Source of oestrogen in early pregnancy in the mare

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Summary. Oestrogen secretion was determined by oestrogen conjugate (EC) analysis of urine in three groups of pregnant mares: Group I (N = 6), animals ovariectomized on Day 18–19 of gestation with pregnancy maintained by daily administration of an oral progestagen, altrenogest; Group II (N = 9), untreated, pregnant mares; Group III (N = 5) intact, pregnant mares treated daily with altrenogest.

The mean EC concentrations in the ovariectomized mares in Group I increased in a constant linear manner from 17 ng/mg Cr on Day 20 to 291 ng/mg Cr on Day 70, with no apparent surge in oestrogen secretion around Day 39. Mean EC concentrations on Days 33, 39 and 44 were respectively 41, 48, and 73 ng/mg Cr. In the intact mares in Groups II and III (shown in parentheses), the mean urinary EC concentrations were 201 (171) ng/mg Cr between Days 20 and 33 of gestation, increased rapidly from 172 (77) ng/mg Cr on Day 33 to a peak of 1066 (895) ng/mg Cr on Day 39, followed by a decline to 637 (719) ng/mg Cr on Day 44. After Day 44, EC concentrations continued to increase in a linear manner to 1191 (842) ng/mg Cr on Day 70. The mean EC concentrations between Days 20 and 70 in Group I were significantly (P < 0.05) lower than in mares in Groups II and III. EC concentrations in Group III mares were significantly lower (P < 0.05) than in Group II mares between Days 28 and 34.

We suggest that the ovary is a major contributor to the oestrogen conjugate concentrations measured between Days 20 and 70 and that the rapid increase between Days 33 and 39 is the result of a change in the rate of ovarian oestrogen synthesis.

Keywords: oestrogen; pregnancy; ovary; horse

Introduction

Determination of oestrone sulphate concentrations in plasma and urine have been used to diagnose pregnancy after Day 40 of pregnancy in the mare and to monitor the viability of the horse fetus (Terqui & Palmer, 1979; Kindahl et al., 1982; Evans et al., 1984; Hyland et al., 1984; Darenius et al., 1988; Kasman et al., 1988; Varner et al., 1988). While oestrogen concentrations during the first 30 days of pregnancy are not different from the non-pregnant mare, plasma concentrations of oestrogen conjugates increase 2–3-fold between Days 35 and 40 of gestation. After this increase, oestrogen concentrations tend to plateau until Day 70–80 when they increase again with values remaining significantly higher for the rest of pregnancy (Nett et al., 1975; Stewart et al., 1982).

The horse embryo is able to synthesize oestrogens as early as Day 7 after conception (Flood et al., 1979; Heap et al., 1982; Zavy et al., 1984). The close temporal relationship between the increase in oestrogen secretion at Day 40 and the onset of horse chorionic gonadotrophin (CG) secretion by the endometrial cups has led to the hypothesis that fetal oestrogen secretion may be stimulated by horse CG (Ginther, 1979). Surgical removal of the fetus and prostaglandin-induced abortion, between Days 44 and 89 of pregnancy, have been shown to result in a significant reduction in urinary oestrone sulphate concentrations, suggesting a correlation between oestrogen production
and viability of the fetus (Kasman et al., 1988). A similar decrease in oestrogen secretion was seen following induction of fetal death at Day 45 by means of injection of hypertonic saline into the fetal sac (Jeffcott et al., 1987). Darenius et al. (1988) showed decreased oestrogen concentrations in association with death and resorption of a fetus around Day 50 of gestation. Termination of pregnancy on Day 90 by different methods, i.e. uterine flushing and PGF-2α administration, consistently resulted in a significant decrease in oestrogen concentrations (Varner et al., 1988). All these observations suggest that the horse fetus is an important source of oestrogens and that changes in oestrogen secretion are a direct reflection of fetal viability.

On the other hand, Terqui & Palmer (1979) have suggested that at least part of the oestrogen secreted around Day 40 of gestation is of ovarian origin. No increase in oestrogen secretion was observed between Days 30 and 40 of pregnancy, as is normally seen in intact pregnant mares, when a pregnant mare was ovarioectomized on Day 34 of gestation. In addition, plasma oestrogen concentrations decreased following ovarioectomy after Day 40 (Terqui & Palmer, 1979; Kasman et al., 1988), suggesting that the ovary also contributes to the increased oestrogen concentrations after Day 35. Based on their observations Terqui & Palmer (1979) proposed that the increase in oestrogen secretion that occurs between Days 30 and 40 is the result of horse CG stimulating follicular growth.

The following study was performed to determine the ovarian contribution to the increase in oestrogen secretion that occurs in early gestation.

**Materials and Methods**

**Experimental design.** Three groups of pregnant mares were used. In Group I, 6 pregnant mares were ovarioectomized before Day 20 of gestation and treated with altrenogest from the day of surgery until Day 70 of gestation. In Group II, 9 intact, pregnant mares, not treated with altrenogest, were used as controls. In Group III, 5 intact, pregnant mares were treated with altrenogest (using the same schedule and dosage as in Group I) and were used as altrenogest controls. Concentrations of oestrogen conjugates in urine were determined to assess oestrogen secretion. Plasma progesterone and horse CG concentrations were determined to confirm luteal activity and development of endometrial cups.

**Animals.** The mares used were of various breeds of light horses, between 3 and 14 years old and with no known reproductive pathology. Mares were mated by the same stallion by natural service, with the day of ovulation being Day 1 of pregnancy. Ovulation and pregnancy were determined every second day by palpation and ultrasonography per rectum (Alaska 210, 5 MHz linear transducer, Corometrics Medical Systems Inc., North Wallingford, CT, USA). Animals were kept initially on grass pasture, from one day before until 8 days after ovarioectomy in box stalls, and subsequently on grass pasture for the remainder of the experimental period.

**Treatments.** Six pregnant mares were ovarioectomized on Day 18 or 19 after ovulation (Group I). Ovarioectomy was performed by the colpotomy technique with a chain ecraseur (Scott & Kunze, 1977). The mares were fasted for 24 h before surgery. Sedation and pain relief were achieved with xylazine (Rompun, Haver, Shawnee, KS, USA) and morphine sulphate (Elkins-Sinn, Cherry Hill, NJ, USA) dosed to effect. After surgery the mares were treated daily for 4 days with 2 g phenylbutazololide (phenylbutazone paste: Coopers Animal Health, Kansas City, MO, USA) and 48 g trimethoprim and 24 g sulphamethoxazole (Sulfamethoxazole and Trimethoprim Tablets DS: Heather Drug Company, Cherry Hill, NJ, USA). All mares recovered without secondary complications.

Daily altrenogest administration per os (44 mg/day: Regu-Mate, Hoechst-Roussel Agri-Vet, Somerville, NJ, USA; chemical name: 17α-allyl-17β-hydroxyoestra-4,9,11-trien-3-one) was used as a progesterone replacement treatment (Shideler et al., 1982). Treatment was started on the morning preceding ovarioectomy or on Day 20 for intact, treated mares (Group III) and was continued until Day 70.

**Sample collection.** Blood and urine samples were collected daily between Days 20 and 70 of gestation from mares in Groups I and II, and 3 times/week from mares in Group III. Blood was collected by jugular venepuncture into heparinized collection tubes (Monoject Vacutainers, Sherwood Medical, St Louis, MO, USA). Plasma was separated and stored at −20°C until analysis. Urine was collected in sterile manner by inserting a 28 French rubber stallion catheter in the urethra and stored without preservatives at −20°C until analysed.

**Hormone assays.** Oestrogen conjugate concentrations in urine were determined with a direct radioimmunoassay (RIA). The antibody (R-583) was produced in rabbits and was directed against oestrone-3-glucuronide which had been conjugated to bovine serum albumin. The antiserum was used at a dilution of 1:8000 in Tris buffer (0·1 M-Tris pH 8·4, 0·1% gelatin, 0·9% NaCl, 0·1% sodium azide). When using oestrone-3-sulphate as the standard (100%), the antibody cross-reacted with oestrone (200%), oestradiol-17β (100%), equilin (50%), oestrone-3-glucuronide (38%), oestradiol-3-sulphate (21%), and oestradiol-3-glucuronide (6·8%); the cross-reaction was less than 0·5% with all non-
oestrogenic steroids tested. For the assay, urine samples were diluted 1:100 in distilled water and 0·05 ml was assayed. Serially diluted urine samples produced displacement curves parallel to the oestrone sulphate standards. The limit of the sensitivity was 0·018 ng/tube or 0·36 ng/ml plasma or 36 ng/ml urine. The inter-assay coefficients of variation were 13% (n = 10) to 21% (n = 15) at 20–30% binding and 10% (n = 15) to 12% (n = 9) at 50–60% binding on the standard curve; the intra-assay coefficient of variation was 6–9%. The details of the assay, with the exception of the antisera used, have been described previously (Shideler et al., 1983a, b).

Plasma progesterone concentrations were determined in plasma using an enzyme immunoassay (Murro & Stabenfeldt, 1984). The antibody cross-reacts with 11α-hydroxyprogesterone (21·4%), 5α-pregnane-3,20-dione (29·5%), and 20β-dihydroprogesterone (2·4%) while the cross-reaction is <0·5% for 17α-hydroxyprogesterone, oestradiol-17β, cortisol, Δ4-androstenedione and testosterone. The limit of sensitivity of the assay was 2·5 pg/ml. The inter-assay coefficient of variation ranged from 1·2 to 4·3% at different concentrations of hormone and the intra-assay coefficient of variation ranged from 2·7 to 8·4%.

Horse CG concentrations were determined by radioimmunoassay (Roser & Lofstedt, 1989).

Creatinine concentrations were used to index the urinary oestrogen conjugate concentrations to compensate for variations in the specific gravity of urine. Urinary creatinine concentrations were determined by modification of a colorimetric method (Taussky, 1954).

Statistical analysis. The temporal pattern in oestrogen concentrations was analysed by Tukey’s HSD comparisons based on grouped and log transformed data.

Results

Group I

In these ovariectomized mares, mean oestrogen conjugate concentrations increased from 17 ± 2·3 ng/mg Cr on Day 20 to 291 ± 69 ng/mg Cr on Day 70 (Fig. 1). Mean concentrations on Days 33, 39 and 44 were, respectively, 41 ± 14, 48 ± 17 and 73 ± 27 ng/mg Cr. During the entire sampling period (Days 20–70), mean oestrogen conjugate concentrations were significantly lower (P < 0·05) than in the intact, pregnant mares in Groups II and III. Progesterone concentrations were low (<0·5 ng/ml) after ovariectomy, indicating an absence of luteal tissue. Horse CG concentrations increased between Days 35 and 40, confirming development of functional endometrial cups. In one mare, ovariectomized on Day 20, fetal death was diagnosed on Day 56 while still being treated with altrenogest. The oestrogen conjugate concentrations of this mare were excluded from the group values because in the 10 days preceding fetal death the concentrations ranged between 99 and 154 ng/mg Cr, and were not significantly different (P > 0·05) from those of the other ovariectomized mares (Fig. 2). On the day after fetal death, concentrations dropped to 27 ng/mg Cr.

Group II

Urinary oestrogen conjugate concentrations in the intact, untreated mares remained constant between Days 20 and 33 except for one mare that had higher values on Days 28–30. Mean concentrations were 114 ± 23 ng/mg Cr on Day 20 and 172 ± 40 ng/mg Cr on Day 33 (Fig. 1). After Day 33 concentrations increased to a peak of 1066 ± 219 ng/mg Cr on Day 39 (3-fold increase), followed by a decline to 637 ± 94 ng/mg Cr on Day 44 and subsequently an increase to 1115 ± 83 ng/mg Cr on Day 70.

Group III

In intact treated mares, oestrogen conjugate concentrations declined from 193 ± 60 ng/mg Cr on Day 20 to a nadir of 77 ± 7·8 ng/mg Cr on Day 33 (Fig. 1). After Day 35, the profiles of oestrogen secretion in Groups II and III were not significantly different (P > 0·05): although the increase in mean concentrations started 1 day later (Day 34), peak values were reached on the same day (Day 39) and were not significantly different (P > 0·05). Mean oestrogen conjugate concentrations were 895 ± 229 ng/mg Cr on Day 39, 719 ± 293 ng/mg Cr on Day 44 and 916 ± 108 ng/mg Cr on Day 70. Between Days 32 and 35, mean oestrogen conjugate concentrations in Group III
Fig. 1. Oestrogen conjugate concentrations in urine of pregnant mares ovariectomized on Day 18–19 of pregnancy and treated with altrenogest (Group I), pregnant mares (Group II) and pregnant mares treated with altrenogest (Group III) between Days 20 and 70 of pregnancy.

Fig. 2. Oestrogen conjugate concentrations in urine of an ovariectomized pregnant mare treated with altrenogest. Fetal death was diagnosed by ultrasonography on Day 56 of pregnancy.

were significantly lower ($P < 0.05$) than those in Group II. In both groups, progesterone concentrations confirmed the presence of active luteal tissue and horse CG concentrations increased at the expected time, between Days 35 and 40, confirming normal development of functional endometrial cups.
Discussion

The increase in oestrogen secretion previously observed between Days 35 and 40 (unpublished data) did not occur in the ovarioctomized, pregnant mares. The oestrogen results indicate that the ovaries represent a major source of oestrogen in the first 70 days of gestation. Oestrogen conjugate concentrations in ovarioctomized, pregnant mares were approximately 13% of the concentrations in the intact, pregnant mares between Days 20 and 33, then 5% on Day 39 and 20% on Day 70. The abrupt increase in oestrogen secretion observed around Day 35 in intact, pregnant mares did not occur in the ovarioctomized mares. Terqui & Palmer (1979) have reported that the oestrogen surge at Day 40 did not occur in a pregnant mare ovarioctomized on Day 34.

Although it is possible that the oestrogen surge on Day 39 is the result of stimulation of follicle growth by horse CG as has been suggested previously (Terqui & Palmer, 1979), we did not observe a significant increase in follicular activity, nor did the increase of oestrogen concentrations coincide with the presence or rapid development of large follicles between Days 33 and 39 in the untreated mares (Group II). In the intact, pregnant mares treated with altrenogest (Group III), oestrogen conjugate concentrations gradually decreased during the first 15 days of treatment. This decrease in oestrogen excretion was probably the result of depressed ovarian activity as indicated by ultrasonography. Despite the low oestrogen conjugate concentrations and the presence of very few follicles before Day 33, the oestrogen profile immediately after Day 33 for Group II was identical to that in Group III, suggesting again that the follicular status has little effect on the oestrogen surge. Squires et al. (1974) reported that follicular dynamics in hysterectomized pony mares between Days 10 and 68 after ovulation paralleled the changes occurring in pregnant mares over the same time span, thus indicating that pregnancy per se had no effect on follicular status.

Lowered oestrogen excretion in mares treated with altrenogest (Groups I and III) may indicate a direct effect of the synthetic steroid on oestrogen synthesis. Since altrenogest is structurally different from progesterone, with 2 extra double bounds and an allyl-group in the 17 position, it may interfere with some progesterone-dependent events. Marsan et al. (1987) have suggested a two-cell system for oestradiol synthesis in the horse embryo. Progesterone produced by one cell is transported to another cell for conversion to oestradiol, as is believed to occur in ovarian follicles. It is possible that altrenogest blocks those enzymes, aromatase and desmolase, necessary for oestradiol production. Although this possibility needs to be investigated, comparison with previous results indicates that the effect of altrenogest on oestradiol synthesis is negligible (Terqui & Palmer, 1979; Kasman et al., 1988).

In the non-pregnant mare, the maturation of Graafian follicles is characterized by an increase in both oestradiol-17β and oestrogen conjugate whereas there is no increase in oestradiol-17β associated with the increase in oestrogen conjugates concentrations before Day 70 of gestation, suggesting that the oestrogens secreted in early pregnancy may be from a source other than antral follicles (Nett et al., 1975). Our preliminary work has shown that the oestrogen peak on Day 39 is absent in pregnant mares in which the primary corpus luteum was eliminated by administration of prostaglandin F-2α and pregnancy was maintained by daily altrenogest administration. It is postulated that horse CG stimulates oestrogen secretion by the primary corpus luteum. The effect of horse CG in the mare appears to be similar to the effect of hCG in pregnant women, in which the secretion of hCG is associated with an increased oestrogen secretion (Lasley et al., 1985). The concept that the corpus luteum is a source of oestrogen after Day 35 of pregnancy is not necessarily in disagreement with the decrease in oestrogen secretion observed after termination of pregnancy in the first third of pregnancy in that the methods used to terminate pregnancy (PGF-2α administration, surgical removal of the fetus or injection of hypertonic solution into the allantoic sac) may have caused regression of the primary corpus luteum (Jeffcott et al., 1987; Kasman et al., 1988).

While oestrogen conjugate concentrations in ovarioctomized, pregnant mares were relatively constant up to Day 45, the curvilinear increase after Day 45 suggests that the development of the feto-placental unit as a source of steroids begins at about this time. Steroid secretion accelerates
after Days 70–80 (Nett et al., 1975; Stewart et al., 1982) and both maternal and fetal gonadectomy have identified the fetal gonads and placenta as the source of oestrogens and progesterogens in mid-gestation (Raeside et al., 1973; Pashen & Allen, 1979a, b; Stewart et al., 1982).

From our data during the first 70 days of pregnancy it appears that the ovary is a major source of oestrogen. The first significant increase in oestrogen concentrations which begins at about Day 35 is an ovarian response (probably luteal) to the secretion of horse CG and thus reflects the formation of endometrial cups rather than an increase in oestrogen secretion by the feto-placental unit. After Day 80, the feto-placental unit becomes the dominant oestrogen source and changes in circulating oestrogen concentrations are a reflection of fetal secretion.

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