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# Morpho-functional studies regarding the pathogenesis of the equine endometrosis with special emphasis on uterine secretions - preliminary results

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**Summary**

The aim of the study was to investigate potential etiologic factors (e.g. endometritis) for the equine endometrosis as well as to determine possible effects by seasonal, cyclical and pregnancy-associated influences. Additionally, immunohistochemical methods were established to characterize the glandular secretion pattern of mares suffering from endometrosis compared to healthy control mares. For the histomorphological and immunohistochemical examinations 952 endometrial biopsies from 620 mares were used. The endometrosis can be divided in a destructive and a non destructive form, which can be either active or inactive. However, concerning quality and quantity there were no seasonal, cyclical or pregnancy-associated influences detectable. While the uterine secretion pattern of uteroglobin, calbindin, uteroferrin, uterocalin and glycogen follows a typical cycle-dependent staining intensity in the intact endometrium, the glandular epithelia within the fibrotic foci failed to. The latter revealed a distinct variety concerning the distribution and the staining intensity. These results indicate that subsequent disturbances of the uterine micro-environment are important factors leading to reduced fertility in mares suffering from endometrosis.

**Keywords:** mare, endometrosis, endometrial biopsy, uterine secretion pattern

**Morphofunktionale Untersuchungen zur Pathogenese der Endometrose des Pferdes unter besonderer Betrachtung des uterinen Sekretionsmusters - vorläufige Ergebnisse**

Ziel der Untersuchung war die Ermittlung potentieller ätiologischer Faktoren (z. B. Endometritis) für die equine Endometrose sowie die Bestimmung eines möglichen Einflusses saisonaler, zyklischer und trächtigkeitsassoziierter Variationen. Darüber hinaus sollte, im Vergleich zu gesunden Kontrollstuten, eine immunhistologische Charakterisierung der glandulären Sekretionsprodukte bei Endometrosepatientinnen erfolgen. Als Material für Histologie und Immunhistologie standen 952 Endometriumbiopsien von 620 Stuten zur Verfügung. Die Endometrose tritt in destruierender und nicht destruierender Form, aktiv oder ruhend auf. Nachweisbar sind weder saisonale, zyklische noch trächtigkeitsassoziierte Einflüsse. Dies betrifft sowohl qualitative als auch quantitative Gesichtspunkte. Während das uterine Sekretionsmuster von Uteroglobin, Calbindin, Uteroferrin, Uterocalin und Glycogen im intakten zyklischen Endometrium einem typischen, zyklusabhängigen Verlauf folgt, ist dies in den glandulären Epithelien fibrotischer Herde nicht nachweisbar. Stattdessen zeichnen sich diese Areale durch eine große Variabilität hinsichtlich Verteilung und Reaktionsintensität aus. Diese Resultate werden von den Autoren als Hinweis für eine fertilitätsmindernde Beeinflussung des uterinen Mikromilieus im Rahmen der Endometrose interpretiert.

**Schlüsselwörter:** Reproduktion, Stute, Endometrose, Endometriumbiopsie, uterines Sekretionsmuster

## Introduction

The term endometrosis describes a periglandular and/or stromal endometrial fibrosis including glandular alterations in fibrotic foci. Single glands and/or glandular nests can be affected (*Schoon* et al. 1995). The degree of endometrosis increases with the age of the mare; however, there is no correlation to the number of foalings (*Schoon* et al. 1997). Until now the etiology as well as the pathogenesis of this important cause for equine infertility remains unknown.

The beginning fibrosis is characterized by large polygonal stromal cells of type I synthesizing collagen fibres. In advanced fibrosis, metabolic active or inactive stromal cells of type II without signs of collagen synthesis as well as myofibroblasts are the predominant cells (*Raila* 2000).

In the initial stages of endometrosis, the epithelia of the fibrotic glands are characterized by a cycle asynchronous differentiation compared to non affected glands (*Raila* 2000). Subsequently an epithelial degeneration and a glandular dilatation can develop (*Schoon* et al. 1992). As a result, marked differences occur in the fibrotic foci concerning the histochemical patterns (*Schoon* et al. 1995) as well as the expression of the steroid hormone receptors, the proliferation intensity (*Aupperle* et al. 1999) and the intermediate filaments (*Aupperle* 1997). These dysfunctions of the fibrotic glands may lead to qualitative and quantitative alterations of the endometrial secretions and consequently to a disturbance of the intrauterine micro-environment (*Hein* 2000).

However, in animal species, like mares, in which the trophoblast is non-invasive the uterine secretions seem consi-

derably more likely to play an important role in the maintenance of the conceptus (*Amoroso* 1951). Throughout the estrous cycle of the mare numerous secretory proteins and carbohydrates are present (*McDowell* et al. 1987, *Freeman* et al. 1990), with a higher accumulation of endometrial proteins in the luteal-phase compared to other mammalian species examined until now (*Beier-Hellweg* et al. 1995). The functions of the molecules identified are different. While glycogen (*Freeman* et al. 1990), the calcium-binding-protein calbindin (*Nikitenko* et al. 1998), the iron-binding protein uteroferrin (Roberts et al., 1986) and the lipocalin uterocalin (*Crosset* et al. 1998) probably supply the conceptus, the role of uteroglobin in the reproductive tract is unknown. In other tissues multiple functions of uteroglobin like anti inflammatory and anti chemotactic activities are discussed (*Mukherjee* et al. 1999).

The aim of this study was the histomorphological and immunohistochemical characterization of endometrosis in order to find out which factors possibly influence the progress of this disease. Uterine secretion patterns were examined in endometrial biopsies collected in mares during the estrous cycle as well as in mares showing degenerative lesions varying in quality and quantity, using histochemical and immunohistochemical techniques.

## Materials and methods

952 endometrial biopsies from 620 mares were studied. All specimens showed signs of endometrosis varying in quality and quantity (active/inactive, mild to severe). The animals examined and the respective targets of the analyses are summarized in Table 1.

The biopsies were fixed in formalin, embedded in paraffin and stained with Hematoxylin-Eosin (H.-E.).

Glycogen was detected by the PAS-reaction with and without a-Amylase digestion. Alcian blue staining before and after a prior treatment with Testes- and Streptomyces-hyaluronidase was used to identify the extracellular matrix in the periglandular fibrosis.

The expression of steroid hormone receptors, Ki-67-antigen, laminin, vimentin, desmin, smooth-muscle-a-actin (*Aupperle* et al. 1999, *Raila* 2000), as well as the endometrial proteins uteroglobin, calbindin, uteroferrin and uterocalin (Hoffmann, unpublished data) were detected in biopsies selected representatively by immunohistochemistry using the PAP-technique. The Immuno Reactive Score, established by *Özgen* et al. (1997) to evaluate the steroid hormone receptor expression, was modified into a Secretion-Score (SSc) to precisely assess the immunohistochemical reactions.

## Results

*Histomorphological classification of the endometrosis*

Using H.E.-stained slides it was possible to distinguish between a destructive and a non destructive appearance of endometrosis. While a non destructive endometrosis is characterized by mild alterations of the glandular epithelia, the glands in destructive endometrosis, occurring in 25% of the cases examined, showed signs of an extensive epithelial degeneration with a loss of the typical glandular architecture. Subtypes of metabolic active (ovoid, hypochromatous nuclei) and inactive (spindle-shaped, hyperchromatous nuclei) stromal cells formed the periglandular fibrosis. The coincidence of both types in one biopsy is frequent.

**Tab 1** Mares investigated and the respective target of analysis. *Übersicht über das Tiergut und die damit verfolgten Untersuchungsziele*

| Number of mares | Target of analysis | Characteristic of the uterine biopsies |
|---|---|---|
| 508 | Characterization of different types of endometrosis and possible seasonal influences | Routine biopsies during the<br>* breeding season (May to July, n=242)<br>* non-breeding season (Nov. to Feb., n=266) |
| 79 | Progress of endometrosis over a longer period of time | Repeated biopsies of the same mare<br>* over a period from 6 months to 5 years |
| 7 | Cyclic variabilities of endometrosis and characterization of the uterine secretion pattern during the estrous cycle | Repeated collection during the estrous cycle<br>* days 0 (ovulation), 5,10,13,16,19, 21<br>* determination of estradiol- and progesterone plasma concentrations |
| 8 | Influence of pregnancy on the development of endometrosis | Biopsies prior to mating and after following pregnancy<br>* days 3, 7, 10 or 28 post partum |
| 20 | Influence of endometritis on the progress of endometrosis | Experimentally induced bacterial endometritis and subsequent treatments<br>* 10 paired biopsies from 20 mares<br>* over a period of 2 years |

*Immunohistochemical and histochemical examination of the different types of endometrosis*

Obvious differences occurred in the stromal cells of periglandular fibrosis as well as in the glandular epithelia depending on type, activity and degree of endometrosis. The results of the immunohistochemical and the histochemical analysis are summarized in Table 2.

*Statistical analysis of the factors possibly influencing the progress of the endometrosis*

A statistically significant coincidence of endometrosis and endometritis is obvious in cases of destructive endometrosis, independent of the degree of the periglandular fibrosis ($p=0.05$) and in cases of severe non destructive endometrosis ($p<0.005$). Additionally, 10 of 20 mares with an experimentally induced endometritis and subsequent treatments showed a temporary metabolic activation of fibrotic stromal cells five days post infection. However, the degree of endometrosis neither increased nor decreased over the 2-year-period of the experiment. On the other hand seasonal and cyclic changes as well as pregnancies did not influence the type and/or progress of the endometrosis.

*Immunohistochemical and histochemical examination of uterine secretion during the estrous cycle*

Histochemical and immunohistochemical reaction products of the uterine secretions were detectable in the cytoplasm of the glandular epithelia, within the lumina of the glands and

sporadically in the cytoplasm of the luminal epithelia. Maximal SSc-values of the uterine secretions examined were observed in the mid and/or basal endometrial glands.

**Tab 2** Immunohistochemical and histochemical examination of periglandular fibrosis, basal lamina and glandular epithelia within fibrotic areas
*Immunhistologische und histochemische Befunde der periglandulären Fibrose, der Basallamina und der glandulären Epithelien innerhalb fibrotischer Herde*

| | | Non destructive endometrosis | | Destructive endometrosis | |
|---|---|---|---|---|---|
| **Tissue structure examined** | Parameter | active fibrosis | inactive fibrosis | active fibrosis | inactive fibrosis |
| **Periglandular fibrosis** | steroid hormone receptors[1] | ⇩ | ⇩ | ⇩ | ⇩ |
| | Ki-67-antigen (proliferation activity) | (+) | -- | (+) | -- |
| | smooth-muscle-α-actin | +(+) | +(+) | ++(+)[2] | +(+) |
| | acid mucopolysaccharides | ++ | + | +++ | + |
| **Basal lamina** | laminin[5] | +(+)[4] | ++[4] | +++ | +++ |
| **Glandular epithelia** | steroid hormone receptors[1] | ⇧ | ⇩ | ⇩⇩[3] | ⇩(⇩)[3] |
| | vimentin | +[4] | + | ++(+) | ++ |

1 compared with the expression of steroid hormone receptors in unaffected glands
2 p<0.005
3 p<0.001
4 depending on the degree of endometrosis
5 degree of discontinuity

⇩ = mild decrease
⇩(⇩) = mild to moderate decrease
⇩⇩ = moderate decrease
⇧ = mild increase
(+) = slight
+ = mild
+(+) = mild to moderate
++ = moderate
++(+) = moderate to severe
+++ = severe

The staining intensity, the percentage and localization of positive cells revealed typical reaction patterns during the estrous cycle. However, the day of the cycle on which a maximum was detected varied between the mares. An overview of the proteins and glycogen reaction patterns during the estrous cycle is presented in Table 3.

*Immunohistochemical and histochemical examination of glandular secretion patterns in endometrosis*

In comparison to non affected glands, most of the epithelia in periglandular fibrosis showed a decreased staining intensity of the uterine secretion products examined, except for uteroferrin.

In contrast, uteroferrin presented a variable reaction pattern; the highest staining-intensities were achieved in the glandular epithelia within areas of a severe non destructive as well as destructive endometrosis. A schematic overview is presented in Table 4. The influence of the fibrosis activity on the expression of the uterine secretion products examined was undetectable.

**Tab 3** Glandular secretion patterns of the proteins and glycogen examined throughout the estrous cycle
*Expression der sekretorischen Proteine und Nachweis des Glykogens innerhalb endometrialer Drüsen im Verlaufe des Zyklus*

| Uterine secretion product | Day(s) of maximal SSc | Predominant glandular localization | Basic staining-intensity throughout the whole estrous cycle |
|---|---|---|---|
| **Uteroglobin** | 21,0 and 13 | mid and basal glands | low values |
| **Glycogen** | 5-10, 19 | mid glands | low values |
| **Calbindin** | 5-10, 21 | apical, mid and basal glands | none |
| **Uterocalin** | 10-13 | mid and basal glands | none or low values |
| **Uteroferrin** | 13 | apical, mid and basal glands | None or low values |

## Conclusions

By the histomorphological and immunohistochemical techniques applied, a distinct differentiation of the endometrosis in destructive and non destructive types was possible.
The high glandular expressions of vimentin as well as the severely damaged basal lamina are features of the advanced epithelial degeneration in destructive fibrosis. This progredient character is likely attributed to the increased number of contractile myofibroblasts compared to that of non destructive fibrosis.

**Tab 4** Glandular secretion patterns of the proteins and glycogen examined within the endometrotic foci
*Sekretionsmuster der Proteine bzw. des Glykogens in glandulären Epithelien endometrotischer Areale*

| | Uteroglobin | Calbindin | Uteroferrin | Uterocalin | Glycogen |
|---|---|---|---|---|---|
| **Glandular epithelia in fibrotic foci[1]** | ⇩/↑ | ⇩/↑ | ⇩/⇧ | ⇩/↑ | ⇩/↑ |

[1] compared with unaffected glandular epithelia
⇩ decreased SSc
⇧ increased SSc
↑ increased SSc, nearly exclusively seen in non destructive endometrosis

Neither a pregnancy nor seasonal and/or cyclical endocrine variations seem to have a remarkable influence on the progress of endometrosis. The hypothesis that fibrotic glands became independent of the uterine control mechanisms (*Schoon* et al. 1995) is confirmed.

Moreover, the high expression of steroid hormone receptors observed only in active non destructive endometrosis refers to a reciprocal correlation between the epithelia and the fibrotic stromal cells. As discussed in previous studies (*Raila* 2000), different growth factors as possible mediators and other cytokines may also be involved.

The proteins investigated as well as glycogen showed typical expression patterns throughout the estrous cycle; however, distinct individual differences between the mares examined were obvious.

Deficient protein secretion patterns in uterine and cervical secretions of mares suffering from moderate or severe endometrosis has been demonstrated by *Hein* (2000) by means of electrophoresis. It has to be considered that numerous of these proteins are not yet identified. Even glycogen and the four proteins investigated in this study showed deviations of the epithelial secretion patterns within the fibrotic areas compared to the unaffected glands. This phenomenon is probably at least one factor leading to a disturbance of the intrauterine micro-environment, relevant in the pathogenesis of endometrosis induced fertility problems in the mare.

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