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Estrogen Metabolism in the Equine Conceptus and Endometrium during Early Pregnancy in Relation to Estrogen Concentrations in Yolk-sac Fluid

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1 **ABSTRACT**

2 Because estradiol (E_2) production by the early equine conceptus is considered crucial to the
3 establishment of pregnancy, the amounts of E_2, estrone (E_1) and their sulfates (E_2S, E_1S) were
4 measured by RIA in yolk-sac fluid of 63 conceptuses collected by transcervical lavage over the
5 period of 11 to 26 days after ovulation. Amounts increased significantly with age of conceptus,
6 especially for E_1S. Then, the metabolism of E_2, which may be highly relevant for its action, was
7 examined in the conceptus and endometrium over the period when the conceptus ceases to
8 migrate within the uterus. Eleven conceptuses collected mainly on Days 12, 15 and 18, with
9 endometrial biopsy samples taken immediately thereafter, were used for steroid metabolic
10 studies. Trophoblastic and endometrial tissues were incubated with [^{3}H]-labeled E_2 or E_1, and
11 with [^{14}C]-E_1 in one experiment. Steroids were recovered from the media by solid-phase
12 extraction (SPE) and eluted separately as unconjugated and conjugated fractions. Conjugation
13 increased from Day 12 for the trophoblast (more so by bilaminar than trilaminar tissues on Day
14 18) and was much greater for endometrium, with almost all as sulfoconjugates. HPLC profiles
15 of free and sulfate fractions were obtained from a gradient of acetonitrile/water. Interconversion
16 ($E_2 \rightleftharpoons E_1$) by trophoblast varied with development; it favored E_2 in older conceptuses, more in
17 bilaminar than trilaminar tissues. Some more polar products were also noted, with loss of tritium
18 seen as [^{3}H]$_2$O at SPE, and confirmed by HPLC in a second system with authentic reference
19 steroids. Almost all radioactivity in the endometrium was present as E_2 in both free and sulfate
20 fractions. It was concluded that 'local metabolism' of E_2 is quantitatively significant and may
21 play an important role in the actions of the large amounts of estradiol produced by the early
equine conceptus.

INTRODUCTION

Estradiol (E_2) production by the early equine conceptus is well recognised and considered very significant to the establishment of pregnancy [1-5]. Its exact role in the process has not yet been determined. Some actions of E_2 are known to result from the formation of estrogen metabolites which function as local mediators in target tissues [6]. These active metabolites may subserve unique biological functions, not directly associated with the parent hormone, and so contribute to the overall action of the estrogen. Moreover, a reduction in the bioavailability of estradiol may result from its local conversion to estrone (E_1, a much weaker estrogen), and also to sulfoconjugation which further weakens its potency and aids in its removal. For these reasons, the study of metabolism of steroid hormones in target tissues, known as intracrinology [7], is considered necessary for a proper understanding of the physiological role of estradiol in early pregnancy.

Steroidogenesis in the equine embryo appears to begin as early as Day 6 after ovulation, based on histochemical evidence of 3β-hydroxysteroid dehydrogenase activity [1]. By Day 12 the equine conceptus produces significant quantities of estrogens [2-4]. This activity increases subsequently and is reflected in the concentrations of estrogens in yolk-sac fluid, especially after Day 20 [5]. A more transient production of estrogens occurs in the pig [8,9] in accord with the limited duration of expression of cytochrome P450 aromatase ($P450_{arom}$) activity in porcine blastocysts [10]. In contrast, estrogen synthesis by the conceptus of the mare is sustained [2,5]. Estrogens serve as the anti-luteolytic factor in the maternal recognition of pregnancy in the sow [11] but may not do so in the mare [12]. They may make an essential contribution to proper

maternal/conceptus communication in the epitheliochorial placenta, which is characteristic of the mare [13]. A simple apposition of the trophoblast and the uterine epithelium from the time of implantation means that the demand for nutrients and oxygen must be met by diffusion across this interface, where efficiency may depend critically on permeability. Perhaps estrogens, acting through a vascular permeability factor (e.g. the vascular endothelial growth factor, VEGF) as agent [14,15], provide a means to this end. One of the earliest reported actions of estrogens is the increase in uterine edema resulting from increased vascular permeability in the mouse [16].

The present study was aimed at providing more complete information on the extent of local metabolism of estrogens by the early equine conceptus and endometrium and, as a second aim, to present more complete data on the amounts of E_2 and E_1 and their sulfoconjugated forms (E_2S and E_1S) in yolk-sac fluid. The stages chosen for examination of estrogen metabolism (Days 12,15 and 18) were selected to represent the beginning and end of the early period of increase in diameter of the conceptus (Days 11-16), and shortly after the time of “fixation” (Day 16) when the migration of the conceptus within the uterus comes to an end [17]. Such knowledge is deemed essential for subsequent studies on the role of estrogen production in the early stages of pregnancy and its relation to early pregnancy loss in the mare. Preliminary reports on some of our data have appeared as abstracts [18,19].

MATERIALS AND METHODS

Chemicals and Reagents

Non-radioactive steroids were purchased from Steraloids Inc.(Newport, RI). The radioactive steroids obtained from NEN Life Sciences Products Inc. (Boston, MA) were [2,4,6,7-^{3}H]-estradiol-17β (71.0 Ci/mmol) and [2,4,6,7-^{3}H]-estrone (74.1 Ci/mmol); and [4-^{14}C]-E_1 (53.4 mCi/mmol) was from the Radiochemical Centre, Amersham, UK. Solvents from Caledon Laboratories, Ltd. (Georgetown, ON, Canada) were glass-distilled, reagent grade and acetonitrile (190) was used for HPLC. All other chemicals were analytical grade from Fisher Scientific (Toronto, ON, Canada) unless otherwise indicated.

Collection of Conceptuses and Endometrial Tissue

Standardbred and Thoroughbred mares (n = 11 each season, for 3 years) from the research herd at the University of Guelph were used to recover conceptuses under conditions approved by the University Animal Care Committee. The timing of ovulation was monitored by transrectal ultrasonography at intervals of 2, or occasionally 3, days and was deemed to have occurred on Day 0, midway between examinations showing an intact follicle and a corpus luteum. Thus, ages of conceptuses were subject to an error of ±1 or ±1.5 days. Conceptuses were collected from mares by transcervical uterine lavage, with Dulbecco PBS, pH 7.4, using a technique based on one described previously [20]. The mares were sedated with xylazine hydrochloride (Rompun;

BAY-VET, Etobicoke, ON), administered i.v., usually 0.3 mg kg^{-1} BW. The flush fluid (1000 ml) was infused by gravity flow and drained into a beaker to recover the conceptus. Endometrial biopsy samples were taken immediately thereafter. Yolk-sac fluid was obtained from most conceptuses by rupture in a dry Petri dish and aspiration into a syringe with a 20-G hypodermic needle. However, samples from conceptuses older than 20 days were taken by direct needle aspiration. Sixty-three conceptuses, ranging in age from 11 to 26 days were used to provide yolk-sac fluids. The fluids were stored at -20 ^{0}C until the time of assay. The amount from each conceptus was determined by measuring the volume of aspirated fluid wherever possible or, otherwise, calculated from ultrasonographic measurements taken immediately before uterine flushing. Eleven conceptuses were used to study estrogen metabolism at Days 12 (n=5), 15 (n=3) and 18 (n=3).

Radioimmunoassay of Estrogens in Yolk-sac Fluid

Steroids in the yolk-sac fluid were recovered by taking an aliquot ($\leq$1 ml) which was diluted with distilled water to a volume of 5 ml for solid-phase extraction (Waters C_{18} Sep-Pak column, Canada Waters Limited, Mississauga, ON), as described previously for equine plasma [21]. Unconjugated and conjugated steroids were eluted from the primed columns with 5 ml of diethyl ether and 5 ml of methanol, successively. The ether and methanol eluates were evaporated separately under nitrogen at < 45°C and the dried ether residue (unconjugated fraction) was used to measure E_2 and E_1. The dried methanol (conjugate) fraction was acid-solvolysed overnight at 45°C with trifluoroacetic acid/ethyl acetate (1/100; v/v) to obtain free steroids from

sulfoconjugated estrogens by means of a second Sep-Pak column. The dried residues were dissolved in methanol and aliquots taken for radioimmunoassays (RIA) of the estrogens as reported previously [22].

Tissue Preparation and Incubations

After removal of yolk-sac fluid, the trophoblastic tissues were divided into 2 to 4 equal portions (about 15-20 mg wet wt) depending on amounts available. For Day 18, the trophoblast was divided into bilaminar (trophectoderm and endoderm) and trilaminar (trophectoderm, mesoderm and endoderm) components by dissection, and these were incubated separately. Lesser amounts of tissue from the day-12 conceptuses precluded incubations in duplicate and so additional conceptuses were used to provide sufficient replication. The tissues were washed with PBS and dispensed into small flasks containing 2.5 ml TCM 199 to which either $[^3H]$-E_2, $[^3H]$-E_1 (1×10^6 cpm) or $[^{14}C]$-E_1 (0.25×10^6 cpm) was added for a 2h incubation in a shaking waterbath at 37 ^{0}C under 5% CO_2 in air. Similarly, the biopsy samples of endometrium were incubated, in duplicate, as minced tissues (30 to 60 mg wet wt). As an inactivated tissue control in some experiments for Days 15 and 18, samples from trophoblast (n=5) and endometrium (n=2), in TCM 199, were placed in boiling water for at least 10 min before incubation with the radiolabeled estrogens.

Analytical Procedures

Steroids in the media were recovered by solid-phase extraction (SPE) as described above. Unconjugated and conjugated steroids were eluted with 5ml diethyl ether and 5ml methanol in succession. The conjugated material underwent two hydrolytic steps, in series, to yield a "sulfate" and a "glucuronidate" fraction obtained in each case as free steroids from Sep-Pak columns [23]. The amounts of radioactive material recovered from each incubation, each hydrolysis step and in each SepPak fraction were determined by liquid scintillation counting in 5ml of Ecolite cocktail (ICN, Costa Mesa, CA). In addition, an indirect assessment of metabolism involving loss of the tritium-label at carbons 2,4,6 and/or7 was made by collecting the initial ("flow-through") and the following ("water-wash") fraction from the SPE. These aqueous fractions were combined and, after treatment with charcoal and centrifugation as done in radioimmunoassay, the measurement of radioactivity in the supernatant was assumed to reflect the formation of $[^3H]_2O$ from oxygenation at the tritium-labeled carbon sites of the estrogen molecule. Further confirmation was obtained from distillation of the supernatants from the charcoal treatment, for two incubations of bilaminar trophoblast from day-18 conceptuses, with almost complete recovery of radioactivity in the distillate (data not shown).

Unconjugated and hydrolyzed steroids in the fractions derived serially from Sep-Pak columns were examined by HPLC. Profiles of steroid metabolic products were generated using a binary solvent gradient of acetonitrile: water (from 28:72 to 90:10 percent, over 35 min) on a Waters HPLC column and system (Waters Corp, Milford, MA) at a flow-rate of 0.7 ml min^{-1} and absorbance monitored at 280 nm. A radiodetector (Packard 505TR, Packard Instrument Co,

Meriden, CT) was used for a direct scan of the effluent for radioactivity (cpm). The areas recorded (as % of total radioactivity) for detected peaks were taken for quantitative comparisons of the products formed. A second HPLC system, with methanol in the gradient, was used to further identify the material in some peaks of the chromatograms. The ternary gradient started with a methanol: acetonitrile: water mix of 46.8: 4.2: 49.0 that was changed to 70:15:15 over a 30 min period using Waters gradient curve #7, at a flow rate of 0.5 ml min^{-1}. Identification of the radiolabeled metabolites was then based on their co-elution with authentic reference steroids from the two column matrices.

Statistical Analysis

Scatter plots were used to present the data for total amounts of E_2, E_1 and their sulfates in yolk-sac fluid. Log transformation of the data for estrogens was done, to accommodate the extreme ranges in values, before fitting regression lines using SigmaPlot5.05. Comparisons for the amounts of radioactivity seen as steroid conjugates from incubations were made by one-way ANOVA with Bonferroni Multiple Comparison post test performed using the Instat Version for Windows 95 (GraphPad software, San Diego, CA). P values < 0.05 were considered to be significant.

RESULTS

The rapidly changing nature of the yolk-sac wall over the period of early pregnancy encompassed in this study is illustrated in Figure 1. The relationship between age and size of conceptus (data not shown) was essentially the same as has been recorded [17]; embryonic vesicle diameter increased linearly until Day 16, with a 4-fold increase, after which a plateau was seen until Day 26.

Estrogens in Yolk-sac Fluids

The amounts of estrogens (concentrations x volumes) found in yolk-sac fluid from conceptuses collected over the period of our steroid metabolism studies, and slightly beyond, are shown in Figure 2. Values for the total amounts of the estrogens ranged from 0.2-128.5 , 0.1-76.6 , 0.26-158.3 and 0.28-720.9 ng, respectively, for E_2, E_1, E_2S and E_1S in conceptuses of 5 to 32 mm diameter (volumes = 0.07 to 17.15 ml). Estrone sulfate clearly became the dominant form as development progressed. The rates of increase for E_2 and E_1 production in the early stages were similar but a plateau was reached earlier for E_2 than for the other estrogens. This was reflected by the concentrations in the yolk-sac fluid (data not shown), where the daily increments in concentrations from Days 11-16 were similar for E_2 and E_1 (0.66 and 0.55 ng/ml, respectively) but differed markedly between those for E_2S and E_1S (0.31 and 2.63 ng/ml). Mean values ($\pm$ SEM) for the concentrations (ng/ml) of endogenous substrates on the days of the metabolism studies (Days 12, 15 and 18; n = 11, 13 and 7, respectively) were 2.03 $\pm$ 0.45, 4.93 $\pm$

0.63 and 4.89 ± 0.73 for E_2; 2.59 ± 0.43, 4.66 ± 1.26 and 7.77 ± 1.41 for E_1; 1.17 ± 0.23, 1.75 ± 0.21 and 3.69 ± 0.77 for E_2S; 3.79 ± 0.69, 10.31 ± 1.75 and 29.92 ± 3.42, for E_1S. These values include data from other conceptuses not used in the metabolism studies.

Conjugation of Steroids

Conjugation of estrogens by the trophoblast was markedly different from that by endometrial samples (Table 1). The data showed an increase in conjugation after Day 12 for the trophoblast, and formation of greater amounts by the bilaminar than by trilaminar tissues on Day 18. Levels of conjugation by the endometrium were higher than by the trophoblast, especially on Day 12, even allowing for the larger amounts of endometrium used. There seemed to be greater quantities formed from E_1 than from E_2 as substrate, but this was statistically significant only for Day 18. It was also noted that conjugation of E_2 by the endometrium was significantly lower on Day 18 than on Day 12. Incubations of boiled tissues from conceptuses on Days 15 and 18 resulted in about 3% of the radioactivity appearing in the conjugate fraction for [^{3}H]-E_2 and [^{3}H]-E_1. No subtractions of these amounts were made for the data in Table 1.

Within the conjugate fractions for the trophoblast, the predominant form in all cases was the sulfate from incubations with either E_2 or E_1, with values of about 60% for each (e.g. Day 18 bilaminar, 60.9 ± 3.6, n = 3, from E_2; and 59.7 ± 2.2, n = 3, from E_1). Using a bacterial β-glucuronidase preparation [23], recovery of radioactivity from the remaining, non-solvolyzed, fraction (about 40 %) was estimated for Day 15 (n = 2) and for Day 18 (bilaminar trophoblast only, n = 2), where sufficient amounts of radioactivity were available. The mean values for

yields of "glucuronidates" for duplicate incubations with E_2 and E_1, respectively, were 28.1 and 24.7 % for Day 15, and 29.4 and 25.6 % for Day 18. No further examination of the remaining radio-activity (about 28% of total conjugated material) was made for other forms of conjugation. Only estrogen sulfates (>95 %) were seen in media from endometrial incubations with both E_2 and E_1.

HPLC Profiles from Estrogen Metabolism

All profiles presented are examples from two or more replicates for each substrate in each age group. Each profile closely represented that of its replicate(s) (data not shown). Chromatography of unconjugated steroids showed extensive metabolism by trophoblast tissue of a day-12 conceptus for each estrogen as substrate (Fig. 3). E_2 was converted to E_1 in large measure; conversely, E_1 was metabolized to E_2 to a lesser extent. Several minor peaks were seen as products from each substrate.

At Day 15, the profiles from metabolism of E_2 and E_1 were remarkably similar to each other (Fig. 4). However, they showed a much lower formation of E_1 from E_2 than noted for trophoblast tissues on Day 12. On the other hand, E_1 was converted mainly to E_2 . This resulted in a similar ratio for the distribution of radioactivity between E_2 and E_1 regardless of the estrogen used as substrate. It was also noted that two lesser peaks had similar retention times (Rt) to those of reference standards of 6α- (or 6β-) OH-E_2 and 6-oxo-E_2 run separately (Fig. 3A). These peaks made up about 6 to 8 % each of the total radioactivity in the profiles and were present in about the same proportions from either E_2 or E_1 as substrate.

Incubation of trophoblast tissues from the day-18 conceptuses resulted in markedly different metabolic profiles for the bilaminar and trilaminar components, with either E_2 or E_1 as substrate (Fig. 5). The amounts of E_1 formed from E_2 were 14 and 57 % for the bilaminar versus trilaminar tissues, respectively. About 5 % of the radioactivity was noted in each of the polar peaks corresponding to Rt of 6α-OH-E_2 and 6-oxo-E_2, but only for the bilaminar tissues. Differences between the two tissue types were again expressed when E_1 was the substrate. The resulting profiles for the metabolites of E_1 were almost identical to those seen with E_2 as substrate. It was concluded that E_2 was the major estrogen resulting from metabolism in bilaminar tissue regardless of which substrate was used. A more even distribution between E_2 and E_1 occurred with the trilaminar material.

Evidence for the formation of 6-oxygenated estrogens was strengthened by further chromatography of the metabolites of [^{3}H]-E_2 and [^{3}H]-E_1, from incubation of day-18 bilaminar tissue, with authentic reference steroids as internal standards. In the first gradient system (acetonitrile/water), two peaks of radiolabeled material were found which corresponded to those of the 6α-OH-E_2 and 6-oxo-E_2 standard compounds (Table 2), but separation of 6α-OH- from 6β-OH-E_2 was barely adequate (Retention times, Rt = 3.2 and 3.6 min). In the second gradient system, with methanol, the two epimers were clearly separated (Rt = 3.9 and 6.6 min, Table 3).

Because losses of [^{3}H], detected as [^3H_2O] at the SPE step, were between 30-50% for day-18 bilaminar tissue, the relative amounts of 6-oxygenated estrogens formed were not fully reflected at chromatography. A comparison of [4-^{14}C]-E_1 with [^{3}H]-E_1 as substrates was made in an additional experiment. The results of chromatography in the first system (HPLC-1) showed clearly that the proportion of radioactivity which appeared as polar metabolites (6-oxygenated

estrogens) was three times higher for the [^{14}C]-E_1 incubation (Table 2), when total areas under the peaks were compared for retention times < 10 min (139.0 versus 46.4 arbitrary units). With the second solvent system (HPLC-2), evidence for both 6α- and 6β-OH-E_2, as well as for 6-oxo-E_2, was obtained in the ^{14}C-labeled material (Table 3). In fact, the data suggest that the 6β-epimer is the major form resulting from 6-hydroxylation by the bilaminar trophoblast.

Profiles of the sulfoconjugated steroids from incubations of E_2 with day-18 endometrial tissues are shown in Figure 6. With >95 % of the radioactivity in a conjugated form from these incubations, it was seen that almost all of the radioactivity was in the sulfate fraction and was present as E_2S, with only trace amounts of E_1S. This was also true for the lesser amounts of free steroids where E_2 was again predominant.

DISCUSSION

Our results should be considered for their significance to the establishment and maintenance of pregnancy in the mare from two standpoints: (1) the accumulation of estrogens, and (2) their local metabolism. Evidence for the production of estrogens by the early conceptus [1-5] has been greatly extended by our assays of yolk-sac fluid. The accumulation of E_1S is one of a number of features of equine pregnancy that are unusual [12,24]. Whatever the response might be to this early presence of estrogens in the pregnant mare, it appears to differ from the action of estrogens on the regulation of prostaglandin $F_{2\alpha}$ secretion in the maternal recognition of pregnancy in the pig [11]. In this regard, it may be noted that the concentrations of prostaglandins E_2 and $F_{2\alpha}$ in the yolk-sac fluid in the horse remain constant [25] over the same

period that the amounts of estrogens are seen to rise in our study. Nevertheless, it seems likely that estrogen secretion by the early equine conceptus is a requirement for the maintenance of pregnancy. For example, studies with exogenous steroids in anestrous mares suggest that conceptus-derived estrogens are likely to be the cause of the increased proliferative activity in some components of the endometrium during early pregnancy [26]. The high content of estrogens in the yolk-sac fluid we have reported lends strong support for this view.

The actual amounts of E_2 that are available to act on the conceptus and endometrium at this critical stage depend on several factors in addition to the production of E_2 by the conceptus itself. Our findings indicate that 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes in the trophoblast make a significant contribution to the bioavailability of E_2 to the tissues, as evidenced by marked changes in the extent to which E_1 is converted to E_2 over the period of rapid expansion of the blastocyst. How these changes favoring E_2 formation in the trophoblast can be reconciled with the higher concentrations of E_1S in yolk-sac fluid in the older conceptuses is unanswered, but might lie in a possible difference in 17β-HSD activity towards the free and conjugate forms. It should be noted, however, that our experimental approach did not address the difficult question of metabolic clearance rates for estrogens in the conceptus. No clear explanation for differences in estrogen metabolism between the bilaminar and trilaminar tissues can be given at this point, but it may relate to differences in their cellular components.

Estrogen sulfates were the major form of conjugation found in the yolk-sac fluid and on incubation of the tissues, especially the endometrium. The biological significance of this estrogen sulfotransferase (EST) activity for early equine pregnancy has yet to be demonstrated, but it would have a bearing on the bioavailability of estrogen. Although we did not include

incubations with radiolabeled estrogen sulfates to test for sulfatase activity, our findings strongly suggest that the dominant activity is EST, leading to relatively reduced amounts of free E_2. Recently, a porcine uterine EST has been characterized [27]. Also, EST rather than sulfatase is the more widely expressed enzyme in various human peripheral tissues, including the syncytiotrophoblast of the placenta [28]. However, it remains to be demonstrated whether estrogen sulfates are active in their own right and not formed simply to protect tissues from possible excessive estrogenic stimulation, or even to serve as a latent precursor for the active free steroid [29].

Some actions of estradiol are dependent not only on the local level of the estrogen in the target tissues and on the presence of its receptors but also, in part, on its metabolism within the target cells themselves [6]. These estrogen metabolites may have unique roles as local mediators and contribute to the overall action of estradiol. It is for this reason that we have examined the metabolism of estradiol in the early equine conceptus and endometrium. Evidence from the HPLC profiles, and from losses as great as 30-50% of [^{3}H] on incubation of radiolabeled E_2 and E_1, clearly show that a significant amount of oxidative metabolism of the estrogens does occur in the trophoblast. As a consequence of the removal of tritium from E_2 and E_1, the radioscan for the HPLC profiles cannot fully reflect the amounts of some of the oxygenated metabolites formed. Use of [^{14}C]-E_1 as substrate allowed not only a better appreciation of the extent of the formation of these metabolic products but also helped towards establishing their identity; formation by the equine trophoblast of both 6α-OH-E_2 and 6β-OH-E_2, as well as 6-oxo-E_2, can reasonably be assumed from our chromatographic findings. The presence of metabolites with oxygenated functions at position C6 (6-OH and 6-oxo-E_2 and -E_1) may have particular relevance insofar as

they have been referred to as "impeded estrogens", from their depression of estrogen-induced uterine growth [30]. To the best of our knowledge, no such studies have been reported for the early stages of pregnancy in the mare.

Estrogen biosynthesis by preattachment embryos, as seen in the mare and pig, has been documented for other species with epitheliochorial placentation [31,32]. Furthermore, a recent report revealed by immunocytochemistry that steroid synthesis enzymes were present in the uninucleate trophoblast in the camelid placenta at Days 14 and 30 after ovulation [33]. This would allow the conversion of cholesterol to estrogen. It was suggested that the capacity for steroidogenesis close to the fetomaternal interface may maintain high concentrations of steroids at this position and indicate a need for localized placental estrogen for a successful camelid pregnancy. Immunocytochemical localization of the aromatase enzyme in the trophoblast of the early equine conceptus from Day 12 to Day 15 [34] could represent a similar requirement for local estrogen synthesis in the pregnant mare.

Estrogens might promote the establishment of pregnancy in the mare through the agency of various growth factors. A relationship between estrogens and growth factors in early pregnancy in the mare has yet to be explored except for the largely negative findings for IGF-1 [35]. Vascular endothelial growth factor/permeability factor (VEGF/PF), an endothelial cell mitogen and permeability factor, acts as an intermediary in a variety of estrogen-responsive tissues. It has been shown that E_2-induced VEGF gene expression in human endometrial cells depends on both estrogen receptors, ERα and ERβ [36]. The rapidly developing vascularization of the mesoderm of the equine conceptus, illustrated in Figure 1, strongly suggests that VEGF may be required for this progressive modification of the yolk-sac wall, and implies that estrogens could well play a

role in this regard. With regard to permeability, the equine conceptus remains spherical, with linearly increasing diameter between Days 11 and 16 [17] despite the hypotonicity of its yolk-sac fluid [24]. For this reason, it may be important to look for factors that can influence permeability rather than, or in addition to, playing a mitogenic role in the epithelium. Such a factor might be found in the discovery of a tissue-specific growth factor which induced proliferation and fenestration in capillary endothelial cells of endocrine glands, the so-called endocrine-gland-derived vascular endothelial growth factor (EG-VEGF) [37,38]. This permeability-enhancing factor is restricted mainly to steroidogenic tissues, including the placenta. Thus, EG-VEGF could serve a dual role as a permeability-enhancing factor and a mitogenic agent in the trophoblast, which functions in some respects as an endothelial tissue as well as producing estrogens. Moreover, the recent demonstration of estradiol regulation of aquaporin-2 in the mouse [39-41] may have relevance in the mare, especially given the demonstrated role of aquaporins in blastocyst expansion [41]. In all of this, our findings suggest a need to consider the role of local estrogen metabolism within the reproductive tissues when examining the possible biological actions of the large quantities of estrogens present in, and formed by, the conceptus at this early stage of pregnancy which includes the critical events of conceptus expansion and fixation.

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FIGURE LEGENDS

Figure 1. The rapid growth of the conceptus during the second and third weeks of pregnancy in horses.

A. A fresh intact conceptus on Day 14±1, seen from the embryonic pole while floating in PBS. Its diameter in this dimension is 20 mm; its depth was 17 mm. The primitive streak is prominent in the embryonic disc at the centre of the photograph. Arrowheads mark the advancing front of the mesoderm.

B. An intact conceptus, 24 mm in diameter, floating in the PBS in which it was recovered transcervically on Day 18±1. The capsule, which was initially tightly applied, has loosened during the 35 min interval since recovery but still envelops the yolk sac completely. Blood islands are visible near the embryo proper at the lower pole of the conceptus in the trilaminar omphalopleure.

C. The border of the bi- and trilaminar omphalopleures of a conceptus of an equivalent developmental stage to that shown in B. The sinus terminalis (S.t.) contains blood cells, and vessels within the mesoderm of the trilaminar portion are prominent. Scale bar = 1 mm.

Figure 2. Correlations were made between age of conceptus and amounts (ng) of total estradiol-17β, estrone, and their sulfoconjugated forms in yolk-sac fluid (n = 63), after log transformation. Vertical line at Day 16 indicates time of fixation.

Figure 3. HPLC profiles of unconjugated metabolites in extracts of media from incubations of trophoblast tissues of day-12 equine conceptuses. A. Estrogen reference standards: E_2 and E_1 along with (1) 6α-OH E2, (2) 6β-OH E2, (3) 6-oxo-E_2, (4) 6-oxo-E_1 and (5) E_2-17α. B. [^{3}H]-E_2 as substrate. C. [^{3}H]-E_1 as substrate.

Figure 4. HPLC profiles of unconjugated metabolites in extracts of media from incubations of day-15 trophoblast.tissues. A. [^{3}H]-E_2 as substrate. B. [^{3}H]-E_1 as substrate.

Figure 5. HPLC profiles of unconjugated metabolites from incubations of day-18 bilaminar (A, C) and trilaminar (B, D) trophoblast tissues, with [^{3}H]-E_2 (A, B) and [^{3}H]-E_1 (C, D) as substrates.

Figure 6. HPLC profiles of unconjugated (A) and sulfoconjugated (B) metabolites from day-18 endometrial tissue incubations with [^{3}H]-E_2 as substrate.

Table 1. Percent radioactivity present in the conjugate fractions from 2h-incubations with estradiol or estrone as substrate.

Age of Conceptus		Trophoblast		Endometrium	
		Substrate		Substrate	
Day		E_2	E_1	E_2	E_1
12		5.9±1.9a	5.9±1.2[a]	67.8±4.9[a]	69.5±9.4[a]
15		14.5±1.1[b,c]	15.2±2.4[b,c]	62.1±13.3 a,b	73.6±14.4[a]
18	bilaminar	18.1±3.2[c]	20.4±5.0[c]	54.2±2.2[b]	65.9±0.7[a]
18	trilaminar	9.0±2.3[a,b]	9.7±1.5[a,b]		

Values are mean ± SD, derived from duplicate incubations in each of 3 experiments for Days 15 and 18, and from single incubations for Day 12 (n=5).
Values with different superscript letters within columns are different (P<0.05).
Values for bilaminar and trilaminar omphalopleure are presented separately for Day 18.

Table 2. Distribution of metabolites of estrone in HPLC system 1, from incubation of bilaminar trophoblast tissue.

Compound	Retention Time (min)			Area (AU)*	
	Reference Standard	**^{3}H**	**^{14}C**	**^{3}H**	**^{14}C**
6α-OH-E_2	3.22	3.28	3.38	17.80	10.55
6β-OH-E_2	3.64	-	3.95	-	42.43
		5.58	5.79	4.46	23.69
6-oxo-E_2	6.01	5.98	6.29	20.69	46.05
6-oxo-E_1	9.91	9.84	10.10	3.47	16.28
		11.93	-	4.08	-
		13.17	-	5.41	-
E_2-17β	15.05	14.88	15.27	134.80	113.18
E_2-17α	16.72	-	-	-	-
		17.29	-	45.80	-
		19.56	-	11.08	-
E_1	20.82	20.56	21.03	100.00	100.00
		31.8	-	21.50	-

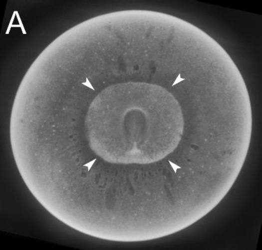
*Area units (AU) are expressed in relation to area under the E_1 peak (E_1=100).

Several radioactive products were unmatched by a retention time for a reference standard.

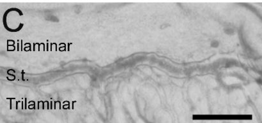
Table 3. Distribution of metabolites of estrone in HPLC system 2, from incubation of bilaminar trophoblast tissue.

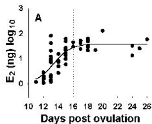
Compound	**Retention Time (min)**		
	Reference Standard	**^{3}H**	**^{14}C**
6α-OH-E_2	3.93	4.07	4.09
6β-OH-E_2	6.57	6.45	6.60
6-oxo-E_2	7.12	-	7.16
6-oxo-E_1	8.39	-	-
	-	17.81	-
E_2-17β	18.93	18.86	18.98
E_1	19.58	19.48	19.67
E_2-17α	20.09	-	-
	-	21.10	21.18

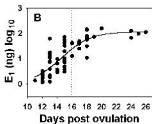
Area units (AU) are not given since comparisons with reference to the area under the E_1 peak could not be made, as in Table 2, because of incomplete separation of E_1 from E_2-17β in the second HPLC system.

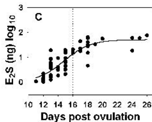

A

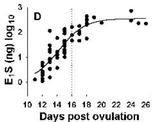
B
Bilaminar
Trilaminar
Capsule

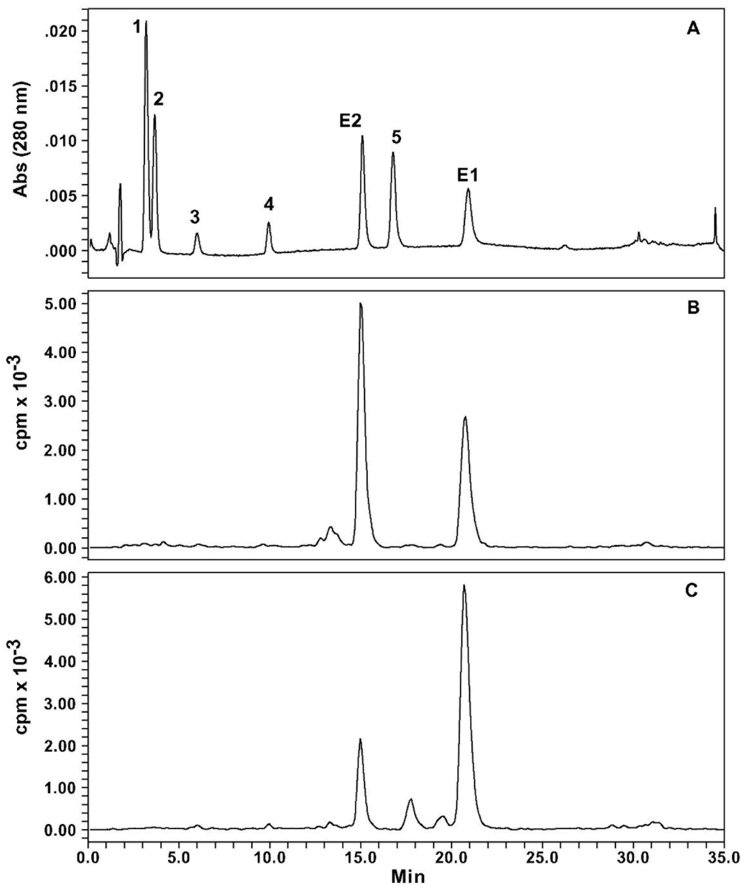

C
Bilaminar
S.t.
Trilaminar


A
E_2 (ng) $\log_{10}$
Days post ovulation

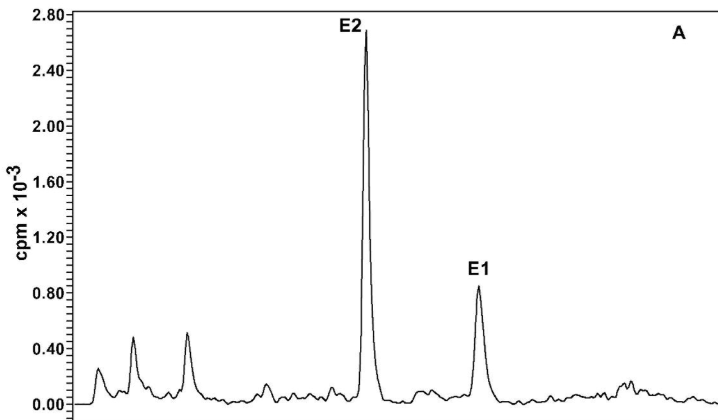

B
E_1 (ng) $\log_{10}$
Days post ovulation

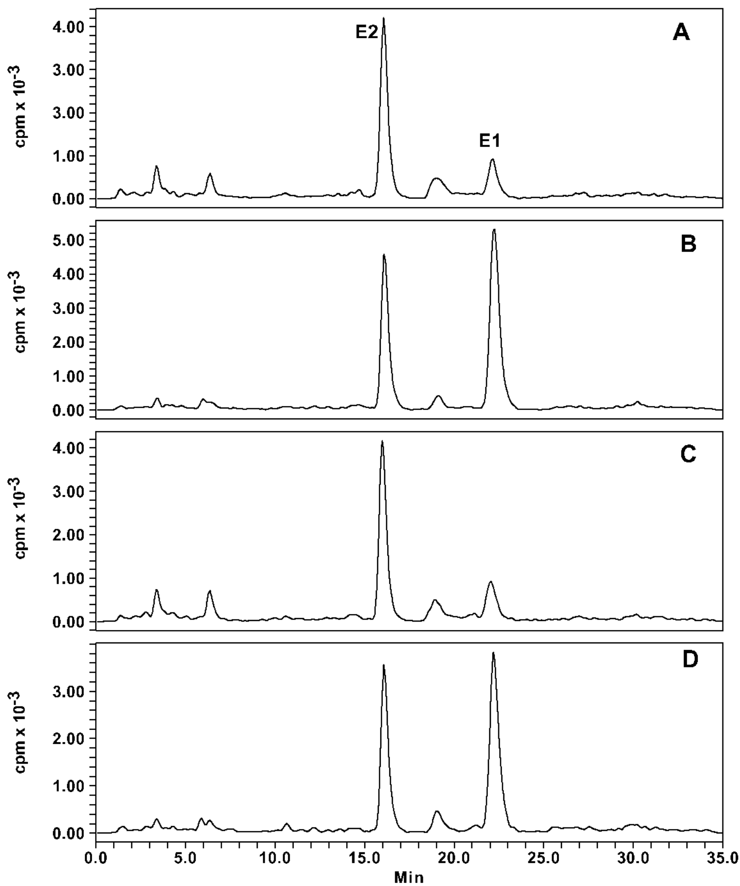

C
E_2S (ng) $\log_{10}$
Days post ovulation


D
E_1S (ng) $\log_{10}$
Days post ovulation

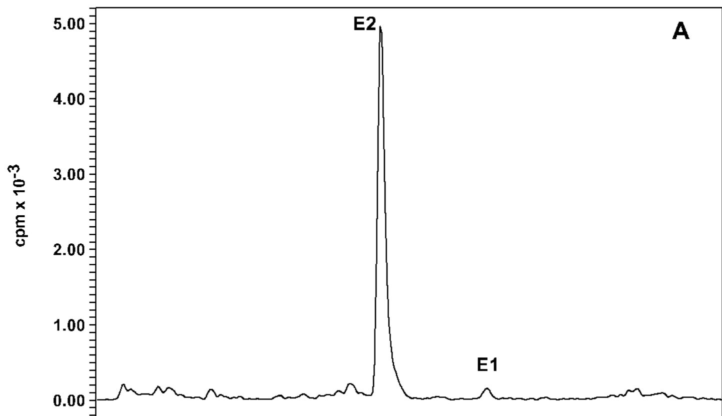


A
B
C
Abs (280 nm)
cpm x 10^{-3}
cpm x 10^{-3}
Min
1
2
3
4
E2
5
E1
.020
.015
.010
.005
.000
5.00
4.00
3.00
2.00
1.00
0.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0


E2
E1
A
cpm x 10-3
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
B
cpm x 10-3
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00
3.50
7.00
10.50
14.00
17.50
21.00
24.50
28.00
31.50
35.00
Min



A
B
C
D
E2
E1
cpm x 10^{-3}
4.00
3.00
3.00
1.00
0.00
5.00
4.00
3.00
2.00
1.00
0.00
4.00
3.00
2.00
1.00
0.00
3.00
2.00
1.00
0.00
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
Min



B
cpm x 10-3
8.00
6.00
4.00
2.00
0.00
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
Min