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Histopathological and immunohistochemical findings during the sexual cycle in female mules and effects of long-term Regumate® administration

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Summary

The aim of the first part of this study was a comparative immunohistochemical characterisation of the uterine secretory proteins uteroglobin (UG), uterocalin (UC), uteroferrin (UF) and of the calcium-binding protein calbindin (CAL) in the endometrium of clinically acyclic (n = 4) and "cyclic" mules (n = 2) considering clinical-gynaecological findings, endometrial functional morphology and blood serum concentrations of progesterone and estradiol. For this purpose a clinical-gynaecological examination was performed in five animals from April to August and in one mule until November 2004. Endometrial biopsies were taken concurrently (acyclic mules n = 46 biopsies, "cyclic" mules n = 51 biopsies). The endometrial proteins UG, UC, UF and CAL showed a very slight expression in the inactively differentiated endometria of the acyclic mules. In the endometria of the "cyclic" mules the secretion of these proteins was highly variable and no correlation between the phases of the sexual cycle, the concentration of the serum hormone values or the endometrial functional morphology could be detected. In the second part of the study two of the acyclic mules received an oral dose of 0.044 mg/kg BW (body weight) Regumate® for 50 days in order to examine the influence of a long-term progestin administration on the endometrial functional morphology. During the treatment period until 27 days post administration the mules were weekly biopsied and examined both gynaecologically and endocrinologically. Two years after the treatment endometrial biopsies were collected once more. The results obtained prior to Regumate® administration were compared with those during and after the treatment. On that account, 28 endometrial biopsies were examined pathohistologically (H. E. staining) and imunohistologically (UG, UC, UF, CAL, estrogen receptor (ER), progesterone receptor (PR), Ki-67 antigen). Prior to Regumate® medication the endometrium of the acyclic mule mares exhibited an irregular inactive differentiation with a very faint immunolabelling of all uterine proteins investigated. During the medication the endometrium developed a highly irregular secretory differentiation associated with a distinctly increased secretion of CAL, while the secretion of the other investigated proteins remained low. After cessation of the Regumate® administration as well as two years later the endometrium was found to be similarly irregular and inactive as before the treatment, the secretion pattern of the endometrial proteins was comparable to that before medication. In the course of the treatment an overall increased and highly variable expression of the steroid hormone receptors was noted. The administration of Regumate® did not show any significant influence on the endometrial proliferative activity. The results of this study clearly show that in mule mares an exogenous long-term administration of progestins causes an exceedingly pronounced secretory differentiation in the endometrium of acyclic mules, and beyond this generates distinctly visible and in principal reversible morphologic and functional alterations (maldifferentiations) as described in mares.

Keywords: mule, progestin long-term administration, endometrium, maldifferentiation

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Histopathologische und immunhiostologische Untersuchungen zum Sexualzyklus weiblicher Maultiere und zu den Auswirkungen einer Regumate®-Langzeitapplikation

Ziel dieser Studie war erstens die vergleichende immunhistologische Charakterisierung der uterinen Sekretionsprodukte Uteroglobin (UG), Uterokalin (UK), Uteroferrin (UF) und des kalziumbindenden Proteins Calbindin (CAL) im Endometrium von klinisch azyklischen (n = 4) und „zyklischen" Maultierstuten (n = 2) unter Berücksichtigung der klinisch dokumentierten Befunde, der endometrialen Funktionsmorphologie und der endokrinologisch bestimmten Serumhormonwerte. Hierzu wurden die sechs Tiere von April bis August, eine Stute bis November 2004, regelmäßig klinisch-gynäkologisch untersucht und Endometriumbioptate entnommen (azyklische Maultiere n = 46 Bioptate, „zyklische" Maultiere n = 51 Bioptate). Die Sekretion der endometrialen Proteine UG, UK, UF und CAL erfolgte bei den azyklischen Maultierstuten bei Vorliegen eines inaktiven Endometriums in Spuren. Bei den beiden „zyklischen" Tieren wurde eine äußerst variable Sekretion der uterinen Proteine beobachtet, wobei keine Korrelation zum klinisch dokumentierten Zyklusstand, den Serumhormonkonzentrationen oder der endometrialen Funktionsmorphologie ermittelt werden konnte. Im zweiten Schritt wurden zwei der azyklischen Maultierstuten über 50 Tage einer oralen Regumate®-Applikation (0,044 mg/kg) unterzogen, um den Einfluss einer Progestagen-Langzeitapplikation auf die endometriale Funktionsmorphologie zu überprüfen. Begleitend zur wöchentlichen Bioptatentnahme fanden klinisch-gynäkologische und endokrinologische Untersuchungen statt. Dieses Vorgehen wurde nach Absetzen des Medikaments über 27 Tage beibehalten, und 2 Jahre nach der Behandlung wurden beide Stuten erneut einmalig bioptiert. Die Ergebnisse vor Behandlungsbeginn wurden sowohl mit den Befunden während der Regumate®-Applikation als auch mit denen post applicationem verglichen. Für diese Untersuchungen standen insgesamt 28 Bioptate zur Verfügung, die (immun-)histologisch (H.-E.-Färbung, UG, UK, UF, CAL, Östrogen- (ER) und Progesteronrezeptoren (PR), Ki-67 Antigen) untersucht wurden. Vor der Gabe von Regumate® stellte sich das Endometrium der zwei azyklischen Maultierstuten irregulär inaktiv differenziert dar, mit Hilfe immunhistologischer Methoden konnte eine äußerst geringgradige Sekretion aller untersuchten endometrialen Proteine nachgewiesen werden. Unter der Progestagen-Langzeitapplikation bildete sich eine hochgradig irregulär sekretorische Diffe-

renzierung aus, bei der partiell eine überdeutliche Sekretion von CAL auftrat, während sich die der übrigen untersuchten Proteine kaum veränderte. Sowohl direkt nach dem Absetzen von Regumate® als auch zwei Jahre nach Beendigung der Studie zeigte sich das Endometrium ähnlich irregulär inaktiv differenziert wie vor der Hormongabe. Die Sekretion der endometrialen Proteine entsprach weitgehend jener vor der Behandlung. Die Expression der Steroidhormonrezeptoren steigerte sich im Verlauf der Untersuchungen insgesamt deutlich, wobei die Expression hochgradig variabel erfolgte. Auf die endometriale Proliferationsaktivität zeigte die Behandlung keinen signifikanten Einfluss. Diese Ergebnisse verdeutlichen, dass exogen über einen längeren Zeitraum zugeführte Progestagene bei azyklischen Maultierstuten zu einer überdeutlichen endometrialen Sekretionsmorphologie führen, und ebenso deutlich erkennbare sowie grundsätzlich reversible morphologisch-funktionelle endometriale Alterationen (Fehldifferenzierungen) verursachen wie bei Pferdestuten.

Schlüsselwörter: Maultier, Progestagen-Langzeitapplikation, Endometrium, Fehldifferenzierung

Introduction

Mules (crossbreds between a male donkey (E. asinus; 2n = 62) and an equine mare (E. caballus; 2n = 64)) are in most cases infertile because of their uneven chromosome set (2n = 63) (*von Gugelberg* und *Bähler* 1994), but the fact that these animals are successfully used as embryo recipients from equine mares and jennies proves that their genital system, in cooperation with the endocrine mechanisms, is basically capable of providing the conceptus an adequate uterine environment that fulfils its nutrient and oxygen requirements (*Möllmann* 1991, *Allen* and *Short* 1997, *Camillo* et al. 2003).

The secretion of endometrial proteins has not yet been studied in mules, whereas in the mare, selective uterine proteins have been characterised (*Hoffmann* 2006).

The lipocalin uterocalin (UC) (*Beier-Hellwig* et al. 1995), uteroglobin (UG), which serves as an anti-inflammatory agent and immune-modulator (*Miele* et al. 1987, *Mukherjee* et al. 1999), and the iron-binding protein uteroferrin (UF) (*Bazer* et al. 1975), belong to a wide group of endometrial secretory proteins, whereas calbindin (CAL) is part of a family of intracellular calcium-binding proteins (*Wooding* et al. 1996).

Hoffmann (2006) detects a distinctly regulative effect of progesterone on the synthesis/secretion of these proteins in the endometrium of mares with maximum staining intensities during diestrus.

Huth et al. (2007) demonstrate in the same group of animals as used in the present study, a histologically cycle-asynchronous and maldifferentiated endometrium in biopsy specimens of mules with clinically erratic sexual cycles. Consistent with the striking variability of the endometrial morphology the authors note a patchy immunolabelling of the estrogen (ER) and progesterone (PR) receptors and a strong variance in the expression of the proliferation marker Ki-67 antigen. Acyclic mules show an irregular inactive endometrial differentiation with a likewise variable expression of ER and PR and an at most low proliferation activity of the endometrium (*Huth* et al. 2007).

In reproductive medicine in the mare, progestins are used with various indications such as exogenous control of the sexual cycle or progesterone substitution during pregnancy (*Ousey* 2002). For these purposes Regumate® is often chosen as its active agent Altrenogest reduces the blood concentrations of the gonadotropins LH (luteinizing hormone) and FSH (follicle stimulating hormone), due to a negative feedback on the hypothalamus-pituitary-axis leading in consequence to a blockade of the sexual cycle (*Klug* et al. 1997).

Contrary to the approved control of the sexual cycle in mares, the use of supplementary progestagens during pregnancy is controversially discussed.

Jackson et al. (1986) describe Altrenogest as a common and efficient drug for this use, in order to avoid an insufficient concentration of the hormone and hence the risk of abortion; other authors regard this routine medication sceptical by (*Allen* 1984, *Ginther* 1985).

The aim of this study was primarily a comparative immunohistochemical characterisation of the endometrial secretory proteins UG, UC, UF and CAL in the endometria of acyclic (n = 4) and "cyclic" (n = 2) mules, taking the gynaecological findings, the endometrial functional morphology and the endocrinologically identified concentrations of serum estradiol and progesterone into consideration. Secondly, two acyclic mule mares were selected for a prolonged oral administration of progestins for 50 days. The results prior to the treatment were compared to the findings during Regumate® administration and to those after cessation of the medication, including the evaluation of the concurrently documented gynaecological data as well as the endocrinological findings.

Materials and methods

Animals

For investigation 46 endometrial biopsies of 4 clinically acyclic and 51 biopsies of 2 "cyclic" mules, aged 13 to 23 years, were obtained. The acyclic animals were examined gynaecologically from April until August 2004 in an approximate ten day interval and every clinical exploration was combined with endometrial biopsy sampling and blood withdrawl to measure the serum levels of estradiol and progesterone (Tabel 1). Following the same examination protocol for the two "cyclic" mules (mule 1, 13 years; mule 2, 20 years) specimens from mule 1 were obtained almost weekly from April to November, but mule 2 was sampled in more irregular intervals from April to August.

Clinical history

The four acyclic mules showed small, inactive ovaries when examined gynaecologically and ultrasonographically as well as low and almost steady serum hormone values (Table 1).

Within the gynaecological and ultrasonographical examination of the two "cyclic" mules the existence and growth of

Table 1 Serum hormone concentrations of acyclic mules (n = 4). *Serumhormonkonzentrationen der azyklischen Maultierstuten (n = 4).*

Mules	Progesterone [ng/ml]	Estradiol [pg/ml]
Mule 4	0.63 – 1.1	10.8 – 14.5
Mule 5	0.59 – 0.85	7.8 – 13.7
Mule 3	0.28 – 1.0	7.0 – 10.8
Mule 6	0.25 – 0.97	9.3 – 14.2

Table 2 Serum hormone concentrations of "cyclic" mules (n = 2). *Serumhormonkonzentrationen der „zyklischen" Maultierstuten (n = 2).*

Phase of cycle	Progesterone [ng/ml]	Estradiol [pg/ml]
Estrus	0.40 – 2.0	4.9 -11.8
Interestrus	0.23 – 9.8	< 1.0 – 15.0
Anestrus	1.0	6.8

Table 3 Biopsy sampling in acyclic mules (n = 2) prior to, during and after Regumate® administration.
Biopsieschema bei azyklischen Maultierstuten (n = 2) vor, unter und nach der Regumate®-Applikation.

Animals	Prior to Regumate® administration (period: 121 d)	During Regumate® administration (period: 50 d)	After Regumate® administration
Mule 4 (21 years)	11 biopsies/animal	9 biopsies/animal	(period: 27 d) 4 biopsies/animal
Mule 5 (19 years)			(after 2 years) 1 biopsy/animal

ovarian follicles, ovulations and the development of corpora lutea could be documented, while the phases of the sexual cycle varied to a great extent (27 to 112 days); estrus, interestrus and anestus occurred very irregular and without any correlation with the measured serum hormone values (*Bartmann* et al. 2007, table 2).

Subsequently, two mules from the acyclic group (mule 4, 21 years; mule 5, 19 years) received a long-term treatment over 50 days with an oral dose of 0.044 mg/kg BW (body weight) Regumate® (Altrenogest) (Table 3). Biopsies were taken almost weekly during the treatment period (n=9 biopsies/animal) as well as in the following 27 days after treatment (n=4 biopsies/animal with the first biopsy taken on day 8 after the end of treatment). The clinical examination protocol con-tinued as described above.

Two years after the termination of the study one biopsy was taken again from each mule.

Histopathology/Immunohistochemistry

Biopsy samples of all animals were fixed in 4% buffered formalin immediately after collection, embedded in paraplast, stained with haemalaun and eosin (H E) and finally examined histopathologically and immunohistochemically (UG[1], UK[2], UF[3], CAL[4]). Furthermore, biopsy samples of the two acyclic mules that received a long-term administration of progestins were immunolabelled for ER[5], PR[6] and Ki-67[7] antigen. Immunohistochemistry was performed via the peroxidase antiperoxidase (PAP) method. The expression of the endometrial steroid hormone receptors was analysed using the immunoreactive score (IRS) established by *Özgen* et al. (1997), and the secretion of the endometrial proteins was evaluated by means of the secretion-score (SSc) established by *Hoffmann* et al. (2003).

Results

Comparative presentation of the results from acyclic and "cyclic" mules

Functional morphology of the endometrium

Consistent with the clinically diagnosed phase of cycle and the comparatively low serum hormone concentrations, the four acyclic mules exhibited an inactive but slightly irregularly differentiated endometrium. Independent of the clinically documented stage of cycle or the endocrinological parameters, the two "cyclic" mules showed a proliferative, secretory or inactive endometrium with concurrent irregular and/or unequal differentiation. Additionally, all six mules displayed an endometrosis varying in type and degree. A detailed characterisation of the endomemetrial functional morphology of all these mules was performed by *Huth* et al. (2007).

Immunohistochemical results

Secretion of the uterine proteins was detectable in all mules during the whole investigation period and was observed diffusely in the cytoplasm of all uterine epithelia; in addition CAL was immunolabelled in the nuclei. Except of UC, protein secretion (especially CAL) was detectable in uneven patterns within as well as between the uterine glands.

Table 4 Serum hormone concentrations in acyclic mules (n = 2) prior to, during, and after Regumate® administration.
Serumhormonkonzentrationen der azyklischen Maultierstuten (n = 2) vor, unter und nach der Regumate®-Applikation.

	Mule 4		Mule 5	
	Progesterone [ng/ml]	Estradiol [pg/ml]	Progesterone [ng/ml]	Estradiol [pg/ml]
Prior to Regumate® administration	0.63 - 1.1	10.8 - 14.5	0.59 - 0.85	7.8 – 13.7
During Regumate® administration	0.62 - 0.97	11.1 - 14.4	0.50 - 0.67	8.2 - 12.9
After Regumate® administration	0.80 - 0.95	10.6 - 14.1	0.64 - 0.79	11.0 - 13.6

Acyclic mules

UG secretion was mostly visible at very low sporadically slight-to-moderate levels (SSc 0.01-3.15), UC exhibited a hinted to at most mild immunolabelling (SSc 0.05-0.6). Besides a hinted UF expression (SSc 0.05-0.06), the production of CAL was highly inconsistent. The secretion intensity varied from very faint (SSc 0.1) to mild-to-moderate (SSc 3.8) without a discoverable correlation to blood hormone concentrations or the endometrial functional morphology.

"Cyclic" mules

Mule 2 showed a highly variable UG secretion pattern (SSc 0.05-6.3); in mule 1 the secretion intensity ranged between hinted and slight-to-moderate values (SSc 0.01-3.15). In both mules UC labelling was barely detectable or with slight intensity (0.05-1.75). Mule 2 exhibited a very low endometrial UF reactivity (SSc 0.05-0.6), whereas the secretion in mule 1 was highly inconsistent (SSc 0.05-6.4). CAL production in mule 2 was documented predominantly with a hinted, sporadically slight intensity (SSc 0.05-2.5). In mule 1 the secretion intensity ranged from very low to maximum levels (SSc 0.1-9.5). On most of the examination dates no coherence could be found between the clinically diagnosed phase of the sexual cycle, the blood hormone concentrations (Table 2), the endometrial functional morphology and the secretion of the uterine proteins.

Regumate® administration in two acyclic mules

Gynaecological findings prior to, during and after Regumate® administration

In the course of the experiment, clinical examinations revealed no signs of estrus or ovarian activity in either animal.

Endocrinological findings prior to, during and after Regumate® administration

Blood concentrations of estradiol and progesterone in both mules showed basal levels and only very slight changes during the entire course of examination (Table 4).

Functional morphology of the endometrium prior to, during and after Regumate® administration

Corresponding to the clinically documented acyclia, the endometria of both mules were inactive but partially irregularly differentiated before the treatment with Regumate® (Fig. 1). During the medication the endometrial morphology changed into a highly irregular secretory differentiation, characterised by polymorphic, very tall columnar glandular epithelia with mainly hypo-, partially hyperchromatic nuclei in basal, middle or apical position, and the appearance of very slender cells with elongated ellipsoid nuclei (Fig. 2). After cessation of treatment as well as two years later the endometrial differentiation equalled the one found prior to Regumate® medication. Neither type nor degree of the endometrosis changed in either of the mules during the entire course of the examinations. On day 4 of the medication, in one mule, a mild superficial purulent endometritis occurred, which resolved four days later, and could not be detected again during the treatment period or post administration.

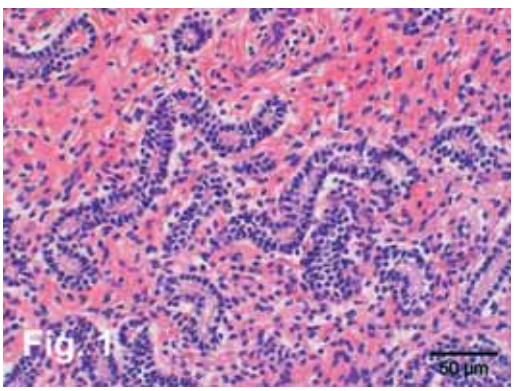

Fig 1 Prior to Regumate® administration: Inactive, slightly irregular differentiation of the endometrium (H E staining).
Vor Regumate®-Applikation: Inaktive, geringgradig irreguläre endometriale Differenzierung (H.-E.-Färbung).

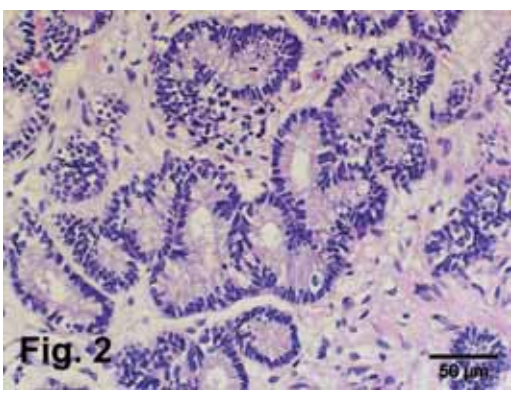

Fig 2 During Regumate® administration: Highly irregular secretory differentiation of the endometrium (H E staining).
Unter Regumate®-Applikation: Hochgradig irreguläre sekretorische endometriale Differenzierung (H.-E.-Färbung).

Table 5 Secretion of the endometrial proteins in acyclic mules (n = 2) before, during, and after Regumate® administration.
Sekretion der endometrialen Proteine bei den azyklischen Maultierstuten (n = 2) vor, unter und nach der Regumate®-Applikation.

	CAL		UC		UG		UF	
	Mule 4 Ø SSc	Mule 5 Ø SSc	Mule 4 Ø SSc	Mule 5 Ø SSc	Mule 4 Ø SSc	Mule 5 Ø SSc	Mule 4 Ø SSc	Mule 5 Ø SSc
Prior to Regumate® administration	0.19	0.28	0.17	0.15	0.02	0.18	0.20	0.11
During Regumate® administration	5.01	5.04	0.42	0.47	0.17	0.49	0.17	0.48
After Regumate® administration	3.21	4.20	0.20	0.19	0.02	0.08	0.02	0.33

CAL = calbindin; UC = uterocalin; UG = Uteroglobin; UF = Uteroferrin; Ø SSc = average Secretion Score

Immunohistochemical findings prior to, during and after Regumate® administration

Before the Regumate® administration only traces of the secretory endometrial proteins UG, UF, UC and the calcium binding protein CAL (Fig. 3) were detectable (Table 5). Corresponding to the development of an exceedingly pronounced secretory morphology the secretion of CAL increased distinctly during progestin treatment and rose up to twenty five- to fifty-fold higher levels than prior to medication (Table 5). Additionally, a "mosaic-like" secretion pattern was noted in the cytoplasm of the glandular epithelia (Fig. 4). In the course of the Regumate® administration the secretion of UC, UG and UF increased only very slightly, with the glands staining almost as faintly as before medication (Table 5).

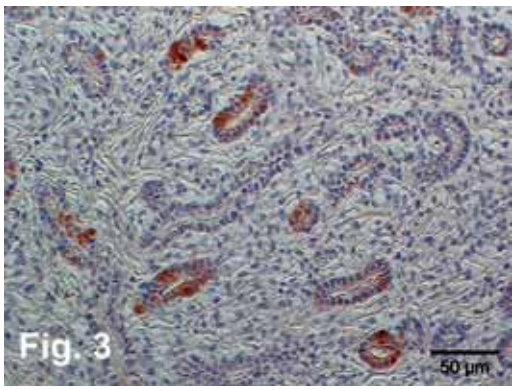

Fig 3 Prior to Regumate® administration: Low secretion of CAL in the uterine glands (Immunohistochemistry, Nomarski-interference-contrast).
Vor Regumate®-Applikation: Geringgradige Sekretion von CAL in den Uterindrüsen (Immunhistologie, Nomarski-Interferenz-Kontrast).

The production of CAL decreased after the termination of medication, but retained higher values than prior to Regumate® administration. The immunolabelling of UC, UG and UF was as faint as before medication (Table 5). The expression of ER (Fig. 5, 6) and PR before, during and after the Regumate® administration was strongly variable (Table 6), and the glandular proliferation activity showed maximal faint values (Table 6). Although there was no clinically traceable influence of the hormonal intervention on the ovarian activity or significant deviations of the serum hormone values during the course of the study, a overall continuous increase of the glandular and stromal expression of steroid hormone receptors was documented (Table 4).

Discussion

The results of this study show that the four acyclic mules have inactive ovaries, low serum hormone values (*Bartmann* et al. 2007) and exhibit inactive and maldifferentiated endometria with an accordingly low secretion of the uterine proteins UG, UC, UF and CAL. The expression of the steroid hormone receptors in these animals varies to a great extent, while the proliferation activity is absent or only sporadically detectable (*Huth* et al. 2007). This resembles the results in equine mares during winter anestrus (*Aupperle* et al. 2003).

In the two "cyclic" mules used in this study a highly inconsistent and unequal secretion of the endometrial proteins is observed; no correlation to the clinical phase of the sexual cycle, the serum hormone values or the endometrial functional morphology could be established. In the same two "cyclic" animals *Huth* et al. (2007) documented endometrial maldifferentiation, a highly variable expression of ER and PR, and a missing to moderate proliferation activity. In contrast to the investigations in cyclic equine mares, where progesterone is regarded as a regulating hormone for the synthesis/secretion of the analysed uterine proteins (*Hoffmann* et al. 2006), this influence cannot be deduced in the "cyclic" mules of this study, as the proteins seem to be secreted independently of the clinical state of cycle, the serum hormone values, and the endometrial functional morphology.

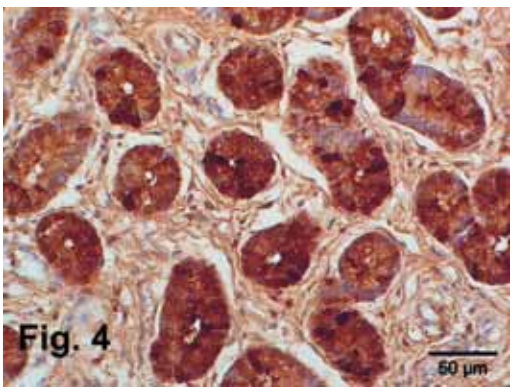

Fig 4 During Regumate® administration: Exceedingly pronounced secretion of CAL in the uterine glands with a "mosaic-like" expression pattern within the glands (Immunohistochemistry, Nomarski-interference-contrast).
Unter Regumate®-Applikation: Hochgradige Sekretion von CAL in den Uterindrüsen mit „mosaikartigem" Expressionsmuster innerhalb der Drüsenquerschnitte (Immunhistologie, Nomarski-Interferenz-Kontrast).

Table 6 Expression of the steroid hormone receptors and Ki-67 antigen in acyclic mules (n = 2) before, during, and after Regumate®-administration.
Hormonrezeptor- und Ki-67 Antigen-Expression bei den azyklischen Maultierstuten (n = 2) vor, unter und nach der Regumate®-Applikation.

	IRS ER				IRS PR				Ki-67 antigen			
	Mule 4		Mule 5		Mule 4		Mule 5		Mule 4		Mule 5	
	G	S	G	S	G	S	G	S	G	S	G	S
Prior to Regumate® administration	0.4 - 4.3	2.0 - 7.1	0.3 - 5.7	0.8 - 6.6	0.3 - 2.2	1.5 - 5.2	0.2 - 2.3	1.3 - 5.1	(+)/+	(+)	(+)/+	(+)
During Regumate® administration	1.4 - 9.5	3.1 - 8.6	1.0 - 8.5	1.4 - 6.7	0.5 - 8.0	3.4 - 7.1	0.1 - 6.7	1.6 - 7.6	(+)/+	--	(+)/+	--
After Regumate® administration	1.4 - 10	5.7 - 8.6	2.7 - 9.0	4.4 - 7.2	2.5 - 8.6	7.1 - 9.0	1.8 - 6.3	1.7 - 8.5	(+)	--	(+)	--

IRS = Immunoreactive Score; ER = estrogen receptors; PR = progesterone receptors; G = glands; S = stromal cells; -- = negative; (+) = very few positively staining cells; + = low number of positively staining cells

Altogether these results resemble the findings in equine mares during the transitional period from autumn to spring where a highly variable expression of the hormone receptors and an unequal endometrial proliferation activity are frequently observed phenomena (*Gockeln* 2006). Until now studies examining the synthesis/secretion of UG, UC, UF and CAL during the transitional period have not been conducted; however, it could be postulated that in these two "cyclic" mules the endometrial maldifferentiation and the great variability of the other investigated parameters impair an equal and appropriate secretion.

In all mules an endometrosis varying in type and degree was found. Corresponding to the results of *Huth* et al. (2007), this is most likely a matter of an irreversible degenerative, age-associated alteration of the endometrium as described for equine mares (*Kenney* 1978, *Schoon* et al. 1995).

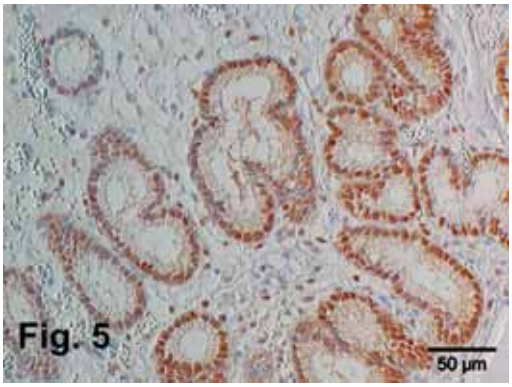

Fig 5 During Regumate® administration: Distinctly variable glandular and stromal expression of ER (Immunohistochemistry, Nomarski-interference-contrast).
Unter Regumate®-Applikation: Deutlich variable glanduläre und stromale Expression der ER (Immunhistologie, Nomarski-Interferenz-Kontrast).

In the second part of the study the comparison between histomorphological and immunohistological results before, during and after a long-term Regumate® administration in two acyclic mules demonstrated that in mules, as in equine mares (*Klug* et al. 1997), exogenously administered progestins cause an exceedingly pronounced secretory morphology with distinct irregularity of the glandular epithelia. In the mule as in the mare, these alterations must be regarded as principally reversible, but it must be considered that both mules already exhibited a slightly irregular endometrial differentiation prior to the treatment with Regumate® which, however, clearly increased during medication.

The non-recurring endometritis in one mule at the beginning of medication is likely to be an endometrial immune reaction to latently resident bacteria, as described by *Klug* et al. (1997), who observed similar findings in mares. Since endometritis occurred only once in one single mule during the following examinations, an aetiological impact of biopsy sampling frequency is more than unlikely.

Both before and after the treatment with Regumate® a very faint secretion of the uterine proteins was detected, consistent with the clinically documented acyclia, the low serum hormone values and the inactive endometrial morphology.

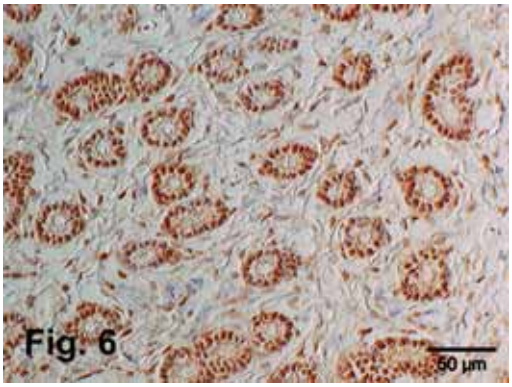

Fig 6 27 days post Regumate® administration: Strong, synchronous expression of ER in endometrial glands and stromal cells (Immunohistochemistry, Nomarski-interference-contrast).
27 Tage nach Regumate®-Applikation: Hochgradige synchrone Expression der ER in Drüsen und Stroma (Immunhistologie, Nomarski-Interferenz-Kontrast).

During Regumate® administration only the secretion of CAL increased noticeably; although, in contrast to the findings in equine mares (*Klug* et al. 1997), neither the clinically documented stage of acyclia changed nor the serum values of estradiol and progesterone significantly fluctuated. Thus it may be concluded that, contradictory to equine mares (*Ellenberger* et al. 2004), in these two acyclic mules exogenously administered progestins merely affect CAL secretion. The

medication did not exert a significant influence on the glandular or stromal proliferation activity disagreeing with the findings of *Klug* et al. (1997) in mares, who describe an obvious variance in the glandular Ki-67 antigen expression after the cessation of Regumate® treatment.

During and after the administration of Regumate® in the present study, the expression of ER and PR distinctly increased compared to the findings prior to treatment, however, the IRS-values varied extremely. These results differ from findings in long-term progestin-treated mares, who showed a down regulation of the steroid hormone receptors during treatment, and a clear peak of ER and PR expression after therapy discontinuation (*Klug* et al. 1997).

Hence, it can be concluded that the exogenous administration of progestins in the two mules investigated causes a transient and exceedingly pronounced secretory endometrial morphology with an increase of CAL secretion. However, beyond this it appears that the regulation of the uterine secretory proteins, the endometrial proliferation activity as well as the expression of the steroid hormone receptors depends on further, more complex mechanisms that could not be revealed within this study.

Footnotes and manufacturer's addresses

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