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Establishment of a new method for isolation and culture of equine endometrial epithelial and stromal cells

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Summary

A new method of in vitro culture of equine endometrial epithelial and stromal cells is described. After dissection, the endometrial tissue was dissociated in collagenase type II, sieved through nylon gauze and the different cell types were separated by centrifugation and differential adhesion. Cells were seeded at a density of $1x10^4$ viable cells per cm^2 on several surfaces and cultured at 37 °C in humidified atmosphere. For both cell types the same serum-supplemented culture medium was utilized whereby confluent monolayer evolved after 10 to 14 days of incubation. Confluent cultures were dissociated using 0.25 % trypsin-EDTA (stromal cells) or alfazyme (epithelial cells) and passaged up to 19 times. Further on, a successful cryoconservation-protocol could be established. With it, we created the precondition to establish a co-culture system for equine endometrial epithelial and stromal cells with the long-term objective to study growth characteristics, effects of steroid hormones and mutual influences between the cell populations, as well as potential pathogenetic factors and etiological influences on equine endometrosis.

Keywords: cell culture, endometrium, epithelial cells, stromal cells, horse

Etablierung einer neuen Methode zur Isolierung und Kultur equiner endometrialer Epithel- und Stromazellen

Eine neue Methode der in-vitro-Kultur equiner endometrialer Epithel- und Stromazellen wird beschrieben. Nach mechanischer Zerkleinerung der Proben erfolgte die enzymatische Auftrennung des Gewebes mittels Kollagenase Typ II, sowie die Trennung der verschiedenen Zellarten durch Filtration, Dichtegradientenzentrifugation und Differenzialadhärenz. Die Zellen wurden dann in einer Dichte von $1x10^4$ lebenden Zellen pro cm^2 auf verschiedene Wachstumsoberflächen ausgesät und in feuchter Umgebung bei 37 °C kultiviert. Für beide Zellarten wurde dasselbe serumhaltige Nährmedium verwendet, womit die Kulturen nach 10 bis 14 Tagen in der Lage waren, konfluente Monolayer auszubilden. Diese wurden dann mittels 0.25 %-iger Trypsin-EDTA Lösung (Stromazellen) beziehungsweise Alfazyme (Epithelzellen) erfolgreich über bis zu 19 Passagen hinweg subkultiviert. Nicht passagierte Epithel- und Stromazellen wurden in flüssigem Stickstoff kryokonserviert und konnten zu späteren Zeitpunkten wieder der Kultur zugeführt werden. Damit wurden essenzielle Voraussetzungen für die Etablierung einer Kokultur equiner endometrialer Epithel- und Stromazellen geschaffen, auf deren Basis wiederum weitere Untersuchungen zur Pathogenese und Ätiologie der equinen Endometrose durchgeführt werden können.

Schlüsselwörter: Zellkultur, Endometrium, Epithelzellen, Stromazellen, Pferd

Introduction

In general, in vitro culture is an appropriate model to investigate the physiology, interaction and differentiation of different cell types under controlled and defined conditions (*Freshney* 1990), for the better understanding of the pathogenesis and the etiology of several diseases (*Findlay* et al. 1990). Because preliminary tests could be accomplished using this technique one can reduce the number of animal experiments, as well.

Up to now, numerous cell culture systems and protocols for isolation and culture of endometrial tissue from humans (*Satyaswaroop* et al. 1979, *Mylonas* et al. 2000) and several animals exist (*Uchima* et al. 1991, *Ordener* et al. 1993, *Sheldrick* et al. 1993, *Piva* et al. 1996, *Groothuis* et al. 2002, *Sharma* et al. 2002, *Yamauchi* et al. 2003). However, little was done to isolate and culture equine epithelial glandular cells and stromal cells (*Watson* et al. 1992, *Brady* et al. 1992, 1993, *Day* et al. 1998, *Buschatz* 2007).

The aim of the present work was therefore to establish and to improve a new cell culture system for equine endometrial epithelial (EEC) and stromal cells (ESC) in order to study the characteristics and growth of different cell types as well as influences due to selected steroid hormones, growth factors and/or hypoxia. Finally, this work shall provide a basis to create a co-culture system as an appropriate model to investigate the etiopathogenesis of equine endometrosis. This disease is one of the most important causes of sub- and infertility in mares. While several histological and in vivo studies were done, only parts of the pathogenesis are understood (*Hoffmann* et al. 2009) and the etiology, as well as possible aspects of treatment still remain unclear (*Kenney* 1978, *Schoon* et al. 1995, *Hoffmann* et al. 2009).

Material and Methods:

Animals

Endometrial tissue was obtained from adult gynecologically healthy mares by transcervical biopsy (n = 9). In two cases whole uteri from slaughtered mares were used for isolation

and one culture was performed from the uterus of a mare that was euthanized due to severe recurrent airway obstruction.

The biopsy specimens were only applied to evaluate the technical procedure. Therefore the animals were not examined gynecologically in detail, but were healthy and had normal cyclic activity. Unfortunately, the exact day of the cycle of the three mares that donated the whole uteri could not be stated as well, given that all uteri were examined by the author at the time of necropsy, respectively after slaughtering. Clinical data regarding the ovarian cycle, especially the time of the last ovulation do not exist. The only available clinical information for the author was that all mares did not show any signs of gynecological diseases. Thus, we estimated the stage of the ovarian cycle on the basis of development and regression of follicles, corpora lutea and corpora haemorrhagica (*Arthur* 1958, *VanNiekerk* et al. 1973, 1975, *Kenney* et al. 1979) in comparison to routine histopathological examination of the endometrial tissue (*Kenney* and *Doig* 1986, *Schoon* et al. 1992, 1997).

Endometrial epithelial and stromal cells were isolated by a modified protocol according to the technique described by *Buschatz* (2007). All of the obtained biopsy specimens were collected as aseptically as possible, cut into pieces of 1.0 x 0.5 x 0.5 cm and placed into cold sterile Ca/Mg-free Hank s balanced salt solution (HBSS) (PAA Laboratories, Cölbe, Germany) containing 2 % Antibiotic-Antimycotic Solution (AB) (PAA Laboratories, Cölbe, Germany) until they arrived at the laboratory. For preparation of endometrium from the whole uterus the surface of the organs was washed with HBSS, opened longitudinally at the corpus uteri and strips of about 2.0 x 0.7 x 0.2 cm were dissected and separated from the myometrium using a disposable scalpel.

Isolation

After two washing steps in HBSS to remove remaining blood cells, debris or mucus the pieces were minced into fragments of about 1 mm^3 and rinsed in HBSS again. The tissue was digested in collagenase solution containing collagenase type II (Biochrom AG, Berlin, Germany) in HBSS with 2 % AB at 37 °C for 3 to 4 hours (5 % CO_2 atmosphere). The tissue fragments were agitated continuously by a laboratory shaker and periodically by gentle, repeated pipetting to disrupt the cells and abbreviate the residence time of collagenase. In the third and fourth hour of the process the digestion progress was monitored every 30 to 45 minutes by decanting a drop of the solution on a microscopic slide and visualized by an inverted microscope (Olympus CK2, Olympus, Tokyo, Japan). When only a few ESC left adhering to the glands, the slurry was filtered through a nylon sieve, rinsed with warm Dulbecco s modified Eagle s medium (DMEM)/Ham s F-12 (PAA Laboratories, Cölbe, Germany) and the arising ESC were centrifuged on a Percoll solution (Amersham Biosciences, Uppsala, Sweden) with 400 x g for 20 minutes (Buschatz 2007). The remaining EEC on the gauze were rinsed and afterwards centrifuged on a Percoll gradient (*Buschatz* 2007) as well. The average cell yield was determined using a Neubauer counting chamber. To avoid higher grades of cross-contamination (more than 5 %), differential adherence was also performed with each cell type. Non-cultured cells from each type were fixed in 4 % neutral buffered formalin, pelletised and embedded in paraplast for immunocytochemistry.

Culture

The isolated cells were allowed to grow in DMEM/Ham s F-12, supplemented with 2.5 % foetal bovine serum (FBS), 1 % AB, 0.275 % Amphotericin B, 1 % ITS (insulin, transferrin, selenium), 1 % HEPES-Buffer (all obtained from PAA Laboratories, Cölbe, Germany), 8 ng/ml epidermal growth factor (human, E. coli) (Biochrom AG, Berlin, Germany) and 0.5 µg/ml hydrocortisone (Sigma-Aldrich, Steinheim, Germany). Cells were plated at a density of $1x10^4$ viable cells per cm^2 (trypan-blue exclusion method) (trypan blue, Sigma Aldrich, Taufkirchen, Germany) in culture flasks, 6-/12-/24-well plates (Becton Dickinson, Franklin Lakes, New Jersey, USA), Millicell® PCF culture inserts (12 mm, 3.0 µm pores, Millipore, Bedford, USA), Chamber Slides™ (Nalge Nunc International, NY, USA) or cover slips (Carl Roth GmbH, Karlsruhe, Germany) and maintained to confluence at 37 °C in a humidified atmosphere containing 5 % CO_2. In general, the medium was changed every two days. Sole exception were the epithelial cells remaining in the medium, they were seeded in for the first four days of culture (primary and following passages). Thereafter, they were handled in the same manner than the ESC.

Subculture

Confluent cultures were passaged from culture flasks by the following protocols depending on the cell type. Twice rinsing with warm HBSS was followed by an enzymatic detachment with alfazyme (EEC) (PAA Laboratories, Cölbe, Germany) and 0.25 % trypsin-EDTA (ESC) (Biochrom AG, Berlin, Germany) in HBSS, both at 37 °C in humidified atmosphere. Epithelial cultures needed a reaction time up to 45 minutes, whereas stromal cells already detached within 5 to 10 minutes. The appropriate enzyme was blocked with an equal amount of FBS-supplemented culture medium and centrifuged at 100 x g for 15 minutes to segregate the used enzyme. Afterwards, the arising cell pellet was dispersed in culture medium and the cells seeded at the same density than in primary culture. However, for the first three passages, epithelial cells had to be seeded at higher density to achieve satisfactory results. As aforementioned, non-cultured cells were embedded in paraplast for immunocytochemistry (*Böttcher* et al. 2010), as well.

Cryopreservation

Cryopreservation was performed with both EEC and ESC according to the sub cultivation protocol described above. The generated pellet from one culture flask was resolved in 1 ml of culture medium and mixed with the same amount of cryomedium, containing 40 % FBS, 40 % culture medium and 20 % dimethyl sulfoxide (DMSO) (Carl Roth GmbH, Karlsruhe, Germany) as preservative agent. Vials of 1 ml volume were frozen at -80 °C for 24 hours and afterwards stored in liquid nitrogen. Depending on the cell type each vial contained approximately $1x10^4$ stromal and $4x10^5$ epithelial cells.

Cryopreserved cells were thawed in a heating bath at 37 °C, mixed with 3 ml of warm culture medium followed by centrifugation with 100 x g for 15 minutes. In general, two 25 cm^2 culture flasks were charged from one vial and allowed to grow to confluence.

Cryomedium was evaluated using the stromal cell population. For this purpose, half of a cell population was frozen at -196 °C in DMSO-containing cryomedium prior seeding in a 6-well plate (density $1x10^4$ cells per well). Then, growth rate was assigned in comparison to non-frozen cultures (control) seeded at the same density, every second day by trypsinization and counting in a Neubauer counting chamber (trypan-blue exclusion method) until the cultures reached confluence. At a later date, epithelial cells from subculture 11 were frozen likewise, but seeded at a higher density ($4x10^5$ per culture flask) and were cultivated to confluence.

Visualization

Cultures were inspected for growth, morphology and cross-contamination every two days on an inverted microscope. In addition, confluent cultures grown on Chamber Slides™ or cover slips were fixed for 10 minutes in pure methanol prior the staining with Giemsa, haemalaun and eosin (H.-E.) and alcian blue (*Romeis* 1989) for microscopic examination.

Results

Routine histopathological examination of the endometrial tissue from the whole uteri showed that one mare was in the transition from proliferatory to secretory phase, while the others were in the early secretory phase. In addition to the findings in the ovaries of each mare (unilateral one Corpus luteum, respectively Corpus haemorrhagicum and on every ovary multiple small follicles and Corpora albicantia), we stated two mares to be in the interoestrus and one to be postovulatory.

Light microscopic examination during the culture period of primary isolated and subcultured endometrial cells pointed out that stromal and epithelial cells differed in morphology. Cross-contamination was judged by light microscopy to be less than 5 %. Whereas ESC came out of the isolation process as single cells, the EEC were seeded in as glands with up to 200 cells per cluster.

The stromal cells were spindle to star shaped during the first days of culture and grew to confluent monolayers revealing typical fibroblast-like morphology in the following period including subculture (Fig. 1).

EEC glands collapsed and flattened after adhesion on the growth surface and started to grow out radially from each cluster. Even in the following subculture, two different types of epithelial cells could be observed: small (up to 10 µm in diameter) polygonal cells with distinct and regular borders growing in clusters with up to 100 cells, and larger (up to 50 µm in diameter) polyhedral, occasionally multinucleated cells with irregular and indistinct borders lain in groups containing up to 20 cells (Figs. 2 and 3).

The morphology of the cultured cells revealed comparable results in every primary and following subculture independently from the origin of the material.

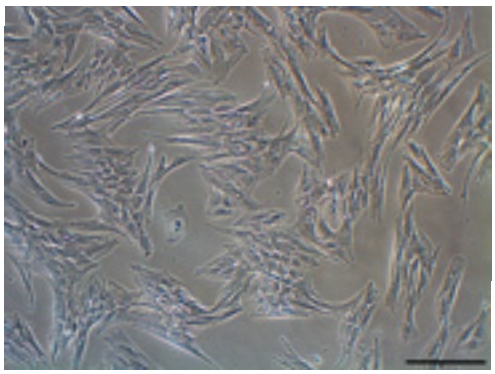

Fig. 1 Stromal cells growing on plastic on day 6 of culture revealing typical fibroblast-like morphology, phase contrast, bar 50 µm.
Stromazellen auf Plastikoberfläche am 6. Kulturtag mit typischer Fibroblasten-ähnlicher Morphologie, Phasenkontrast, Balken 50 µm.

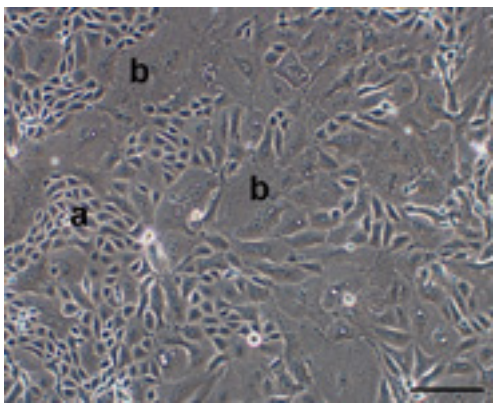

Fig. 2 Epithelial cells growing on plastic, small polygonal cells with regular and distinct borders (a), larger polyhedral cells with irregular borders (b), passage 10, day 6 of culture, phase contrast, bar 50 µm.
Epithelzellen auf Plastikoberfläche, kleine polygonale Zellen mit regulären und deutlichen Zellgrenzen (a), größere polyedrische Zellen mit irregulären Zellgrenzen (b), Passage 10, 6. Kulturtag, Phasenkontrast, Balken 50 µm.

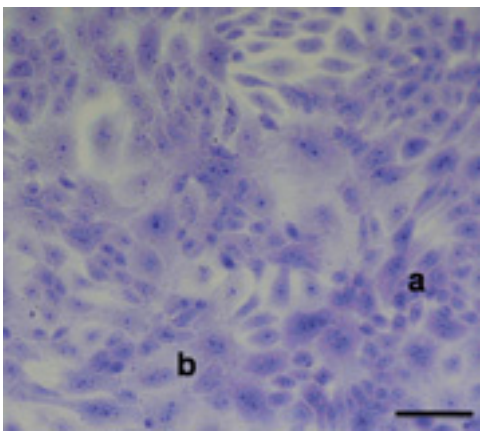

Fig. 3 Epithelial cells growing on Chamber Slides™, small polygonal cells with regular and distinct borders (a), bigger polyhedral cells with irregular borders (b), passage 12, day 6 of culture, Giemsa, bar 100 µm.
Epithelzellen auf Chamber Slides™, kleine polygonale Zellen mit regulären und deutlichen Zellgrenzen (a), größere polyedrische Zellen mit irregulären Zellgrenzen (b), Passage 12, 6. Kulturtag, Giemsa, Balken 100 µm.

Viability of primary isolated cells depended directly on the time elapsed from preparation of the tissue to further processing in the laboratory. Biopsy specimens were usually taken in close proximity to our institute providing high viability, but fewer numbers of average cell counts, due to limited material. On the contrary, whole uteri yielded disproportionately more cells, but either they could only be obtained from slaughter houses farther away or during necropsy – both prospects took more time from the point of death of the horse to tissue preparation with decreased numbers of viable cells.

Subculture of stromal cells could be accomplished in this study without any challenge. However, subculture of epithelial cells revealed no satisfying results as cultures were increasingly contaminated with ESC. In case of absence of stromal cells, passages up to 19 times and more were possible without any changes in proliferation rate, growth behaviour and morphology (Figs. 2 and 3).

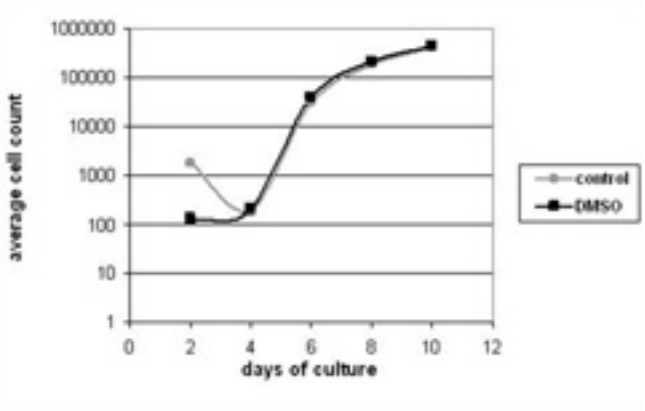

Fig. 4 Average cell count of ESC frozen in DMSO-supplemented cryomedium (DMSO) compared to non-frozen ESC (control), assigned every second day.
Mittlere Zellzahl an ESC, welche in DMSO-haltigem Kryomedium (DMSO) eingefroren wurden, im Vergleich zu nicht kryokonservierten Stromazellen (control). Die Bestimmung erfolgte an jedem zweiten Kulturtag.

As pointed out in Fig. 4, cell count of cryopreserved and conventionally seeded ESC decreased until day 4 and accelerated in the same manner the following days, until DMSO-group and control were confluent on day 10. Epithelial cells from subculture 11 which were frozen likewise, reached confluence after 10 days of culture.

Discussion

Isolation and culture of equine endometrial epithelial and/or stromal cells has previously been described only by a handful of authors (*Watson* et al. 1992, *Brady* et al. 1992, 1993, *Burghardt* et al. 1995, *Day* et al. 1998, *Buschatz* 2007). The morphology of EEC and ESC cultured in this study reveal matchable results compared to the findings in other species (women and animals) (*Satyaswaroop* et al. 1979, *Ricketts* et al. 1983, *Zhang* et al. 1991) and the mare (*Watson* et al. 1992, *Buschatz* 2007) without the prejudice of the usage of a serum-reduced culture medium.

As indicated by several authors, for example *Gruenert* et al. (1995) and *Shibeshi* et al. (2008), removal of fibroblasts (stromal cells) is a key factor for establishing long-term primary epithelial cultures and sub cultivation. For this purpose, two possible procedures exist: on one hand selective removal of fibroblasts by magnetic cell sortening or antibody mediated complement reaction (*Singer* et al. 1989) and on the other hand differential cell adhesion. In addition, serum-free (or at least serum-reduced) culture medium can suppress the growth of remaining stromal cells. Given that the aforementioned procedures require higher amounts of technical equipment and are proved not suitable for equine fibroblasts (*Shibeshi* et al. 2008), this study focused on the development of a serum-reduced culture medium, a gentle detachment and adapted subculture. In combination with the formerly described method by *Buschatz* (2007) this resulted in high pureness of cell lines (<5 % cross-contamination), the suitability of the same culture medium for EEC and ESC and further on the possibility of sub cultivation and cryopreservation. For this reason, we created the precondition to establish a co-culture system for both primary culture and subculture (for example as described by *Arnold* et al. 2001 and *Lu* et al. 2008) with the long-term objective to study growth characteristics, effects of steroid hormones and mutual influences between the cell populations, as well as potential pathogenetic factors and etiological influences on equine endometrosis.

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