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Ultrastructural and immunohistochemical characterization of the physiological and pathological inactivity of the equine endometrium

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Summary

The objective of this study was the characterization of the endometrial inactivity during winter anestrus compared to pathological inactivity during the breeding season. Endometrial biopsies from 20 mares in winter anestrus were investigated histologically, immunohistochemically (estrogen receptor (ER), progesterone receptor (PR), Ki-67 antigen, desmin), and ultrastructurally. Additionally, endometrial biopsies from five mares suffering from an endometrial inactivity during the physiological breeding season were examined. During winter anestrus the inactive endometria were characterized by a reduced number of non tortuous glands, mainly consisting of non ciliated cells with short microvilli, round nuclei, and a few filamentous mitochondria. Some of the inactive stromal cells showed incorporated cilia. In all the endometria a very low Ki-67 antigen and an intense stromal desmin expression was detected, but there was a distinct variety in the epithelial and stromal ER and PR expressions with no correlation to the morphological findings. During the breeding season all inactive endometria showed no expression of Ki-67 antigen and very weak expressions of ER and PR. An intense stromal desmin expression occurred in only one of the mares. In conclusion, during the breeding season, the detection of Ki-67 antigen, ER and PR is a worth full tool in the diagnosis of endometrial inactivity. However, during winter anestrus the endometrial morphology is uniform, but the immunohistochemical findings are variable and atypical expression patterns at this time have to be interpreted critically.

Keywords: reproduction, endometrium, inactivity, mare, morphology, immunohistochemistry

Ultrastrukturelle und immunhistochemische Charakterisierung der physiologischen und pathologischen Inaktivität des equinen Endometriums

Ziel dieser Studie war die Charakterisierung und der Vergleich der endometrialen Inaktivität im Winteranöstrus und während der physiologischen Decksaison. Endometriumbiopsien von 20 Stuten im Winteranöstrus wurden histologisch, immunhistologisch (Östrogenrezeptoren (ER), Progesteronrezeptoren (PR), Ki-67 Antigen, Desmin) und transmissionselektronen-mikroskopisch beurteilt. Außerdem wurden Endometriumbiopsien von fünf Stuten mit einer endometrialen Inaktivität während der physiologischen Decksaison untersucht. Das physiologisch inaktive Endometrium weist eine geringe Anzahl gestreckt verlaufender Drüsen auf, die überwiegend aus nicht zilierten Zellen mit kurzen Mikrovilli, runden Zellkernen sowie wenigen filamentösen Mitochondrien bestehen. Die inaktiven Stromazellen exprimieren Desmin und einzelne Zellen enthalten Zilien. Während alle Endometriumbiopsien eine sehr schwache Expression von Ki-67 Antigen aufweisen, variiert die Expression der Hormonrezeptoren in den Drüsen und im Stroma stark, unabhängig von den morphologischen Befunden. Die endometriale Inaktivität während der physiologischen Decksaison ist gekennzeichnet durch das Fehlen einer Expression von Ki-67 Antigen und durch eine sehr schwache Expression von ER und PR. Eine stromale Desminexpression kann lediglich bei einer Stute nachgewiesen werden. Die Darstellung der Steroidhormonrezeptoren ist ein nützliches Hilfsmittel bei der Diagnose der endometrialen Inaktivität während der physiologischen Decksaison. Da aber die Expression der Hormonrezeptoren im Winteranöstrus, trotz einheitlicher endometrialer Morphologie sehr variabel ist, ist während dieses Zeitraumes eine kritische Interpretation atypischer Expressionsmuster erforderlich.

Schlüsselwörter: Reproduktion, Endometrium, Inaktivität, Stute, Morphologie, Immunhistochemie

Introduction

In mares, the winter anestrus extends in the northern hemisphere from the last ovulation in September/October until the first ovulation of the subsequent ovulatory season (March/April) (*Ginther* 1992). In general, during winter anestrus, the ovarian follicles are smaller than 20 mm in diameter, serum LH, estradiol and progesterone values are basal, whereas the FSH level is unchanged compared to the breeding season and the melatonin concentration is high (*Ginther* 1992). Numerous factors controlling the equine anestrus, as melatonin (*Fitzgerald* and *Smith* 1995), length of day light (*Palmer* and *Guillaume* 1992), opioids (*Aurich* et al. 1994) and others have been identified, though the mechanisms were not completely understood until now. One important fact is the wide range of indiviual variations regarding the clinical behaviour and the ovarian activity (*Fitzgerald* and *Smith* 1995). However, estrus activity occurs even during the anovulatory season in pregnant and in ovariectomized mares, possibly due to androgen synthesis by the adrenal glands (*Ginther* 1992).

During the breeding season, anestrus behaviour may be the result of pregnancy, bad body condition, chromosomal abnormalities, persistent corpora lutea, or ovarian tumors (*Allen* 1977, *Hughes* and *Stabenfeld* 1977). Furthermore, endometrial inactivity at this time may be caused by ovarian

disorders (*Ellenberger* et al. 2002) or endometrial steroid hormone receptor insufficiency (*Schoon* et al. 1999, 2000). During winter anestrus the endometrium shows signs of inactivity, which are reversible with the onset of spring (*Ginther* 1992). Until today, lightmicroscopical descriptions (*Kenney* 1978) but no detailed ultrastructural and immunohistochemical studies of the equine endometrium during winter anestrus are available, however, numerous detailed studies of the normal actively cycling endometrium have been published (*Brunckhorst* et al. 1991, *Strankmeyer* 1993, *Raila* et al. 1997, *Schoon* et al. 1997, *Aupperle* et al. 2000, 2003). The objective of this study is the characterization of morphological and functional parameters in inactive endometria during the physiological anovulatory season, as well as in pathologically inactive endometria during the breeding season.

Material and Methods

Endometrial biopsies of 20 mares (4 to 19 years old), which had been sent to the "Institut für Veterinär-Pathologie Leipzig" for routine investigation between October to February were investigated lightmicroscopically (n=20) and ultrastructurally (n=6). All mares showed anestrus behaviour and the ovarien follicles were smaller than 20 mm. Additionally, the endometrial biopsies of five mares (10 to 14 years old), whose endometria were classified as inactive in May and July, were investigated lightmicroscopically. They showed active ovaries and in three of the mares estrous behaviour was reported, while no information was available in two cases.

Endometrial biopsies were fixed in 4% formalin, embedded in paraplast, and routinely stained with Hematoxylin-Eosin. Immunohistochemistry was performed using the Peroxidase anti-Peroxidase technique. Mouse anti-human estrogen receptor[1], mouse anti human-progesterone receptor[2], mouse anti-human Ki-67 antigen[1], and mouse anti-human desmin[3] served as primary antibodies. The evaluation of the Immunoreactive Score (IRS) of the steroid hormone receptor expression refers to the method described by *Özgen* et al. (2002). Specimens of six mares (winter anestrus, inactive endometrium) were dissected; small pieces were post fixed in 3% glutaraldehyde and prepared for transmissionelectronmicroscopy (TEM).

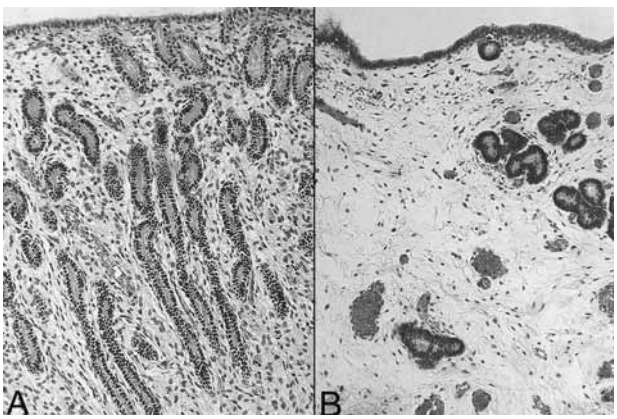
Results

Endometrial findings during winter anestrus

Lightmicroscopy

The endometria of 15 mares were classified as "inactive" during winter anestrus. They showed a uniform morphology characterized by one-layered, cuboidal luminal, and glandular epithelia with small round hyperchromatic nuclei. The number of glands was low and they appeared to be non tortuous (Fig. 1A). Glandular lumina were hardly visible or dilated with inspissated remnants of secretion. The endometrial stroma was dense and the stromal cells showed large oval hyperchromatic nuclei and scanty amounts of pale cytoplasm. Furthermore, in five mares with clinical findings of winter anestrus, the endometria revealed signs of mild proliferative (n=3) or secretory activity (n=2).

Fig 1 A: The inactive endometrium during winter anestrus is characterized by an one-layered, cuboidal luminal epithelium, a few straight minimal branched glands with hardly visible lumina and a dense stroma with large stromal cells. (H.-E. stain, magnification 31.25x)
Das inaktive Endometrium besitzt im Winteranöstrus ein einreihiges kubisches luminales Epithel, wenige gerade, kaum verzweigte Drüsen mit engen Lumina und ein dichtes Stroma mit großen Stromazellen. (H.-E. Färbung, Gerätevergrößerung 31,25x)
B. The inactive endometrium during the physiological breeding season is characterized by columnar luminal epithelia and a few irregularely inactive glands, lying in an edematous endometrium. (H.-E., magnification 31.25x)
Das inaktive Endometrium während der physiologischen Decksaison ist gekennzeichnet durch ein hochprismatisches luminales Epithel und sehr wenige Drüsen mit einer irregulär inaktiven Differenzierung, die in einem ödematisierten Stroma liegen. (H.-E. Färbung, Gerätevergrößerung 31,25x)



Transmissionelectronmicroscopy

The inactive endometria (n=6) showed an uniform ultrastructure corresponding to the lightmicroscopically findings: The luminal epithelia were composed of numerous cuboidal secretory cells with short microvilli (0.35 µm), some secretory vacuoles, and few ciliated cells (Fig. 2A). Both cell types showed mildly invaginated oval euchromatic nuclei, small oval mitochondria (0.80 x 0.36 µm), small vesicles of Golgi´s apparatus, minute amounts of rough endoplasmic reticulum and numerous polyribosomes.

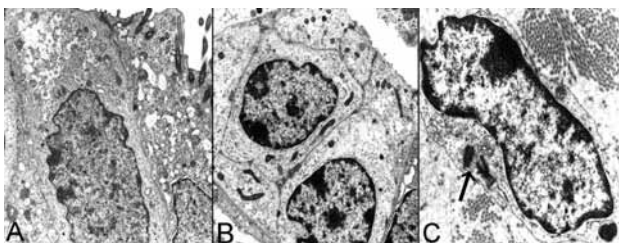
The inactive glands mostly consisted of cuboidal non ciliated cells with a few, very short microvilli (0.23 µm) and the lumina contained secretory remnants and debris (Fig. 2B). The glandular epithelia showed oval euchromatic nuclei (5.6 x 4.5 µm), single filamentous mitochondria (1.8 x 0.23 µm), small parts of rough endoplasmic reticulum, and an inactive Golgi´s apparatus as well as single secretory vacuols and secondary lysosomes.

The stromal cells appeared to be spindle shaped with a few short processes. They contained oval heterochromatic nuclei (5.7 x 3.4 µm) with mild invaginations, a few oval mitochondria (0.8 x 0.4 µm), some ribosomes and single lysosomes. In three mares some stromal cells showed incorporated cilia (Fig. 2C).

Immunohistochemistry

In all mares (n=20), independent of the endometrial activity, Ki-67 antigen was expressed only in a few luminal epithelia

Fig 2 A. During winter anestrus the luminal epithelium of the inactive endometrium is composed of numerous cuboidal non ciliated cells containing a few secretory vacuols and a few ciliated cells. Both cell types show oval euchromatic nuclei, some oval mitochondria, numerous polyribosomes, and single vesicles of the Golgi´s apparatus (TEM, magnification 4400x).
Während des Winteranöstrus besteht das luminale Epithel des inaktiven Endometriums überwiegend aus kubischen nicht zilierten Zellen, die einzelne Sekretvakuolen enthalten und wenigen zilierten Zellen. Beide Zelltypen enthalten ovale euchromatische Zellkerne, einzelne ovale Mitochondrien und zahlreiche Polyribosomen sowie einzelne Vesikel des Golgi-Apparates. (TEM, Gerätevergrößerung 4400x).
B. The cuboidal glandular epithelia mostly show non ciliated cells with a few short microvilli, the lumina contain secretory remnants, and debris. The epithelial cells show oval euchromatic nuclei, single filamentous mitochondria, small parts of rough endoplasmic reticulum, and the cellular membranes are straight (TEM, magnification 4400x).
Die kubischen Drüsenepithelien bestehen überwiegend aus nicht zilierten Zellen mit kurzen Mikrovilli, in den Drüsenlumina liegen Sekretreste und Debris. Die Epithelzellen enthalten ovale euchromatische Zellkerne, einzelne filamentöse Mitochondrien, wenig raues endoplasmatisches Retikulum und die Zellgrenzen verlaufen gerade. (TEM, Gerätevergrößerung 4400x).
C. The spindle shaped stromal cell contains an oval euchromatic nuclei with mild invaginations. The cytoplasm is scanty and includes a few oval mitochondria, some ribosomes and single lysosomes. In this stromal cell single cilia (arrow) are present. (TEM, magnification 7000x)
Die spindelige Stromazelle enthält einen ovalen euchromatischen Zellkern mit geringgradigen Einkerbungen. In dem schmalen Zytoplasmasaum finden sich wenige ovale Mitochondrien, Ribosomen und Lysosomen sowie vereinzelte Zilien (Pfeil). (TEM, Gerätevergrößerung 7000x)



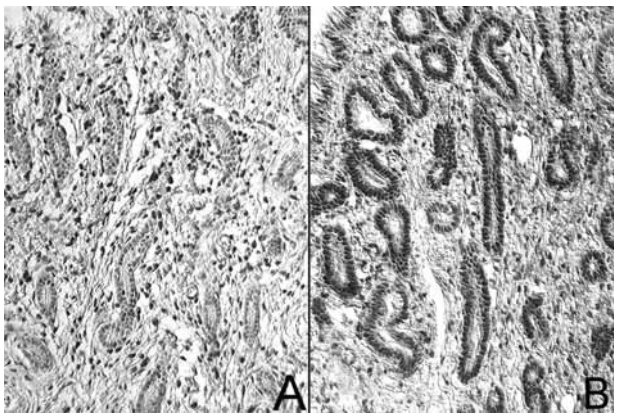
and was very rarely found in glandular and stromal cells. Intense desmin expression occurred in 70% to 100% of the stromal cells of all mares.

Whereas the endometria showed uniform expression patterns of Ki-67 antigen and desmin, the hormone receptor expression (Fig. 3) presented a very distinct variety. The expression of ER and PR was not synchronously high or low in the stromal respectively the glandular cells and no typical pattern was obvious. The Immunoreactive Score of hormone receptor expression varied within the glands between IRS 0.7-10.0 (ER), respectively IRS 0.2-7.5 (PR), and in the stromal cells IRS values were between 1.0-8.1 (ER), respectively IRS 0.2-4.8 (PR). No correlation could be detected between the lightmicrocopical or ultrastructural findings and the expression pattern of hormone receptors.

Endometrial inactivity during the breeding season

The biopsies of five mares showed an unequal inactivity during the physiological breeding season. The stratum glandulare was thin and contained a few glands or glandular nests embedded in a moderately edematous stroma (Fig. 1B).

Fig 3 Variable expression patterns of estrogen receptor (ER) in the inactive endometrium during winter anestrus of two different mares: A. Intense stromal and weak glandular expression of ER. B. Intense glandular and moderate stromal expression of ER. (Immunohistochemistry, estrogen receptor, magnification 62.5x)
Variables Expressionsmuster der Östrogenrezeptoren im inaktiven Endometrium während des Winteranöstrus bei zwei Stuten: A. Intensive stromale und schwache glanduläre ER Expression. B. Intensive glanduläre und mittelgradige stromale ER Expression. (Immunhistologie, Östrogenrezeptor, Gerätevegrößerung 62,5x)



The two-layered luminal epithelium was mostly columnar with oval hyperchromatic nuclei. The lumina of the glandular ducts were of a variable diameter and often contained inspissated secretory remnants. The glandular epithelia were irregularely cuboidal or columnar with small round or oval hyperchromatic nuclei. The slender stromal cells showed thin or spindle shaped hyperchromatic nuclei.

The immunohistochemical investigations revealed a uniform picture: No expression of Ki-67 antigen was detected and the expression of ER and PR was very weak in epithelial (ER/PR IRS 0.1-0.9) and stromal cells (ER/PR IRS 0.1-0.6). Stromal desmin expression occurred only in one mare.

Discussion

The results of the present study show that the lightmicroscopical findings in inactive endometria during physiological winter anestrus corresponded to the findings reported by *Kenney* (1978). The ultrastructural morphology of the endometria was similar in parts to that described in involution on day 16 post ovulationem (*Raila* et al. 1997, 1999). Although during winter anestrus epithelial and stromal cells were quiet inactive, and ciliated stromal cells appear to be normal during winter anestrus. Whereas ciliated stromal cells have been observed before but only in areas of endometrosis in the active endometrium (*Raila* et al. 1998, 1999). Stromal desmin expression, observed during winter anestrus, has been reported before in endometrial maldifferentiation (*Häfner* 1999, *Ellenberger* et al. 2002) and in endometrosis (*Raila* et al. 1998), but not in the actively cycling endometrium (*Aupperle* et al. 2003).

During the breeding season the endocrinological values as well as the morphological and immunohistochemical findings in the endometrium are closely related (*Aupperle* et al. 2000). The most striking result of this study was the fact, that during anestrus, despite of an almost uniform inactive endometrial

morphology, the expression of ER and PR varies individually in an unexpected extent. The cause of this phenomenon remains unclear in the present study and further investigations are neccessary for a better understanding of these results.

The morphology of the inactive endometria during summer is different from that, characterized as "endometrial atrophy during the physiological breeding season" (*Kenney* 1978, *Schoon* et al. 1997). The morphology and the immunohistochemical results (the lack of Ki-67 antigen, ER and PR expression) correspond to the cases described as "unequal inactivity" by *Häfner* (1999) and are quiete different from the findings during winter anestrus.

As reported in previous studies, the investigation of ER and PR expression is an important tool to diagnose endometrial maldifferentiation of various causes (*Schoon* et al. 1999, *Ellenberger* et al. 2002). However, the present investigation reveals, that this method is useful only during the breeding season. During winter anestrus the findings are variable and the atypical expression patterns at this time have to be interpreted critically and repeated biopsies may be necessary to confirm the diagnosis of maldifferentiation.

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