Pferdeheilkunde 28 (2012) 1 (Januar/Februar) 8-13

Diagnosis of equine endometritis – Microbiology, cytology and histology of endometrial biopsies and the correlation to fertility

Jesper Møller Nielsen[1], Frederik Høgsberg Nielsen[2] and Morten Rønn Petersen[2]

Ansager Dyrehospital, Ansager[1] and Veterinary Reproduction and Obstetrics, Department of Animal Science, Faculty for Life Sciences, University of Copenhagen, Frederiksberg[2], Denmark

Summary

The objective of this study was to examine the relationship between pregnancy results and the presence of inflammation detected by PMNs (cytology or histology) and microbial growth from an endometrial biopsy. Following recovery of the endometrial biopsy a quick stained cytology slide and bacterial culture was performed. After this was done the biopsy was fixed in 4% formaldehyde. Histology was evaluated for presence of PMNs and a Kenney score was assigned to each mare using the guidelines proposed by Kenney and Doig (1986). Significant correlation was detected between bacterial growth and PMNs detected by histology ($p=0.0082$) and PMNs detected by histology and day 70 pregnancy rate ($p= 0.0022$), but no significant correlation could be determined between cytology or bacterial growth and day 70 pregnancy rate or cytology and presence of PMNs detected by histology. In a logistic regression model following factors were evaluated to determine impact on pregnancy rate at day 70: 1) Histological detection of PMNs, 2) Cytology, 3) Bacteriological culture, 4) Mare age and 5) Kenney score. The only significant factor with impact on day 70 pregnancy rate was histological detection of PMNs ($p=0.003$). Significant correlation between Kenney groups and pregnancy rates was found in both the 75 initially included mares and in 74 mares from the same two stud farms that were retrospectively included in the study ($p=0.0069$ and $p <0.0001$), respectively. The study suggests the use of histological detection of presence of PMNs to diagnose endometritis, due to the statistical association and strong negative impact of PMNs present within the endometrium on chances of establishing a pregnancy.

Keywords: Reproduction, endometritis, cytology, microbiology, histology.

Diagnostik der Endeometritis beim Pferd – Mikrobiologie, Zytologie und Histologie von Endometriumsbiopsien und Korrelation mit der Fruchtbarkeit

Ziel der Studie war es, die Beziehungen zwischen Abfohlraten, dem Auftreten von neutrophilen Granulozyten (PMNs) (Zytologie und Histologie) und dem Bakterienwachstum an einer Endometriumbiopsie zu untersuchen. Nach Entnahme der Endometriumbiopsie wurden ein zytologisches Präparat (Schnellfärbeverfahren) und eine Bakterienkultur angefertigt. Danach erfolgte die Fixierung der Biopsie in 4%igem Formaldehyd. Histologisch wurde die Anwesenheit von PMNs geschätzt und jeder Stute ein Kenney Score zugeordnet (Richtlinien nach Kenney und Doig 1986). Eine signifikante Korrelation wurde zwischen dem bakteriellem Wachstum und den histologisch erfassbaren PMNs ($p=0,082$) sowie den histologisch erfassbaren PMNs und der Trächtigkeitsrate am 70. Tag ($p=0,0022$) ermittelt. Keine signifikante Korrelation lag hingegen zwischen der Zytologie oder dem bakteriellem Wachstum und der Trächtigkeitsrate am 70. Tag oder der Zytologie und der Anwesenheit von PMNs im histologischen Präparat vor. In einem logistischen Regressionsmodell wurden folgende Faktoren, die einen Einfluss auf die Trächtigkeitsrate am 70. Tag haben könnten, überprüft: 1. histologisch erfassbare PMNs, 2. Zytologie, 3. Bakterienkultur, 4. Alter der Stute und 5. der Kenney Score. Als einziger signifikanter Faktor mit einem Einfluss auf die Trächtigkeitsrate am 70. Tag erwies sich die Präsenz von PMNs bei der histologischen Auswertung der Biopsie. Eine signifikante Korrelation zwischen den Kenney Gruppen und der Trächtigkeitsrate wurde bei den 75 Stuten, die initial in diese Studie eingingen, sowie bei weiteren 74 Stuten aus denselben zwei Gestüten, die retrospektiv mit aufgenommen wurden, gefunden ($p=0,0069$ bzw. $p<0,0001$). Aufgrund der vorliegenden Studie wird für die Diagnose einer Endometritis die histologische Untersuchung auf PMNs anhand einer Endometriumbiopsie vorgeschlagen, da eine statistische Verbindung und ein starker negativer Einfluss der PMNs bezogen auf die Etablierung einer Trächtigkeit bestehen.

Schlüsselwörter: Reproduktion, Endometritis, Zytologie, Mikrobiologie, Histologie

Introduction

Diagnosis of infertility problems in the mare by sampling of material from the uterus was initially described more than 90 years ago in Germany and Kentucky, USA (*Schiebel* 1920, *Dimmocks* and *Edwards* 1923). Uterine disease, primarily endometritis, is still one of the major causes for infertility in the mare and a substantial problem in the modern breeding industry (*Riddle* et al. 2007).

Uterine samples have been used to diagnose infertility problems, in particular endometritis and endometrosis, using microbiological culture (*Ricketts* et al. 1993, *Nielsen* 2005), cytology (*Knudsen* 1964, *Nielsen* 2005), and histology (*Kenney* 1975, *Ricketts* 1975, *Doig* et al. 1981), respectively.

The finding of polymorph nuclear leucocytes (PMNs) in a uterine sample demonstrates the presence of endometrial inflammation, but without identification of the causal agent, an optimal treatment protocol cannot be proposed (*Ricketts* et al. 1993). Using histological examination of tissue from an endometrial biopsy a differentiated diagnosis of both inflammatory and degenerative changes, hallmarks of endometritis and endometrosis respectively, can be obtained (*Kenney* 1975 and *Ricketts* 1975). The severity of the end-

ometrial inflammation and degenerative changes, as described in the classification system described by *Kenney* and *Doig* (1986), is correlated to the capacity of the uterus to support an established pregnancy to term.

Several authors have speculated, whether the degree of fibrosis or the presence of infiltrative cells and hence inflammation, is detrimental to the establishment of pregnancy in the mare (*Kenney* 1975, *Doig* et al. 1981, *Kenney* and *Doig* 1986, *Schoon* et al. 1997), but the question remains open to an answer. It has been suggested that inflammation, characterized by the presence of infiltrative cells in the endometrium, is primarily a problem for the early establishment of pregnancy, whereas the degree of fibrosis is considered to be a problem for the long term maintenance of pregnancy (*Love* 2011). The relationship between endometritis and endometrosis is unclear and has to be investigated further. Using immunohistochemical characterization of the different stages of fibrotic changes and endometrosis it was however recently demonstrated that endometritis can lead to activation of fibrotic stromal cells and hereby induce endometrosis (*Hoffmann* et al. 2009).

The classification systems proposed by most of these authors try to combine both the presence of inflammatory findings and the degree of fibrosis into a single factor. The relationship between pregnancy results and results of bacteriology, cytology and histology is however so far received little attention.

This study was therefore conducted to examine the relationship between pregnancy result on day 70 and the presence of inflammation detected by PMNs and bacterial growth from endometrial biopsies diagnosed by cytology and histology and microbiological procedures commonly used in modern equine breeding industry (*Kenney* and *Doig* 1986, *Nielsen* 2005).

Material and Methods

Animals

A total of 75 mares of light horse breeds were initially included in this study. All the mares were presented during one single breeding season on two different stud farms for "routine swabbing and endometritis diagnosis". The mares were included in the study whether they had clinical signs or history of endometritis. The veterinary management on the two farms was identical. It was in this study not possible to correlate results of endometritis treatment and pregnancy, due to a lack of consistency in the treatment records.

Retrospectively pregnancy results of 74 other mares from two previous breeding seasons from the same two stud farms were also included in the study. These 74 mares had all a record of an endometrial biopsy for histological examination and grouping as described by *Kenney* and *Doig* (1986).

Microbiology, cytology and histology

Endometrial biopsies from the 75 mares initially included in the study were immediately after collection examined for growth of microorganisms on a blood agar and for the presence of PMNs in a quick stained cytological slide as described by *Nielsen* (2005). All the biopsies were then saved in 4 % formaldehyde, and had a histological examination performed by the end of the breeding season.

The presence of PMNs by light microscopy in a Hematoxilin-Eosin (H&E) stained histology slide was recorded. All mares were grouped, as described by *Kenney* and *Doig* (1986), based on the findings in the histological examination. The identity of the mare was blinded for the examiner when the histology was evaluated.

Statistics

Evaluation of association between different test results and pregnancy results was performed using Fisher´s exact test. Comparison of proportions between independent groups was performed using chi square. Agreement between two paired tests was demonstrated by Cohens kappa. Effect of several risk factors on a dichotomous outcome was done by logistic regression. Significance level was set at $p<0.05$.

Results

Day 70 pregnancy result was available for 72 of the 75 mares included in the study. The pregnancy rate per season was 65 %. The pregnancy rate per cycle was 35 % (Table 1).

In more than half of the mares (53 % (26/49)) with negative cytology PMNs could be detected at the histological examination. It was in 31 % (8/26) of the cytology positive mares not possible to detect PMNs in a histological slide. The Cohen´s kappa value of 0,139 shows a poor agreement between the results of the two tests (Table 2).

There is no significant correlation between result of cytology and day 70 pregnancy status.

A significant correlation between positive bacterial growth and presence of PMNs in a histological slide from the endometrium could be demonstrated. In 46 % (22/47) of the mares with negative bacteriology PMNs within the endometrium could be demonstrated and no bacterial growth was

Table 1 Relationship between positive or negative cytology (+,-) and presence of PMNs (+,-) detected by histology / *Beziehung zwischen positiver oder negativer Zytologie (+/-) und den histologisch erfassbaren PMNs (+/-)*

		PMNs detected on histology		
		+	-	Total
Cytology	+	18	8	26
	-	26	23	49
	Total	44	31	75

Cohen´s kappa: k = 0.139

found in 50 % (22/44) of the mares with PMNs detected by histology. In 21 % (6/28) of the bacteriology positive mares it was not possible to detect PMNs in the histological slide. The relative risk of having PMNs detected by histology was 1.68 higher for bacteriology positive mares compared to mares with no bacterial growth (Table 3). There is no significant correlation between the result of bacteriology and the chance of becoming pregnant (Table 4).

There is a significant correlation between pregnancy result and the result of detection of PMNs in a histological slide prepared from an endometrial biopsy. If PMNs could be detected by histology 50% (21/42) of the mares became pregnant, whereas 87 % (26/30) of the mares became pregnant if no PMNs were detected. If PMNs were detected by histology the chance of becoming pregnant was 42 % lower than if no PMNs were detected (relative risk=0.577)(Table 5).

There is a significant overall correlation between Kenney score after evaluation of a histological slide and day 70 pregnancy result (p=0.0069). There is however only significant difference in pregnancy rates between the Kenney groups I versus IIb and between IIa and IIb (Table 6).

Logistic regression test: To determine risk factors of impact on day 70 pregnancy status a logistic regression was performed, in which the following factors were included in the model: Histological detection of PMNs, cytology, bacteriological culture, mare age and Kenney score. Histological detection of PMNs had, as the only risk factor, a significant impact on day 70 pregnancy status (p = 0.003). Odds Ratio was 0.15. This means the odds of becoming pregnant is 6.5 times less likely if PMNs are detected by histology than if no PMNs are detected.

There is a significant correlation between Kenney score after evaluation of a histological slide and day 17 pregnancy result (p<0.0001). There is however only significant difference between the single Kenney groups I versus IIb, I versus III, and between IIa and III (Table 7)

Table 2 Relationship between positive or negative cytology (+,-) and day 70 pregnancy status (+,-) / *Beziehung zwischen positiver oder negativer Zytologie (+/-) und der Trächtigkeitsrate am 70. Tag (+/-)*

		Pregnant day 70		
		+	-	Total
Cytology	+	15	11	26
	-	32	14	46
	Total	47	25	72

Fisher´s exact test: p = 0.3184

Table 3 Relationship between positive or negative bacterial growth (+,-) and presence of PMNs detected by histology (+,-). / Beziehung zwischen positivem oder negativem Bakterienwachstum (+/-) und den histologisch erfassbaren PMNs (+/-)

		PMNs detected by histology		
		+	-	Total
Bacterial growth	+	22	6	28
	-	22	25	47
	Total	44	31	75

Fisher´s exact test: p = 0.0082

Table 4 Relationship between positive or negative bacterial growth (+,-) and day 70 pregnancy status (+,-) / *Beziehung zwischen positivem oder negativem Bakterienwachstum (+/-)und der Trächtigkeitsrate am 70. Tag (+/-)*

		Pregnant day 70		
		+	-	Total
Bacterial growth	+	14	12	26
	-	33	13	46
	Total	47	25	72

Fisher´s exact test: p = 0.197

Table 5 Relationship between presence of PMNs detected by histology in an H&E stained slide (+,-) and day 70 pregnancy status (+,-) / *Beziehung zwischen der Anwesenheit von PMNs, histologisch erfasst anhand eines HE-gefärbten Schnittpräparates, (+/-) und der Trächtigkeitsrate am 70. Tag (+/-)*

		Pregnant day 70		
		+	-	Total
PMNs detected by histology	+	21	21	42
	-	26	4	30
	Total	47	25	72

Fisher´s exact test: p = 0.0022

Table 6 Relationship between Kenney score (I, IIa, IIb, III) (Kenney and Doig 1986) and day 70 pregnancy status (+,-) / *Beziehung zwischen dem Kenney Score (I, IIa, IIb, III) (Kenney und Doig 1986) und der Trächtigkeitsrate am 70. Tag (+/-)*

		Pregnant day 70		
		+	-	Total
Kenney score	I	14	2	16 [a]
	IIa	18	6	24 [a]
	IIb	11	16	27 [b, c]
	III	4	1	5 [a, c]
	Total	47	25	72

Different letters [a,b,c] indicate significant differences in pregnancy results between Kenney groups ($p<0.05$) (Chi square). Fisher´s exact test: $p = 0.0069$

Table 7 Relationship between Kenney score (I, IIa, IIb, III) (*Kenney* and *Doig* 1986) and day 17 pregnancy status (+,-). Results from these 74 mares were retrospectively found in the records of the two participating stud farms after the initial study. / *Beziehung zwischen dem Kenney Score (I, IIa, IIb, III) (*Kenney *und* Doig *1986) und der Trächtigkeitsrate am 17. Tag (+/-). Die Ergebnisse von diesen 74 Stuten wurden retrospektiv ermittelt anhand der Daten der zwei beteiligten Gestüte der initialen Studie.*

		Pregnant day 17		Total
		+	-	
Kenney score	I	11	0	11 [a]
	IIa	18	4	22 [a,c]
	IIb	11	9	20 [b,c]
	III	6	15	21 [b]
	Total	46	28	74

Different letters [a,b,c] indicate significant differences in pregnancy results between Kenney groups ($p<0.05$) (Chi square). Fisher´s exact test: $p < 0.0001$.

Discussion

As shown in previous studies, different diagnostic tests for detection of infection and inflammation in the equine uterus has different reliability (*Doig* et al. 1981, *LeBlanc* 2007, *Nielsen* 2005, *Nielsen* et al. 2010, *Overbeck* et al. 2011). The different diagnostic sampling techniques vary in the endometrial surface area evaluated, and tests performed on endometrial biopsies are the only currently available approach to diagnose infection and/or inflammation within the endometrium. Pathogens apply different approaches to establish and maintain infection (*Quinn* et al. 1994). The localization of pathogens is one aspect of pathogenesis (*Petersen* et al. 2009). It is therefore not likely that one diagnostic sampling technique is adequate to determine the true status of the endometrium in all mares.

Mares in this study, that was diagnosed with endometritis (positive on culture and/or cytology) or demonstrated clinical symptoms of endometritis, received treatment. Information on whether a mare was treated for endometritis or not was not included in the study. It should be highlighted that treatment is a potential confounder, which could affect fertility. Future studies should evaluate the impact of treatment on presence of PMNs within the endometrium and fertility.

Cytology

The use of cytology from a quick stained slide has been described in several studies (*Nielsen* 2005, *Nielsen* et al. 2010, *Overbeck* et al. 2011). The sensitivity and specificity of this test compared to detection of PMNs by histology has in these studies been reported moderate to poor. In this study the same tendency was present. In 53% of the mares with negative cytology endometrial inflammation was present (PMNs detected on histology), whereas 39 % of the cytology positive samples had no PMNs detected by histology. No significant correlation between pregnancy status on day 70 and result of the cytological examination could be detected.

Microbiology

Bacteriological examination of material from the uterus collected by endometrial swab, cytobrush, endometrial biopsy and uterine lavage is well described in the literature (*LeBlanc* et al. 2007, *Nielsen* et al. 2010, *Overbeck* et al. 2011). Sensitivity and specificity of bacteriological tests are reported good to moderate. Highest sensitivity and specificity of bacterial culture, compared to detection of PMNs by histological examination as best standard, has been reported when material from endometrial biopsy or low volume uterine lavage was examined. In this study a significant correlation between bacteriology and detection of PMNs by histology was demonstrated ($p=0.0082$). The correlation between pregnancy result and bacteriology was however not significant. The lack of information on treatment in the study might a influence this correlation. If bacteria is detected and treated, a mare's chance of becoming pregnant will enhance (*Nielsen* et al. 2008). In 46 % (22/47) of the mares with negative bacteriology PMNs could be detected within the endometrium. No bacterial growth was found in 50 % (22/44) of mares with PMNs detected by histology. This indicates that false negative results always should be considered when bacteriological tests are evaluated.

Histology

The presence of PMNs in the subepithelial tissue of the endometrium evaluated in a histological slide has been used as best standard for detection of endometritis (*LeBlanc* et al.

2007, *Nielsen* et al. 2010, *Overbeck* et al. 2011). Several different markers of uterine immune response in the mare has been suggested (*Nash* et al., 2010), but currently none of these markers are used routinely in clinical practice. In this study there was a significant ($p=0.0022$) correlation between detection of PMNs in the subepithelial tissue on histology and pregnancy result. Only 50 % of the mares positive for PMNs on histology became pregnant, whereas 87 % of the mares negative for PMNs on histology became pregnant. Logistic regression demonstrated that presence of PMNs detected on histology was the only factor significantly correlated to pregnancy status on day 70 ($p=0.003$). Presence of PMNs in the endometrium reduces the odds of becoming pregnant 6.5 times, compared to mares in which no PMNs were detected in the endometrium.

Kenney score

In the scoring system proposed by *Kenney* and *Doig* (1986) mares are grouped into four categories based on the histological findings of inflammatory cells, fibrosis and lymphatic lacunae in the endometrial tissue. The scoring system is based on the pregnancy results published in several papers (*Kenney* 1975, *Doig* et al. 1981). In this study, mares were grouped as proposed by *Kenney* and *Doig* (1986). A strong correlation between pregnancy result day 70 and 17, and the four Kenney groups was found in both studies ($p=0.0069$ and $p<0.0001$), respectively.

Several studies have suggested that other pathological conditions within the endometrium besides "the Kenney score" should be considered when endometrial evaluation is performed (*Schoon* et al. 1997, *Hoffmann* et. al. 2009 and *Lehmann* et al. 2011). None of these studies have however demonstrated as strong a correlation to fertility as the papers by *Doig* et al. (1981) and *Kenney* and *Doig* (1986).

Conclusion

In this study a strong significant correlation between the presence of PMNs on histology, Kenney score and pregnancy on day 70 was established. However presence of PMNs in the endometrium was the only factor with a significant impact on pregnancy on day 70, as determined by logistic regression. The Kenney score determines the ability of a mare to carry a foal to term and not the capacity to become pregnant (Love 2010). This is in agreement with our data, since Kenney score did not have a significant impact on pregnancy rate at day 70. Whether the Kenney score would have significant impact on live foal rates can in this study not be determined.

It is relatively uncomplicated to examine a HE-slide for the presence of sub epithelial PMNs by light microscopy. We suggest using histological detection of presence of PMNs to diagnose endometritis, due to the strong negative impact of PMNs within the endometrium on chances of establishing a pregnancy (day 70). A down side of the histological evaluation is the time it takes for getting an endometrial biopsy to a diagnostic lab, processed and evaluated - Time that unfortunately not always is available in the middle of a breeding season. This disadvantage should however be outweighed by the advantages of improved diagnosis.

References

Dimmocks W. W. and *Edwards P. R.* (1923) Bacteria of the genital tract of mares and the semen of the stallions and their relation to breeding efficiency. J. Am. Vet. Med. Assoc. 64, 288-298

Doig P. A., McKnigth J. D. and *Miller R. B.* (1981) The use of endometrial biopsy in the infertile mare. Can. vet. J. 22, 72-76

Hoffmann C., Ellenberger C., Mattos R. C., Aupperle H., Dhein S., Steif B. and *Schoon H. A.* (2009) the equine endometrosis: New insights in the pathogenesis. Anim. Reprod. Sci. 111, 261-278

Kenney R. M. (1975) Prognostic value of endometrial biopsy of the mare. J. Reprod. Fert. 23, 347-348

Kenney R. M. and Doig P. A. (1986) Equine endometrial biopsy. Current therapy in Theriogenology, vol 2. W.B. Saunders; 723-729.

Knudsen O. (1964) Endometrial cytology as a diagnostic aid in mares. Cornell Vet. 54, 415-422

Leblanc M. M., Magsig J. and *Stromberg A. J.* (2007) Use of a low volume uterine flush for diagnosing endometritis in chronically infertile mares. Theriogenology 68, 403-412

Lehmann J., Ellenberger C., Hoffmann C., Bazer F. W., Klug J., Allen W. R., Sieme H. and *Schoon H. A.* (2011) Morpho-functiona studies regarding the fertility prognosis of mares suffering from equine endometrosis. Theriogenology 76, 1326-1336

Love C. C. (2011) Endometrial biopsy. In Equine Reproduction. Willey-Blackwell 1929-1939

Nash D. M., Sheldon I. M., Herath S. and *Lane E. A.* (2010) Markers of the uterine innate immune response of the mare. Anim. Reprod. Sci. 119, 31-39

Nielsen J. M. (2005) Endometritis in the mare: A diagnostic study comparing cultures from swab and biopsy. Theriogenology 64, 510-518

Nielsen J. M., Bock T. S. K. and *Ersbøl A. K.* (2008) Factors associated with fertility in horses in a Danish equine practice after artificial insemination with frozen-thawed semen. Anim. Reprod. Sc. 107, 336-337

Nielsen J. M., Troedsson M. H., Petersen M. R., Bojesen A. M., Lehn-Jensen H. and *Zent W. W.* (2010) Diagnosis of endometritis in the mare based on bacteriological and cytological examination of the endometrium: Comarison of results obtained by swabs and biopsies. J. Equine Vet. Sci. 30, 27-30

Overbeck W., Witte T. S. and *Heuwieser W.* (2011) Comparison of three diagnostic methods to identify subclinical endometritis in mares. Theriogenology 75, 1311-1318

Petersen M. R., Nielsen, J. M., Lehn-Jensen H. and *Bojesen A. M.* (2009) Streptocuccus Equi Subspecies Zooepidemicus resides deep in the chronically infected endometrium of mares. Theriogenology 1, 161-167

Quinn P. J., Carter M. E., Markey B. and *Carter G. R.* (1998) Clinical veterinary microbiology. Wolfe London, U.K.

Ricketts S. W. (1975) Endometrial biopsy as a guide to diagnosis of endometrial pathology in the mare. Equine vet. J. 7, 102-108

Ricketts S. W., Young A. and *Medici E. B.* (1993) Uterine and clitorial cultures. In: McKinnon and Voss, editors. Equine reproduction. Lea & Fibiger 234-245

Riddle W. T., Leblanc M. M. and *Stromberg A. J.* (2007) Relationship between uterine culture, cytology and pregnancy rates in a thoroughbred practice. Theriogenology 68, 395-402

Schiebel G. (1920) Untersuchungen über bakterienflora im uterus sterile stuten. Duss. Med. Vet. Hannover

Schoon H. A., Schoon D. and *Klug E.* (1997) Die endometriumbiopsie bei der stute im klinisch-gynäkologischen context. Pferdeheilkunde 13, 453-464

Jesper Møller Nielsen
Ansager Dyrehospital
Gartnerhaven 5
6823 Ansager,
Denmark
jazz.miller@newmail.dk