Effects of age and altrenogest treatment on conceptus development and secretion of LH, progesterone and eCG in early-pregnant mares

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Abstract

The treatment of early pregnant mares with a history of repeated early embryonic loss with the progestin altrenogest has become routine; however no controlled studies on the efficiency of altrenogest to prevent embryonic losses are available so far. In the present study, we have investigated effects of altrenogest treatment in mares on conceptus development and the secretion of LH, progesterone, and eCG until day 100 of pregnancy. In addition, differences related to age of mares were assessed. Mares were treated with altrenogest (0.044 mg/kg per os once daily) or sunflower oil (10 ml per os once daily) from day 6 to day 100 after ovulation. Blood samples for analysis of LH, progesterone, and eCG were collected. The size of the embryonic vesicle and embryo/fetus was determined by ultrasound. No difference in the per cycle pregnancy rate between altrenogest-treated (75\%) and sunflower oil-treated mares (74\%) was detected (n.s.). A significant effect of age but not of altrenogest treatment on mean diameter of the embryonic vesicle was found between days 12 and 22 of pregnancy (e.g. day 15: control, 4 – 8 years: 22.9 ± 1.0 mm, >8 years: 22.0 ± 1.7 mm, altrenogest, 4 – 8 years: 26.1 ± 2.0 mm, >8 years: 20.4 ± 1.0 mm, P < 0.05). A significant effect of age and treatment on size of the embryo proper between days 30 and 45 was detected (P < 0.05). In the control group but not in the altrenogest group, size of the embryo proper respective fetus was negatively correlated with age of the mares (day 30: r = −0.834, P < 0.05; day 35: r = −0.506, P < 0.05). Plasma concentrations of LH and progesterone were neither effected by age nor by treatment of mares, but significant effects of age and altrenogest treatment on eCG concentrations between days 40 and 130 were detected (P < 0.05). The present study demonstrates for the first time a positive influence of altrenogest-treatment on a retarded development of the embryo respective fetus around the beginning of placentation in mares older than 8 years.

Keywords: Horse; Pregnancy; Age; Conceptus development; Altrenogest

1. Introduction

Early pregnancy loss until approximately day 20 after ovulation is a major reason for low reproductive efficiency in subfertile mares [1,2]. Treatment of early pregnant mares with a history of repeated early embryonic loss with the progestin altrenogest has become routine [3]. Altrenogest has been shown to efficiently maintain pregnancy in ovariectomized embryo recipient mares until the onset of placental progesterin synthesis [4,5]. Similarly, altrenogest prevents embryonic loss in early pregnant mares after ovariectomy or induction of luteoly-
sis [6,7,8]. However, no controlled studies on the efficiency of altrenogest administration for prevention of embryonic loss in fertile and subfertile mares are available so far.

After ovulation, pregnancy is maintained by progesterone from the corpus luteum. During the initial phase of gestation in mares, maximal concentrations of progesterone are reached on day 8 after ovulation and subsequently slowly decrease [9]. This is paralleled by a progressive decrease in the mean cross-sectional area of the CL from day 4 of the cycle [10]. Maternal recognition of pregnancy in the mare occurs between days 10 and 14 after ovulation [9]. However, its exact mechanism is still unknown in the horse. After recognition of pregnancy, the primary corpus luteum is responsible for maintenance of pregnancy for the next 3 to 4 weeks. During this period, progesterone secretion may decrease to concentrations much lower than during the first 10 days of pregnancy [3]. Development of accessory corpora lutea as a result of endometrial cup formation and associated eCG synthesis starting approximately on day 37 [11] leads to a second pronounced increase in progesterone concentration from day 40 onwards [12]. A gradual degeneration of the endometrial cups and consequently a loss of eCG synthesis capacity is initiated after day 70 of pregnancy by an immune reaction of the endometrium, subsequently also resulting in regression of the accessory corpus lutea [13]. The primary corpus luteum itself is maintained until day 160 to 180 after ovulation [14,12]. However, placental progesterone synthesis in the pregnant mare starts as early as day 60 after ovulation. Therefore, from day 70 onwards, circulating progesterone concentration in the pregnant mare is considered a mixture of luteal progesterone and placental progestins. From day 160 of pregnancy onwards, progesterone itself can no longer be detected in the maternal circulation [15].

In approximately 10% of fertile mares, early embryonic loss occurs. In subfertile mares, this condition is seen even more frequently and also occurs over a longer period of time [1]. Insufficient growth and development of the conceptus are considered major reasons for early embryonic losses. Thus in embryos with subnormal size, the loss rate is higher than in embryos with normal size [1,16,17,18]. In aged mares, the quality of early embryos is inferior than in young mares [19]. Approximately 60% of early pregnancy losses occur between days 15 and 35 [2]. However, it is unclear whether subnormal progesterone concentration contributes to failure of embryonic development in the horse [3]. In cows, a positive relationship between progesterone concentration in maternal plasma and development of the embryo, resulting in a stronger antiluteolytic signal has been demonstrated [20,21,22]. In the mare, the presence of progesterone seems to be a prerequisite for mobility of the conceptus as well as its subsequent fixation and orientation in the uterus [23].

In the present study, we have investigated effects of altrenogest treatment of early-pregnant mares on conceptus development and secretion of the reproductive hormones LH, progesterone, and eCG. Differences between altrenogest-treated and non-treated mares in relation to age of the mares were assessed.

2. Materials and methods

2.1. Experimental animals and breeding management

2.1.1. Animals

Mares were warmblood mares (n = 32) of the Brandenburg breed belonging to the broodmare herd of the Brandenburg State Stud at Neustadt (Dosse), Germany. Age, weight, and reproductive history of mares are shown in Table 1. Mares were kept in groups of 8 to 10 animals. Until the end of April, mares were housed in spacious group stables. During this time they were fed oats (3 kg per mare and day) and minerals thrice daily. Hay was given ad libitum. During daytime, the mare groups spent several hours in outdoor paddocks. From

<table>
<thead>
<tr>
<th>Age group</th>
<th>Treatment</th>
<th>Number of mares (n)</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Status in season used for experiment (Maiden/lactating/barren)</th>
<th>Number of breeding seasons* (n)</th>
<th>Number of pregnancies carried to term* (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4–8 years</td>
<td>Control</td>
<td>11</td>
<td>5.9 ± 0.4*</td>
<td>532.8 ± 9.3</td>
<td>1/8/2</td>
<td>3.7 ± 0.6*</td>
<td>3.0 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td>Altrenogest</td>
<td>8</td>
<td>4.7 ± 0.6*</td>
<td>533.9 ± 8.5</td>
<td>2/6/0</td>
<td>3.1 ± 0.9*</td>
<td>3.1 ± 0.9*</td>
</tr>
<tr>
<td>&gt;8 years</td>
<td>Control</td>
<td>5</td>
<td>12.4 ± 1.5*</td>
<td>551.7 ± 13.2</td>
<td>0/2/3</td>
<td>10.6 ± 1.6*</td>
<td>8.4 ± 1.0*</td>
</tr>
<tr>
<td></td>
<td>Altrenogest</td>
<td>8</td>
<td>11.3 ± 0.7*</td>
<td>556.0 ± 17.1</td>
<td>0/4/4</td>
<td>8.9 ± 0.7*</td>
<td>7.1 ± 0.5*</td>
</tr>
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* Including season/pregnancy included into the experiment.

a,b Significant differences between age groups (P < 0.05).
the end of April, mares spend the daytime on pasture and the night in spacious group stables. During this time of the year, they were fed oats (2 kg per mare and day) and minerals twice daily, at night hay was available ad libitum. They always had access to water.

2.1.2. Breeding management

Mares were controlled for oestrous behavior with a stallion three times per week. At the time of expected foal heat (approximately 6 days after foaling) or when the mares showed signs of oestrus, ovaries were examined for the presence of follicles and the uterus for the presence of endometrial oedema by rectal palpation and scanning with a 7.0 MHz linear scanner (DP-6600Vet, Mindray, Shenzhen, China). When a follicle of 35 mm in diameter together with uterine oedema was detected mares were bred with semen from a stallion chosen by the studfarm director. Seventeen different stallions with proven fertility were selected. One stallion was used to breed 8 mares that belonged to all mare groups. For the other stallions the number of mares bred ranged between one and three, but the number of mares per group that was bred to the same stallion never exceeded two. All insemination doses met quality criteria as defined by the World Breeding Association for Sport Horses (2009). In case mares did not ovulate within 48 hours, insemination was repeated at 48 hour-intervals with semen from the same stallion. The day of ovulation (= day 0) was defined as the day before the first detection of a corpus luteum by ultrasound.

2.2. Experimental design

Mares were assigned to control and altrenogest treatments in alternating order when they were bred for the first time. The animals were further grouped into mares between 4 and 8 years of age (n = 19) and mares of 9 years and older (n = 13; see Table 1) to determine possible effects of age and interactions between age and treatment on development of the conceptus and secretion of reproductive hormones. Mares were treated with altrenogest (0.044 mg/kg per os once daily, Regumate equine, Intervet, Beaucouzé, France) or placebo (sunflower oil, 10 ml per os once daily) from day 6 to day 100 after ovulation. Day 1 of pregnancy for the whole group of experimental animals was between 2 March and 22 May in the same breeding season. The experiment was performed according to German animal welfare legislation and was approved by the Brandenburg State Ministry for Rural Development, Environment and Consumer Protection.

2.3. Experimental procedures

Blood samples from the animals were collected by venipuncture into heparinized tubes (Vacutte, Greiner, Kremsmünster, Austria) on days 1, 3, and 5, daily from day 6 to day 18, at two day-intervals from day 20 to day 34, daily from day 35 to day 45 and at two day-intervals from day 47 to day 99 after ovulation. The sampling schedule takes into account two critical phases for the maintenance of pregnancy: maternal recognition of pregnancy (days before day 18) and the beginning of placentation and eCG secretion (days 35–45). Additional blood samples were collected on days 110, 120, and 130 after ovulation. Immediately after collection, all blood samples were centrifuged at 1200 g for 10 min, the plasma was decanted and frozen at −20 °C until assayed. Only blood samples from mares found pregnant on day 12 after ovulation by ultrasonographic detection of a conceptus were included into the experiment.

Transrectal ultrasound examination of the conceptus was performed on days 12, 15, 18, 20, 22, 30, 35, 40, and 45 after ovulation. On days 12 to 22, the diameter of the embryonic vesicle was determined in two dimensions using the electronic calipers of the ultrasound machine. The mean of the two measurements was calculated as mean diameter of the vesicle. Embryonic vesicles were rated as undersized when their diameter was smaller than the mean diameter of all embryos minus 2 standard deviations [16]. On days 30, 35, 40, and 45, the largest length and height of the embryo proper or fetus was determined using the electronic calipers. The mean of the two measurements was calculated as mean size of the embryo/fetus. On days 60, 80, and 100, mares were again checked for the presence of a healthy fetus by rectal ultrasonography.

2.4. Hormone analysis

2.4.1. Equine LH

LH was determined by RIA as described previously [24] using equine LH (Biogenesis, Poole England) as standard and for iodination and an antibody raised in rabbits against equine LH (A 543, Biogenesis). Intra- and interassay variations of the assay were 5.7% and 8.2%, respectively; the minimal detectable concentration of the assay was 0.5 ng/ml. Cross-reactivity of the antibody with equine FSH (E276B, Dr. H. Papkoff, University of California, San Francisco, CA, USA) was less than 2.8%, cross-reactivity with eCG was 23%.

2.4.2. Progesterone

Progesterone was determined using a commercial chemiluminescence-based method (ACS: 180 Automated System with kit PRGE, Bayer Vital, Fernwald, Germany). The validity of this method in horses was confirmed by previous comparative measurements using a well established RIA method after sample extraction with hexane [25]. The minimum detectable concentration was at 0.1 ng/ml. Inter-assay coefficients of variation were 14.3 and 5.2%, for the low (1.29 ng/ml) and high (8.74 ng/ml) control points, respectively.

2.4.3. eCG

Assessment of PSMG (eCG)-levels was performed using a commercially available enzyme-linked immunosorbent assay (DRG Instruments, Marburg, Germany) following the manufacturers instructions. The minimum detectable concentration was 25 mIU/ml, interassay variation was 18%.

2.5. Statistical analysis

Statistical analysis was performed with the programme SPSS for windows version 17.0. The pregnancy rate between treatment groups was compared by Chi-Square analysis. To evaluate effects of age and treatment group on the different parameters analysed (size of the embryonic vesicle, size of the embryo proper or fetus and concentrations of LH, progesterone and eCG) the general linear model for repeated measures procedure was used. For evaluation of LH concentration by analysis of variance for repeated measures, the experimental period was divided into the following periods: 1) formation of the corpus luteum (day 1 to day 6 after ovulation); and 2) the time from day 6 until day 34 after ovulation. After day 34 a significant increase in LH concentration was determined due to cross-reactivity of the LH-antibody with eCG. Concentration of LH was therefore not further analysed after day 35. For evaluation of progesterone concentration over time, the experimental period was divided into the following periods: 1) day 1 to day 6 after ovulation (formation of the corpus luteum); 2) day 6 to day 35 (until beginning of eCG production); 3) day 35 to day 51; 4) day 51 to day 99 after ovulation. Correlations were evaluated using the Pearson correlation test. A P-value of < 0.05 was considered significant. All values given are means ± SEM.

3. Results

3.1. Fertility of mares and development of the conceptus

No difference in the pregnancy rate per cycle between altrenogest-treated (75%) and placebo-treated mares (74%) was detected (n.s.). None of the mares pregnant on day 12 after ovulation subsequently lost her pregnancy and all mares gave birth to healthy mature foals during the next spring.

The size of the embryonic vesicle increased significantly from day 12 to day 22 after ovulation irrespectively of age and treatment (P < 0.05, Fig. 1). A significant effect of age on mean diameter of the embryonic vesicle was found. An undersized embryonic vesicle was detected in one control mare aged 12 years on days 12, 15, and 20, but subsequently size and development became normal. In one altrenogest-treated mare aged 12 years, the embryonic vesicle was found to be undersized on days 20 and 22 after ovulation, but showed normal development thereafter. In an 18 year old control mare, the vesicle was found to be undersized on day 20 after ovulation only. No significant effects of altrenogest treatment on diameter of the embryonic vesicle could be detected until day 22. The size of the embryonic vesicle on day 12 was correlated with the size on day 15 (r = 0.729, P < 0.05) and the size of the vesicle on day 20 was correlated with the size on day 22 (r = 0.756, P < 0.05). Furthermore, a negative correlation between size of the vesicle on day 20 and the age of the mare (r = −0.475, P < 0.05) was found irrespective of treatment.
The mean size of the embryo respective fetus (from day 35 onwards) increased significantly between days 30 and 45 after ovulation in all groups (P < 0.05, Figure 2). A significant influence of age and treatment between days 30 and 45 was detected (P < 0.05). Mean size of the embryo proper on day 30 was 12.4 ± 0.6 mm in control mares aged 4–8 years, 8.9 ± 0.6 mm in control mares older than 8 years, 11.1 ± 0.6 mm in altrenogest-treated mares aged 4–8 years and 10.9 ± 0.8 mm in altrenogest-treated mares older than 8 years (Figure 2).

In the control group, size of the embryo proper respective fetus was negatively correlated with age of the mares (day 30: r = −0.834, P < 0.05; day 35: r = −0.506, P < 0.05) as well as the number of active breeding seasons per mare (day 30: r = −0.820, P < 0.05; day 35: r = −0.533, P < 0.05). These correlations were lost in altrenogest-treated mares. The size of the embryo proper on day 30 was positively correlated to fetal size on day 35 (r = 0.767, P < 0.05).

3.2. Concentration of reproductive hormones

3.2.1. Luteinising hormone

Mean concentration of LH significantly decreased from day 1 to day 6 after ovulation in all mares irrespective of age and treatment (P < 0.05). Thereafter, the concentration of LH was at a constant low level and neither affected by age nor by treatment of mares (Figure 3).

3.2.2. Progesterone

Plasma progesterone concentration significantly increased from day 1 to day 6 after ovulation (P < 0.05) irrespective of age and treatment. A significant decrease in progesterone from day 6 to day 34 (P < 0.05) occurred in all mares and was followed by a second increase in progesterone concentration from day 35 to 51 (P < 0.05). No effects of age or treatment on progesterone secretion could be determined. After day 51, progesterone concentration was relatively constant and not affected by treatment or age (Figure 4).
Moreover, the per cycle pregnancy rate of approximately 75% in the present study is high in comparison to a 60% per cycle pregnancy rate in a rather unselected broodmare population [2]. However, it cannot be excluded that fertility and also rate of embryonic death might differ if a higher number of experimental animals would have been used for the study. Before the experiment started, the mares were not examined for their endometrial status, but none of the mares showed signs of susceptibility to endometritis during breeding. Together this may reflect that the endometrial status of the experimental animals used in the present study is good and potentially allows an unimpaired early development of the conceptus. The smaller size of the embryonic vesicle respective embryo proper and fetus in older mares may be caused by a lower oocyte quality in these mares [19] resulting in delayed embryonic development but not necessarily leading to pregnancy loss if overcome by a favourable uterine environment. Thus, even undersized embryonic vesicles survived. Similarly, day 2 embryos from young, fertile and old, subfertile mares reached the blastocyst stage to the same extent when cultured in an in vitro system. The quality score of the blastocysts was not different when they were co-cultured with oviductal epithelial cells from young mares. In contrast, co-culture of day 2 embryos from the old, subfertile mares with oviductal epithelial cells from old mares resulted in inferior quality of the blastocysts after 7 days [19].

Most interestingly, a smaller size of the embryo proper on day 30 and the fetus between days 35 and 45 in older mares was improved by treatment with altrenogest. This is to our knowledge the first time that a direct positive influence of this progestin on conceptus development has been demonstrated. In agreement with a study by Voller et al [27], the size of the embryonic vesicle was not affected by treatment with altrenogest. Therefore, progestin substitution in the presence of an intact corpus luteum did not stimulate growth of the blastocoeel in early pregnancy. This finding is in contrast to the situation in cattle where high progesterone concentrations in maternal plasma facilitate growth of the trophoblast and expression of the embryonic signal for maternal recognition of pregnancy [20,21,22]. The present data suggest that altrenogest supports development of the embryo and fetus from days 30–45 of pregnancy. This period corresponds to completion of organogenesis and beginning of placentation which is a critical phase of pregnancy [28]. Positive effects of altrenogest on the secretion of uterine milk which ensures nutrition of the conceptus until placentation [28] are feasible. Furthermore, this phase of pregnancy co-

### 3.2.3. Equine chorionic gonadotropin

A significant interaction of age and treatment over time (P < 0.05) on eCG concentration was detected (Figure 5). Concentrations of eCG on day 79 were 851 ± 234 ng/ml in 4-8 year-old control mares, 1156 ± 725 ng/ml in control mares >8 years, 1691 ± 262 ng/ml in altrenogest-treated 4–8 year old mares and 725 ± 152 ng/ml in altrenogest-treated mares >8 years.

### 4. Discussion

In the present study, effects of age and altrenogest treatment on conceptus development and the secretion of reproductive hormones during early pregnancy in mares were investigated. One important finding is that conceptus development is limited in mares aged >8 years in comparison to mares between 4 and 8 years of age. In the older animals, size of the embryonic vesicle as well as size of the embryo proper respective fetus was smaller than in the younger mares. Embryonic vesicles that subsequently undergo early embryonic death on average are smaller than control vesicles in maintained pregnancies [16]. Besides retarded growth, also cellular degeneration and extensive cellular necrosis are found to a higher degree in embryos from subfertile than from fertile mares [26]. An inferior size of the conceptus in mares from an age of 9 years onwards is in agreement with a higher pregnancy loss rate in Thoroughbred mares of similar age [2]. Interestingly, none of the mares in the present study lost her pregnancy. However, all of them have to be considered as mares of high fertility that are kept as broodmares on a commercial studfarm and were used for breeding continuously from an age of 3 years. Mares with low fertility or repeated pregnancy loss are usually excluded from that herd. Moreover, the per cycle pregnancy rate...
The conclusion of Day and Rowlands, a decrease in eCG secretion with successive pregnancies of mares is not supported by the results of the present study. Interestingly, recent results showed that even immunological sensitization of mares to antigens of the stallions they were subsequently bred to did not shorten endometrial cup function [33]. It is unclear by which mechanism altrenogest can change eCG secretion in mares. It could be speculated that altrenogest might modulate the maternal immunological reaction to foreign trophoblast-specific antigens [34,35,36] that is finally leading to cessation of endometrial cup activity. Immunosuppressive effects of progesterone [37] and also altrenogest [38] have been shown recently. However, effects of altrenogest on endometrial cup function in mares remain to be determined in more detail.

In conclusion, the present study demonstrates for the first time a positive influence of altrenogest-treatment on delayed conceptus development in pregnant mares. This effect did not occur during early pregnancy at the time of maternal recognition of pregnancy, but became apparent when development of the embryo or fetus was monitored around the beginning of placentation. Beneficial effects of the progesterone treatment are suggested. In addition, development of the conceptus in fertile broodmares older than 8 years was retarded and this delay was completely overcome by altrenogest substitution. This may justify altrenogest-treatment of older mares or those with a history of early embryonic death to support development of their conceptus.

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References


