium alone was significantly higher than in control (11% = 16 of 148; $p < 0.01$). In both the OECM+OECM-conditioned medium and the OECM+fresh medium groups, oocyte penetration rates by sperm cocultured with OECM derived from the isthmic segment were significantly lower than those by sperm cocultured with OECM derived from the ampullary segment (44% = 43 of 98 vs. 72% = 68 of 94, and 42% = 49 of 117 vs. 66% = 72 of 110, respectively; $p < 0.01$). Sperm penetration rates were low after insemination in the OECM-conditioned medium alone derived from either the isthmic or the ampullary segments (20% = 20 of 100 and 19% = 17 of 91, respectively). However, the sperm penetration rate was improved significantly when OECM-conditioned medium was obtained from whole-oviduct OECM (57% = 60 of 105; $p < 0.01$). The effect of OECM derived from different segments on ability of sperm binding and maintaining motility was also evaluated in vitro. After 2 h of coculture, more than half the sperm attached to OECM regardless of their origin. Sperm were gradually released from OECM in the whole oviduct and ampullary segments after 3 h of coculture; however, sperm remained attached to isthmic OECM after 12 h of coculture. After 48 h of coculture, the motility of unattached sperm in either isthmic OECM or whole-oviduct OECM was significantly higher than in ampullary OECM (52% ± 3, 55% ± 4, and 25% ± 4, respectively; $p < 0.01$). When sperm were cocultured with ampullary OECM, the percentage of motile sperm was significantly lower from 24 h after coculture than percentages obtained after coculture with whole-oviduct OECM and isthmic OECM (26% ± 4, 63% ± 3, and 61% ± 4, respectively; $p < 0.01$).

These results suggest that sperm capacitation was synergistically induced by means of both attachment to OECM and exposure to OECM-conditioned medium, and that sperm attachment to the ampullary epithelium enhanced sperm capacitation. These results also suggest that the isthmus epithelial cells are important in maintaining sperm motility and that attachment of sperm in the isthmus may act to reduce the number of sperm arriving at the ampulla in vivo.

INTRODUCTION

The mammalian oviduct provides the microenvironment where sperm capacitation, fertilization, and the initial stages of embryonic development occur. It is also the site where sperm await the arrival of eggs [1, 2]. Before sperm become capable of fertilizing, however, they must undergo the process of capacitation [3, 4]. The site where capacitation is initiated and completed may vary from species to species, although the oviductal isthmus acts as a functional sperm reservoir during the preovulatory period in the mouse [5], guinea-pig [6], hamster [1], rabbit [7], sheep [8, 9], pig [10], and cow [11]. Recently, it has been shown that spermatozoa may be held in the oviduct by binding to mucosal epithelial cells [12, 13]. Contact between spermatozoa and the mucosa is known to be beneficial for sperm survival both in vivo [13] and in vitro [14]. In the hamster, sperm remain attached to the isthmic mucosa until they become capacitated, at which time they detach and migrate to the ampulla to fertilize the eggs in vivo [15]. In the cow, sperm-fertilizing capacity may be maintained and hyperactivated motility may be induced by the oviductal epithelium [11, 16, 17]. In vitro investigations have also indicated that sperm capacitation may be stimulated by interactions with oviduct epithelial cell monolayers (OECM), suggesting that the oviduct can promote survival and storage of sperm and, at the same time, induce sperm capacitation [18].

Although little is known of the specific function(s) of the oviduct epithelial cells, several reports have demonstrated the capacity of these cells and oviductal fluid to assist in the capacitation of bovine [18–21], equine [22], porcine [23], and ovine [24] spermatozoa. The observation that certain oviductal proteins associate with spermatozoa [25–27] supports the hypothesis that some of these proteins have a role in sperm survival and/or capacitation. However, the mechanism by which such sperm survival and capacitation are induced is not fully understood.

In vitro cell culture techniques have become vital in the study of animal cell function. Oviduct epithelial cell culture introduces new possibilities for the analysis of fertilization mechanisms in large domestic animals. Bovine cumulus oophorus and oviductal cells can support and maintain the viability and motility of frozen-thawed bovine sperm over time [14, 28]. In addition, our laboratory has previously demonstrated that bovine granulosa cells and oviductal cells significantly improve the motility scores of sperm samples maintained in vitro for extended periods of time [29]. The

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objectives of the present study were to determine the effects of oviduct epithelial cells and conditioned medium in coculture on the in vitro fertilizing ability of bovine spermatozoa, and to assess the kinetic interaction of bovine spermatozoa with oviductal epithelial cell monolayers in vitro.

MATERIALS AND METHODS

Preparation of OECM

Bovine oviduct epithelial cells were collected and prepared according to a modified procedure of Eyestone and First [30]. Briefly, oviducts were collected one or two days after ovulation in a normal estrous cycle. They were transported to the laboratory on ice (all the following procedures were carried out at 4°C unless otherwise indicated), washed three times in 0.9% NaCl, and dissected free from the ovaries and other tissues. Oviducts were then washed three times in saline, and both ends were trimmed after each wash. Finally, they were washed once in Hanks' medium (containing 5% fetal calf serum [FCS], 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) without calcium and magnesium. Mucosal tissue was extracted from the oviduct by compressing with a glass slide from the isthmus toward the infundibulum. Mucosal tissue (1.0 ml) was transferred to a 50-ml conical tube containing 15 ml Hanks' medium and washed twice at room temperature. The epithelium sheets were dispersed by passage through a 27-gauge needle several times. The dissociated epithelial cell suspensions were transferred to each well of a 4-well multidish (Nunc, Cat. No. 176740, Roskilde, Denmark) at a final cell concentration of 1.0~1.5 × 10^6 cells/ml (90~95% of live cells; trypan blue dye-negative staining) in 1 ml Tissue Culture 199 medium (TC-199; GIBCO BRL, Grand Island, N.Y.) medium supplemented with 10% heat-treated FCS (TC-199+FCS) and 50 μg gentamicin/ml. The oviduct epithelial cells were incubated at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. Monolayers derived from the different oviduct segments were formed (60~70% confluent) within 5 days (120 h), at which time the original medium was replaced with 1 ml fresh TC-199+FCS. After 7 days (160 h) of culture, mucosal epithelial cells derived from the different oviduct segments had formed confluent monolayers (Fig. 1).

Maturation of Oocytes In Vitro

Ovaries from cycling or pregnant heifers or cows were removed within 30 min after slaughter and transported to the laboratory at approximately 35°C in 0.9% NaCl aqueous solution containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). Cumulus-oocyte complexes (COC) were aspirated from 1–5-mm follicles with an 18-gauge needle, pooled, and selected as described previously [31]. The COC were rapidly washed 4 times in HEPES-buffered Tyrode's medium [32] supplemented with 10% FCS, 0.25 mM pyruvic acid, and 50 μg/ml gentamicin. After washing, 10–15 oocytes were cultured in 50-μl droplets of TC-199 medium under 9 ml mineral oil in 60-mm Petri dishes. For oocyte maturation, TC-199 was supplemented with 10% (v/v) FCS, 0.2 mM pyruvate, 50 μg/ml gentamicin, 1 μg/ml estradiol-17ß (Sigma Chemical Co., St. Louis, MO), 0.5 μg/ml FSH (oFSH; NIADDK-oFSH-17), and 1.0 μg/ml LH (bLH; NIADDK-LH-B9, generously donated by the National Hormone and Pituitary Program, Baltimore, MD). Oocytes were incubated at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After maturation for 24 h, the oocytes were used for in vitro fertilization.

Preparation of Spermatozoa

Frozen semen, pooled from five bulls, was donated by the Centre d'Insémination Artificielle du Québec (C.I.A.Q. Inc., St-Hyacinthe, PQ). Straws of semen were thawed in a water bath (35°C) for 30 sec and processed by swim-up as described Parrish et al. [33]. The sperm were then washed twice in modified Tyrode's albumin lactate pyruvate medium used for sperm culture (Sp-TALP) [34] containing 6 mg/ml fatty acid-free BSA (Sigma), 1.0 mM pyruvic acid, and 50 μg/ml gentamycin. The sperm suspension was used for either in vitro fertilization or evaluation of kinetic interaction of spermatozoa with OECM in vitro.

Experimental Design

Experiment 1. OECM in each well of a 4-well culture dish were rinsed gently twice with modified Tyrode's medium (m-TALP) [34] containing 6 mg/ml BSA, 0.25 mM pyruvic acid, and 50 μg/ml gentamycin, and were cultured with 0.5 ml of m-TALP medium for 3 days (72 h) to obtain conditioned medium. After 3 days, four different sperm treatments were used to capacitate the spermatozoa: 1) OECM with OECM-conditioned medium (M + CM), 2) OECM with fresh m-TALP medium (M + FM), 3) OECM-conditioned medium alone (CM), 4) fresh m-TALP medium containing 2 μg/ml heparin, and 5) fresh m-TALP medium alone (m-TALP). Each well received 15 COC for insemination. The washed sperm suspensions were added directly to each well containing oocytes. The final concentration of spermatozoa in the fertilization medium was adjusted to 1 × 10^6 sperm/ml. Eighteen to 24 h after insemination, oocytes were fixed for evidence of sperm penetration.

Experiment 2. OECM were derived from different segments: whole-oviduct, isthmic, and ampullary segments. The OECM from the various segments in each well of a 4-well culture dish were gently rinsed twice with m-TALP, then cultured with 0.5 ml of m-TALP medium for 3 days (72 h) to obtain conditioned medium. After 3 days, the five different treatments for sperm capacitation described for experiment 1 were applied. Aliquots of sperm suspension were

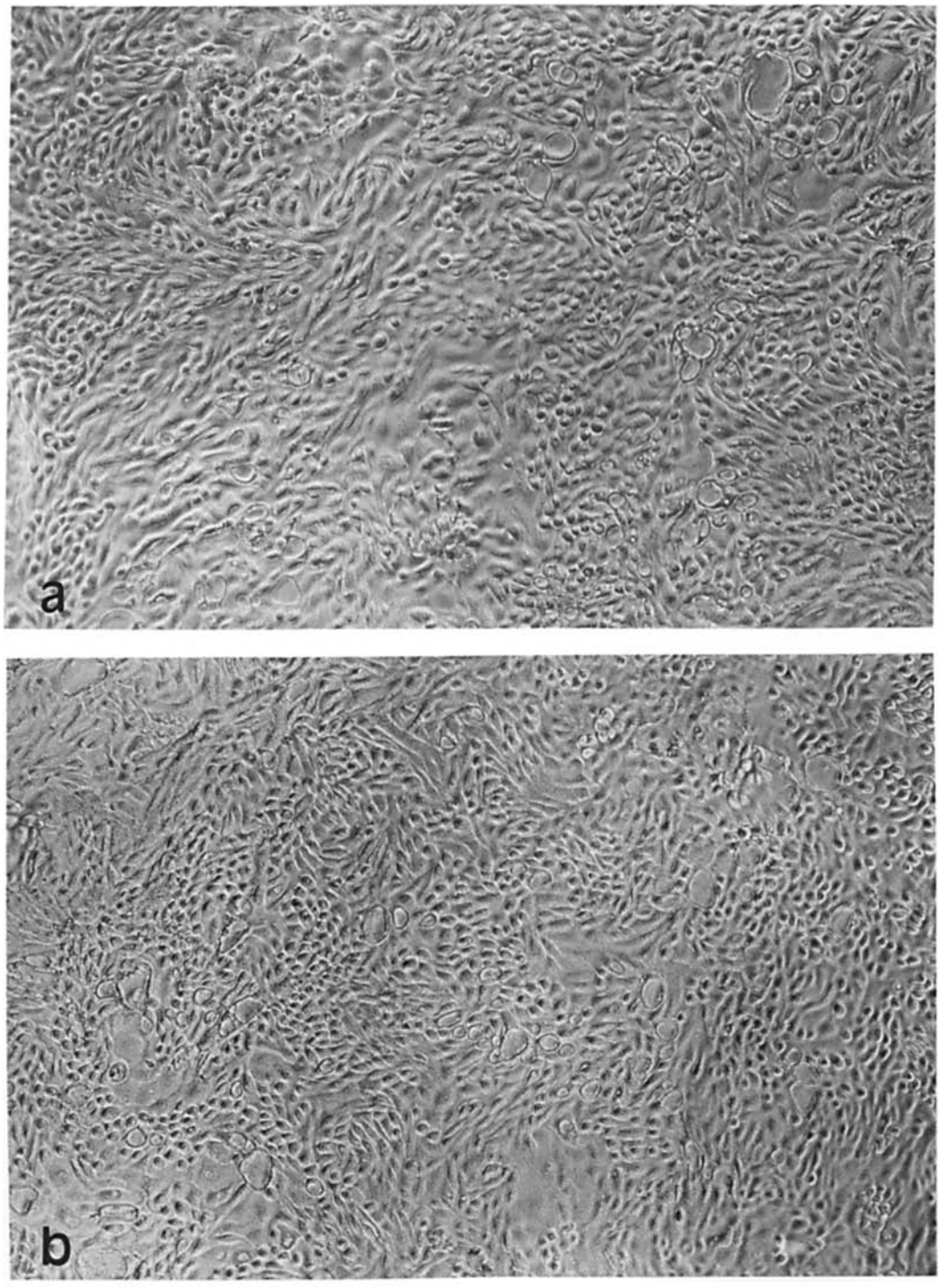

FIG. 1. The different segments of oviduct epithelial cells formed confluent monolayers at 7 days (160 h) after culture. ×293. a) Ampullary segment of epithelial cell monolayer; b) isthmic segment of epithelial cell monolaye..

transferred to each well containing 15 COC for a final concentration of 1×10^6 spermatozoa/ml in the fertilization medium. Eighteen to 24 h after insemination, oocytes were fixed for evidence of sperm penetration.

Experiment 3. The numbers of spermatozoa attached to OECM derived from whole-oviduct, isthmic, and ampullary segments, and the motilities of unattached spermatozoa during coculture were assessed. The OECM derived from different segments were gently rinsed twice with m-TALP medium containing 6 mg/ml BSA, 0.25 mM pyruvic acid, and 50 μg/ml gentamycin. After washing, 0.5 ml of fresh m-TALP medium was added to each well of a 4-well mul-

tidish. After sperm coculture (1×10^6 sperm/ml) with the OECM (1.77 cm^2), the number of unattached spermatozoa and the percentage of motile spermatozoa in the overlying medium were evaluated at 1-h intervals until 6 h and then at 12 h, 24 h, and 48 h with use of a hemocytometer at 30°C. Briefly, at each time point, the 4-well multidishes were carefully moved onto a microscope stage, and the interaction between spermatozoa and the OECM was observed with different focal planes. After observation, the cocultured samples were gently mixed by pipetting several times, and 10-μl samples were obtained from each treatment group to determine the concentration and motility of the unattached sperm. The percentage of motile sperm was calculated by scoring 100 spermatozoa as motile or immotile at each time point. The motile spermatozoa included those that were moving rapidly and progressively, and those with the tail beating and attached by the head to the coverslip.

Fixation of Oocytes

At 18–24 h after insemination, oocytes were freed from cumulus cells by bovine testicular hyaluronidase (Sigma) treatment (0.1% in PBS) for 3–5 min, then passed repeatedly through a fine pipette. Completely denuded oocytes were transferred into small drops on glass slides. Four dots of a vaseline-paraffin mixture (9:1) were used to keep the coverslip in contact with the oocytes without excessive pressure. The slides were immersed in a fixative solution (ethanol:acetic acid, 3:1) for a minimum of 48 h at room temperature, stained with 1% orcein in 45% acetic acid, and examined for evidence of sperm penetration [35]. Oocytes were considered to be penetrated when decondensed (or enlarged) sperm nuclei or pronuclei and their associated tails were seen in the cytoplasm.

Statistical Analysis

Sperm penetration results were analyzed by chi-square (χ^2-test; n = 4) evaluation. Means (n = 4) of sperm concentration and motility at each time point for each treatment were determined and compared by analysis of variance using the Newman-Keuls' test [36].

RESULTS

The effect of sperm coculture with OECM under various conditions on sperm penetration is shown in Table 1. The percentage of penetrated oocytes in M + CM was the same as with the heparin treatment (93% vs. 94%). However, the penetration rate of spermatozoa in M+FM was significantly lower than in M+CM (67% vs. 93%; $p < 0.01$). There was also a significant difference in penetration rates between M+FM and CM (67% vs. 52%; $p < 0.05$). Furthermore, the penetration rate in CM was significantly higher than in control (52% vs. 11%; $p < 0.01$).

As shown in Figure 2, when sperm and COC were cocultured with OECM derived from the isthmic segment, the

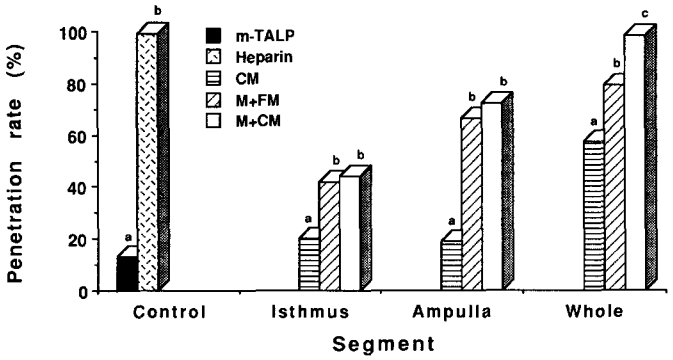

FIG. 2. Effect of OECM derived from different segments of oviduct on sperm penetration under various conditions (total of 1181 oocytes; n = 4). m-TALP: fresh m-TALP medium alone containing 6 mg/ml BSA, 0.25 mM pyruvic acid, and 50 μg/ml gentamycin; heparin: m-TALP medium containing 2 μg/ml heparin, 6 mg/ml BSA, 0.25 mM pyruvic acid, and 50 μg/ml gentamycin. Different letters within representation of each segment denote significant differences ($p < 0.01$).

percentage of penetrated oocytes in either M+CM or M+FM was significantly lower than after coculture with OECM derived from the ampullary segment (44% vs. 72% and 42% vs. 66%, respectively; $p < 0.01$). Although sperm penetration rates were low after insemination in CM derived from either the isthmic or the ampullary segments, the percentage of penetrated oocytes was improved significantly when CM was obtained from OECM derived from the whole oviduct (20%, 19%, and 57%, respectively; $p < 0.01$). Sperm penetration rates in either M+CM or M+FM were also improved significantly after insemination when OECM were derived from whole oviduct compared to OECM derived from only the ampullary segment (98% vs. 72% and 78% vs. 66%, respectively; $p < 0.01$).

As shown in Figure 3, after 2 h of coculture, more than half the spermatozoa were attached to OECM regardless of derivation of the monolayers. Spermatozoa were gradually released from either the OECM derived from the whole oviduct or those derived from the ampullary segment 3 h after coculture. However, spermatozoa remained attached

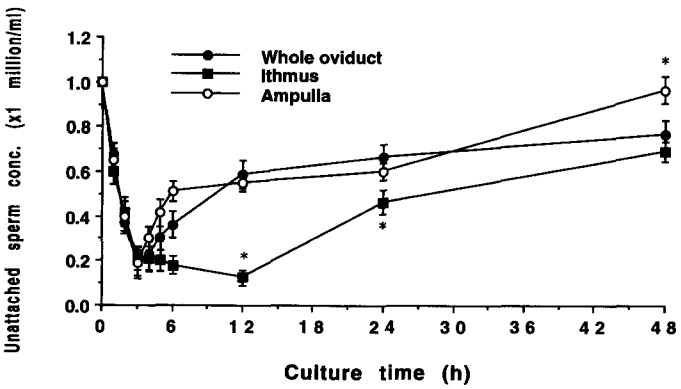

FIG. 3. Concentration changes of unattached spermatozoa during coculture with OECM derived from whole oviduct and isthmic and ampullary segments (n = 4). *Mean at coculture time point compared to other two treatments is significantly different ($p < 0.05$).

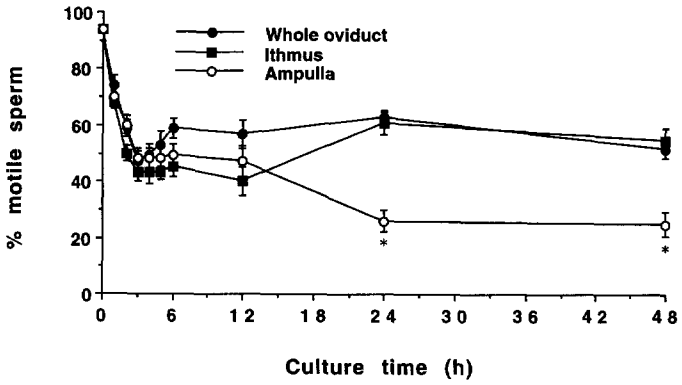

FIG. 4. Changes in motility of unattached spermatozoa cocultured with OECM derived from whole oviduct and isthmic and ampullary segments at various times (n = 4). *Denotes a significant difference at time point compared to other two segments ($p < 0.01$).

to OECM derived from the isthmus after 12 h of coculture (0.88 ± 0.03 million/ml; mean ± SD). Although almost all spermatozoa were released from ampullary OECM after 48 h of coculture, 31% (0.31 ± 0.06 million/ml) remained attached to isthmic OECM ($p < 0.01$).

As shown in Figure 4, after 3–48 h of coculture with whole-oviduct OECM, the motility of unattached sperm was maintained at about 50% (from 47% ± 2 to 63% ± 3; mean% ± SD). However, the motility of unattached sperm decreased continuously during the first 12 h of coculture with isthmic OECM. After 48 h of coculture, the motile proportion of unattached spermatozoa in either isthmic OECM or whole-oviduct OECM was significantly higher than in ampullary OECM (52% ± 3, 55% ± 4, and 25% ± 4, respectively; $p < 0.01$). The percentage of motile sperm was significantly decreased from 24 h after coculture with ampullary OECM compared to whole-oviduct or isthmic OECM (26% ± 4, 63% ± 3, and 61% ± 4, respectively; $p < 0.01$).

DISCUSSION

Contact between spermatozoa and OECM may induce alterations in the sperm surface that are possibly involved in sperm capacitation [18]. This contact between spermatozoa and OECM in vitro may alter the spermatozoa in a way similar to the changes that occur in vivo [21]. Recent evidence suggests that sperm attachment to OECM in culture may stimulate the synthesis and secretion of a cohort of proteins from OECM [27]. In the present study, the oocyte penetration rate in OECM-conditioned media was significantly higher than in control (Table 1), suggesting that OECM may secrete a sperm capacitation factor(s) into the medium. Indeed, it has previously been suggested that a heparin-like protein from the oviduct may be involved in capacitation of bovine spermatozoa in vivo [19]. There was a significant increase in the penetration rate when spermatozoa were exposed to both OECM and the conditioned medium (Table 1), indicating that sperm capacitation may be synergistically induced by attachment to OECM and exposure to conditioned medium. Although oviduct epithelial cells are different after 1 wk of in vitro culture from those in in vivo conditions, we can postulate from the present results that sperm capacitation may occur in an analogous manner in vivo.

It is well known that sperm-egg interaction occurs in the ampullary region of the oviduct. Smith and Yanagimachi [15] suggested that hamster sperm remain attached to the isthmic mucosa until they become capacitated, then detach and migrate to the ampulla to fertilize the eggs. Using bovine gametes in the present study, we found the percentage of penetrated oocytes after coculture with ampullary OECM to be greater than the percentage of those cocultured with isthmic OECM. However, sperm penetration rates after coculture with isthmic and ampullary OECM were significantly lower than with whole-oviduct OECM (Fig. 2), suggesting that both isthmic and ampullary components may be required for efficient capacitation. There are histological differences between the isthmus and the ampulla with regard to cell type and relative abundance; the isthmic mucosa is mostly composed of the nonciliated cells, whereas the ampullary mucosa consists of densely distributed ciliated cells [37]. Percentages of penetrated oocytes in either M+CM or M+FM were significantly lower in OECM derived from the isthmic segment than in OECM derived from the ampullary segment (Fig. 2). However, sperm penetration rates did not differ for isthmic and ampullary OECM-conditioned medium. This indicates that, in the bovine species, sperm attachment to ampullary epithelium enhances sperm capacitation. It seems likely that as spermatozoa move from the isthmus to the ampulla, capacitation is brought

TABLE 1. Penetration in vitro of bovine oocytes by frozen-thawed spermatozoa cocultured with OECM and/or OECM-conditioned medium (n = 4).

Sperm treatment	No. of oocytes examined	No. of oocytes penetrated		No. of polyspermic oocytes	
		Total (%)	% Range	Total (%)	% Range
m-TALP	182	20 (11)[a]	3–15	1 (5)	0–8
Heparin	167	157 (94)[b]	83–99	14 (9)	7–10
M + CM	166	155 (93)[b]	85–98	15 (10)	6–12
M + FM	173	115 (67)[c]	50–79	10 (9)	3–12
CM	167	87 (52)[d]	44–57	7 (8)	0–12

[abcd]Different letters within column denote significant differences (X^2-test; $p < 0.05$).

about both by attachment to the oviduct epithelium and by contact with secreted products in the oviductal fluid. It is also possible that the sperm undergo some final biochemical changes in the ampulla before effecting fertilization. Sperm penetration rates after coculture with OECM plus OECM-conditioned medium were the same as with heparin treatment (Table 1 and Fig. 2), further indicating that sperm coculture with whole-oviduct OECM may provide another efficient system for fertilization in vitro.

Ellington et al. [18] have previously described the interactions of bovine spermatozoa with OECM in vitro. Forty-three percent of the 12×10^6 spermatozoa added to 2 cm^2 of OECM had bound within 4 h of coculture. By 7 h of coculture, 19% of the previously bound spermatozoa had been released from the OECM and were mostly dead, or had limited motility. The spermatozoa remaining on the monolayer at 7 h showed vigorous tail motion and were gradually released from the OECM over a 48-h period. In another study, Goldman et al. [38] indicated that frozen-thawed spermatozoa were limited in their binding potential compared to fresh sperm. Although frozen-thawed spermatozoa were used in the present research, we did not observe the same result, probably because we used a different sperm concentration (1×10^6/ml vs. 12×10^6/ml) over about an equal surface area. However, we did observe that more than half the sperm remained attached to the OECM from whole oviduct, isthmus and ampulla following 2 h of coculture. Sperm were gradually released from the OECM from whole oviduct and ampulla 3 h after coculture. It is possible that the spermatozoa had become capacitated 3 h after coculture with the OECM, as we did observe some hyperactivated sperm and a few penetrated oocytes after 3 h of coculture with the OECM from whole oviduct (data not shown). Taken together, the results shown in Figure 3 and Figure 4 indicate that the motile spermatozoa were gradually released from the whole-oviduct OECM after 3 h of coculture. It has been suggested that hyperactivated motility enables sperm to be released from the epithelial cell surface [39] and facilitates sperm passage through the highly folded oviductal lumen [40]. A study in sheep [24] showed that interactions leading to the acrosome reaction were observed only when sperm were cocultured with homologous or heterogenetic OECM compared to other epithelial cell monolayers. In the present study, we did not evaluate the acrosomal status of unattached spermatozoa. Whether changes in acrosomal status of attached spermatozoa are related to the gradual release from the OECM remains to be determined.

The oviduct has traditionally been divided into the isthmus for sperm storage and the ampulla for fertilization. Therefore, with regard to sperm, the isthmus acts as a site for sperm storage during the preovulatory period and as a sieve to decrease the number of sperm reaching the site of fertilization. In cattle, a large number of spermatozoa are stored in the caudal isthmus of the oviduct and may function to ensure a high fertilization rate [16]. The majority of spermatozoa stored in the hamster isthmus die before ovulation [13], and only a very small number of the stored spermatozoa ascend to the ampulla [15]. Our data suggest that when spermatozoa pass through the long and narrow isthmus lumen they may become attached to the epithelial cells, possibly reducing the number of spermatozoa arriving at the ampulla. Our observation of the strong potential of the isthmus in the maintenance of sperm binding and motility supports the notion that it may function to reduce the number of sperm arriving at the ampulla and to increase the quality of those arriving at the site of fertilization.

In the hamster, sperm attachment to the epithelial cells of the isthmus appears to play an important role in maintaining sperm viability in the oviduct [13]. Pollard et al. [14] showed that OECM not only bind spermatozoa but also maintain sperm viability and fertilizing capacity. They suggested that sperm motility may be maintained by physical interactions with the OECM and that the specific factors in the oviduct add to this effect. Our results show that sperm can be continuously attached to isthmic OECM for 12 h and that sperm motility can be maintained for over 48 h during coculture. These data suggest that only isthmic epithelial cells can efficiently maintain the sperm motility. Furthermore, our previous study [29] indicated that oviduct cells secrete sperm motility-maintaining factor(s). We hypothesized that the increase in sperm motility may be due to stimulation of metabolic activity, and that the prolongation of sperm motility may be due to stabilization of the sperm membrane or a delay in capacitation or both. How the isthmic cells perform these functions are not clear; however, at least two factors may contribute to the maintenance of sperm motility. First, spermatozoa are physically bound to the OECM, and second, spermatozoa may be nurtured by a motility-maintaining factor(s) secreted by isthmus OECM. In conclusion, sperm capacitation may be synergistically induced by means of both attachment to OECM and factors in the conditioned medium. The ampulla may perform a role in the completion of sperm capacitation. The oviduct isthmus may function to maintain sperm motility and to reduce the number of sperm arriving at the ampulla in vivo.

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