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Determination of conjugated oestrogens in maternal blood plasma and urine for pregnancy diagnosis and monitoring of foetal well-being in the mare

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Summary

Due to the distinct differences of oestrogen concentrations in pregnant and non-pregnant animals the radioimmunological determination of conjugated oestrogens in maternal blood plasma or serum is a highly reliable method for pregnancy diagnosis in mares during the interval corresponding to the phase of placental oestrogen production between around days 70–80 after mating/insemination and parturition. As placental oestrogen biosynthesis depends on precursors provided by the foetal gonads, any loss of foetal vitality is strictly associated with a decreased placental oestrogen production. Therefore, oestrogen concentrations are a useful parameter for the monitoring of foetal well-being in cases of unclear symptoms of impaired pregnancy. Prior to the onset of placental oestrogen production determination of ovarian estrone sulfate can also be applied (> day 40). However, pregnancy diagnosis may be problematic due to slightly overlapping values between 1 ng/ml to 2.5 ng/ml likewise occurring in mares at oestrus and a minor fraction of pregnant mares. The determination of urinary conjugated oestrogens can be used as a non-invasive alternative to the measurement in blood plasma within the interval corresponding to the phase of placental oestrogen production (> day 80). Preliminary results also suggest that ovarian oestrogen production between days 40 and 80 after mating (insemination) may be monitored for pregnancy diagnosis, but the preliminary cut-off level at 175 ng/ml needs further confirmation.

Keywords: horse, pregnancy diagnosis, oestrogens, blood, urine

Messung konjugierter Blut- und Harnöstrogene zur Graviditätsdiagnose und Überwachung der Fetusvitalität beim Pferd

Die trächtigkeitsspezifische Östrogenproduktion der Stute kann entsprechend der Östrogenquellen in zwei Phasen eingeteilt werden. In der ersten Phase findet sich im peripheren maternalen Plasma nach einem ersten Anstieg um den 40. Graviditätstag eine leichte Erhöhung der Estronsulfatkonzentrationen von Basalniveau (< 0,5 ng/ml) auf 1,5–10 ng/ml bis zum ca. 70.–80. Graviditätstag. Als Östrogenquelle fungiert hierbei das Ovar. In der folgenden plazentaren Phase kommt es im maternalen Blutplasma zu einem steilen Anstieg der Konzentrationen konjugierter Östrogene auf Maximalwerte um 700 ng/ml in der Graviditätsmitte, gefolgt von einem allmählichen Abfall auf Werte um 50–100 ng/ml unmittelbar vor der Geburt. Unter der Geburt fallen die Östrogenkonzentrationen weiter steil auf Basalniveau ab. Aufgrund der außerordentlich hohen Östrogenkonzentrationen in der plazentaren Phase stellt die radioimmunologische Bestimmung konjugierter Östrogene in Blutplasma oder -serum ab dem 80. Tag nach der Bedeckung/Besamung eine äußerst zuverlässige Methode zur hormonellen Trächtigkeitsdiagnose dar. Da die Präkursoren der plazentaren Östrogene den fetalen Gonaden entstammen, ist eine Beeinträchtigung der Vitalität des Fohlens stets mit einem Abfall der plazentaren Östrogene verbunden. Daher eignet sich die Östrogenbestimmung auch zur Trächtigkeitsüberwachung in Fällen unklarer Symptome eines drohenden Abortes. Auch zwischen dem 40.–80. Graviditätstag ist in den allermeisten Fällen eine Trächtigkeitsdiagnose anhand der Bestimmung konjugierter Östrogene möglich. Schwierigkeiten können sich hier in einzelnen Fällen durch die geringfügig überlappenden Meßwerte rossiger bzw. frühgravider Stuten im Bereich zwischen 1,0 und 2,5 ng/ml ergeben. Neben der Bestimmung im Blutplasma kann in dem der plazentaren Phase entsprechenden Zeitraum auch die Messung konjugierter Östrogene im Urin als nichtinvasive alternative Methode angewandt werden. Vorläufige Ergebnisse deuten an, daß auch in der der ovariellen Phase entsprechenden Zeitspanne (Tag 40–80) anhand der Östrogenkonzentrationen im Urin eine Aussage hinsichtlich des Bestehens einer Gravidität möglich ist. Der vorläufige Grenzwert von 175 ng/ml bedarf jedoch einer weiteren Bestätigung durch eine größere Anzahl von Tieren.

Schlüsselwörter: Pferd, Trächtigkeitsdiagnose, Östrogene, Blut, Urin

Introduction

Due to the limited breeding season an accurate pregnancy diagnosis as soon as possible after the last mating or insemination is of outstanding importance for horse breeders. Ultrasonography-aided rectal palpation has become the standard method which allows precise and early confirmation of singleton and twin pregnancies from day 18 onward concomitant with the provision of information about the viability of the foetus *(McKinnon et al., 1993; Shideler, 1993)*. However, in spite of this situation there is still room for hormonal pregnancy diagnosis which may be even more advantageous in certain situations such as pregnancy diagnosis in vicious mares or if the investigation must be performed under otherwise unsuitable conditions, in small races and donkeys and in animals with injured rectum. Furthermore, hormonal pregnancy diagnosis can be used to verify the diagnosis in cases of uncertain rectal diagnosis, especially at later stages of pregnancy when the enlarged uterus is not sufficiently accessible to examination. Hormonal pregnancy diagnosis may also be an alternative for less experienced, non-specialised practitioners in doubtful cases. Moreover, there is an increasing interest of horse owners in non-invasive and therefore more economical me-

thods. Non-invasive methods are also of special interest in wild equids or zoo animals. Several methods of hormonal pregnancy diagnosis in horses are available such as progesterone measurement in blood at the time of the next oestrus in case of unsuccessful breeding, the detection of equine chorionic gonadotrophin in blood *(Hoffmann et al., 1996)*, the measurement of pregnancy-associated oestrogens in blood, urine *(Schuler, 1998)* or faeces *(Möstl et al., 1983; Palme et al., 1989)* or the determination of faecal concentrations of pregnancy-specific gestagens *(Schwarzenberger et al., 1989)*. In our laboratory, hormonal pregnancy diagnosis is routinely performed by radioimmunological determination of total estrone in blood plasma or serum, and – as a non-invasive alternative – in urine.

In the mare large amounts of oestrogens are produced during pregnancy with sulfoconjugated forms dominating by far over free ones *(Hoffmann et al., 1996)*. Equine pregnancy associated oestrogen production can be divided into two phases according to the source of oestrogens. A first rise of ovarian origin from basal levels below 0.5 ng/ml up to plasma concentrations between 1.5–10 ng/ml commences around day 40 of gestation *(Daels et al., 1990; Hoffmann et al., 1996)*, followed by a plateau until around day 80, when placental oestrogen production increases sharply providing plasma concentrations up to values around 700 ng/ml at midgestation. Thereafter oestrogen concentrations decline progressively reaching values of about 100 ng/ml in the last week of gestation. The final drop occurs immediately prior to parturition and baseline levels are reached 1–2 days post partum *(Hoffmann et al., 1996)*. Since in the horse oestrogens are predominantly excreted via the urine *(Palme et al., 1996)*, the urinary oestrogen profile is basically identical to the one in blood plasma but on a 100–1000-fold higher level *(Evans et al., 1984; Monfort et al., 1991)*.

Material and methods

Determination of pregnancy-specific oestrogens in blood plasma or serum

The radioimmunoassay (RIA) used for the determination of pregnancy associated oestrogens was adopted from *Gentz (1994)* and modified for routine performance. Since in the mare sulfoconjugated forms dominate by far over the free ones, no separate estimation of free and conjugated oestrogens was intended and they were determined as total estrone equivalents after hydrolysis of the conjugates. For hydrolysis of conjugated oestrogens, 160 µl 625 mM acetate buffer pH 4.8 and 50 µl diluted β-glucuronidase-arylsulfatase from helix pomatia (Serva Feinbiochemika GmbH&Co, D-69155 Heidelberg; diluted 1:25 in 0.15 M NaCl), were added to plasma or serum samples (0.4 ml). After an overnight incubation at 37°C oestrogens were extracted with toluene. To ensure redissolution of the extracted oestrogens, 160 µl 0.1 N NaOH with 0.1% rat serum albumin were added to the evaporated samples which were then incubated for 20 min at room temperature. Neutralisation and adjustment to RIA conditions were achieved by the addition of 20 µl 0.8 N HCl and 20 µl concentrated phosphate buffer (8.356 g Na_2HPO_4, 2.686 g KH_2PO_4, and 0.325 g NaN_3). Depending on the stage of gestation the redissolved samples were then further diluted prior to RIA. When taking 1.0 ml as a starting position the final dilutions varied between 1:5 and 1:1000. In order to obtain exact values in case of low oestrogen concentrations, the 1:5-dilution was measured of each sample, irrespective of the assumed stage of gestation. Paired dilutions of 1:5 and 1:500 were chosen for samples provided without any anamnestic information. The antiserum used was directed against estrone *(Hoffmann et al., 1994)* and exhibited the following cross reactions: estrone: 100%, equilenine: 23.83%, equiline: 7.28%, estradiol-17β: 1.94%, estradiol-17α: 1.02%, estriol: 0.06%. Concentrations of estrone equivalents were calculated by comparison with a standard curve consisting of eight standards between 20 fmol and 3200 fmol estrone/tube. Intra- and interassay coefficients of variation varied between 9.4% and 12.5%. Classification of estrone concentrations as indicative for pregnancy was based on the data of *Hoffmann et al. (1996)*.

Determination of pregnancy-specific oestrogens in urine

The procedure for the measurement of pregnancy associated oestrogens in urine was identical to the method described above for the determination in blood plasma and serum. Due to the considerably higher oestrogen concentrations in urine, dilutions of 1: 500 and 1:50000 were chosen for all urine samples.

Results and discussion

Due to the distinct differences of oestrogen concentrations in pregnant and non-pregnant mares the determination of conjugated oestrogens in maternal blood plasma is a highly reliable method for pregnancy diagnosis during the phase of placental oestrogen production lasting from around day 70–80 of gestation until parturition. As placental oestrogen biosynthesis depends on precursors provided by the foetal gonads (reviewed by *Möstl, 1994*), any loss of foetal vitality strictly coincides with a decreased placental oestrogen production *(Kasman et al., 1988; Hoffmann et al., 1996)*. Thus, oestrogen concentrations are a useful parameter for the monitoring of foetal well-being in cases of unclear symptoms and suspected impaired pregnancies. Accordingly, with the exception of one case, all mares exhibiting estrone concentrations clearly below the respective normal range aborted within one to five days. The exception refers to one mare which gave birth to an abnormally small but viable foal after a prolonged gestation of 420 days. There subnormal concentrations of estrone equivalents around 3–6 ng/ml (normal range: 50–200 ng/ml) were observed in the last two weeks of gestation. On the other hand, mares with threatened abortions showing normal oestrogen concentrations

maintained pregnancy throughout the time of observation in the clinic. Apart from subnormal oestrogen concentrations also extraordinarily high values can be indicative for impaired gestation *(Hoffmann et al., 1996)*. Furthermore, twin pregnancies are associated with oestrogen levels in the upper range.

Prior to the onset of placental oestrogen production determination of estrone sulfate of ovarian origin can also be applied. However, pregnancy diagnosis may be problematic due to slightly overlapping values between 1 to 2.5 ng/ml likewise occurring in mares at oestrus *(Makawiti et al., 1983; Koskinen et al., 1989)* and a minor fraction of eCG-positive mares *(Schuler, 1998)*. However, since the detection of eCG and not the positive outcome of gestation was used to classify animals as pregnant, no information is available if pregnancy was still intact at the time of testing as eCG production can persist for a relatively long time after foetal death *(Hoffmann et al., 1996)*. Hence the „true" cut-off level might be somewhat higher. This is indicated by preliminary results from the additional progesterone determination in cases of doubtful oestrogen concentrations; all samples tested for progesterone because of oestrogen levels within the overlapping range exhibited concentrations which were not consistent with early pregnancy (< 1.5 ng/ml; *Hoffmann et al., 1996)*.

The same radioimmunological method is applicable for the determination of urinary oestrogens which allows the replacement of the overcome chemical assays of *Cuboni (1934)* or *Rommel (1964)*. Due to the extremely high concentrations exceeding 56 µg/ml at midgestation the determination of urinary oestrogen levels is a very reliable method for non-invasive pregnancy diagnosis.

First results of urinary oestrogen determination also suggest that ovarian oestrogen production between days 50 and 80 after mating (insemination) may be monitored for pregnancy diagnosis; however, the preliminary cut-off level at 175 ng/ml needs further confirmation.

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