Pferdeheilkunde 24 (2008) 1 (Januar/Februar) 74-78

Persistent mating-induced endometritis susceptibility: the role of uterine secretion

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Summary

The aim of this study was to compare the protein profile of endometrial secretions from estrous mares resistant or susceptible to persistent post-mating endometritis (PPME). The hypothesis was that before insemination in susceptible mares the uterine environment is disturbed. Endometrial secretions from 17 estrous mares were collected before artificial insemination (AI), when a pre-ovulatory follicle and a characteristic ultrasonic uterine image from estrous was observed, using a regular vaginal tampon. Immediately after the remove of the tampon an endometrial biopsy was obtained. The biopsies were evaluated by immunohistochemistry. Another examination was performed 36-48h after AI to confirm ovulation and to detect intrauterine fluid accumulation (IUF). The mares were classified into 2 groups, according to the findings: "Resistant" (n = 11), no IUF was detected 36-48 h post AI and "Susceptible" (n = 6), mares presenting more than 2 mm of IUF 36-48 h after AI. An aliquot of endometrial secretions was centrifuged and the supernatant was transferred to cryovials for storage in liquid nitrogen until assay. The endometrial samples were recentrifuged and then submitted to 2D-PAGE using 12% acrylamide gels. Protein (100 mg) was loaded into the gels. Gels were stained in a 0.15% Comassie Brilliant Blue R-250. The Optiquant Acquisition & Analysis software was used to determine the relative protein content of the spots. The image analysis software identified 33 protein spots, with a molecular weight (MW) ranging from 15 to 105 kDa and isoeletric point (Ip) from 4.3 to 10.0. Twelve proteins showed higher relative protein content and eight proteins showed frequency differences in endometrial secretions from susceptible compared to resistant mares. Based on MW and Ip, the proteins may be related to inflammatory processes and uterine contractility. A higher (P = 0.07) estrogen and progesterone receptor staining intensity in stromal and luminal epithelium cells in susceptible mares was also observed. In conclusion, there is a difference in the uterine environment between resistant and susceptible mares to PPME, probably affecting the inflammatory response to spermatozoa. This difference can be related to ovarian steroid receptors expression.

Keywords: mares, post-mating endometritis, susceptibility, proteins, reproduction

Zur Bedeutung der uterinen Sekrete bei der persistierenden, durch die Belegung induzierten Endometritis

Ziel der Studie ist, die Proteinprofile der endometrialen Sekrete bei östrischen Stuten, die empfänglich oder unempfänglich gegenüber einer persistierenden, durch die Belegung induzierten Endometritis sind, zu vergleichen. Die Arbeitshypothese war, dass das uterine Milieu empfänglicher Stuten vor der artifiziellen Insemination (AI) gestört ist. Von 17 östrischen Stuten wurden endometriale Sekrete mit Hilfe eines konventionellen Vaginaltampons vor der AI gewonnen. Bedingung war ein präovulatorischer Follikel und ein charakteristisches östrisches Ultraschallbild. Unmittelbar nach Entfernung des Tampons erfolgte die Entnahme einer Endometriumbiopsie, die immunhistologisch untersucht wurden. 36-48 Stunden nach der AI erfolgte eine weitere Untersuchung, um zu überprüfen, ob eine Ovulation stattgefunden und sich eine intrauterine Flüssigkeitsansammlung (IUF)gebildet hat. Die Stuten wurden wie folgt in zwei Gruppen eingeteilt: „resistent" (n=11), wenn sich 36-48 Stunden post AI keine IUF gebildet hat, „empfänglich" (n=6) bei Vorliegen von mindestens 2mm intrauteriner Flüssigkeit. Ein Aliquot des endometrialen Sekretes wurde zentrifugiert und der Überstand in flüssigem Stickstoff bis zur Untersuchung gelagert. Die zweidimensionale SDS-Gelelektrophorese (12% Acrylamid-Gel, NEFGHE – nicht-äquilibrierter pH Gradient) erfolgte mit 100µl der rezentrifugierten Proben. Als Färbung diente 0,15%iges Comassie Brilliant Blau R-250. Mit Hilfe einer Software (Optiquant Acquisition & Analysis) wurde die relative Zusammensetzung der Proteine bestimmt. Das Bildanalysesystem identifizierte 33 Protein-Lokalisationen mit einem Molekulargewicht von 15 bis 105 kDa und einem isoelektrischen Punkt von 4,3 bis 10,0. Zwölf Proteine wiesen einen höheren Gehalt auf und acht Proteine einen Unterschied in der Häufigkeit des Vorkommens zwischen empfänglichen und resistenten Stuten. Die anhand ihres Molekulargewichtes und isoelektrischen Punktes charakterisierten Eiweiße könnten Proteine sein, die an Entzündungsprozessen und der uterinen Kontraktilität beteiligt sind. Zudem wurde bei empfänglichen Stuten eine höhere (P = 0,07) Östrogen- und Progesteronrezeptorintensität der luminalen Epithelien und Stromazellen nachgewiesen. Zusammenfassend wird festgestellt, dass resistente und empfängliche Stuten ein unterschiedliches uterines Milieu aufweisen und dies die Entzündungsreaktion auf Spermien beeinflussen kann. Zudem besteht ein Zusammenhang zu der Expression der ovariellen Steroidhormonrezeptoren.

Schlüsselwörter: Stute, Post-mating Endometritis, Empfänglichkeit, Proteine, Reproduktion

Introduction

Endometritis is a normal physiological event after mating (*Watson*, 2000) being a response to intrauterine deposition of semen in the mare (*Kotilainen* et al. 1994, *Fiala* et al 2007). This inflammatory reaction removes excess of semen and bacterial contamination inoculated at the time of breeding (*Kotilainen* et al. 1994, *Troedsson* et al. 1995, *Fiala* et al. 2007). However, if inflammation persists, the resulting environment is not compatible with establishment of pregnancy (*Watson*

2000). Mares that fail to clear the inflammation within the first 36 hours after mating and accumulate fluid in the uterine lumen (*LeBlanc* 2003) are classified as susceptible and are believed to have an impaired physical clearance. Accumulation of uterine fluid during estrus was associated with compositional changes in the uterine secretions. The mean total protein concentration in uterine secretion in mares with IUF was one-third of that found in mares without IUF. This presumably indicates that the uterine fluid is composed of both glandular secretions and of a transudate (*Reilas* 2001). The aim of this study was to compare the protein profile of endometrial secretions from estrous mares resistant or susceptible to persistent post-mating endometritis (PPME). The hypothesis was that before insemination in susceptible mares the uterine environment is disturbed. The possible differences in the endometrial secretion can help to understand the PPME mechanism of pathogenicity and to determine susceptibility markers.

Materials and Methods

The study was conducted in mares from a breeding center located in Southern Brazil during the 2006 breeding season. A total of 17 estrous warmblood mares, aged between 5 and 22 years were used in the experiment. Animals were pastured and supplemented with oats and hay. Mares were previously examined for reproductive soundness, including evaluation of perineal conformation, palpation per rectum and ultrasound of the genital tract. Only clinically normal mares were used. Mares were examined by means of palpation and ultrasound per rectum daily in order to evaluate follicular growth, grade of uterine edema and presence of intrauterine fluid accumulation (IUF). AI was performed with 500 x 10^6 sperm cells, with a total volume of 20 mL, when a pre-ovulatory follicle (> 35 mm) and an ultrasonographic image characteristic for estrus uterus were observed. Only mares without presence of IUF were inseminated.

Sample collection and processing

Uterine secretion was collected using a regular size tampon (Ob, Johnson & Johnson) before the insemination. The technique was a modification of the procedure described by *Reilas* (2001). A 50 cm umbilical tape was attached to the tampon, which was then passed through the cervix into the uterus using a modified doubleglove technique. The distal part of a rectal glove was cut off from the wrist to make a plastic tube. The gloved hand with the tampon was placed inside the tube, and then closed by gathering its end with one's fingers. The gloved hand was set free from the plastic tube before reaching the cervix. The tampon was allowed to absorb uterine fluid for 30 min and was then withdrawn with a glove. After removal, each tampon was placed inside a 20-mL syringe, and the absorbed fluid was squeezed out into a sterile plastic tube. An aliquot of 2.0 mL of endometrial secretions were centrifuged at 1500 x g for 15-20 minutes. The supernatant was transferred to cryovials and stored in liquid nitrogen until assay.

Reproductive management

Immediately after the remove of the tampon an endometrial biopsy specimen was obtained to perform histhopathology and immunohistochemistry. Mares were inseminated with fresh semen 2 to 12 h after the removal of the tampon. Another examination was performed per rectum 36-48 h after breeding in order to confirm ovulation and to identify the presence of IUF. According to the presence of IUF mares were classified into two groups. Resistant: mares without presence of any amount of IUF 36-48 h after AI and Susceptible: mares presenting more than 2 mm of IUF 36-48 h after AI.

Electrophoresis

Frozen samples were thawed, recentrifuged at 10.000 g for 60 min at 4°C and 50 μL were taken from the supernatant and transferred to cryovials for storage at 70°C. Protein concentration was assessed according to *Lowry* et al. (1951) using bovine serum albumin (1 mg/mL) as a standard. Endometrial secretion samples were subjected (in duplicate) to the two-dimensional gel electrophoresis technique described by *O'Farrel* (1977) and modified by *Rodnight* (1988). Gels were immersed in a solution of 0.15% Comassie brilliant blue R-250 (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 53% methanol, 7% acetic acid and water, and were stained overnight. The gels were destained in a mixture of 50% methanol, 7% acetic acid and water, with a minimum of five solution changes per gel. Destained gels were equilibrated in a mixture of 50% methanol, 1% glycerol and water, for 2 h. The gels were then placed between two cellophane sheets until dry.

After drying, gels were scanned (Hewlet-Packard 6100C, Palo Alto, CA, USA) and analyzed using software (Optiquant Acquisition & Analysis, version 02.00, Canberra, Australia) to determine the intensity of the spots, which were expressed in pixels. The relative intensity of the spots was expressed as relative percentage, considering 100% the total of the spots (in pixels) within a defined area (constant for all gels). To match the analyzed protein spots and specific spots to equine endometrial fluid, the approximate molecular weight and isoelectric point were used.

Histopathology and immunohistologic examination

Endometrial biopsies were classified according to the distribution and severity of inflammation. All samples were evaluated in a blind manner as described by *Kenney* and *Doig* (1986) modified (*Schoon* et al., 1997). Receptors for oestrogen and progesterone were stained with the peroxidase-anti-peroxidase-technique to determine the receptor status in luminal epithelial, stromal and glandular structures. The immunohistologic reaction was classified according to *Özgen* et al. (1997).

Statistical analysis

Susceptibility was tested in mares grouped according to the post-breeding examination. Data from the relative protein content in the gels were analyzed using ANOVA with a statistical significance of $p<0.05$. Differences between means were tested by Tukey's t-test. The relative frequency from the different spots presented in susceptible and in resistant mares were analyzed using the Chi Square Test. Hormonal expression receptors were analyzed by T-Student test.

Results

Samples from 17 mares were collected, 11 classified as resistant and 6 as susceptible. Samples from two mares classified as susceptible presented insufficient protein content to perform the 2-D eletrophoresis. A total of 22 gels from resistant mares and 12 gels from susceptible mares were used. A total of 33 protein spots with molecular weight ranged from 15 to 105 kDa and isoeletric point (Ip) from 4.3 to 10.0 were observed (Figure 1).

Twelve proteins (Figure 2) showed higher (P > 0.04) relative protein content and eight proteins (Table 1) showed quantitative frequency differences in endometrial secretions from

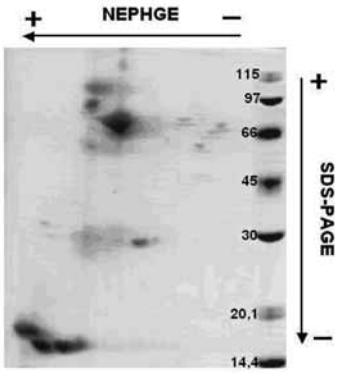

Fig 1 Two-dimensional polyacrylamide electrophoretic gel of equine endometrial secretion proteins. Two-dimensional 12% SDS-PAGE gel stained with Comassie blue. The arrow on the top shows the direction of the non-equilibrated pH gradient (NEPHGE) from the basic end (+) to the acid end (-) in the first dimension. Molecular weight markers and the corresponding weights are to the right.
Zweidimensionales Polyacrylamidelektrophorese-Gel (12%ig, gefärbt mit Comassie-Blau) sezernierter equiner endometrialer Sekrete. Der oben positionierte Pfeil zeigt die Richtung des nicht äquilibrierten pH Gradienten (NEPHGE) vom basischen (+) zum sauren (-) in der ersten Dimension. Rechts sind die Molekulargewichtsmarker und die entsprechenden Molekulargewichte zu sehen.

susceptible mares compared to resistant mares. No differences (P >0.5) were observed in the percentage of resistant mares presenting inflammation (44.4%) when compared with the percentage of susceptible mares (50%) by histopathology.

A tendency of higher expression of oestrogen receptors was observed in the luminal epithelium (P <0.07) and of progesterone in the stroma (P < 0.07).

Discussion

An impaired physical clearance is the main factor involved in susceptibility to persistent mating-induced endometritis (*Troedsson* 1997). During prostaglandin synthesis the cyclooxygenase enzyme is involved in the transformation of arachidonic acid in prostaglandin H2 (PGH2). Prostaglandin E synthase (PGE synthase), that may correspond to protein spot 7 (16-17 KDa, Ip: 9.7-10), is the enzyme responsible for the final transformation of PGH2 in prostaglandin E2 (PGE2) (*Tizard* 1998).

The expression of the enzymes involved in PGE2 synthesis like PGE synthase is apparently coordinated by IL-1ß in labours in

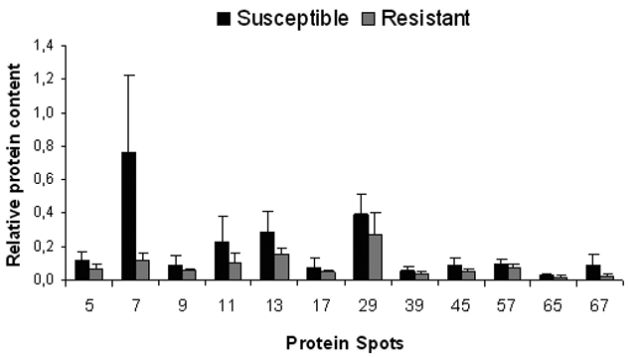

Fig 2 Means and standard deviation of the relative intensity of 12 protein spots presenting significant difference between susceptible and resistant mares (P < 0.04).
Mittelwerte und Standardabweichungen der relativen Intensität von 12 Protein spots. Es besteht ein signifikanter Unterschied zwischen resistenten und empfänglichen Tieren (p < 0,04).

women (*Sooranna* et al. 2006). Immunohistochemical analysis reveals that PGE synthase is located in the luminal and in the glandular epithelial cells and, at a lower level, in stromal cells (*Parent* and *Fortier* 2005). PGE2 presents an effect in myoelectrical activity of the uterus (*Troedsson* et al. 1995) and increases both uterine contractility and tone when applied on day 12 after ovulation. PGE2 is probably responsible to the uterine tone increase at days 11 to 15 of gestation (*Gastal* et al. 1998). The uterus of dioestrus mares exhibits prolonged bursts of low amplitude electromyographic activity compromising the physical clearance (*Evans* et al. 1987, *Jones* et al. 1991). Susceptible mares release lower PGF2α after oxytocin administration probably due to a defect at the oxytocin receptor or post-receptor level (*Nikolakopoulos* et al.

Table 1 Molecular Weight (MW), isoeletric point (Ip) and frequency (%) of the protein spots in susceptible and resistant mares.
Molekulargewicht (MW), isoelektrischer Punkt (Ip) und Häufigkeit (%) von Protein spots empfänglicher und resistenter Stuten.

Spot	MW (kDa)	*Ip*	Susceptible (n=12) (%)	Resistant (n=22) (%)	*P*
7	16 - 17	9.7 – 10.0	100.0	68.2	0.03
19	37 - 38	9.5 – 9.6	100.0	72.2	0.05
21	30 - 32	9.2 – 9.3	100.0	68.2	0.03
23	33 - 35	8.8 – 8.9	100.0	72.2	0.05
25	47 - 49	8.8 – 9.0	100.0	72.2	0.05
63	81 - 82	7.4 – 7.5	91.6	50.0	0.02
65	81 - 82	7.6 – 7.7	83.3	45.4	0.01
67	99 - 100	6.3 – 7.0	100.0	54.4	0.03

2000). The higher PGE synthase suggested in the present study can be associated to a higher release of PGE2 and a consequent lower release of PGF2α because both prostaglandins are originated from PGH2. This shift could result in a contractility pattern which reduces the uterine physical clearance. In the present study a higher relative protein content in 12 proteins spots and a higher frequency in 8 proteins spots were observed in susceptible mares. In 11 spots was found a correspondence with proteins described in equines and 6 of them can be related to inflammatory process.

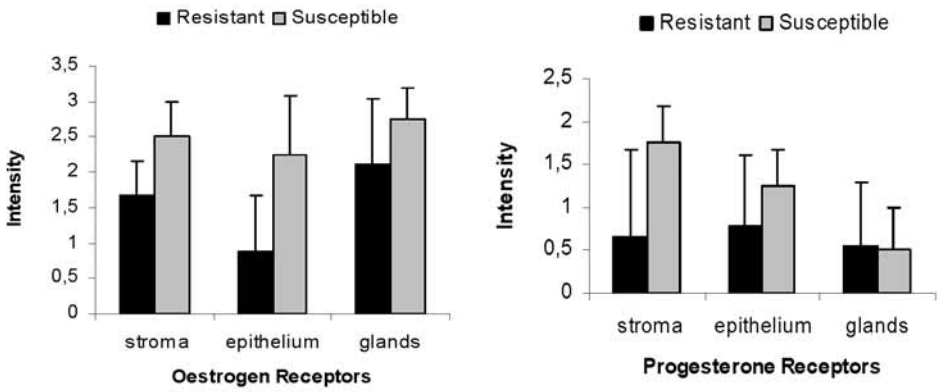

Fig 3 Means and standard deviation of the intensity of oestrogen and progesterone receptors from susceptible and resistant mares (epithelium = luminal epithelium).
Mittelwerte und Standardabweichungen der Intensität von Östrogen- und Progesteronrezeptoren bei empfänglichen und resistenten Stuten mares (epithelium = luminal epithelium).

IL-4, probably spot 5 (15-16 KDa, Ip: 8.9-9.2), is a well-characterized cytokine known to be produced by normal T cells and have an impact on normal B cells differentiation and proliferation (*Kay* and *Pittner* 2003) regulating the expression of the major histocompatibility complex class II (MHC-II). MHC II, probably spot 11 (26-28 KDa, Ip 8.9-9.3), presents antigens to CD4+ T lymphocytes (T helper), which are the main regulators of the immune response (*Drozina* et al. 2005). Chronic inflammation of the endometrium is characterized by infiltrations of lymphocytes (*Kenney* and *Doig* 1986). The distribution of CD4+ T lymphocytes was examined immunocytochemically and increase in the subfertile mares (*Tunón* et al. 1997). These cells are needed to provide activating signals for macrophages and B cells which increases the antibody production. In mares with history of chronic metritis, immunoglobulins concentration was significantly elevated in uterine secretions (*Widders* et al. 1984).

Based on molecular weight and Ip, spot 45 (46-47 KDa, Ip: 4.9-5.9), may be Caspase-1 [precursor] (CASP-1). This protein cleaves Interleukin-1β (IL-1β), a proinflammatory cytokine that plays a central role in the regulation of inflammatory reactions, including fever, acute phase response, and connective tissue degradation and remodeling (*Dinarello* 1996). Under basal conditions susceptible mares had higher endometrial IL-1β expression than resistant mares in estrus and in diestrus (*Fumuso* et al. 2003).

Spot 39 (47-48 KDa, Ip: 7.3-7.5) may be Interleukin-1 receptor type II [Precursor] (IL-1 RII). IL-1RII is the main IL-1-binding protein in neutrophils, and B cells (Mantovani et al., 2001). IL-1RII acts as a decoy receptor, binding and inhibiting the effect of IL-1 (*Bessis* et al. 2000). The major mechanism of IL-1RII release is cell-surface shedding mediated by an MMP or TNFα (*Mantovani* et al. 2001). The release of these soluble receptors is connected to the beginning of the inflammatory process. The same stimuli that generate proinflammatory responses also evoke the release of anti-inflammatory or regulatory molecules, like IL-1RII, that serve to rein in the inflammatory response (*Mackay* 2000). The activity of these pro-inflammatory molecules was demonstrated in the mare endometrium (*Fumuso* et al. 2003, *Oddsdottir* et al. 2006). Therefore it is possible that the presence of IL-1RII is an indicator of IL-1 b activity, which is cleaved by Casp-1 and had higher activity in susceptible mares.

Protein spot 13 (26-28 KDa, Ip: 8.3-8.6) probably corresponds to a transforming growth factor beta (TGF-β). TGF-β is synthesized by a wide variety of cell types, including platelets, macrophages, fibroblasts and tumor cells. Their pleiotropic activities include context-specific inhibition or stimulation of cell proliferation, control of extracellular matrix (ECM) synthesis and degradation, control of mesenchymal–epithelial interactions during embryogenesis, mediation of cell and tissue responses to injury, and modulation of immune functions (*Javelaud* and *Mauviel* 2004).

Protein spot 9 (24-25 kDa; Ip: 8.7-8.9) probably corresponds to TIMP-3 [precursor] (tissue inhibitor of metalloproteinase-3), which binds to and inhibits MMP-9 on a 1:1 basis (*Gomez* et al. 1997). TIMP-3 is bound to the extracellular matrix and has been suggested to act as an additional regulatory stop point for MMP action (*Leco* et al. 1994). The collagenase MMP-9, which has an important role destroying the extra cellular matrix during inflammation, was detected in equine endometrium after experimental infection and artificial insemination (*Oddsdottir* et al. 2006). Findings from *Oddsdottir* et al. (2006) associated with the unexistence of differences in inflammation between susceptible and resistant mares observed in the present study, reinforce the hypothesis from *Fumuso* et al. (2003), that in resistant mares stimulus should exceed a threshold to induce a coordinate transcription of cytokines, and that in susceptible mares, such a threshold would be lower.

Based on the molecular weight (30-32 kDa) and Ip (9.2-9.3), protein spot 21, may correspond to GAP junction beta-1 protein (GAP b1) or connexin-32. In the present study this spot was present in 100% of the samples from susceptible mares but only in 68.2% of the resistant mares. GAP junctions, which provide contacting cells with a common pool of regulatory and informational molecules, are involved in the integration of uterine function in cycling and early pregnant mares (*Brady* et al. 1995) and this communication may be important for the coordinated secretory activity of the endometrium. An increase on connexin activity may be related to a higher expression of other proteins observed in susceptible mares or to an impaired control in the secretions of the endometrial glands. Fluid accumulation observed in susceptible mares can also be rela-

ted to the expression of oestrogen and progesterone receptors in endometrium. A higher expression of these receptors was observed in mares with hydromucometra (*Özgen* et al. 1997). In the present study a tendency to a higher expression of oestrogen and progesterone receptor staining intensity in stromal and luminal epithelium cells in susceptible mares was also observed, which can affect the secretion of the endometrial glands. Further studies including a greater number of animals are required to verify the receptor expression in the endometrium from susceptible mares. In conclusion, there is a difference in the uterine environment between resistant and susceptible mares to PPME, probably affecting the inflammatory response and the uterine contractility. It was not possible to elect a protein spot as a marker to susceptibility due to the interaction between the inflammatory molecules and their different functions (*Tizard* 1988). However, it would be interesting to investigate the use of caspase-1 inhibitors to block IL-1α activity for the treatment of susceptible mares as it is done in inflammatory diseases in humans (*Dinarello* 2004).

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