Equine oviduct explant culture: a basic model to decipher embryo–maternal communication

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Abstract. Equine embryos remain for 6 days in the oviduct and thus there is a need for an \textit{in vitro} model to study embryo–oviductal interactions in the horse, since this subtle way of communication is very difficult to analyse \textit{in vivo}. Until now, no equine oviduct explant culture model has been characterised both morphologically and functionally. Therefore, we established a culture system for equine oviduct explants that maintained epithelial morphology during 6 days of culture, as revealed by light microscopy and transmission electron microscopy. We demonstrated the presence of highly differentiated, tall columnar, pseudostratified epithelium with basal nuclei, numerous nucleoli, secretory granules and apical cilia, which is very similar to the \textit{in vivo} situation. Both epithelium and stromal cells originating from the lamina propria are represented in the explants. Moreover, at least 98% of the cells remained membrane intact and fewer than 2% of the cells were apoptotic after 6 days of culture. Although dark-cell degeneration, which is a hypoxia-related type of cell death, was observed in the centre of the explants, quantitative real-time PCR failed to detect upregulation of the hypoxia-related marker genes \textit{HIF1A}, \textit{VEGFA}, \textit{uPA}, \textit{GLUT1} and \textit{PAI1}. Since the explants remained morphologically and functionally intact and since the system is easy to set up, it appears to be an excellent tool for proteome, transcriptome and miRNome analysis in order to unravel embryo–maternal interactions in the horse.

Additional keywords: dark-cell degeneration, horse.

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Introduction

Co-culture of bovine embryos with somatic cells has been used extensively in the early 1990s; since at that time, it has been the only way to overcome the 8- to 16-cell block (Eyestone and First 1989; Eyestone \textit{et al.} 1991). This indicates that the culture media used then were suboptimal for bovine embryo development and had to be ‘conditioned’ first by somatic cells. For a few years, co-culture became a routine technique to produce embryos \textit{in vitro} and in many cases, oviduct epithelial cells were used e.g. in cattle (Van Soom \textit{et al.} 1992) and in pigs (Hardy and Spanos 2002). Also in the horse, the benefits of oviduct co-culture were investigated using \textit{in vivo}-collected equine embryos (Ball \textit{et al.} 1991, 1993; Ball and Miller 1992; Brinsko \textit{et al.} 1994). At the end of the 1990s, semi-defined media were developed that were equally suited to culture embryos from the zygote to the blastocyst stage \textit{in vitro}, with blastocyst rates of ~35% reported for cattle embryos (in modified SOF with BSA; Lonergan \textit{et al.} 1991; Holm \textit{et al.} 1996). Horse embryos produced by intracytoplasmic sperm injection (ICSI) can be successfully cultured to the blastocyst stage in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) plus serum (Yuan \textit{et al.} 2003; Choi \textit{et al.} 2004, 2006; Hinrichs and Choi 2005; Smits \textit{et al.} 2012) with percentages ranging between 10 and 35%. Since then, embryo–somatic cell co-culture has been abandoned and is considered to be a less desirable technique because it is labour intensive, may introduce pathogens and is less defined than cell-free medium (Bavister 1995; De Pauw \textit{et al.} 2003). However, in view of the fact that the
oviductal environment represents the optimal environment for early embryo development, we want to reconsider the use of oviduct epithelial explants, especially for co-culture in in vitro-produced horse embryos to enable the study of embryo–maternal communication in the horse at the level of gene expression in an in vitro model that approximates the in vivo situation as closely as possible.

At present, molecular biology has progressed enormously compared with twenty years ago, making it possible to analyse subtle changes in gene expression at the level of single embryos or using very low amounts of tissue (Merkel et al. 2010). Unfortunately, this does not allow us to study embryo–maternal interactions in vivo, “because some interactions are very short or very local, making it very challenging to localise an embryo of only a few hundred µm in diameter in a large-size reproductive tract” (Ulbrich et al. 2013). For the reasons mentioned above, we think it is timely now to reintroduce oviduct explants in horse-embryo culture, not for the purpose of increasing embryo production, but to study signalling events during the first embryonic cleavage divisions in vitro. To this end, the most important prerequisite is to preserve the 3D architecture of the oviduct by preventing epithelial cell dedifferentiation. Since monolayers dedifferentiate with a concomitant loss of important morphological characteristics, an explant culture system more closely resembles the in vivo situation (Walter 1995), since it is produced by stripping epithelial folds from the oviduct, which consist of both epithelial and stromal cells. In order to be used as an in vitro model to study embryo–oviduct interactions in the horse, the oviduct explants have to remain viable and exert normal gene expression for ~6 days, which is the time spent in the oviduct by the horse embryo. One salient feature of the equine oviduct explants was that many of them showed central darkening after a few days of culture, while still displaying vigorous ciliary activity, a feature that we had never observed with bovine oviduct explants (De Pauw et al. 2002). Therefore the aims of this study were: (1) to characterise equine oviduct explants morphologically by means of light, immunofluorescence and transmission electron microscopy over time and (2) to assess whether the central dark zones in the explants were induced by hypoxia by quantifying the expression of hypoxia-related genes with an embryotrophic function in explants at Day 6, that the dark zones are caused by dark-cell degeneration and that hypoxia-related gene expression is not changed during culture, indicating that culture conditions are not hypoxic. The equine oviduct explant model is at present being used to study the importance of endocrine and paracrine signalling during early embryo development in the horse.

Materials and methods

Animals

Oviducts (three replicates of 7 to 10 oviducts) were obtained at a slaughterhouse from healthy warmblood mares aged 5–22 years and without any visible reproductive tract pathologies. Only ipsilateral oviducts of mares showing a recent corpus luteum or a corpus hemorrhagicum on the ovaries, indicating ovulation had occurred not later than 5 days earlier (Pierson and Ginther 1985), were used. Determination of progesterone concentration by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC/MS-MS) in another study (Nelis et al. 2012) revealed a positive predicting value of 94% between the presence of a corpus hemorrhagicum and a progesterone concentration lower than 2 ng mL⁻¹.

Preparation and morphological evaluation of oviduct explants

Oviducts of mares were trimmed of excess connective tissue, closed at both ends and transported in sterile 0.9% saline and gentamycin (50 µg mL⁻¹; Invitrogen, Merelbeke, Belgium) on ice. In the laboratory oviducts were washed in phosphate-buffered saline (PBS) and the epithelial cells were obtained by scraping the ampullary–isthmic region of the longitudinally opened oviduct.

The harvested cellular material was put in a tube containing home-made 10× 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Tyrode’s albumin–lactate–pyruvate (TALP) and was left to settle for 10 min, after which the cell pellet was resuspended in 3 mL of fresh HEPES-buffered TALP washing medium. The process of sedimentation was repeated twice. Afterwards, the harvested cellular material was washed and cultured in DMEM/F12 (Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen), 50 µg mL⁻¹ gentamycin and 2.5 µg mL⁻¹ amphotericin-B (Fungizone; Invitrogen) at 38.5°C in a humidified atmosphere with 5% CO₂ in air. The time-span from slaughter of mares to seeding of cells was ~3 to 4 h. The DMEM/F12-FBS culture medium was refreshed the first and the fourth day after cell isolation. Only cultures with more than 99% of membrane-intact cells, determined by Trypan blue staining (Sigma-Aldrich, Diegem, Belgium), 2 to 4 h after start of culture (= Day 0) were used. In the first experiment we compared two culture systems: the first were 50-µL droplets under oil containing ~5 explants with a diameter less than 200 µm per droplet and the second was identical to the first but contained only one vesicle with a diameter larger than 600 µm. Frozen damaged explants were used as a membrane-damaged control. These explants were subjected to three subsequent freezers (~20°C) – thaw (38°C) cycles in PBS. Every 24 h, 20 to 30 explants were fixed in 4% formaldehyde for 24 h and pro- nessed for haematoxylin–eosin staining. Next, ciliary activity and central dark-zone formation, as shown in Fig. 1a–c, was evaluated every 24 h in the whole number of explants present in at least 20 droplets, containing 20–30 explants each. Explants were considered to show ciliary activity when bordered by vigorously beating cilia, clearly seen on the inverted microscope (400× magnification). To lower interpretative bias, all explants were counted by two researchers and the mean value was used for statistical analysis.

Fluorescence microscopy

To determine the presence of membrane-damaged cells, 10 explants were stained after every 24 h of culture with the nucleic stain SYBR14 and propidium iodide (PI; LIVE/DEAD Sperm Viability Kit; Molecular Probes, Leiden, The Netherlands; Garner et al. 1994). The explants were washed in HEPES-buffered washing medium, incubated in 5 µL of a 1 : 50 SYBR14
dilution in HEPES-buffered washing medium, for 15 min at 37°C, followed by a 5 min incubation with 5 μL PI. The stained explants were mounted in 1,4-diazabicyclo[2.2.2]octaan (DABCO) on siliconised glass slides. Explants that were frozen in PBS at −20°C for 24 h, subsequently thawed at 38°C and stained were used as a membrane-damaged control. To detect apoptotic and necrotic cells, epithelial explants showing dark zones after 6 days of culture were stained with a combination of Hoechst, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and anti-caspase based on previous publications (Gjørret et al. 2007; Vandaele et al. 2007; Wydooghe et al. 2011). Explants were fixed in 4% paraformaldehyde for 20 min at room temperature and stored in PBS containing 0.5% bovine serum albumin (PBS–BSA; Sigma–Aldrich, Bornem, Belgium) at 4°C until the staining was performed. Positive-control explants were incubated overnight with 0.5 μM staurosporine. Fixed explants were permeabilised with 0.5% Triton X-100 (Sigma–Aldrich, Bornem) in PBS for 1 h and washed again in polyvinylpyrrolidion (PVP) solution. The explants were held for 1 h in 0.5% Triton X-100 in PBS at room temperature. Subsequently, the explants were washed three times for 2 min in PBS–BSA. After washing, the explants were blocked overnight in 10% goat serum (Invitrogen) and 0.05% Tween 20 in PBS at 4°C. Explants serving as negative controls remained in blocking solution. The test explants were washed two times for 15 min at room temperature and incubated overnight at 4°C in rabbit active caspase-3 antibody (0.768 μg mL⁻¹ in blocking solution; Cell Signalling Technology, Leiden, The Netherlands). After another wash step (two times for 15 min), test explants and negative controls were transferred to goat anti-rabbit Texas Red antibody (20 μg mL⁻¹ in blocking solution; Molecular Probes, Merelbeke, Belgium) for 1 h at room temperature. For TUNEL staining, positive and negative controls were treated with DNase (50 U mL⁻¹ in PBS) for 1 h at 37°C to ensure

Fig. 1. (a, c) Inverted microscopic image of equine oviduct explants after 1 day of culture and (b) after 6 days of culture, showing central dark zones. (d) Trypan blue and (e) SYBR14 staining revealed that more than 98% of the cells were membrane intact after 6 days of culture (green in SYBR14, translucent in Trypan blue staining). (f) Very few membrane-damaged cells were stained orange by propidium iodide. (g) Confocal transmission view of the combined staining of TUNEL (green) and anti-caspase-3 (orange; arrow). (h) Confocal 3D view of oviduct explants stained with Hoechst, TUNEL and anti-caspase-3. Fewer than 2% of the cells in the explants were TUNEL and/or anti-caspase-3 positive after 6 days of culture. (i) Staining negative confocal transmission view for background autofluorescence subtraction. (a, b, h) Bar = 100 μm; (c) Bar = 50 μm; (d–g, i) Bar = 20 μm.
Table 1. Primers for target genes
For each gene the NCBI GenBank accession number, the sequence of both forward and reverse primers, the size of the amplicon and the optimal primer annealing temperature are listed. F, forward primer; R, reverse primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Primer sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>Ta (°C)</th>
<th>Efficiency (%)</th>
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<tr>
<td>uPA LOC100072886</td>
<td>XM_001502951.3</td>
<td>F: AAAGTCCTCCTCCTCCTC&lt;br&gt;R: CGAAGAAGGAGGACTACATT</td>
<td>249</td>
<td>61</td>
<td>92</td>
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<tr>
<td>VEGFA</td>
<td>NM_001081821.1</td>
<td>F: ACTCCGGTCAAATCGAGA&lt;br&gt;R: ATACAAACCTCACAAAGCCA</td>
<td>193</td>
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<td>97</td>
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<tr>
<td>Hypoxia-inducible factor 1-α-like LOC100061166</td>
<td>XM_001493206.3</td>
<td>F: TGCTGGGACACAATCATA&lt;br&gt;R: GAGTTTCAGAGGCAGGTAAT</td>
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<td>59</td>
<td>87</td>
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<tr>
<td>GLUT1</td>
<td>NM_001163971.1</td>
<td>F: CCGAAGGTTGATCGAGGAAT&lt;br&gt;R: CAGTTTTGGAAGGCCCATGGA</td>
<td>238</td>
<td>60</td>
<td>118</td>
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<tr>
<td>PAI1</td>
<td>XM_001492517.3</td>
<td>F: ACTCCGGAGAGGATCAGAGA&lt;br&gt;R: CAGGTTGGACTTTCAGAGGGT</td>
<td>223</td>
<td>61</td>
<td>87</td>
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</tbody>
</table>

Detection of strand breaks by TUNEL (In Situ Cell Detection kit; Boehringer, Mannheim, Germany). After washing, positive controls and samples were incubated in fluorescein (FITC)-dUTP and terminal deoxynucleotidyl transferase for 1 h at 37°C in the dark. Meanwhile, the negative control was incubated in nucleotide mixture only in the absence of transferase. After a second washing, controls and samples were incubated in RNase in PBS) for 1 h at room temperature.

The explants were washed twice and transferred to Hoechst 33258 (50 µg mL⁻¹ in PBS–BSA; Molecular Probes, Life Technologies Europe B.V., Ghent, Belgium) for 10 min at room temperature. Evaluation of the explants was performed the next day by fluorescence microscopy, with a U-M3DAFITR filter for 488 nm laser, FITC (525/50 filter, 488 nm laser) and Texas Red (595/50 filter, 561 nm laser). The number of cells of at least 10 explants from each of the three replicates was determined by counting the Hoechst-positive cells. Next, the percentage of anti-caspase-3 and TUNEL-positive cells was determined by counting all the Hoechst-, TUNEL- and anti-caspase-3-positive cells in at least 10 explants. After counting, per explant, the ratio of TUNEL- and/or anti-caspase-3-positive cells of the Hoechst-positive cells was determined. Images were acquired with a C1si confocal laser-scanning microscope (Nikon, Brussels, Belgium) using a Plan Apo 40× objective with a numerical aperture of 0.95 (Nikon). All filters and lasers were purchased from CVI Melles Griot (Albuquerque, NM, USA).

Transmission electron microscopy
At Days 0, 3 and 6 of culture, oviduct explants were fixed in 0.2 M sodium cacodylate-buffered formaldehyde and post-fixed with osmium tetroxide. After rinsing, cells were pelleted by centrifugation (for 5 min at 200g at room temperature) in 10% BSA supplemented with 1% glutaraldehyde. The pellet was then dehydrated and embedded in epoxy resin (LX-112; Ladd Industries, Williston, ND, USA). Sections were made with a Reichert Jung (Depew, NY, USA) Ultracut E ultra-microtome.

Semi-thin sections (2 µm) were stained with toluidine blue to select the most appropriate regions for ultrathin sectioning. Next ultra-thin sections (90 nm) were made and stained with uranyl acetate and lead citrate solutions before examining under a JEOL EX II transmission electron microscope (JEOL Europe, Zaventem, Belgium) at 80 kV.

RNA extraction and quantitative RT-PCR
Prime design and validation, RNA extraction and quantitative reverse transcription–polymerase chain reaction (RT-PCR) were performed according to the MIQE-guidelines (Bustin et al. 2009). Oviduct explants were cultured in 50-µL drops in DMEM/F12–10% FBS. At Days 0 and 6 of culture, between 40 to 70 explants per sample (n = 12 per group) were washed in Dulbecco’s Phosphate-Buffered Saline (DPBS; Gibco BRL Invitrogen, Life Technologies Europe B.V., Ghent, Belgium) and conserved at −80°C in RNase-free water containing 10% RNasin Plus RNase inhibitor (Promega, Leiden, The Netherlands), 5% dithiothreitol (Promega) and 0.8% Igepal CA-630 (Sigma-Aldrich, Bornem) until analysis.

The primers (Table 1; Integrated DNA Technologies, Leuven, Belgium and Sigma-Aldrich, Bornem, Belgium) for the genes of interest (VEGFA, HIF1A, GLUT1, PAI1, uPA) were designed by means of Primer3 software (Misener et al. 1999; http://frodo.wi.mit.edu/primer3/, accessed 10 May 2011), based on horse sequences found in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/, accessed 10 May 2011). To distinguish genomic DNA amplification, to provide specificity and to avoid secondary structures in the primer region, primers were selected over intron–exon boundaries, tested using a BLAST analysis against the NCBI database and characterised with MFold (Zuker 2003; http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form, accessed 10 May 2011). The amplicons were run on a 2% agarose gel and confirmed by nucleotide sequencing. All primers are listed in Tables 1 and 2.

All RT-qPCR reactions were performed in duplicate with 2.5 µL of sample, 7.5 µL of the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA), 0.6 µL of 5 or 10 µM forward and reverse primer and 3.8 µL water. A blank, a melting curve and a 5- or 10-fold serial dilution series of pooled oviductal cDNA were included for each gene to check for contamination and specificity and to acquire
PCR efficiencies (Tables 1, 2) based on a relative standard curve. Calculation of the Cq values, PCR efficiencies, correlation coefficients and analysis of the melting curves was performed by means of iCycler iQ Optical System Software Version 3.0a (Biorad, Nazareth, Belgium). All quantification cycle (Cq) values were converted into raw data using these PCR efficiencies and normalised by dividing them by their respective normalisation factors. This normalisation factor was determined per sample by calculating the geometric mean of the validated reference genes as determined for each type of explant. Therefore, a set of stable reference genes was identified in 10 samples of post-ovulatory oviduct explants at Day 0 and in 10 samples after 6 days of culture. Eight reference genes were selected based on previous studies (Goossens et al. 2005; Bogaert et al. 2006; Cappelli et al. 2008; Smits et al. 2009). The selected genes (ACTB, GAPDH, HPRT1, RPL32, SDHA, TUBA4A, 18S and UBC; Table 2) belonged to different functional classes, which reduced the chance of co-regulation. Primers for ACTB, GAPDH, HPRT1, RPL32, SDHA, TUBA4A and UBC were provided by Bogaert et al. (2006). Primers for GAPDH, SDHA and 18S were available from previous research in equine blastocysts (Smits et al. 2009).

The mRNA expression of five genes – glucose transporter 1 (GLUT1), vascular endothelial growth factor (VEGFA), hypoxia-inducible factor 1a subunit (HIF1A), plasminogen activator inhibitor 1 (PAI1) and urokinase plasminogen activator inhibitor (uPA) – was evaluated. These genes are upregulated under hypoxic culture conditions and can be used as marker genes for hypoxia. Moreover, these factors play an important role in early embryonic development (Carmeliet et al. 1996; Wrenzycki et al. 2001; Buhi 2002; Liao et al. 2007), so down-regulation of these genes could indicate a loss of functionality of the cells.

**Table 2. Primers for reference genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Primer sequence (5′–3′)</th>
<th>Amplicon size (bp)</th>
<th>Ta (°C)</th>
<th>Efficiency (%)</th>
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<td>ACTB</td>
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<td>HPRT1</td>
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<td>RPL32</td>
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<td>R: CTAACAGGCACCCCTGAGA</td>
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<td>18S</td>
<td>AJ311673</td>
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**Measurement of glucose and lactic acid consumption by oviduct explants**

Oviduct explants were cultured in 5% CO2 in air or in 5% CO2, 5% O2 or in 90% N2 at 38°C as described earlier. At Days 0, 1, 3 and 6 of culture, samples of culture medium were taken and frozen at −80°C until analysis using an UV enzymatic method using the Roche Cobas 8000 according to the manufacturer’s recommendations (Roche Diagnostics, Mannheim, Germany). A calibration line to determine the efficiency was drawn up along with 10 samples per day of sampling. The efficiency of the UV enzymatic analyses was 98% for glucose and 99% for lactic acid. The limit of detection was 0.1 mM for glucose and 0.2 mM for lactic acid.

**Statistical analysis**

For analysis of the Gaussian-distributed data of the morphological evaluation of the explants and glucose and lactic acid concentration, repeated-measures with Greenhouse–Geisser correction with the general linear model procedure was implemented. Post hoc test for multiple comparisons with Bonferroni correction was performed. For gene-expression studies, analysis of variance (ANOVA) and calculation of standard errors of means (s.e.m.) were performed with the general linear model procedure as implemented in SPSS 19 for Windows (SPSS IBM, Brussels, Belgium). Paired-samples t-test and Wilcoxon-signed rank test was used in comparisons of target gene expression differences throughout the culture period, depending on whether or not a Gaussian distribution was obtained after logarithmic transformation of the data. Differences were considered to be significant at P < 0.05. Statistical analysis and graph plotting were performed with SPSS 19. Power analysis and sample-size calculation (α = 0.05; power = 0.90–0.95) for gene-expression studies and other experiments were performed using Piface.
version 1.7 (Lenth 2007; University of Iowa; http://homepage.stat.uiowa.edu/~rlenth/Power/; accessed 28 March 2011), and G*Power 3.1.3 (Faul et al. 2007; Heinrich Heine Universität Düsseldorf; http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/download-and-register; accessed 1 August 2012), respectively.

Results

Morphological features of equine oviductal explants during culture

From 6 h of culture the formation of spherical structures was observed. More than 95% of the explants smaller than 200 μm or larger than 600 μm were still showing ciliary activity after 6 days of culture. Over time, we saw that progressively more explants were showing central darkening (Fig. 1a, b). At Days 5 and 6, a significantly lower percentage of smaller explants was showing central dark zones compared with the larger-sized vesicles ($P = 0.01$; Fig. 2). There was no significant difference ($P = 0.9$) in ciliary activity and the percentage of explants showing central black zones related to culture in low-oxygen atmosphere (90% N₂, 5% CO₂, 5% O₂) and in 5% CO₂ in air (data not shown).

Haematoxylin–eosin staining demonstrated that between Day 0 and Day 6 of culture the explants (Fig. 3a) were bordered by highly differentiated tall columnar epithelial cells with basal nuclei, numerous nucleoli, apical cilia and secretory granules, very similar to ex vivo samples (Fig. 3b). In the spherical explant structures, stromal cells originating from the lamina propria were also observed. Trypan blue (Fig. 1d) and SYBR14/PI (Fig. 1e, f) staining demonstrated that more than 95% of the cells remained membrane intact after 6 days of culture.

Hoechst, TUNEL and anti-caspase-3 staining revealed that only 2% of the cells, scattered over the whole explants, contained fragmented DNA (Fig. 1g, h; green TUNEL-positive cells) and fewer than 1% showed caspase-3-positive cells (Fig. 1g; orange arrow) after a culture period of 6 days.

Transmission electron microscopy confirmed the presence of cell polarity during the 6 days of culture as evidenced by the presence of cilia and microvilli on the apical cell surface and of tight junctions on the lateral cell surfaces (Fig. 4). In explants showing central darkening, originating from mares in the pre- and the post-ovulatory cycle stage, conspicuous dark nuclear and cytoplasmic regions were visible without the hallmark ultrastructural features of either apoptotic or necrotic cell death as described previously (Krysko et al. 2008). In some cases these dark cells were engulfed by neighbouring healthy cells.
Gene expression of equine oviduct explants during culture

Quantitative real-time PCR results of post-ovulatory oviduct explants on Day 0 and Day 6 of culture were normalised against the geometric mean of an optimal number of reference genes.

Results of the reference gene stability, as determined by geNorm, are shown in Fig. 5. The optimal number of genes was determined with geNorm by means of the pairwise variations (V_{n+1}/n) between the sequential normalisation factors (NF_n and NF_{n+1}) after successive inclusion of less-stable reference genes (Fig. 6). The value of the pairwise variations reduces until 0.137 for V_{4.5}. This suggests that the inclusion of a fifth reference gene contributes to the stability. Therefore it is recommended to use the five most-stable genes for RT-qPCR in post-ovulatory oviduct explants. When segregating Day 0 and Day 6, on Day 0 the most stable reference genes were ACTB, UBB, 18S, TUBA4A, SDHA, HPRT and on Day 6, ACTB, SDHA, UBB, RPL32, HPRT were the most stable (data not shown). When both groups of explants (Day 0 and Day 6) were included, the most stable genes were UBB, ACTB, 18S, RPL32 and SDHA. The geometric mean of these latter genes was used for normalisation of the test genes. The M values (Fig. 5) ranged between 0.4 and 0.8, which indicates relatively good stability.

A highly significant downregulation of GLUT1 \((P < 0.0005)\) and a slightly significant downregulation of PAI1 (paired-samples t-test) and uPA (Wilcoxon-signed rank test) was observed after 6 days of culture \((P = 0.03\) and \(P = 0.01\)). Normalised expression values of GLUT1 and PAI1 were logarithmically transformed to obtain a Gaussian distribution. There were no differences in expression levels of HIF1A and VEGFA between Day 0 and Day 6 of culture (Fig. 7).

Fig. 4. Transmission electron microscopic images of oviduct explants. (a) Ex vivo explant, bar 500 nm; (b) explant cultured for 1 day, bar = 500 nm; (c) explant cultured for 5 days, bar = 1 μm; (d) explant cultured for 6 days with dark zone, bar = 2 μm. During the whole culture period, the explants were bordered by highly differentiated epithelial cells with apical cilia (ci) and microvilli (Mv), intact junctional complexes (arrowheads) and numerous mitochondria (M) and rough endoplasmic reticulum (RER). Explants showing central dark zones contain cells undergoing dark-cell degeneration (D), hallmarked by their shrunken and very electron-dense appearance without recognisable organelles in the cytoplasm, their nuclei containing condensed chromatin and areas of vacuolisation in the cytoplasm.
Glucose and lactic acid changes during culture

After 3 days of culture glucose consumption was 5.4 ± 0.8 mM, so the glucose concentration in the culture medium had significantly dropped from 17 mM to 11.6 ± 0.8 mM. Lactic acid concentrations increased from 0 to 4.4 mM in 5% CO2 in air (P < 0.005). Glucose and lactic acid concentration did not change during the subsequent 3 days of culture (P = 0.9). In 5% O2, 5% CO2 and 90% N2, glucose consumption significantly increased to 8.3 mM on Day 3 and to 11.3 mM on Day 6 (P < 0.005 and 0.0005, respectively) whereas lactic acid concentration increased to 18.8 mM (P < 0.0005) on Day 3 and did not change during the following 3 days of culture (Fig. 8).

Discussion

This study is the first to describe the morphology, ultrastructure, glucose consumption, lactic acid production and related gene expression of an equine oviduct explant culture system showing preservation of epithelial differentiation for 6 days of culture, which is equal to the time spent in the oviduct by the equine embryo. This is a significant finding since this model will be used for the further study of differentiation markers and signalling molecules to unravel embryo–maternal interaction at the level of gene expression in the horse. The first objective of this study was to obtain equine oviduct explants with preservation of morphological and ultrastructural features. Oviduct explants have several advantages when compared with monolayers. First of all, it has been proven that in cattle (Thibodeaux et al. 1992; Walter 1995) and horses (Thomas et al. 1995; Dobrinski et al. 1999) proliferating oviductal cells grown in monolayers dedifferentiate with a concomitant reduction in cell height, loss of beating cilia and loss of secretory granules and bulbous protrusions. Despite the fact that the use of monolayers and resulting cell lines minimises the risk of disease transmission and batch-dependent variations (Menck et al. 1997), they are a less solid reflection of the in vivo situation (Thibodeaux et al. 1992; Walter 1995; Reischl et al. 1999). In our hands, beating cilia in equine oviduct cell aggregates were still detected after 20 days in culture (data not shown). Transmission electron microscopy confirmed the presence of healthy cilia and
in different species, such as DMEM/F12, Menezo’s in vitro actions was preserved. A limitation in studying embryo–oviduct interactions after 6 days of culture, indicating that the functional integrity of the explant suspension. Next, the oviduct is a biosynthetic active and secretory organ that releases macromolecules throughout the oestrous cycle (Buhi et al. 2000). The explants in our culture system were showing numerous secretory granules and the lactic acid production of the explants in the low-oxygen environment was significantly higher than in higher-oxygen conditions. ***P < 0.001; repeated-measures ANOVA with Greenhouse–Heisser correction; post hoc tests with Bonferroni correction.

Fig. 8. Mean glucose consumption and lactic acid production (mM) measured in medium containing equine oviduct explants over 6 days of culture under high- or low-oxygen atmosphere. The glucose consumption and the lactic acid production of the explants in the low-oxygen environment were significantly higher than in higher-oxygen conditions. ***P < 0.001; repeated-measures ANOVA with Greenhouse–Heisser correction; post hoc tests with Bonferroni correction.

numerous microvilli (Fig. 4). Furthermore, the cells bordering the explants maintained their ultrastructural highly differentiated morphology, including numerous mitochondria and rough endoplasmic reticulum, highly similar to the oviduct epithelium ex vivo (Fig. 4). Since the ciliation process is stated to be the endpoint of differentiation that cannot be induced in an in vitro system (Thibodeaux et al. 1992), this means an important benefit of the explant suspension. Next, the oviduct is a biosynthetic active and secretory organ that releases macromolecules throughout the oestrous cycle (Buhi et al. 2000). The explants in our culture system were showing numerous secretory granules after 6 days of culture, indicating that the functional integrity was preserved. A limitation in studying embryo–oviduct interactions in vitro is the impossibility to study regional and temporal differences. Furthermore, in the horse, it is rather difficult to determine the exact postovulatory cycle stage. To lower this bias, 7–10 oviducts ipsilateral to ovulation were pooled. A major advantage of explants compared with monolayers is of practical importance: explants can be used within 6 to 12 h after harvest whereas monolayers can be used only after several days (Rottmayer et al. 2006).

Different media have been used to culture oviduct cells in vitro in different species, such as DMEM/F12, Menezo’s B2 medium (MB2), Roswell Park Memorial Institute (RPMI-1640 medium), Tissue Culture Medium-199 (TCM-199), Connaught Medical Research Laboratories 1066 (CMRL 1066) (Abe and Hoshi 1997; De Pauw et al. 2002; Ulbrich et al. 2003). Since equine embryos apparently benefit from high glucose concentrations during early development (Choi et al. 2004), DMEM/F12 medium was selected for explant culture since it contains high levels of glucose (17.5 mM) and is a suitable medium for equine embryo culture (Hinrichs 2010; Smits et al. 2011). Interestingly, glucose consumption by the oviduct explants was almost 2-fold higher under low oxygen tension (5% CO₂, 5% O₂, 90% N₂) compared with 5% CO₂ in air (Fig. 8), indicating that cellular activity is much higher (Leclerc et al. 2003). Concomitantly, we observed more than 3-fold higher lactic acid production in the low oxygen tension condition. This can be explained by the enhanced generation of lactic acid by anaerobic glycolysis when oxygen is limited (Vander et al. 2009).

One salient finding of this study was that, over time, the number of explants showing central dark zones increased gradually, reaching 85% in explants smaller than 200 µm and 98% in explants larger than 600 µm at Day 6 of culture (Figs 1b–f). This finding has never been reported before in oviduct explant culture. These dark zones could not be attributed to membrane-damaged cells, since Trypan blue and SYBR14/PI staining showed that the percentage of membrane-intact cells was over 95% during the whole culture period (Fig. 1d–f). Acceptable values of 90% membrane-intact cells are reported as an adequate criterion for cell isolation and culture (Cox and Leese 1997; Reischl et al. 1999; Mishra et al. 2003; Ulbrich et al. 2003). Since the dark zones did not seem to consist of membrane-damaged cells, the presence of apoptotic cells was evaluated. Over the whole culture period, fewer than 2% of the cells in the explants were TUNEL positive and fewer than 1% were anti-caspase-3 positive. The difference in the percentage of cells containing fragmented DNA (TUNEL positive) and anti-caspase-3 positive cells can be explained by the fact that TUNEL is prone to artefacts (Hardy and Spanos 2002) and that only a portion of the TUNEL-positive cells stain for the caspase-3 cleavage product, suggesting caspase-3 activation may not be required for apoptosis in all cells (Namura et al. 1998). These results indicate that the central dark zones did not consist of a cluster of apoptotic nor necrotic cells since TUNEL is a marker for DNA fragmentation (Stadelmann and Lassmann 2000). Transmission electron microscopy (TEM) was performed to elucidate the ultrastructural features of the oviduct explants. This technique revealed only a few cells with ultrastructural changes typical of apoptosis such as plasma membrane blebbing but also showed epithelial cells undergoing dark-cell degeneration, characterised by strong cytoplasmic condensation, chromatin clumping and ruffling of the cell membrane but no blebbing of the nucleus or plasma membrane (Leist and Jaattela 2001).

Dark-cell degeneration is stated to be an apoptosis-like hypoxia-related type of cell degeneration described hitherto in Huntington’s disease, in neuronal cell degeneration (Leist and Jaattela 2001) and chondrocytes (Roach and Clarke 2000). In neuronal cells, dark-cell degeneration is stated to be caused
by hypoxia (Barenberg et al. 2001). Destructive levels of glutamate have been reported to mediate hypoxic-induced neuronal death, caused by the positive feedback of glutamate on its own release (Hardy et al. 2002).

We hypothesised that the dark-cell degeneration in the explants was caused by hypoxic culture conditions as described in neuronal cells. Although a concentration of 5% oxygen, as applied in our experiments, is the physiological oviductal concentration of mammals (Fischer and Bavister 1993), due to the lack of microvasculature, the explants might (partly) suffer from hypoxia. Therefore, we evaluated the mRNA expression of five hypoxia-related genes. Hypoxia-inducible factor (HIF1A) is a transcription factor with a central role in the hypoxia response. Its activity is regulated by the oxygen-dependent degradation of the HIF1A protein (Chi et al. 2006). Hypoxia induces not only the expression of HIF1A in mammalian cells but also regulates the expression of growth factors such as VEGFA, a potent angiogenic factor with an essential role in embryonic vasculogenesis and angiogenesis in mice (Carmeliet et al. 1996; Ferrara et al. 1996). In cattle, VEGFA secretion is increased before ovulation and therefore creates an optimal environment for gamete maturation, fertilisation and early embryonic development (Wijayagunawardane et al. 2005). Furthermore, VEGFA enhances in vitro maturation of bovine oocytes and accelerates early embryonic development (Gabler et al. 1999; Luo et al. 2002). Wijayagunawardane et al. (2005) hypothesise that VEGFA regulates oviductal embryo transport in cattle. The promoter region of the VEGFA gene has hypoxia-responsive elements that respond to HIF1A (Raleigh et al. 1998). VEGFA mRNA levels are dramatically increased within a few hours of exposing different cell cultures to hypoxia (0% O₂; Shweiki et al. 1992), which stimulates neovascularisation (Fan et al. 2009). In this study, there was no change in expression of HIF1A nor VEGFA after 6 days of culture under different oxygen tensions.

The expression of glucose transporter 1 (GLUT1) is also upregulated under hypoxic conditions and mediated by HIF1A. Expression of the GLUT1 gene is frequently used to describe differences between different culture systems (Wrenzyci et al. 1998, 2001). Furthermore, GLUT1 plays an important role in the transfer of glucose from the oviduct epithelium into the lumen and in maintaining adequate glucose concentration in the oviductal fluid (Tadokoro et al. 1995). It also mediates cellular glucose incorporation into embryonic cells and is necessary for transition from the morula to blastocyst stage (Leese et al. 1995). Our results revealed a 5-fold downregulation of GLUT1 after a culture period of 6 days. This can be explained by the autoregulatory mechanism of cells by downregulating or induction of degradation of the principal glucose transporter GLUT1-mRNA to protect against deleterious effects of hyperglycaemic culture conditions (Hahn et al. 1998). Despite the high glucose consumption by the explants and the concomitant drop in glucose concentration (Fig. 8), there was a downregulation of GLUT1. This can be explained as a negative feedback caused by the non-physiologically high glucose concentration in the medium, even after 6 days of culture (7–12 mM), since the in vivo glucose concentration in oviductal fluid of the mare is only 2.84–5.92 mM (Campbell et al. 1979). However, modified DMEM/F12 medium with a high glucose concentration of 17 mM is superior to media with no glucose or low glucose concentration to support equine embryonic development in vitro (Choi et al. 2004). Since the explants, especially in the low-oxygen embryo-culture environment, seem to consume high amounts of glucose (Fig. 8), it may be advisable to add glucose daily in embryo–oviduct co-culture experiments.

Plasminogen activator inhibitor 1 (PAI1) is also regulated by hypoxia and HIF1A. It may have a function in protecting the zona pellucida, the preimplantation embryo and oviductal tissue from proteolytic degradation by active proteases such as urokinase plasminogen activator (uPA) and matrix metalloproteinases present in the oviductal environment (Kouba et al. 2000; Buhi 2002). Further, it may have a function in the regulation of extracellular matrix turnover and remodelling and may be involved in early cleavage-stage embryonic development (Kouba et al. 2000). In this experiment, both PAI1 and uPA were slightly downregulated after 6 days of culture under atmospheric oxygen (0.01 < P < 0.05). This is another indication of the fact that the explants did not suffer hypoxia, since under hypoxic conditions these factors are supposed to be upregulated (Liao et al. 2007). In bovine oviducts ex vivo, uPA expression was significantly higher during the preovulatory phase compared with Days 1 to 5 after ovulation (Gabler et al. 2001), indicating hormonally regulated expression. Therefore, the downregulation observed in our experiment might be caused either by the presence of uPA inhibitors such as PAI1, PAI2 and nexitin (Blasi 1997) in the serum or produced by the explants, or by the lack of hormonal stimulation in our culture system.

In conclusion, our culture system sustains equine oviductal explants bordered by highly differentiated, functional and intact epithelial cells showing vigorous ciliary activity during 6 days of culture. Furthermore, only a negligible percentage (1–2%) of the cells in the explants shows features of apoptosis or necrosis and therefore the explants mimic the in vivo situation very closely. Although dark-cell degeneration, which is a hypoxia related type of cell death, was observed, no proof of hypoxia could be observed at the level of mRNA expression.

Since our culture system is lacking hormonal stimulation and since in vivo oviductal hormone concentrations in the horse are high experiments with steroid hormone supplementation are ongoing and promising (Nelis et al. 2013). Furthermore, in order to finally validate our model, co-culture experiments with ICSI-produced embryos are planned. So, although the cause of the dark-cell degeneration needs to be clarified, the oviduct explant system is definitely the basis of a robust model suitable to study embryo–oviduct interactions in the horse.

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