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De Novo Protein Synthesis by Bovine Uterine Tube (Oviduct) Epithelial Cells Changes during Co-Culture with Bull Spermatozoa[1]

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ABSTRACT

Polypeptides secreted by uterine tube epithelial cells (UTEC) may facilitate sperm cell capacitation in vivo. This experiment evaluated the effect of sperm-UTEC co-culture on de novo protein synthesis by epithelial cells of the tubal isthmus. Comparisons of the patterns of proteins secreted into medium were made between four culture groups incubated for 24 h in the presence of ^{35}S-methionine: group 1, sperm cells alone; group 2, control UTEC monolayers; group 3, UTEC co-cultured with sperm cells; and group 4, UTEC partitioned by a diffusible membrane from sperm cells during culture. Two-dimensional PAGE followed by fluorography was used to analyze conditioned medium containing secreted proteins from each group. The experiment was replicated four times. Sperm cells alone secreted no detectable proteins, whereas control UTEC monolayers produced a wide array of polypeptides. Sperm cells attached to UTEC in co-culture within minutes, and the resultant protein profile for these UTEC differed markedly from that of the control UTEC. Several new proteins were seen only from co-cultured cells, whereas other protein groups that were present with UTEC alone were absent in the co-culture medium of group 3. The protein pattern expressed by UTEC partitioned from sperm cells (group 4) was intermediate between that of the group 2 controls and that of co-cultured UTEC (group 3). In summary, the attachment of sperm cells to the UTEC during co-culture changed the types and quantities of proteins secreted into the conditioned medium as compared to those of control UTEC monolayers. Exposure to sperm cells without attachment stimulated some, but not all, of the changes seen in protein production from the UTEC.

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INTRODUCTION

The mammalian uterine tube (oviduct) is the physiologic site of sperm cell capacitation, gamete fertilization, and subsequent early embryonic development [1]. Secretory epithelial cells of the tube produce a wide array of glycoproteins, and this production is mediated by the steroidal milieu of the female's cycle [2–4]. Glycoproteins secreted in the tube bind to sperm cells, the oocyte, and the early embryo [4–7]. Physiologic functions of these products are not as yet understood.

Secretory products from the uterine tube epithelial cells (UTEC) may coordinate sperm cell capacitation in vivo. There has been much interest in identifying the components of tubal fluid and the mechanisms controlling their production [2, 4, 8–11]. Tubal, estrus-associated proteins have been described in many species including cattle [2, 7, 12, 13]. In cattle, the Golgi apparatus is at maximum volume, the endoplasmic reticulum is dilated, and maximum polyribosomal activity is seen in UTEC under the influence of estrogens at estrus [14]. The production of proteins unique to the time of ovulation in the cow has been reported for UTEC both in vitro and in vivo [3, 15].

Recently, UTEC co-culture has been described as a method to capacitate bull sperm cells in vitro [16–18]. The present study was done to evaluate the effect of sperm cell co-culture with UTEC on de novo protein synthesis profiles of the epithelial cells. Two-dimensional gel electrophoresis was used to analyze conditioned medium from UTEC monolayers both with and without exposure to sperm cells. Further studies evaluated the effect of sperm cells on polypeptide synthesis by the epithelial cells when the sperm cells were partitioned from the UTEC monolayers during culture.

MATERIALS AND METHODS

Monolayer Production

Uterine tubes from cows in the follicular phase of their cycle were collected at the abattoir and transported to the laboratory in PBS. The distal 4 cm of the tubal isthmus ipsilateral to the dominant follicle was removed and the lumen was lavaged to collect UTEC [19]. For each replicate, cells from four animals were pooled, washed by centrifugation at 300 × *g* for 10 min, and cultured at 38.5°C in 5% CO_2 and air. The culture medium used was M199 with 10% fetal bovine serum (FBS), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 10 ng/ml epidermal growth factor, plus 1% antibiotic/antimycotic (Gibco, Grand Island, NY). Clumps of UTEC were cultured directly in 35-mm Petri dishes, without any extracellular matrix, for 1 wk until confluent monolayers had formed. Immediately before use in this experiment, monolayers were washed in a glucose-free modified Tyrode's medium (TALP [20]) and then covered with an additional 3 ml of TALP.

Treatment Groups

Three treatment groups were initially established: 1) control sperm cells in TALP alone; 2) control UTEC with

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TALP alone; and 3) co-cultured UTEC and sperm cells. After two replications another group was added: 4) UTEC and sperm cells, separated by a diffusible membrane insert (0.4 μm) but with shared medium. All four groups were then run in two additional replicates. Freshly ejaculated bull semen was washed in TALP, and 10×10^6 sperm cells/ml were added to each treatment group that included sperm cells (groups 1, 3, and 4). For group 1 (sperm cells alone), sperm cells were left in TALP for 4 h; they were then collected by centrifugation, resuspended in protein and methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 50 μCi/ml ^{35}S-methionine, and incubated at 38.5°C for 24 h. For group 2 (control UTEC), monolayers were covered with TALP for 4 h and then incubated with 1 ml of DMEM supplemented with ^{35}S-methionine for 24 h. Group 3 (co-culture) showed sperm cell attachment to the UTEC within minutes of beginning incubation, as previously described [16]. After 30 min, monolayers were rinsed to remove any nonattached sperm cells, and 3 ml of fresh TALP was added to cover the co-cultured cells. After 4 h the TALP was removed and the radiolabeled DMEM was placed over the cells (sperm and UTEC together) for the 24-h incubation. For group 4, a diffusible membrane insert (Millipore, Bedford, MA) was placed over the UTEC in the culture dishes and a suspension of sperm cells placed within the insert. The TALP medium of a given dish was then exposed to both sperm cells and UTEC; however, there was no direct contact between the two cell types. After 4 h, TALP was removed and DMEM added to the dishes for the 24-h labeling incubation. Semen of high quality from three different bulls was used. One bull, utilized twice, was used once in the initial two replicates and once in the succeeding replicates where all four groups were represented.

Sample Preparation

Conditioned medium containing radiolabeled polypeptides released by cultured cells was removed after 24 h of incubation. The protease inhibitor PMSF was added to a final 1 mM concentration; then debris and detached cells were removed by centrifugation at $700 \times g$ for 5 min. The clarified medium was concentrated, desalted, and simultaneously diafiltered to water via Centricon microconcentrators (Amicon Corp., Lexington, MA; 10 kDa molecular mass cutoff) followed by lyophilization. Residues were dissolved in sample buffer containing 10 M urea, 3% (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 5% (v/v) 2-mercaptoethanol, and 2% (w/v) ampholines, pH 3.5–10 (LKB Pharmacia, Rockville, MD). Incorporation of ^{35}S-methionine was measured by liquid scintillation spectrometry. Aliquots containing 50 000 dpm were subjected to two-dimensional PAGE for each treatment group.

Two-Dimensional PAGE and Fluorography

Proteins recovered from the conditioned medium were resolved in the first dimension by isoelectric focusing to 12 000 volt-hours by the procedure of O'Farrell [21] with the following modifications. Tube gels (2×120 mm) consisted of 4.4% acrylamide, 0.8% bisacrylamide, 10 M urea, 3% CHAPS, and 2% ampholines (pH 3.5–10, 5–7, and 7–9; 0.5:1:1, by volume, respectively). After focusing, gel rods were extruded and equilibrated for 20 min in 0.125 M Tris-HCl (pH 8.8), 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) 2-mercaptoethanol; they were then affixed to the second dimension SDS slab gel [22] consisting of a 13-cm separating gel (12% total acrylamide [T], 2.6% cross-linked acrylamide [C]) overlaid with a 5-mm stacking gel (3.5% T, 2.6% C). To determine the pH range of the first dimension, several blank gels were also run, cut into 4-mm slices, and equilibrated for 4 h in 1 ml of deionized water; pH was then measured with a glass electrode. Electrophoresis in the second dimension was performed at 30 mA per gel, after which the gels were fixed and then stained with Coomassie Blue R-250 to visualize molecular mass markers. Gels were next processed for fluorography by impregnation with 2-,5-diphenyloxazole [23], dried, and exposed for 8 days at −70°C to preflashed x-ray film [24].

RESULTS

Control sperm cells (group 1), incubated alone in TALP plus ^{35}S-methionine, did not synthesize or secrete labeled polypeptides (not shown). In contrast, conditioned medium from control UTEC (group 2) contained an array of newly synthesized proteins (Fig. 1A). The UTEC labeled in the presence of co-cultured, bound sperm cells (group 3, Fig. 1B) also released numerous proteins into the culture medium. There were, however, notable differences between controls and co-cultured UTEC. Several polypeptides (A, B, C, F, and G) and the closely-spaced groups D and E were less abundant or entirely absent (Table 1) from conditioned medium after co-culture. Other proteins, numbered 1–11, were present in increased amounts or were seen only after co-culture. The bracketed group of polypeptides numbered 7 (Fig. 1B) may represent a family of related proteins with essentially identical molecular masses but differing in their individual pI values. Similarly, groups D and E may each reflect charge modifications of an individual polypeptide. There did not appear to be an effect of semen from different bulls on the protein profiles observed.

When sperm cells were separated from epithelial monolayers by a diffusible membrane insert (group 4), the effect on the appearance of labeled proteins in the conditioned medium led to protein profiles (Fig. 2C) exhibiting characteristics of both control UTEC and co-culture incubations in which sperm cell binding had occurred. Protein 4, which appeared with sperm cell binding (Figs. 1B and 2B), was not evident when sperm cells were partitioned from UTEC during culture. Levels of proteins A, B, and F, which were decreased in the co-culture (group 3), were unaffected by

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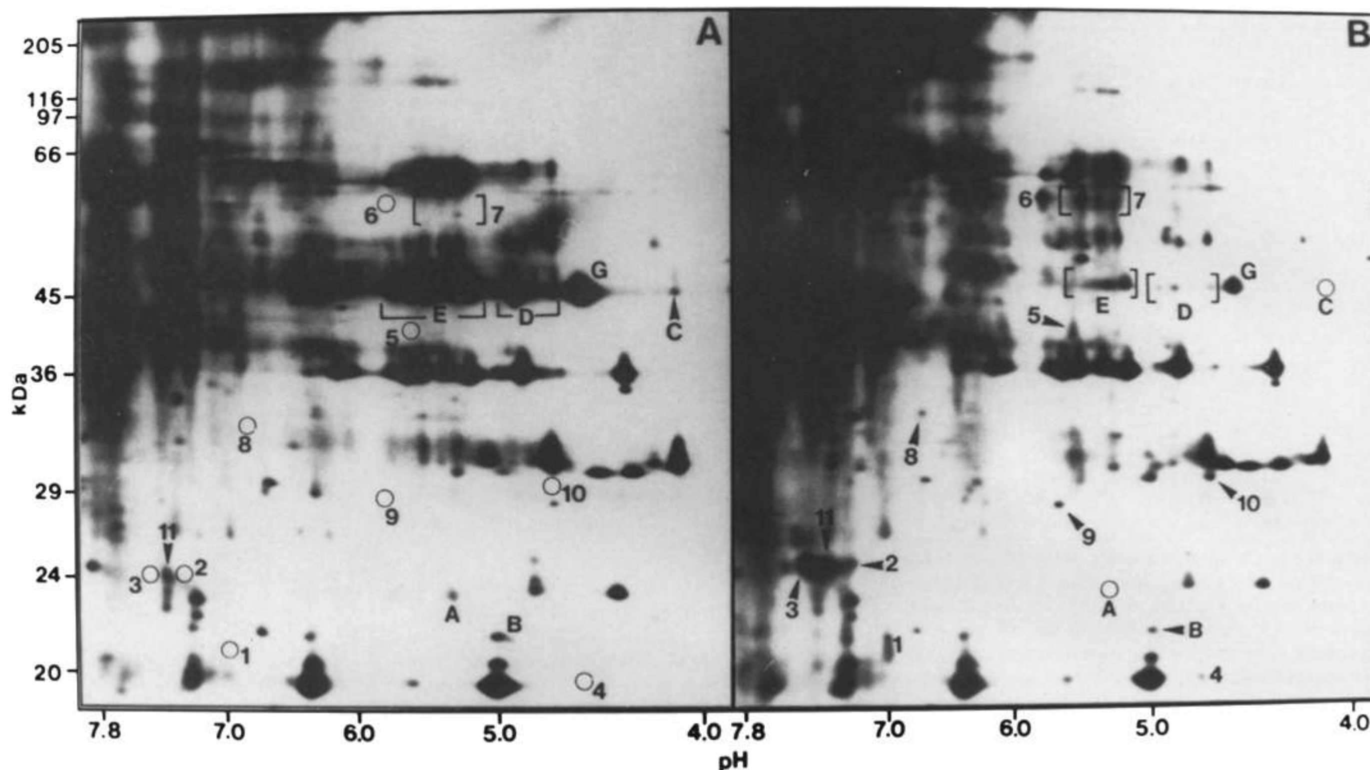

FIG. 1. Radiolabeled polypeptides synthesized and released into culture medium by bovine uterine tube epithelial cells of the isthmus. Cultures were incubated for 24 h in protein and methionine-free DMEM supplemented with 50 μCi/ml ^{35}S-methionine. Secreted and/or released proteins were resolved and visualized by two-dimensional PAGE and fluorography. Panel A: group 2, control UTEC. Panel B: group 3, UTEC-sperm cell co-culture labeled in the presence of adherent sperm cells. Proteins numbered 1–11 were newly synthesized or exhibited increased synthesis after sperm cell binding (see Table 1). Proteins A through G showed decreased synthesis after sperm cell co-culture. Open circles indicate the positions of proteins absent in either group.

culture with separated sperm cells (group 4). The decreased synthesis of proteins D and E in group 4 paralleled the decrease seen after sperm cell binding in group 3, while decreases in levels of peptides C and G for group 4 were not as great as the decrease in group 3, where actual sperm cell contact was permitted to occur.

Polypeptides 8 and 10, which were present after sperm binding (group 3), were only barely detectable using the partitioned co-culture system (group 4). New or increased synthesis of proteins 1 through 11 (with the exception of protein 4, which remained at control group 2 levels), seen after sperm binding (group 3), was generally observed when sperm cells were prevented from binding to the epithelial cells (group 4). While proteins 7 and 9 were elevated for group 4 above control group 2 levels, the increases observed were not as great as those seen when sperm cells were allowed to bind to UTEC cultures before and during the labeling period (group 3). Similarly, protein 1, which was newly seen after co-culture (group 3), did not appear to be as abundant when sperm cells were physically separated from the epithelial monolayers (group 4).

DISCUSSION

These studies indicate that actual physical contact of sperm and UTEC appears to trigger production of a new class of polypeptides in vitro (Table 1). Sperm cells in the partitioned cultures may have had on their surfaces diffusible products that also stimulated synthesis and secretion of some of these UTEC-derived polypeptides. Attachment of sperm cells to monolayers increases intracellular calcium levels in the UTEC, and may enhance cell-to-cell communication through gap junctions between the epithelial cells [25]. These changes could be involved in stimulating increased protein synthesis from the UTEC. It has been suggested that tubal factors control sperm cell capacitation in order to synchronize gamete maturation and union [26–29]. Proteins produced in direct response to the presence of sperm cells in the uterine tube, as demonstrated here, might well indicate this type of synchronization.

The new proteins found after uterine tube epithelial cells were exposed to sperm cells, either directly in co-culture or by membrane separation, may also represent enzymatic degradation products of UTEC proteins. Proteases released either from the sperm cell surface or from acrosomal contents during cell degeneration in culture could influence the protein profiles found in this study. However, the high-molecular-mass polypeptides observed did not differ between the control and co-cultured UTEC, suggesting that at least total degradation of a large class of proteins did not occur. Further studies will need to identify the mRNA for

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TABLE 1. UTEC proteins modulated by sperm cell co-culture.

Protein[a]	kDa	pI	Group 3 UTEC + Bound sperm	Group 4 + Partitioned sperm
1	21	7.0	new	new**
2	24	7.2	new	new
3	24	7.5	new	new
4	19	4.6	new	nc
5	41	5.6	new	new
6	59	5.8	new	new
7	59	5.3–5.6*	inc	inc**
8	33	6.5	new	new**
9	28	5.7	inc	inc**
10	30	4.5	new	new**
11	24	7.3	inc	inc
A	23	5.4	dec	nc
B	22	5.0	dec	nc
C	45	4.2	dec	dec**
D	45	4.5–4.9*	dec	dec
E	45	5.3–5.6*	dec	dec
F	49	6.0	dec	nc
G	45	4.4	dec	dec**

[a]Radiolabeled polypeptides were resolved and visualized by two-dimensional PAGE and fluorography (Figs. 1 and 2). Detailed visual appraisal of the resulting fluorograms revealed that the synthesis and subsequent appearance of the above proteins in the culture medium were affected by co-culture with sperm cells. Responses relative to control UTEC cultures are denoted as either newly synthesized (new), increased synthesis (inc), decreased synthesis (dec), or unchanged (nc).

*Proteins 7, D, and E each represent several polypeptides having small pI differences but essentially identical kDa values.

**Response to co-culture with partitioned sperm cells less than that obtained with attached sperm cells.

these novel proteins in the UTEC to confirm that actual synthesis is the reason for their occurrence after sperm cell co-culture. The present study was not designed to determine, on the basis of the numbers of sperm bound, whether or not a quantitative response of UTEC existed. Clumps of UTEC obtained by tubal lavage were used for monolayer establishment, making it impossible to ascertain the number of UTEC used to seed each culture well. Furthermore, the number of sperm cells that bound to the UTEC during the initial 30-min incubation was not determined. In previous studies, 43% of the sperm cells added to co-culture wells bound to the UTEC under similar culture conditions [16]. Culture of UTEC on an extracellular matrix results in superior polarization of the cells, resulting in more sperm bound to these UTEC than to UTEC cultured on plastic [18]. However, UTEC cultured on plastic as well as frozen-thawed and passaged UTEC have been used by the authors to capacitate sperm cells in vitro [16]. The long-term objective

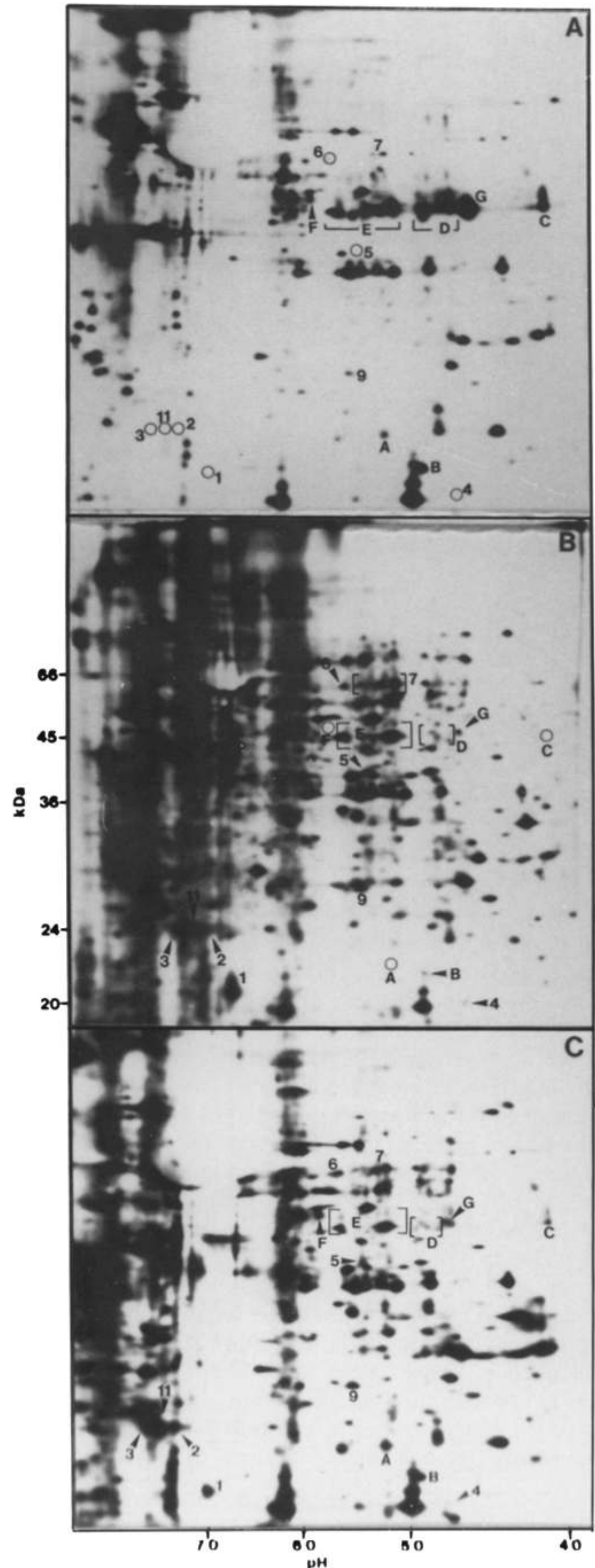

FIG. 2. The effects of sperm cell co-culture and/or sperm cell binding on UTEC protein synthesis. Proteins are identified as in Figure 1 and Table 1. Panel A: group 2, control UTEC proteins released into culture medium. Panel B: group 3, UTEC-sperm cell co-culture labeled in the presence of adherent sperm cells. Panel C: group 4, UTEC with sperm cells that were partitioned from the epithelial monolayer by a diffusible membrane insert during the labeling period. The presence of partitioned sperm cells resulted in an intermediate protein profile, with the production of several new polypeptides in response to the unattached sperm cells.

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of this work has been to develop an efficient, highly repeatable co-culture system using UTEC cell lines for in vitro capacitation of sperm cells. For this reason, the interaction of sperm cells with UTEC cultured on plastic was evaluated.

Previous experiments have shown that UTEC have similar secretory profiles in vivo and in vitro [4, 30, 31]. However, total output of polypeptides appeared lower in culture, at least when uterine tubal epithelia from estrus-phase cows were examined. In comparison with findings from previously published studies of de novo protein synthesis and secretion by tubal explants, an unexpectedly high number of labeled proteins are seen in conditioned medium from cultured UTEC monolayers. Several explanations for this difference are possible. The possibility exists that not all of the proteins detected here represent legitimate secretory proteins; some may be released into the medium upon cell death or damage during co-culture and subsequent washings. An additional explanation may be that during establishment of UTEC monolayers from tubal isthmic lavage materials, the ratio of secretory to nonsecretory cells is altered from that found in vivo, resulting in a greater secretory potential for the monolayers than in intact tubal epithelium. We must also consider that qualitative changes in protein synthesis resulted from the long-term culture required to establish stable monolayers (7–10 days). Although the tubal isthmus was isolated from follicular-phase cows, no attempt was subsequently made to maintain a steroidal/hormonal environment that would mimic in vivo conditions. Nonetheless, the protein profiles presented in the current study were consistently found when culture medium was analyzed by two-dimensional PAGE; and the polypeptides identified in Figures 1 and 2 have been shown repeatedly (four replicates) to be influenced by sperm cell co-culture. Subsequent to collection of the data presented in this report, an additional four replicates of each group were performed. The additional studies confirmed the results presented here.

Polypeptides that were seen with control UTEC (group 2), but not in the co-culture conditioned medium, may have been absent for one of two reasons. Either their synthesis and/or secretion was decreased by sperm cell attachment to the UTEC, or these proteins adhere to sperm cell membranes and are hence removed from the medium. Tubal proteins do bind to sperm cells in other species studied [5, 6]. It has been hypothesized that proteins that adhere to sperm cells may enzymatically enhance receptors on the sperm surface that are involved in gamete union, such as galactosyltransferase [32]. The UTEC proteins D, E, and G, which exhibit marked decreases in response to sperm cell co-culture irrespective of cell contact and binding, may be similar or identical to oviductal secretory proteins previously reported from cows [12, 15], sheep [33], and pigs [34]. Each of those studies demonstrates that uterine tubal epithelial cells and/or explants synthesize and secrete a protein or family of related polypeptides having molecular mass values between 43 and 47 kDa and a pI range of approximately 4 to 5.8. In the cow and ewe, peak production of these proteins occurs between Days 3 and 5 of the estrous cycle, while in the gilt a similar polypeptide group appears by Day 2 and diminishes only after Day 15. Gandolfi et al. [33] also demonstrated binding of the 46-kDa secretory protein to the zona pellucida of sheep embryos both in vivo and in vitro. In preliminary studies involving equine UTEC and stallion sperm cell co-culture, we have observed a 45–47-kDa tubal isthmic secretory protein that binds to sperm cells. This protein has been detected both by exposing sperm cells to radiolabeled medium conditioned by UTEC and by surface iodination of sperm cells previously bound to UTEC monolayers. The similarity in both pI and molecular mass of the proteins suggests that they are related. However, the significance and possible biological role of polypeptides that bind to both sperm cells and oocytes or embryos is at this time unresolved.

Tubal fluid obtained in vivo from estrous cows that have not been inseminated can induce capacitation of sperm cells in vitro, in a dose-dependent fashion [9, 35]. Therefore, the new proteins triggered by the presence of sperm cells in the present experiment are not necessarily required for capacitation to occur. Capacitation requires the sperm cells to be present in the female tract for a finite amount of time, which varies among species, irrespective of whether ovulation has occurred [36]. In fact, the tubal environment may be required not for the induction of capacitation, but rather for its regulation. In many species, including humans, viable sperm cells can be stored in the female tract for days before ovulation [1]. Therefore, mechanisms must act to prevent sperm from capacitating or degenerating before fertilization occurs. A temporal arrest of sperm cell capacitation before ovulation has been shown [28, 29, 37]. Glycoproteins from the UTEC associated with sperm cells may help prevent premature acrosome breakdown, inhibit complement-mediated antibody formation against sperm cells, and enhance gamete recognition by improving cell-to-cell recognition [6, 12, 35]. Furthermore, longevity of bull sperm cells is enhanced when they are in contact with UTEC in vitro [16, 18]. New proteins synthesized by the UTEC in response to the sperm cells may maintain viability of the sperm cells in the female at internal body temperatures.

In conclusion, sperm cell attachment to isthmic uterine tube epithelial cells in culture appeared to stimulate synthesis and secretion of a cohort of proteins from the epithelial cells. A portion of these proteins were also produced in response to the presence of sperm cells that were separated from the UTEC by a diffusible membrane. Concomitantly, the synthesis of some UTEC proteins appeared to be decreased or abolished in the presence of sperm cells. Tubal epithelial cell proteins presumptively produced in response to sperm cell co-culture will be evaluated in the future in terms of their role in sperm cell storage, capacitation, and subsequent gamete union.

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