Early pregnancy failure in the mare

Embryonaler Frühtod bei der Stute

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Pregnancy failure occurs in around 15% of all confirmed pregnancies in Thoroughbred mares (*Sanderson* and *Allen* 1987) and over 60% of these losses take place during the first 45 days of gestation (*Morris* and *Allen* 2002). The advent of high resolution ultrasound scanners has greatly improved the diagnosis and monitoring of early conceptus development and untoward losses but even with this increased diagnostic capability there is rarely anything the veterinary clinician can do to prevent the failure from occurring. So, what might be the causes of these early losses?

Surveys over the years in aborting women and some domestic and laboratory animals species have consistently shown that 40-60% of spontaneous first trimester pregnancy losses can be attributed to the lethal effects of accidental chromosome misalliance at the time of fertilisation (see *Creasy* 1988 for review). The studies by *Woods* et al. (1990) and *Koskinen* (1991), which both showed a sharp increase in early pregnancy losses in mares deliberately inseminated after, rather than before, ovulation, lend strong support to the suggestion that the non-embryonic trophoblastic vesicle which is commonly observed ultrasonographically by studfarm veterinary surgeons to fade away and disappear between 20 and 30 days after ovulation is indeed the equine equivalent of the chromosomally aberrant „blighted embryo" conceptus of human pregnancy (see *Hunter* 1980 for review).

Unlike those of the cow, sheep and pig, the equine conceptus remains spherical due to its envelopment by the tough, glycoprotein equine blastocyst capsule between days 6 and 24 of gestation (*Betteridge* 1989). This gives it strength to withstand the strong uterine contractions which must propel it constantly throughout the uterine lumen between days 6 and 17 in order that it can liberate the embryonic maternal recognition of pregnancy signal required on to the endometrium to suppress the latter's normal cyclical release of prostaglandin F2a (PGF 2a) and, instead, induce luteostasis to continue the supply of luteal progesterone to maintain the pregnancy state and drive the myriads of endometrial glands to produce copious amounts of protein-rich histotroph („uterine milk", *Amoroso* 1952). The unattached free-living conceptus must rely entirely for its sustenance upon uterine milk until at least day 40, when interdigitation of fingers of allantochorion with accommodating crypts in the endometrium at last begins the establishment of the definitive placenta that will support fetal growth during the remainder of gestation. Failure of an embryo to liberate its maternal recognition message to a sufficiently large area of endometrium, perhaps because it is inhibited from traversing the entire uterine lumen by the physical obstruction created by large or multiple endometrial cysts (*Bracher* et al. 1992), or by age-related myometrial atony leading to unresponsiveness to the embryo's liberation of PGF2a and PGE2 to effect its transuterine migration (*Stout* and *Allen* 2001), will clearly lead to early pregnancy loss simply because the mare is unable to recognise biochemically she is pregnant and therefore brings into play her cyclical luteolytic mechanism.

In a similar vein, if the free-living young and rapidly expanding conceptus is unable to bathe in and imbibe sufficient uterine milk during its first 40 days in the uterus, either by failure of its movement between days 6 and 17 as described above, by some developmental inadequacy of the blastocyst capsule to adhere the uterine milk and pass it into the conceptus or, most likely, because of age-related degenerative changes in the endometrial glands, the source of uterine milk, diminishing the supply of this vital food, it will clearly become malnourished and will die (*Bracher* et al. 1996). A good example of this is shown by the high rate of spontaneous reduction of one of twin conceptus when they become „fixed" together in the same uterine horn on day 17 after ovulation (*Ginther* 1985; *Morris* and *Allen* 2002). This death of one conceptus is induced by the fact that its absorptive choriovitelline membrane becomes abutted to the membranes of its co-twin rather than to the uterine milk supplying maternal endometrium.

A valid treatment of aged mares suffering degenerative changes to the endometrium (endometrosis) and general uterine atony is chemical curettage induced by infusing 50 ml of commercially available kerosene into the lumen of the dioestrous uterus. The therapy was originated over 30 years ago by Mr Charles Roberts, a New Zealand veterinary surgeon who determined that short-acting aromatics in the kerosene stimulate an acute but transitory inflammatory response in the endometrium which includes an outpouring of accumulated gland secretions and a vigorous regeneration of the glandular epithelium. Conception rates are markedly improved in kerosene-treated endometrotic mares, although the later abortion rate in these animals remains high, as the kerosene therapy is unable to improve the underlying fibrotic changes to the glands and endometrial stroma (*Bracher* et al. 1991).

Finally, and contrary to popular horse breeder and equine veterinary opinion around the world, a shortage of progesterone in the mare's blood during the first 40 days of gestation is not a significant cause of early pregnancy loss in this species. In their seminal study, *Irvine* et al. (1990) monitored maternal plasma progesterone profiles and early pregnancy loss rates in over 289 commercial Standardbred mares maintained on one studfarm. They encountered 17 cases of early pregnancy failure (9% of all pregnancies) and a lack of progesterone could be associated with the fetal death in only one case. Coupled with this, most synthetic progestagens administered to pregnant mares as a „panacea preventative of pregnancy loss" have been shown to have minimum biological activity in the mare due to their inability to bind to her endometrial progesterone receptor (*McKinnon* et al. 2000). Thus, this widespread practice of treating pregnant mares with progestagens should be branded the quackery that it clearly is and should be stopped.

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Transferred extraspecific donkey-in-horse pregnancy: A valuable model of early pregnancy failure in the mare

Die transferierte extraspezifische Esel-Pferd-Trächtigkeit: Ein geeignetes Modell für embryonale Frühverluste bei der Stute

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Around 75% of donkey embryos (Equus asinus, 2n = 62) transferred on the 7th or 8th day after ovulation to the uteri of synchronised horse mares (E. caballus, 2n = 64) develop normally to around day 65 of gestation but thereafter degenerate and die before being expelled from the surrogate horse uterus between days 80 and 95; the degeneration is associated with inadequate interdigitation of the donkey allantochorion with the horse endometrium and the accumulation of large numbers of lymphocytes and other immune cells in the stroma of the endometrium that is contact with the failing donkey placenta (*Allen* 1982). The other 25% of mares carrying donkey embryos seem able to implant the xenogeneic conceptus successfully and carry it to term, where the foals born range from very poor and dysmature in body development to remarkably mature, healthy and robust due to the larger size of the surrogate horse uterus in which they have gestated. Thus, placentation appears to range from complete failure in the majority, through moderately to highly successful in the remainder in this one model of xenogeneic extraspecific pregnancy.

A notable feature of the donkey-in-horse pregnancy model is the minimal development of the donkey chorionic girdle when in the horse uterus and its complete failure to invade the maternal endometrium to form endometrial cups. The resulting absence of equine Chorionic Gonadotrophin (eCG) in maternal blood leads to a failure of secondary luteal development after day 40 but the primary corpus luteum persists until death of the fetus, or until the ongoing placenta is itself producing appreciable quantities of progestagens and oestrogens from around day 80.

Administration of large quantities of either partially purified eCG ot the orally active synthetic progestagen, Regumate, to mares carrying donkey pregnancies between days 20 and 80 of gestation did not reduce the pregnancy loss rate compared to untreated controls. However, passive immunisation by repeated infusion of large volumes of serum from mares carrying normal interspecies horse conceptuses at equivalent stages of gestation, and active immunisation with parental or unrelated donkey lymphocytes, both reduced the pregnancy loss rate, to 50% and 33% respectively (*Allen* et al. 1987). This suggested that maternal immunological recognition of foreign xenogeneic donkey antigens expressed by the trophoblast cells causing the observed vigorous cytotoxic immune response could be the primary cause of the stage-dependent abortion of most of these pregnancies.

Immune memory was demonstrable in the model by virtue of the fact that 11 of 12 mares which aborted a first donkey fetus at the expected time aborted 2nd and 3rd donkey embryos transferred to them at successively earlier stages of gestation. Conversely, all of the 10 mares that carried one donkey conceptus past 150 days of gestation carried further donkey conceptuses transferred to them without any immunisation or other form of treatment. It was therefore reluctantly concluded that indeterminable genetic differences between the surrogate mares was probably the major underlying cause of the observed differences between those animals that could, and those that wouldn't, implant and carry a donkey embryo to term. The situation showed similarity to the related observation that, whereas all fertile horse mares will conceive readily to insemination with Jack donkey semen to carry a mule pregnancy, only about 15% of the Jenny donkey population will conceive to insemination with horse stallion semen to carry a hinny pregnancy (*Allen* et al. 1993). It begs the question of whether similar, genetic incompatibilities between partners may underlie at least some of the unexplained conception and/or pregnancy failures in otherwise apparently normal mares.

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Effects of unilateral uterine artery ligation on the uterine perfusion monitored by endometrial echotexture analysis and Doppler sonography of the uterine blood flow in horses

Auswirkungen einer unilateralen Ligatur der uterinen Arterie auf die uterine Perfusion - eine Untersuchung des uterinen Blutflusses der Stute mittels Analyse der endometrialen Echotextur und Doppler Sonographie

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Adequate maternal blood flow is essential for fertility and fetal growth. Vascular insufficiency may result in degenerative endometrial disease and retardation of intrauterine growth in horses. Arterial occlusion can be observed due to angiopathies or following rupture of the uterine artery, particularly in older mares.
On the other hand, uterine artery occlusion is an effective surgical intervention in the management of major haemorrhage and for avoiding excessive blood loss and hysterectomy in women. In horses, ligation of the uterine artery or its branches is performed during partial or radical hysterectomy. Complete ligation of the uterine artery could be an effective procedure in the therapy of arterial rupture and life-threatening haemoperitoneum or uterine haemorrhage. To determine the effect of uterine arterial ligation, uterine perfusion was monitored by endometrial echotexture analysis and Doppler sonography in horses after unilateral uterine artery ligation and after partial hysterectomy including resection of one uterine horn and ligation of the ipsilateral uterine artery.

Nine healthy and gynaecologically soundless mares were used for the investigation. Depending on the surgical procedures performed mares were assigned to three groups. Mares of group 1 (n = 4) were control animals without surgical procedures. In the mares of group 2 (n = 3), unilateral ligation of the uterine artery and of the uterine branch of the ovarian artery was performed during laparotomy under general anaesthesia. In mares of group 3 (n = 2), unilateral ligation of those arteries was performed in addition to resection of the ipsilateral uterine horn. The imaging of uterine perfusion was focussed on the perioperative period and on the periovulatory period of the consecutive sexual cycles. Texture analysis of ultrasonographic images of the uterus was performed by one-dimensional computerised grey-scale analysis. For evaluation of local effects the uterus was divided into the compartments left uterine horn, right uterine horn and uterine body. All mares showed a cyclic pattern in endometrial echotexture with a highly significant decrease followed by an increase of the mean median grey level (mGL) before ovulation. At no point did the comparison of the three uterine sections result in significant differences of the mGL. Immediately after surgery, changes of the mGL of different quality and degree were registered in mares of group 2. During the consecutive sexual cycles, the mGL measured by grey-scale analysis of the mares of groups 2 and 3 showed patterns prior to ovulation comparable to those seen in the mares of group 1 and in all the mares prior to surgery. No significant differences could be demonstrated. Furthermore, there were no differences between the uterine sections corresponding to those from mares of group 1.
The Doppler sonographic findings of the uterine blood flow and peripheral resistance (PI) prior to surgical intervention showed no significant differences between the left and right uterine arteries. Six months following surgery, these findings were confirmed except in one mare of group 2. In this mare, peripheral resistance was significantly lower in the uterine artery with peripheral ligation than in the contralateral vessel.

These results suggest that ligation of the uterine artery or partial hysterectomy as described here do not cause long-term effects of uterine perfusion measurable by Doppler ultrasonography or grey-scale analysis. These findings correspond to/are in accordance with the clinical findings of preserved fertility following arterial ligation in women.

Relaxin is an important factor for implantation und early pregnancy maintenance

Relaxin, ein wichtiger Faktor für die Implantation und Aufrechterhaltung der Frühgravidität

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The peptide hormone relaxin (RLX) is known to be a pregnancy-related hormone, associated with uterine and cervical changes during implantation and process of birth. RLX has the ability to induce and modify stromal tissue components by influencing the expression of the members of matrix metalloproteinase family (MMPs), which facilitate invasion through dissolution of the basement membrane. As RLX is involved in endocrine changes as in the regulation of MMP and VEGF-expression it is tempting to speculate a role of RLX and its tissue remodeling and angiogenic ability during the process of implantation. Object of the study: Further experiments were undertaken to analyse the hypothesis that RLX is the supportive factor for endometrial differentiation and therefore implantation.
In our primate model, the marmoset monkey (Callithrix jacchus), RLX could be localized within the ovary and the placenta. There

is a major increase in the levels of RLX in peripheral blood during implantation, whereas implantation failure correlates with reduced circulating RLX concentration. Furthermore, both RLX receptors (LGR7 & LGR8), MMPs- and VEGF expression are markedly up-regulated during early pregnancy in the marmoset monkey, but become down-regulated in the event of implantation failure. Marmoset monkeys with a known breeding history were divided into two groups: a) RLX treated animals and b.) placebo treated animals. These two groups of females, which were housed with fertile males, were implanted with alzet minipumps, filled with either Ringer solution (placebo) or recombinant human RLX (0,25µl/h) for 28 days around the period of ovulation. The course of pregnancy was followed by hormone measurements, ultrasound examination with color Doppler analysis. The RLX-treated group (n=15) showed a more rapid endometrial proliferation during the early pregnancy period than the control group(n=10), and their blood supply system was more developed. However, under RLX treatment, there was a decline in the Resistance-Index, and therefore an increase in blood flow. In general, the RLX treated animals gave birth to healthy babies after an average of 137 ± 2,2 days, whereas the control group gave birth only after 144 ± 3,2 days. Babies from treatment group a had similar birth weights, gender distribution and postnatal developmental status.

In summary, RLX positively supports endometrial proliferation, angiogenesis and invasion of the embryo during early pregnancy in primates, also implying that RLX could act as an useful therapy for implantation problems.

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Histomorphological and immunohistochemical characterization of equine granulosa cell tumors compared to normal ovaries of the mare

Histomorphologische und immunhistologische Charakterisierung equiner Granulosazelltumoren im Vergleich zum normalen Ovar der Stute

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Hormonally active granulosa cell tumors (GCT) are the most common ovarian tumors of the mare (*Hughes* et al. 1980). From the clinical point of view these equine ovarian neoplasms are important concerning differential diagnosis because they may result in changes in behaviour, irregularity of the estrous cycle and infertility (*Bartmann* et al. 2001). An inactive and atrophic contralateral ovary is already clinically demonstrative for an endocrine activity of a GCT (*McCue* 1992). Furthermore equine GCT are mostly characterized as benign neoplasms. The aim of this study was the histomorphological and immunohistochemical characterization of equine GCT compared to normal ovaries of mares with respect to clinical signs and endocrinological data. Furthermore it should be evaluated, if conventional histopathological and immunohistochemical methods can be regarded as a useful contributory diagnostic technique to characterize the biological value of GCT.

For this purpose, GCT (benign neoplasms: n=33, metastasizing tumor: n=1) from 34 mares (3-21 years of age) and normal ovaries from 9 autopsied mares (3-10 years of age) were collected, fixed in 4% formalin, routinely embedded in paraplast, sectioned and investigated by means of histology (H-E) and immunohistochemistry (inhibin α, glutathione S–transferase α (GSTα), c-erbB-2 oncoprotein (cerb), cytokeratin, vimentin, desmin and α-actin). Serum hormone values of estradiol, progesterone and testosterone were examined in 26 patients before and as far as available after ovariectomy. In addition inhibin B levels, determined in serum or fluid out of the tumor cysts taken from six patients with ovarian GCT before or after ovariectomy were compared with inhibin B values of five gynaecologically healthy mares.

Unilateral, benign granulosa cell tumors occur predominantly in five to nine year-old horses and mainly in maiden mares. Clinically, most mares with GCT showed persistent anestrus or acyclia (n=18) and 11 patients exhibited stallion like behaviour. A high variability of serum hormone levels (estradiol, progesterone, testosterone, inhibin) within the group of mares occurred prior to ovariectomy. Testosterone values were elevated especially in virile mares. Not in all mares the routinely determined serum hormone levels (estradiol, progesterone, testosterone) are indicative for the presence of an endocrinologically active GCT. The examination of inhibin B values in serum may be helpful verifying the clinical tentative diagnosis. The endocrinological findings reveal high serum inhibin levels to be suggestive of a GCT, but low inhibin values do not exclude the presence of such a neoplasm.

Histologically, the most common patterns of neoplastic granulosa cells were macro- and microfollicular, tubular and trabecular. In mares with stallion like behaviour and/or elevated serum testosterone levels numerous "Leydig-like cells" were seen in between theca cells and beneath nests of granulosa cells. Immunohistochemically, neoplastic granulosa cells of benign tumors were characterized by an intracytoplasmic intense coexpression of vimentin, cerb and inhibin α. According to the immunohistochemical and endocrinological findings it is supposed that GCT produce abnormally high levels of inhibin, which reduces the release of FSH leading to an atrophy of the contralateral ovary, a clinical finding in 27 mares. GSTα, known as a marker for steroid producing cells, was detectable in granulosa cells and "Leydig-like cells". The expression of inhibin α and GSTα as well as the earlier reported endometrial maldifferentiation in dyshormonal mares with endocrine active ovarian dysfunctions (*Ellenberger* et al. 2002) support the assessment that equine GCT are endocrinologically active.
Whereas theca externa cells in normal ovaries exhibited an intense α-actin expression, theca interna cells reacted negative. In

GCT all theca cells showed a positive staining with a-actin and a morphological differentiation between theca interna and theca externa was not possible. In one single case the very rare phenomenon of a malignant GCT with wide spread metastases through the abdominal cavity was diagnosed. In H-E-stained slides there were no morphological differences between the metastasizing and benign neoplasms, whereas by means of immunohistochemistry a deviating expression pattern of vimentin, cerb and GSTa in the granulosa cells of the malignant GCT was visible. These immunohistochemical markers were detected in a much smaller number of cells than in benign tumors.

In conclusion with conventional histopathology a final statement regarding the biological value of GCT is not possible but the deviating immunohistochemical expression pattern of the metastasizing neoplasia in this study might be an indication to malignancy. Therefore further investigations are necessary.

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Production of mitogenic factor(s) by equine corpora lutea

Produktion mitogener Faktoren durch das equine Corpus luteum.

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Ovarian impairment, resulting in deficient progesterone (P_4) production by the corpus luteum (CL) has been related to early embryonic mortality in the mare. This dysfunction might be associated with a deficient vascularization, since the CL formation and its endocrine function are closely dependent on the growth of new capillaries. During the ovarian/uterine cycle, luteal structures exhibit a physiologic growth and regression accompanied by fast vascular changes. Angiogenic and anti-angiogenic factors have been referred in other species as regulators of angiogenesis and vascular regression. Knowledge of this process might help understanding infertility in the mare due to primary luteal function impairment. The objective of this study was to evaluate the effect of hormones on the production of angiogenic factors by equine luteal structures.

During the breeding season, luteal tissue and blood were obtained post mortem from randomly assigned cycling mares (n=16). Luteal tissue was characterized as corpus hemorraghicum (CH)(n=2); mid luteal phase CL (n= 7) and late luteal phase CL (n=7), according to morphological structure and plasma P_4 levels. Luteal explants were cultured for 6h with no hormone added, or with PGF_{2a} (1µg/ml), LH (1µg USDA-bs/ml), PGE_2 (1µg/ml), LH+PGE_2 (1µg/ml each) or LH+PGF_{2a} (1µg/ml each). Media conditioned by luteal tissues were assayed for P_4 and tested for their ability to stimulate mitogenesis of bovine aortic endothelial cells (BAEC). Vascular endothelial growth factor (VEGF) was used as a positive control. Proliferative response of BAEC to samples was evaluated by determining the number of cells in each well using a neubauer chamber. Plasma and conditioned media P4 concentrations were determined by RIA.

Progesterone production in culture medium without exogenous hormones showed a tendency to rise from CH to Mid-CL, and to decrease in Late-CL, according to plasma P_4 pattern. No significant difference was observed after any hormonal treatment.

Equine luteal tissue alone showed the capability to increase BAEC proliferation, when compared to negative controls. However, no difference was observed among luteal structures on their mitogenic capacity, for any treatment used. Nevertheless, Late-CL conditioned media with PGF_{2a} showed a significant decrease in BAEC proliferation ($p<0.05$) and LH+ PGF_{2a} a tendency to reduce mitogenesis ($p=0.07$), when compared to controls. Our data show that equine luteal tissue from different stages of the estrous cycle stimulates angiogenic factor(s). Furthermore, prostaglandin F_{2a} may play a role on vascular regression of the CL during the late luteal phase in the mare.

Persistent Anovulatory Follicles in the Mare

Persistierende anovulatorische Follikel bei der Stute

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In this study the hormonal levels in the follicular fluid of a group of mares (n=20) with persistent anovulatory follicles were compared to a control group (n=11) of mares with normal preovulatory follicles. The diagnostic criteria for persistent anovulatory follicles were: the dominant follicle had an average diameter >40 mm; there was no softening of the follicular wall; no change in follicular shape; no corpus luteum visible on ultrasound; and the endometrial oedema was reduced or missing. In the control group all mares showed good oestrus signs for several days; had a preovulatory follicle with an average diameter of >40 mm, a tendency to softening of the follicular wall and the start of regeneration of the endometrial oedema. Follicular fluid was collected following transabdominal puncture of the follicles or trans-

vaginally with the aid of ultrasound. Of the mares with persistent anovulatory follicles, 72 % of the punctures were conducted in March and April and only 28 % of the punctures were carried out in May, June and July. The persistent follicles showed echogenic particles in the follicular lumen on ultrasound in 71.4 % of all cases. Hormone concentrations were measured by specific immunoassays and are expressed as mean ± SD, ng/ml of follicular fluid:

	anovulatory group	control group	significance
Oestradiol	941±1425	3361±1653	p<0,01
Inhibin B	6.56±7.67	19.10±7.67	p<0,01
Progesterone	16.2±14.7	29.4±10.2	p<0,05
Testosterone	3.29±4.92	3.35±1.71	n.s.

The concentration of oestradiol was positively correlated with that of inhibin in the follicular fluid of the anovulatory group, whereas in the control group there was a negative correlation between inhibin and progesterone. Of the hormones in peripheral plasma, testosterone was significantly higher in patients as compared to controls (0,02 vs. 0,01 ng/ml, p<0,05); other hormone concentrations were similar in both groups. The lower concentrations of oestradiol, inhibin, and progesterone in the follicular fluid of the anovulatory group suggest an impairment of granulosa cell function in this condition. Puncture of the persistent follicles is a suitable treatment for affected mares resulting in a normal oestrous cycle.

Incidence and morphology of anovulatory haemorrhagic follicles in the mare

Inzidenz und Morphologie anovulatorischer hämorrhagischer Follikel bei der Stute

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Retrospective data from 47 experimental mares monitored during a total of 737 oestrous cycles over 3 years were analysed. During the breeding season (April-August), development of an anovulatory haemorrhagic follicle (AHF), with a mean diameter of 48.1 ± 8.0 mm, occurred in 11.9 % of the oestrous cycles. In most cases, the dominant follicle grew normally to a diameter ≥ 45 mm (range 38-65 mm), oedema developed in the uterus as expected but ovulation did not occur. Instead, the follicular fluid became hazy in appearance and increasingly filled with echogenic flecks and strands. The serum progesterone concentration; i) rose slowly and progressively (50.7 %); ii) remained < 1 ng/ml (11.9 %); iii) plateaued between 1 and 4 ng/ml (10.4 %); iv) rose suddenly from 0 to > 10 ng/ml in one day (10.4 %). Most AHFs that were accompanied by a high serum progesterone concentration exhibited a pronounced echogenicity and they responded well to an intramuscular injection of a prostaglandin F analogue by returning to oestrus and subsequently ovulating normally. A total of 50.5 % of the mares exhibited an AHF during at least one oestrous cycle, and around 25 % produced an AHF during at least 2 oestrous cycles. A definite tendency for recurrence of AHF formation from one breeding season to the next was noted in individual mares. Aged mares tended to develop unusually large (> 50 mm) and hazy follicles, whereas young maiden fillies tended to produce medium-sized AHFs that became more echogenically dense. During the transitional phase in Autumn, AHF formation occurred in 22.2 % of oestrous cycles during the months of September and October to reach a peak occurrence of 58.8 % in November.

Effects of inseminate volume of cooled stallion semen on post breeding endometritis in mares

Auswirkungen des Inseminationsvolumens gekühlten Hengstspermas auf die durch die Bedeckung induzierte Endometritis bei Stuten

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The present study focuses on initiation and persistence of post breeding endometritis (PBEI) analysing endometrial biopsies taken from oestrous mares after insemination with constant sperm doses, suspended in different volumes of extender.

15 oestrous mares (3 to 19 years of age, Hanoverian warmblood) were inseminated once with 600 x 10^6 progressively motile spermatozoa (pms) suspended in either 1 ml (group A: 5 mares); 20 ml (group B: 5 mares) and 50 ml (group C: 5 mares) of skim milk based extender. Semen from fertile warmblood stallions was collected and centrifuged (10'; 600 g). The sperm pellets were further diluted until 600 x 10^6 pms/ml, filled in 0.5 ml straws, and stored at +5°C in a refrigerator until use. Inseminates were prepared immediately before inseminations, which were carried out by resuspending 600 x 10^6 pms with skim milk based extender until the desired inseminate volume. Endometrial biopsies were taken before 0h, 6-8 h and 48 h after insemination, fixed in 4% buffered formalin and examined by light microscopy (enzym-, immunohistochemistry) allowing selective labelling of inflammatory cells. 6-8 hours after treatment 93% of the examined mares showed an acute PBEI (mild, moderate, severe). Cellular infiltrations consisted predominantly of PMNs and lymphocytes and were found in the stratum compactum (SC) as well as in the stratum spongiosum (SS). Influx of PMNs in the SC and the SS increased in all groups ($p<0.05$) at 6-8 hours. PMN infiltrations in the SC, although lower than at the 6-8 h, persisted until 48 hours after treatment in 47% of the biopsy samples. In the SC, there was no detectable difference on the PBEI evolution (0h, 6-8h, 48 h) between the three volume groups. Analyses of uterine clearance by comparing effectiveness of reduction of PMN concentrations from 6-8 until 48 hours after treatment showed no differences between the groups A, B and C.

Conclusion: under the conditions of the present experiment initiation and persistence of PBEI is not affected by the volume of extended inseminate when mares are inseminated with commercial doses of cooled stallion semen.

Detection of abnormal ploidy in early horse embryos by fluorescent in-situ hybridisation (FISH)

Bestimmung abnormer Ploidie bei equinen Embryos in der FRühträchtigkeit mittels Fluoreszenz-in-situ-Hybridisation (FISH)

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In mares, 10-20 % of detected pregnancies are lost during the first 6 weeks of gestation, and the likelihood of early embryonic loss (EEL) increases with maternal age. EEL is thus a significant cause of economic loss to the equine breeding industry. By analogy to the situation in women, chromosomal abnormalities are often put forward as a potential cause of EEL. However, there is as yet little evidence that this is actually the case, not least because the average embryo has only a few nuclei that are in metaphase and thus suitable for karyotypic analysis. Recently, FISH with chromosome-specific probes has been used on interphase nuclei to demonstrate that even macroscopically normal human and bovine embryos contain cells with abnormalities of ploidy (e.g. 3n or 4n). The aim of the current study was to develop FISH staining with chromosome-specific probes for equine interphase nuclei and to use this technique to examine the incidence of abnormal ploidy in early horse embryos.

Twenty-two embryos were recovered on days 6 or 7 after ovulation by uterine lavage. After washing, the embryos were lysed in a drop of spreading solution at the centre of a glass microsope slide. The nuclei were then spread over the entire slide and fixed in a 3:1 (v:v) solution of methanol and acetic acid. Once the slide had been dried, the nuclei were counted and the slides were stored at -80°C until staining. For the development of FISH probes, bacterial artificial chromosomes (BACs: a gift from the BAC-YAC Resource Center of the Animal Genetics Department of the INRA) specific for equine chromosomes 2 (ECA 2) and 4 (ECA 4) were cultured and, after DNA isolation, labelled with biotin-14-dUTP or digoxigenin-11-dUTP, respectively, so that they could be distinguished with an epifluorescence microscope as red (avidin-CY3) or green (sheep-antiDIG-FITC) signals on a blue background (DAPI-stained nuclear DNA). The veracity of the probes was tested using a standard FISH protocol on metaphase, and then interphase, cultured equine lymphocytes.

Of the 22 embryos, one had more than 60% triploid cells and 3 others had up to 15% cells with either triploid or tetraploid nuclei, thereby demonstrating that abnormalities of ploidy do occur in "normal" equine embryos, albeit at a relatively low rate (18%) and in only a proportion of the cells. In only one of these cases, was the abnormality likely to have rendered the embryo nonviable. Currently, 32 in vitro produced horse embryos are being examined to determine whether IVF increases the incidence of abnormal ploidy, as it does in human and cattle embryos.

Grey-scale analysis of the endometrial echotexture during the periovulatory period in correlation with peripheral progesterone and estradiol levels in horses

Graustufenanalyse der endometrialen Echotextur während der periovulatorischen Periode im Vergleich mit den peripheren Progesteron- und Östrogenwerten beim Pferd

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It is known that endometrial echogenicity varies during the oestrous cycle in horses assessed by visual means, and the influence of steroidal hormones has been suggested. It has been demonstrated in previous studies that the endometrium shows the greatest dynamics in echotextural changes during the periovulatory period. The aim of this study was to objectivate and quantify these changes in echotexture during the periovulatory period by computer-assisted one-dimensional grey-scale analysis (GSA) and to consider the correlation with progesterone and estradiol plasma levels.

A total of 19 oestrous cycles of eight generally healthy and gynaecologically soundless mares (4 warmbloods, 4 trotters) were investigated, with the focus on the periovulatory period. Ultrasonographic images were obtained on days -5, -4, -3, -2, -1, 0, and 1 (0 = day of ovulation) by means of a 7.5 MHz linear probe under standardised conditions. Left uterine horn, right uterine horn and uterine body were assessed separately. During each examination three images of each uterine compartment were recorded on S-VHS videotapes. After digitalisation two endometrial regions of interest (ROI) were marked on each image and analysed by a one-dimensional computer-assisted grey-scale analysis programme. The result was expressed as the median grey level (GL) for each ROI (0 = black, 255 = white).
The comparison of the three uterine sections did not result in significant differences in the mean median grey level (mGL) at any point ($p > 0.05$). The endometrial echotexture of all mares followed a course of significant decline and subsequent rise of the mean median (mGL) before ovulation. The lowest mGL was 42.92 ± 2.07, which was reached on day -3.58 ± 1.07. After ovulation, the mGl continued to show a tendency to increase/rise. During this periovulatory period the mGL correlated highly significantly with the plasma concentration of estradiol

17-ß (r = - 0.79), whereas the concomitant plasma progesterone levels showed no significant correlation.

This study demonstrates that computer-assisted one-dimensional grey-scale analysis is a non-invasive method of high sensitivity for the objective assessment of cyclic changes in endometrial echotexture in horses. In contrast to examinations concerning the whole oestrous cycle, here no correlation was found between plasma progesterone concentrations and the endometrial mean median (mGl) during the periovulatory period. On the other hand the influence of estradiol 17-ß on endometrial echotexture during this narrowly restricted time frame was shown to be statistically significant.

Incidence of embyonic and fetal losses in the Hanoverian warmblood breed

Inzidenz embryonaler und fötaler Verluste beim Hannoveraner Warmblut

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The objective of this study was to investigate the incidence of embryonic- and fetal loss rates in the Hanoverian warmblood breed. Analyses were based on data from breeding records derived from 40 AI-stations of the National Stud of Lower Saxony, Celle, Germany. Mares were bred by artificial insemination (AI) with cooled semen (93%) and by natural service (NS; 7%). End of season embryonic- (ELRS) and fetal loss rates (FLRS) were analyzed using data from 7.247 (range: 7.069 – 7.932) brood mares per breeding season over a period of five years (1997 to 2001). Live and registered foals were born by 67.1 % of the mares. 1.8 % of the foals died within the first 6 month of life (colts: 58.9%; fillies: 41.1%). FLRS was 3.8 % and ELRS 5.4 %. No pregnancy was noticed in 21.9 % of the mares.

For investigations on fertility per cycle data from 9.431 brood mares, 33.664 estrous cycles and 74.950 coverings were used over a period of three years (1997 to 1999). Embyronic- (ELRC) and fetal loss rates (FLRC) per cycle were estimated by comparing nonreturn rates (NRR) on days 33, 55 and 77 after last service with pregnancy rate per cycle (PRC) and foaling rate per cycle (FRC). To estimate the incidence of embryonic- and fetal losses differences between PRC and NRR (PRC-NRR), between NRR at different times (NRR-NRR) and between NRR and FRC (NRR-FRC) were calculated. NRR were 52.9% ± 49.9 on day 33, 41.6% ± 49.3 on day 55 and 38.0% ± 48.5 on day 77. PRC was 37.2% ± 44.5, and FRC was 32.7% ±48.5. PRC-NRR33 was 15.7%, NRR33-NRR55 was 11.3%, NRR55-NRR77 was 3.6%, PRC–FRC was 4.5%, and NRR33-FRC was 20.2%.

In conclusion, despite that a high number of mares showing an embryonic loss conceived and give birth to a foal at term after repeated breeding, the rate of pregnancy failure between initial pregnancy per cycle and Day 55 represents the major loss in the Hanoverian warmblood breed.

Early embryonic death - Experimental investigations in the bitch

Embryonaler Frühtod - Experimentelle Untersuchungen bei der Hündin

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Resorption of the embryonic or fetal structures in cases of early embryonic or fetal death in the mare is supposed to be a common incidence. Chromosomal alterations, endometrosis, ovarian dysfunctions and twinning are discussed as possible causes for the early pregnancy loss. The uterine mechanisms involved in placental and embryonic/fetal resorption were not investigated morphologically in the mare until today. Although the placentation sites of the bitch and the mare are quite different, the dog was used as a model for embryonic/fetal and placental resorption in the present study. The objective of this study was to investigate the morphology of canine placental sites after experimentally induced embryonic/fetal and placental resorption in comparison to the normal placental morphology at the same stage of gestation.

In four clinically healthy beagle bitches resorption was induced by administration of luteolytic or antigestagenic drugs (n=1: Cloprostenol - 1μg/kg s.c. and Cabergolin - 5 μg/kg p.o.; n=3: Aglepristone - 10 mg/kg s.c.) starting on day 24 after ovulation (the day of ovulation is defined as day 1 of pregnancy). Additionally, two pregnant beagle bitches served as controls. From day 20 of pregnancy, all dogs were submitted to daily clinical and sonographic examinations. The first signs of experimentally induced resorption could be detected by ultrasound on day 26 of pregnancy, the bitches were ovariohysterectomized between day 30 and day 33 of gestation. Placental sites (n=43) were fixated in formalin and investigated morphologically, by enzyme histochemistry (Naphthol-AS-D chloracetate-esterase (CLAE) and Alpha-Naphthylacetate-esterase (ANAE), acid and alkaline phosphatase) and immunohistochemistry (laminin, collagen IV, estrogen-receptor (ER), progesterone-receptor (PR), Ki-67 antigen, lysozyme, MAC 387, pan-cytokeratine). In 16 placentation sites of the treated dogs embryonic/fetal death and resorption was sonographically visible, whereas 13 embryos/fetuses were alive on the day of ovariohysterectomy.

With regard to morphology in control bitches and in the placental sites of the treated dogs containing a living embryo/fetus, the placental labyrinth appeared to be normal and the physiological necrosis zone underlying this area of fetomaternal attachment could be detected. Alterations occurred in placental sites sonographically identified as resorptions in the treated dogs: the longer the time after the onset of resorption, the thinner the placental labyrinth became. Simultaneously, the necrosis zones were more extended.

In the epithelial cells of the glandular chambers, expression of lysozyme was detected in all bitches, but after induced resorption the number of cells expressing this enzyme increased. Infiltration of the placental stroma by macrophages occurred in all placental sites. In the control dogs and in the dog treated with Cloprostenol and Cabergolin the placental sites showed a high PR expression in maternal stromal cells and myometrial myocytes. In contrast, in all dogs that achieved Aglepristone (a progesterone-receptor antagonist), a lower expression of PR could be seen in placental sites with a living fetus. PR expression was very weak in those localizations where a resorption had been detected by sonography.

It can be summarized that the drugs administered in this investigation successfully induced embryonic/fetal and placental resorption in bitches, causing similar morphologic features of the resorption sites independent of the method used. The preliminary results of the present study show, that the maternal glandular chambers and the maternal stromal cells and myometrial myocytes seem to play an important role during resorption in the bitch. Further experimental investigations will be carried out in mares to compare the results with those in dogs with special emphasis on comparative placental pathology.

Relationships between changes in uterine blood perfusion and nitric oxide during estrous cycle and early pregnancy in mares.

Zur Beziehung zwischen Veränderungen der uterinen Blutperfusion und Stickoxid während des Zyklus und der Frühträchtigkeit bei Stuten

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The aim of the present study was to estimate the influence of nitric oxide on uterine blood supply during estrous cycle and early pregnancy in mares. Five trotter mares were examined during estrous cycles and early pregnancy. The investigations contained colour Doppler- and ultrasound recordings as well as collection of endometrial biopsies. Investigations in cycling mares were carried out on Days 0 (= ovulation), 1, 5, 11, 15 and once during estrus. In pregnant mares colour Doppler recordings and blood samples were taken on Days 0, 1, 5, 11 and 15, while endometrial biopsies were only gained on Day 15. The analysis of uterine blood flow was based on the time-averaged maximum velocity (TAMV). Total RNA was isolated from endometrial biopsies. Transcriptes encoding the endothelial as well as the inducible nitric oxide-synthase (eNOS and iNOS) were quantified by RT-PCR-analysis. Uterine blood flow velocity followed a consistent wavelike pattern ($P < 0.05$) during estrous cycle. Low TAMV values occurred on Days 0, 1 and on Day 11 and high TAMV values were observed on Days 5, 15 and during estrus. Levels of iNOS-mRNA were independend from the stage of estrous cycle ($P > 0.05$), but a characteristic cyclic pattern occurred in changes of eNOS-mRNA ($P < 0.05$). There was a positive correlation between the expression of the eNOS-mRNA and uterine blood flow ($p < 0.05$) in cycling mares. The levels of eNOS mRNA were low on Days 0, 1, 11 and 15 and high on Days 5 and during estrus. There was a positive correlation ($r = 0.46$; $P < 0.05$) between alterations of eNOS and TAMV values. On Day 15 of pregnancy, an increase in uterine perfusion as well as an enhancement in iNOS-mRNA expression was observed.

In conclusion, the results of this study indicate that nitric oxide plays an important role in the regulation of uterine blood supply during estrous cycle as well as during early pregnancy of mares.

Effects of treatments to prevent early pregnancy loss in the mare

Erfolge der Maßnahmen zur Vorbeugung des Frühträchtigkeitsverlustes bei der Stute

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Approximately 10-20% of detected horse pregnancies are lost before day 35 of gestation. As a result, there is considerable interest in treatments to reduce the likelihood of early pregnancy loss (EPL). Currently used therapies include progestagen supplementation and one-off treatments to stimulate or protect the corpus luteum, such as mid-dioestrus GnRH analogue administration. It has been claimed that increased circulating progesterone levels stimulate conceptus growth and, as a result, maternal recognition of pregnancy signal secretion. In cattle, both GnRH injection and progestagen supplementation appear additionally to weaken the luteolytic drive. The aim of the current study was to examine the effects of progestagen supplementation and GnRH analogue injection on conceptus growth, maternal plasma progesterone concentrations and oxytocin-induced PGF2a release. To this end, pregnant and non-pregnant mares were treated with 40mg altrenogest (Regumate) daily during days 6-20 after ovu-

lation (4 and 5 mares, respectively) or 40 µg buserelin on day 10 after ovulation (5 and 8 mares), or kept as controls (7 and 8). Blood samples collected during oestrus and until day 20 after ovulation were assayed for progesterone concentrations and, on days 12 and 20, conceptus size was measured and 20 i.u. oxytocin were injected intravenously. Concentrations of PGFM were measured in blood samples collected at 15 min intervals before and after oxytocin injection.

Administration of altrenogest tended to suppress endogenous progesterone levels in pregnant mares, in one case to 1-2 ng/ml. Although progesterone levels rose on the day after buserelin injection in all non-pregnant mares, there was no sustained increase and the difference was neither statistically significant nor accompanied by ultrasonic evidence of secondary luteinisation. Neither conceptus size nor the PGFM response to oxytocin challenge were affected by either treatment. However, the stage of pregnancy ($p<0.05$) and, in non-pregnant mares, the timing of luteolysis did affect PGFM release ($p=0.0001$). Indeed, in pregnant mares, the PGFM response was minimal on day 12 but significantly higher on day 20 when it was comparable in magnitude to that in day 12 non-pregnant mares with a "normal" dioestrus. This confirmed previous reports that oxytocin-induced PGF2α release is inhibited during the period of conceptus migration but reappears after fixation. The survival of a pregnancy during days 18-35 may thus depend on avoidance of oxytocic stimuli. Additionally, these studies demonstrate that neither GnRH injection nor progesterone administration suppress the luteolytic pathway and question whether administering progesterone really accelerates conceptus growth. It is suggested that a relatively low threshold progesterone level is required for pregnancy maintenance and that more is not always better.

The effect of equine granulosa-theca cell tumors (GCT) on the pituitary-ovarian endocrine axis.

Zur Auswirkung von Granulosazelltumoren auf die endokrine Achse zwischen Hypophyse und Ovarien

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Equine granulosa-theca cell tumors (GTCT) are hormonally active neoplasms with a suppressive effect on the estrous cycle. An enlarged neoplastic ovary and an inactive contra lateral ovary clinically characterize affected mares. It has been suggested that the contra lateral ovary is inactive due to increased inhibin secretion, which suppresses FSH. The objective of this study was to characterize the hormonal activity in mares with GTCT, and to determine how the tumor affects the pituitary-ovarian axis.

Serum concentrations of α-inhibin (all α-subunit-containing forms of inhibin) and dimeric $\alpha\beta_A$-inhibin (forms that contain both an α- and β_A-subunit) were determined in mares with GTCT ($n=22$), normal cycling mares ($n=31$; 23 in estrus / 8 in diestrus), and pregnant mares ($n=5$). FSH, LH, estradiol, and testosterone were determined in mares with GTCT ($n=22$), and normal cycling mares ($n=31$). In addition, blood samples were collected from one mare with a GTCT at 12 hour intervals for 21 days. Concentrations of α-inhibin, FSH, LH, estradiol, and testosterone were determined using RIA. Student's t-test was used to detect differences between hormone concentrations between the two groups of mares. Pearson's correlation coefficients were calculated to determine associations between concentrations of hormones, and Chi-square analyses were used to determine if differences existed in the proportion of GTCT and control mares with detectable and nondetectable hormone concentrations. Significance was set at $P<0.05$. Concentrations of α-inhibin were detected in 95% of mares with GTCT and in 19% (3 estrus/2 diestrus) of cycling mares ($P<0.001$). Serum concentrations of α-inhibin were elevated in mares with GTCT compared to normal cycling mares with detectable concentrations of α-inhibin (0.4 ± 0.1 ng/mL vs 0.05 ± 0.003 ng/mL; $P<0.01$). Concentrations of $\alpha\beta_A$-inhibin were detected in 91% of mares with GTCT and in 23% (4 estrus/3 diestrus) of cycling mares ($P<0.001$). Serum concentrations of α-inhibin and $\alpha\beta_A$-inhibin were nondetectable in all of the pregnant mares. Concentrations of FSH tended to be lower in mares with GTCT compared to normal cycling mares ($P=0.08$), but FSH was not completely suppressed in mares with GTCT. FSH and $\alpha\beta_A$-inhibin tended to be negatively correlated ($r = -0.41$; $P=0.08$), but no correlation was observed between FSH and α-inhibin. Concentrations of LH were greater in mares with GTCT compared to normal cycling mares ($P<0.02$). A positive correlation between LH and testosterone was observed in mares with GTCT ($r = 46$; $P<0.05$). Concentrations of estradiol-17β, and testosterone were similar for mares with GTCT and normal cycling mares. Testosterone concentrations had a wide range within both groups. In the mare with GTCT from which blood was collected every 12 hours for 21 days, α-inhibin concentrations were consistently elevated with little variation over time. In contrast, serum concentrations of dimeric $\alpha\beta_A$-inhibin were variable over time, and testosterone concentrations fluctuated widely during the 21-day period. It was concluded that GTCT secrete α-inhibin and $\alpha\beta_A$-inhibin, but that α-inhibin is a more reliable and consistent indicator of GTCT. It was also included that other factors than a negative feedback by inhibin on FSH may be involved in the suppression of ovarian activity in mares with GTCT.

The excess secretion of LH in mares with GTCT may be the result of low circulating progesterone, or it may be involved in the formation of the tumor as has been suggested in other species.