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Original Research Article

# Bovine oviductal epithelial cells: Long term culture characterization and impact of insulin on cell morphology

S. Palma-Vera *, R. Einspanier, J. Schoen

*Freie Universität Berlin, Institute of Veterinary Biochemistry, Oertzenweg 19b, 14163 Berlin, Germany*

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ABSTRACT

*In vitro* models that resemble cell function *in vivo* are needed to understand oviduct physiology. This study aimed to assess cell functions and insulin effects on bovine oviductal epithelial cells (BOECs) cultured in an air–liquid interface. BOECs ($n = 6$) were grown in conditioned Ham's F12, DMEM or Ham's F12/DMEM with 10% fetal calf serum (FCS) for 3 weeks. After selecting the most suitable medium (Ham's F12), increasing insulin concentrations (1 ng/mL, 20 ng/mL and 5 μg/mL) were applied, and cell morphology and trans-epithelial electrical resistance (TEER; $n = 4$) were evaluated after 3 and 6 weeks. Keratin immunohistochemistry and mRNA expression of oviductal glycoprotein 1 (OVGP1) and progesterone receptor (PGR) were conducted ($n = 4$) to assess cell differentiation. BOECs grown without insulin supplementation or with 1 ng/mL of insulin displayed polarization and secretory activity. However, cells exhibited only 50% of the height of their *in vivo* counterparts. Cultures supplemented with 20 ng/mL insulin showed the highest quality, but the 5 μg/mL concentration induced massive growth. TEER correlated negatively with insulin concentration ($r = -0.459$; $p = 0.009$). OVGP1 and PGR transcripts were still detectable after 3 and 6 weeks. Cellular localization of keratins closely resembled that of BOECs *in vivo*. Cultures showed heterogeneous expression of PGR and OVGP1 in response to estradiol (10 pg/mL). In summary, BOECs grown for long term in an air–liquid interface expressed markers of cell differentiation. Additionally, insulin supplementation (20 ng/mL) improved the cell morphology *in vitro*.



## 1. Introduction

The increase in milk yield observed over the last 40 years in dairy cattle has been accompanied by a decline in cow fertility. As reported by Diskin et al. [1], the majority of Holstein Friesian cows are not able to successfully carry pregnancy after insemination. In this study, reproductive failures resulted from early embryonic death (43%), fertilization failure (10%) and late embryonic death (7%), leaving only 40% of inseminations ending

* *Corresponding author*. Tel.: +49 30 838 62507; fax: +49 30 838 62584.
E-mail addresses: sepalma@zedat.fu-berlin.de, serpalma.v@gmail.com (S. Palma-Vera).
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in successful calving. The oviduct is the first site of contact with the early embryo and provides important factors that directly affect fertility [2]. Final maturation and transport of gametes, fertilization and the early cleavage-stage of embryonic development are events that occur in the microenvironment provided by the oviduct (for review, see [3]).

Cell culture models that resemble the *in vivo* morphology and functions of the oviduct are needed for better understanding of oviductal physiology. Monolayer and suspension cultures of bovine oviductal epithelial cells (BOECs) have been frequently described. Monolayer cultures show rapid loss of cilia (3–4 days of culture) and secretory granules as they become adherent and start to proliferate. In suspension cultures, cells do not attach to the plate, and mitosis does not occur [4,5]. As a result, dedifferentiation is prevented, and cells maintain cilia and secretory granules. In such conditions, cells can be grown up to 12 days without losing their differentiation state [4].

Under physiological conditions, hormones transported by the blood reach the oviductal epithelium from the basolateral surface and regulate cell functions [6]. For instance, during the estrous cycle, the oviduct is under the influence of ovarian sex steroids, which induce morphological and biochemical changes to establish a suitable environment for fertilization and embryonic development. In particular, during the follicular phase, under the influence of estrogens, protein synthesis in the oviduct reaches its highest levels [7], including the expression of oviductal glycoprotein 1 (OVGP1) and progesterone receptor (PGR) [3,8]. In order to recreate this environment *in vitro*, suitable culture conditions must be employed.

Compartmentalized cell culture systems are suitable to induce cell differentiation *in vitro* [9–11]. These systems provide an apical and a basolateral compartment, resembling the *in vivo* state of epithelial cells, allowing hormones to affect the cells in a way comparable to natural conditions. Recently, Miessen et al. [11] successfully established a protocol for the long-term culture of porcine oviductal epithelial cells (POECs) using a compartmentalized air–liquid interface culture system. As a result, cells could be grown for a long period, achieving an *in vivo*-like phenotype. In a similar study, bovine oviductal explants, grown in an air–liquid system showed long-term viability and differentiation, demonstrated by the presence of cilia [12]. In this study, we attempted to adapt the protocol initially developed for porcine oviduct cells as described earlier [11] to the bovine system in order to induce BOEC differentiation *in vitro*. We assessed the differentiation state of cultures by morphological evaluation, expression of specific marker genes and cell responses to estradiol up to six weeks in culture.

## 2. Materials and methods

All media, antibiotics and sera for cell culture were supplied by Biochrom AG, Berlin, Germany. All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated.

### 2.1. Isolation of BOECs and tissue collection

Bovine oviducts were collected in a local slaughterhouse from healthy cows (Holstein Friesian), approximately 15 min after death. Based on ovarian morphology [13], only cycling cows in the luteal phase of the estrous cycle were included in the study. Oviducts were trimmed free of surrounding tissue, washed in Dulbecco's PBS and transported on ice to the laboratory within 2 h. Isolation of oviductal epithelial cells was conducted as previously described [11]. Briefly, ipsi- and contralateral oviducts were filled with collagenase 1A (1 mg/mL Ham's F12 medium) and incubated at 37 °C. After 1 h, oviducts were squeezed and epithelial cells of each individual were pooled. The cell suspension was diluted in Ham's F12 and passed through a 40-μm pore size strainer to capture the cell fraction containing clusters of epithelial cells. This fraction was collected, suspended in Ham's F12 and washed with Dulbecco's PBS. After centrifugation (200 × *g*; 10 min), cells were incubated with 0.5% trypsin/EDTA at 37 °C for 10 min, the reaction was stopped with pure fetal calf serum (FCS) and cells were filtered again. The flow-through was collected in vials and suspended in freezing medium (Cryomaxx NT, PAA Laboratories GmbH, Cölbe, Germany) at a density of $1 \times 10^6$ cells/mL and frozen at a rate of 1 °C/min down to −80 °C before storage in liquid nitrogen until further use. In addition, tissue samples (ampulla) from oviducts ipsilateral to the active ovary were collected and fixed in Bouin's solution.

### 2.2. Media selection for long-term culture of BOECs

Based on the results of preliminary trials (data not shown), three media (Ham's F12, DMEM and DMEM/Ham's F12) were tested to determine the most suitable long-term culture conditions for BOECs ($n = 6$, independent experiments). Each medium was completed with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin, 1 μg/mL amphotericin B, 10 μg/mL reduced glutathione and 10 μg ascorbic acid and combined with conditioned medium (2:1; v/v). Conditioned medium was produced as described previously [11]. The media were named as M1 (complete Ham's F12), M2 (complete DMEM) and M3 (complete DMEM/Ham's F12). BOECs were grown for 3 weeks as described in the following section. Selection of medium was based on cell height and cell morphology: presence of secretory granules, cilia, microvilli, apical protrusions and polarization (defined as cells whose vertical axes were larger than their horizontal axes).

### 2.3. Culture of BOECs and selection of growth medium

Frozen vials were thawed in warm water (37 °C), diluted in 5 mL of pre-warmed medium (M1, M2 or M3) and centrifuged (200 × *g*; 10 min). The supernatant was discarded, cells were resuspended in M1, M2 or M3 at a concentration of $2 \times 10^5$/200 μL and seeded on millicell hanging inserts (PET membrane, 0.4-μm pore size, Millipore, Temocura, Canada). Each insert was previously placed into a well (24-well plate, Greiner Bio-One GmbH, Frickenhausen, Germany) containing 1 mL of the corresponding medium. After seeding the cells, 200 μL of medium was inside the insert and 1 mL was on the outside (basolateral compartment). Cells were incubated in a humid chamber at 37 °C and 5% $CO_2$. After one week of preculture, the medium from inside the insert was removed, allowing the cells to be in contact with the medium only from the basolateral