Estrogen metabolism by the equine embryo proper during the fourth week of pregnancy

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Abstract

Estrogen production by the trophoblast is considered important in early equine pregnancy and leads to high concentrations in yolk-sac (Y-S) fluid. The embryo proper is a potential site for their action. We examined estrogen metabolism in the embryo proper because some actions of estrogens are derived from locally formed metabolites. The embryo proper, as well as separated extraembryonic tissues, of conceptuses collected about day 25 of pregnancy, were incubated with ^3H -estrone (E_1) and ^3H -estradiol (E_2). Steroids were recovered from media by solid-phase extraction and eluted separately as unconjugated and conjugated fractions. Profiles of free and sulfo-conjugated fractions were obtained by HPLC. Some differences and similarities were noted for the embryo proper as compared to the extraembryonic tissues. No reduction of E_1 to E_2 was noted for the embryo proper and allantois, but some was seen with the bilaminar Y-S wall. Less conversion of E_2 to E_1 occurred in the embryo proper than in the extraembryonic tissues. Profiles for hydrolyzed sulfates from incubation of the embryo proper were very similar for both substrates, mainly with E_1 present. Thus, low levels of reductase and high levels of oxido- activities were apparent for the 17β -hydroxysteroid dehydrogenase enzymes. Further evidence of an active role for the embryo proper was seen as minor, polar products, and an unknown compound eluting between E_2 and E_1 . These findings show, for the first time, that the embryo proper can metabolize estrogens that are found in Y-S fluid – a function of potential significance at this stage in its development.

Reproduction (2009) **138** 953–960

Introduction

The early conceptus in the mare synthesizes estrogens in increasing amounts from about day 12 of pregnancy (Heap *et al.* 1982, Zavy *et al.* 1984, Flood *et al.* 1987, Choi *et al.* 1997, Raeside *et al.* 2004). Although the full significance of this synthesis has not yet been established, it is presumed that estrogens play a role in the maintenance of pregnancy. A similar situation occurs in early pregnancy in the pig, where estrogen synthesis by the blastocyst is seen around days 10–12 of pregnancy (Perry *et al.* 1973), a time when 17β -estradiol (E_2) has been shown to influence intrauterine migration of embryos (Pope *et al.* 1982) and uterine prostaglandin $\text{F}_{2\alpha}$ secretion (Bazer & Thatcher 1977). Estrogen formation by the early pig embryo is limited in duration with a transient expression of cytochrome P450 aromatase ($\text{P450}_{\text{arom}}$) enzyme in the trophoblast, between days 10 and 12 (Conley *et al.* 1992, 1994). In contrast, the equine conceptus continues to synthesize estrogens, and in greater quantities, well beyond the initial apposition of trophoblast and uterine endometrium (Heap *et al.* 1982, Raeside *et al.* 2004). A role for the large amounts of estrogens present in yolk-sac (Y-S) fluid remains to be determined. More interest has been

focused on their potential indirect effects on embryonic development – the participation of estrogens in an effective ‘dialogue’ between the conceptus (trophoblast) and the uterine endometrium. However, it is also clear that the embryo proper is exposed to high concentrations of estrogens in the Y-S fluid over a protracted period in the early stages of equine pregnancy. The exposure is entirely through the vitelline stalk at first, but then becomes systemic as the vitelline blood circulation gets established. This raises the question of whether the estrogens could also have a direct effect on the embryo’s development. Information on the amounts of estrogen that might be seen by the embryos in early stages in most mammalian species, other than the horse and pig, is extremely limited. Technical difficulties in obtaining material for measurement of steroids at the level of the conceptus, particularly in small laboratory animals, may be partly responsible.

There is now general consensus that metabolism of steroid hormones in target tissues may play a significant part in their overall action in many types of tissues; this is acknowledged by the introduction of the term intracrinology (Labrie *et al.* 2000). Many actions of E_2 are known to be due to its metabolites which act as local mediators in target tissues to effect changes not seen with

E_2 in its unaltered state (Zhu & Conney 1998). The antiproliferative and antiangiogenic properties of the 2-methoxy derivative of E_2 are examples of a product of local estrogen metabolism having profoundly different activities from those of the parent compound (D'Amato *et al.* 1994, Fotsis *et al.* 1994). Furthermore, the presence of specific enzymes in some target tissues can reduce the biological potency of E_2 by local conversion to estrone (E_1), a much weaker estrogen in most instances, or by forming steroid conjugates such as sulfates and glucuronides. Thus, knowledge of the metabolic fate of E_1 and E_2 in the embryo proper is a prerequisite for investigations on the possible direct action of estrogens on embryonic development.

In the present study we have examined the ability of the embryo proper to metabolize estrogens, using conceptuses (greater than day 20 of pregnancy) in which clear separation of the embryo proper from its extraembryonic membranes is feasible. Emphasis was placed on the metabolism of E_1 because it is present at higher concentrations than E_2 in Y-S fluid. Estrogen metabolism in the embryo proper was clearly demonstrated. Thus, the interest in the early secretion of estrogens by the Y-S wall is extended beyond its effects on the endometrium, to that of a direct role in early embryo development.

Results

The stage of development of an equine conceptus at day 25.5 of pregnancy, and of its contained embryo proper, is illustrated in Fig. 1. Note the development of the heart and the vascular system in the embryo proper. All specimens of the embryo proper used in this study were devoid of extraembryonic tissues.

Estrogens in Y-S fluids

The amounts of estrogens in Y-S fluid from the only conceptus that was recovered intact were 39.67, 7.74, 4.14, and 2.01 ng/ml for E_1 sulfate (E_1S), E_1 , E_2 sulfate (E_2S), and E_2 respectively. Measurements were made by

RIA as previously described (Raeside *et al.* 2004). These values are consistent with those reported in that study and with those from RIA on Y-S fluid from mares at days 24–26 of pregnancy ($n=9$), used in other aspects of our work (39.67 ± 19.13 , 8.20 ± 3.75 , 4.27 ± 0.77 , and 2.27 ± 0.99 s.d. ng/ml). E_1 sulfate was clearly the predominant form at this stage in development.

Conjugation of steroids

The extent of conjugation of estrogens by the different components of the conceptus is given in Table 1. With the embryo proper, no difference was found in the extent of conjugation of the two substrates. Other tissues were included in the study only for qualitative comparison with metabolism by the embryo proper. All tissues showed a notable level of activity with the suggestion of less conjugation by bilaminar tissues with either substrate. More pertinently, the embryo proper was able to 1) form estrogen conjugates, and 2) do so to a degree matching that observed with extraembryonic tissues. The distribution of radioactive metabolites in the conjugate fraction after incubations with the embryo proper is given in Table 2, which shows that sulfo conjugation predominated. No comparable examination was made for the other tissues.

HPLC profiles from estrogen metabolism

The profiles illustrated are examples of the metabolism of E_1 or E_2 by the embryos proper. For the unconjugated steroids, distribution of radioactivity on chromatography for the metabolites formed by the embryo proper was clearly different for E_1 than that for E_2 (Fig. 2). The radioactivity from incubation of E_1 remained largely as unaltered substrate, with a minor amount occurring as a very polar product. No metabolism to E_2 was seen. Metabolism of E_2 was marked by a major conversion to E_1 and to products considerably more polar than the substrate. Although the embryo proper had little or no ability to convert E_1 to E_2 under these conditions,

Figure 1 An equine embryo aged 25.5 ± 0.5 days. (A) Within the entire conceptus. Note particularly the vascularity of the splanchnic mesoderm of the trilaminar yolk-sac wall. (B) After stripping off the embryonic membranes. The future eye, heart (H), somites (S), and fore-limb bud (FLB) are apparent but the hind-limb bud is obscured. The brain has differentiated into the mesencephalon (Mes) and metencephalon (Met). The maxillary process (Max) and the mandibular (Mand) and hyoid (Hyd) arches are prominent. Two 1 mm squares are shown in the bottom left of the image.

Table 1 Percent radioactivity in the conjugate fractions from incubations of embryo proper and extraembryonic tissues with estrone or estradiol.

Tissue	³ [H]-Estrone	³ [H]-Estradiol
Embryo proper	19.3 ± 5.5 (n=6) ^a	20.6 (n=2)
Allantois	22.1 (n=2)	20.1 (n=2)
Bilaminar yolk-sac wall	12.2 ± 4.5 (n=3)	12.5 (n=2)
Trilaminar yolk-sac wall	19.1 ± 4.8 (n=3)	16.2 (n=2)

^aValues are mean ± S.D.; n, number of conceptuses.

it demonstrated nonetheless a considerable capacity for E₂ metabolism even at such an early stage in development.

Other components of the equine conceptus were examined for comparison with estrogen metabolism in the embryo proper. For E₁ metabolism, the distribution pattern of radioactivity was almost indistinguishable between the embryo proper and the other tissues, with the exception of the bilaminar tissue (Fig. 3). As in our earlier study (Raeside *et al.* 2004), the bilaminar tissue exhibited a modest conversion of E₁ to E₂ (Fig. 3C). All showed small to trace amounts of polar products. A small, unidentified peak between E₁ and E₂ had a retention time differing from that of 17 α -estradiol. The profiles of the embryo proper from two conceptuses (Figs 2A and 3A) revealed a very high level of consistency.

Metabolism of E₂ was marked by a major conversion to E₁ and to products considerably more polar than the substrate (Fig. 4). All extraembryonic tissues showed a greater conversion of E₂ to E₁ than was noted for the embryo proper; this was especially so with the trilaminar tissues. A comparison of the profiles for E₂ metabolism by the embryo proper from two conceptuses shows a high level of agreement (Figs 2B and 4A). The repeatability in estrogen metabolism by the embryo proper is illustrated further by an approximation of the distribution of radioactivity from HPLC chromatography (Table 3). The totals of the unknown and polar products (28.3 and 27.5%) formed from E₁ by the two embryos proper were in close agreement; however, the amounts of the unknown compound (X) produced were different.

HPLC profiles from solvolysis of the lesser amounts of radioactivity available in the conjugate fractions (about 20%, see Table 1) were remarkably similar (Fig. 5), with only a trace of E₂ detected in the solvolysed material in

each. Metabolism of E₁ resulted in E₁S formation with only traces of other products. E₂ metabolism gave rise mainly to E₁S and to several small peaks of polar products as sulfo conjugates (Fig. 5B). Only a small portion of E₂ as substrate had been metabolized to its sulfate form. The reductive action of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) appeared extremely low for the sulfate form of E₁ (Fig. 5A) and absent for the unconjugated steroid (Figs 2A and 3A). Similarly, E₁ was evident on HPLC with the small amount of hydrolyzed material from the glucuronidate fractions, but no peaks for E₂ were seen from either estrogen as substrate (data not shown). The form of conjugation for the remaining non-hydrolyzed radioactive material has not yet been examined.

Discussion

These results are, to the best of our knowledge, the first to demonstrate estrogen metabolism by a mammalian embryo proper. The study was based on the premise that the embryo proper, in addition to the endometrium, might be a target for estrogen action. From our current findings and our earlier report (Raeside *et al.* 2004) on the large amounts of estrogens in Y-S fluid of equine conceptuses, it is inferred that estrogen production by the conceptus continues well beyond the phase of transuterine migrations that cease around day 16 of pregnancy. This differs markedly from the more transient production of estrogens in early pregnancy in the pig (Conley *et al.* 1992, 1994). A role for estrogens in the maternal recognition of pregnancy in the pig seems to have been established (Bazer & Thatcher 1977) but the biological function of estrogen secretion by the equine conceptus remains to be defined. The continued presence of high levels of estrogens in Y-S fluid indicates that their action could extend beyond the maintenance

Figure 2 HPLC profiles of unconjugated metabolites in extracts of media from incubations of an embryo proper of a day 24.5 ± 1.5 conceptus with ³[H]-E₁ (A) or with ³[H]-E₂ (B). Estrogen reference standards are shown as a thin line.

Table 2 Distribution of radioactivity (percent)^a in conjugates formed by the embryo proper from estrone and estradiol.

Substrate	Sulfate	Glucuronidate	Other
[³ H]-E ₁	17.1	1.8	4.6
[³ H]-E ₂	12.6	2.5	7.3

^aMean values for embryos proper (n=4) are expressed as percentage of total radioactivity recovered from duplicate incubations in two experiments for each substrate.

Figure 3 HPLC profiles of unconjugated metabolites in extracts of media from incubations of the components of a day 25.5 ± 0.5 conceptus with ^3H -E₁. Estrogen reference standards are shown as a thin line.

of mobility of the conceptus within the uterus, as has been suggested (McDowell *et al.* 1988, Sharp *et al.* 1989). Nutritional support from the maternal endometrium may well be influenced by the conceptus-derived estrogens for an extensive period in early pregnancy of the mare in which placentation (in the sense of a stable, microvillous attachment of the non-invasive trophoblast of the allantochorion to the endometrium) is not established until about day 40 of gestation (Allen 2001).

The exposure of the embryo proper to high concentrations of estrogens over a prolonged period in early development (present results and Raeside *et al.* 2004) has not been reported for any other mammalian species, as far as we could determine. For more than 60 years, marsupials have provided a unique mammalian model to investigate the influence of estrogens on early gonadal development without the need for intrauterine surgery (Burns 1939, Fadem & Tesoriero 1986). However, the possible action of estrogens during organogenesis – before the early stages of formation of the gonads – has received little or no attention in either marsupials or eutherian mammals. Our study of the embryo proper involved specimens that were at a point well before sexual differentiation of the gonads in the horse (Merchant-Larios 1979, Knospe 1998).

The literature is extensive on the role of steroid hormones in sexual development in the lower vertebrates. Attention has been focused mainly on aberrations resulting from exposure of embryos to natural and synthetic estrogens in the environment, along with other endocrine disrupting agents (vom Saal *et al.* 1998, Safe *et al.* 2002). More relevant to our interest are the reports on the presence of steroids transferred naturally from the mother to the yolk in oviparous species. Although this was suspected more than 70 years ago (Altmann & Hutt 1938), the first demonstration of naturally occurring yolk steroids was made many years later in birds (Schwabl 1993). There followed other similar studies in birds and in other oviparous vertebrates, as illustrated by the single citations chosen as examples for alligators (Conley *et al.* 1997), turtles (Bowden *et al.* 2000), lizards (Lovern & Wade 2003), and fish (Khan *et al.* 1997). Again, the emphasis was on the possible effect of yolk steroids on sexual development. However, if yolk and embryo steroid contents change during incubation, when eggs have no further maternal contact, then an examination of the dynamics of yolk and embryo steroid content will yield insight into potential steroid production or metabolism by the embryo. In the green anole (*Anolis carolinensis*), for example, total yolk content of testosterone and E₂ generally declined over the course of incubation, indicating metabolism by the embryos during early stages of development; a later increase in testosterone, but not in E₂, also gave evidence of steroid synthesis (Lovern & Wade 2003).

The female reproductive endocrinology of elasmobranchs (sharks, skates, and rays) is considered by some to be the archetype for amniote vertebrates; and placentation in elasmobranchs approaches the eutherian condition (Callard *et al.* 1993). A study of the placental viviparous bonnethead shark (*Sphyrna tiburo*) allowed the investigation of both yolk and placental hormone transfers of steroid hormones in a single organism (Manire *et al.* 2004). In this species, nutrition is provided by yolk for the first half of embryonic development and by placental transfer for the second half. The pattern of yolk E₂ concentrations was most interesting. The dramatic decline in yolk E₂ levels between the post-ovulatory and the early pregnancy stages is consistent with the utilization of this hormone during embryonic development. A dramatic increase of E₂ after that, i.e. during the pre-implantation stage, would appear to be due to hormone production by the embryo concurrent with, or following, sex differentiation, especially given that maternal serum E₂ concentrations at the time are at baseline levels. No data on metabolism of E₂ were provided in any of these studies in spite of evidence of a decline in E₂ concentrations in the yolk and embryo proper.

The outstanding feature of estrogen metabolism in the equine embryo proper was the preferential expression of the oxidative form of 17β-HSD (type 2), converting E₂ to E₁, has also been found in studies on fish embryos in both

Figure 4 HPLC profiles of unconjugated metabolites in extracts of media from incubations of the components of a day 25.0 ± 1.0 conceptus with ^3H -E₂. Estrogen reference standards are shown as a thin line.

Arctic charr (Khan *et al.* 1997) and rainbow trout (Petkam *et al.* 2002). This conversion was less pronounced than seen with the extraembryonic tissues, especially the trilaminar Y-S wall, but clearly demonstrated an ability of the embryo proper to regulate the degree of exposure to the more biologically active estrogen. Indeed, when E₁ was the substrate there was essentially no E₂ formed by the embryo proper in contrast to the amounts of E₂ noted for the bilaminar tissues. In all tissues, E₂ was further depleted by metabolism to highly polar products as yet not identified. They are most likely to be 6-oxygenated metabolites since their retention time on HPLC

Table 3 Distribution of radioactivity (percent)^a for unconjugated steroids in HPLC profiles from metabolism by the embryo proper.

Profile	Substrate	E ₁	E ₂	X ^b	Polar
1	[³ H]-E ₁	49.1	1.1	3.7	24.6
2	[³ H]-E ₁	49.5	1.6	10.4	17.1
3	[³ H]-E ₂	21.8	23.1	1.8	27.0
4	[³ H]-E ₂	16.6	24.1	1.5	30.2

^aNot all radioactivity above background could be clearly designated as metabolic products. ^bX denotes an unidentified metabolite eluted between E₂ and E₁.

chromatography relates closely to products tentatively identified in our previous study with tissues of equine embryos from earlier stages (Raeside *et al.* 2004). The presence of these polar products was less noticeable when E₁ was the substrate; metabolism to the unidentified peak (not 17 α -E₂) between E₁ and E₂ may have been partly responsible. Lastly, evidence of enzyme activity leading to conjugation of estrogens, as steroid sulfates, glucuronides, and other forms (perhaps with glutathione) by the embryo proper has a parallel in the formation of similar products by fish embryos in Arctic charr (Khan *et al.* 1997), and in rainbow (Petkam *et al.* 2002), and steelhead (Yeoh *et al.* 1996) trout. A marked conversion of E₂ to E₁, followed by preferential metabolism of E₁ to its conjugated forms provides protection from exposure to potentially mutagenic products derived from E₂. The qualitative support for this view is considered substantial in the consistency of the metabolic profiles; and despite the severe limitations in obtaining sufficient material for replication of experiments of this nature, there are reasonable grounds for concluding that the embryo proper is actively engaged in estrogen metabolism at this stage of development.

Whereas it is clear that the embryo proper is an active participant in establishing and regulating its endocrine environment with respect to endogenous estrogens, the biological significance and possible activity of some of the metabolites formed remains unknown. The putative 6-oxygenated metabolites produced by the equine embryo proper, from both E₂ and E₁, have been referred to as 'impeded' estrogens from their repression of uterine growth (Huggins & Jensen 1955). A possible role for the unknown compound formed from E₁ by the embryo proper, and by all of the extraembryonic tissues, represents another example of metabolism. However, quantitatively more important is the metabolism of E₂ to

Figure 5 HPLC profiles of sulfo-conjugated metabolites in extracts of media from incubations of an embryo proper of a day 24.5 ± 1.5 conceptus with ^3H -E₁ (A) and with ^3H -E₂ (B). Estrogen reference standards are shown as a thin line.

E_1 and E_1S . While E_2 itself might act in the embryo proper in its well-known role as an angiogenic agent (note the vascular development in Fig. 1) the presence of greater concentrations of E_1 and E_1S could also contribute in a significant manner. Both E_1 and estriol are designated as 'weak' estrogens in genomic actions but have recently been shown to be strong estrogens in nongenomic signalling pathways and in evoking functional responses in pituitary cells (Watson *et al.* 2008). This may be true for E_1 in vascular tissues also (Rauschemberger *et al.* 2008). The dominant presence of E_1S in Y-S fluid and its formation by the embryo proper raises questions about its relevance to early embryo development. Does it represent an endpoint in metabolism of E_2 , to an inactive product, or does it serve as a source for later retrieval of an active estrogen by means of an estrogen sulfatase? A recent report provides an alternative answer based on a demonstration of a receptor-independent effect of physiological levels of E_1S (Kakusaka *et al.* 2009). Lastly, the presence of other forms of conjugation, accounting for about one-third of conjugates, suggests further activity on the part of the embryo proper in regulating its exposure to biologically active estrogens.

The absence of information on the occurrence, type, and location of estrogen receptors (ESR) at this stage of development places limits on interpretation of the biological importance of local enzyme activity on steroids. At a slightly earlier stage in development of the equine conceptus, day 10–16, no ESR1 could be detected; however, it was shown that *ESR2* gene expression begins on or around day 10 of gestation, with translation to protein by day 14 (Rambags *et al.* 2008). Immunoreactive protein was localized to the extraembryonic trophoctoderm; no evidence for either ESR was recorded for the embryonic disc or embryo proper. Whereas further studies along these lines should be informative, nongenomic actions of the several products of estrogen metabolism by the embryo proper might also have to be considered.

In conclusion, we have shown that the equine embryo proper displays a remarkable ability to exercise some control over its endocrine environment with respect to the high levels of estrogens in the surrounding Y-S fluid. It remains to be determined if species with a similar placental type (Wooding *et al.* 2003) have the same metabolic capabilities for estrogen metabolism displayed by the equine embryo proper. A study of the direct action of estrogens on the embryo proper, however, is never likely to be done with the horse. While our findings of estrogen metabolism are probably the first of this nature in a eutherian species, they suggest that studies on direct effects of estrogens (e.g. angiogenesis) in the early embryo proper in other species may lead to a more complete appreciation of their role in early development.

Materials and Methods

Chemicals and reagents

Nonradioactive steroids were purchased from Steraloids Inc (Newport, RI, USA). The radioactive steroids (NEN) obtained from Perkin–Elmer (Shelton, CT, USA) were [2,4,6,7- 3H]- E_2 (71.0 Ci/mmol) and [2,4,6,7- 3H]- E_1 (74.1 Ci/mmol). Solvents from Caledon Laboratories, Ltd (Georgetown, ON, Canada) were glass-distilled and reagent grade; and acetonitrile (HPLC grade 190) was used for HPLC. Medium 199 and supplements were supplied by Sigma. All other chemicals were analytical grade from Fisher Scientific (Toronto, ON, Canada).

Collection of conceptuses

Standardbred and thoroughbred mares from the research herd at the University of Guelph were used to recover seven conceptuses at days 24–25 under conditions approved by the University Animal Care Committee. The timing of ovulation was monitored by transrectal ultrasonography either daily ($n=3$) or at intervals of 2 ($n=1$) or 3 days ($n=3$) and ovulation 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 ± 0.5 –1.5 days. Conceptuses were collected from mares by transcervical uterine lavage, with Dulbecco's PBS, pH 7.4, using a technique based on one previously described (Raeside *et al.* 2004). The mares were sedated with xylazine hydrochloride (Rompun; BAY-VET, Etobicoke, ON, Canada), administered i.v., usually 0.3 mg/kg body weight. The flush fluid (1000 ml, repeated as necessary) was infused by gravity flow and drained into a beaker to recover the conceptus. Y-S fluid was obtained from only one of the seven conceptuses used.

Dissection to recover the embryo proper

Under a dissecting microscope, the embryo proper in PBS was dissected free of its extraembryonic membranes (Y-S, allantois and amnion) using ophthalmic scissors and forceps.

Tissue preparation and incubation

After separation, the embryo proper was washed, minced, and incubated (20–30 mg) with the radiolabeled estrogens. Duplicate incubations were performed (except for single incubations in two instances) with E_2 or E_1 as substrate. The Y-S wall was divided into bilaminar (trophoctoderm and endoderm) and trilaminar (trophoctoderm, mesoderm, and endoderm) components by dissection, and these were incubated separately (10–20 and 35–70 mg of tissue, respectively, in duplicate). Allantoic tissues (10–20 mg, in duplicate) were also incubated but the amnion, which was sparse, avascular, and shredded by the dissection, was not. All tissues were washed with PBS and dispensed into small flasks containing 2 ml Medium 199 to which either [3H]- E_1 or [3H]- E_2 (1×10^6 c.p.m.) was added for incubation in a shaking water-bath at 37 °C under 5% CO_2 in air for 2 h.

Analytical procedures

Steroids in the media were recovered by solid-phase extraction as previously described by Raeside *et al.* (2004). Briefly, medium from the incubation was diluted with water to a volume of 5 ml for solid-phase extraction (Waters C18 Sep-Pak column, Canada Waters Ltd, Mississauga, ON, Canada). 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; the dried ether residue (unconjugated fraction) was used for HPLC. 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 (Raeside *et al.* 1999). The amounts of radioactive material recovered from each incubation, each hydrolysis step and in each Sep-Pak fraction were determined by liquid scintillation counting in 4 ml of Ecolite cocktail (MP Biochemicals, Santa Ana, CA, USA). In addition, an indirect assessment of metabolism involving loss of the tritium-label at carbons 2, 4, 6, and/or 7 was made by measuring the amount of radioactivity in the initial (flow-through) and the following (water-wash) fraction from the solid-phase extraction. These aqueous fractions were deemed to reflect the formation of [³H]₂O from oxygenation at the tritium-labeled carbon sites of the estrogen molecule (Raeside *et al.* 2004).

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 (Waters # 8) of acetonitrile:water (from 28:72 to 90:10, over 40 min) on a Waters HPLC C18 column and system (Waters Corp., Milford, MA, USA) at a flow-rate of 1.0 ml/min and absorbance monitored at 280 nm. Fractions were collected at 0.5 min intervals. The total radioactivity in the detected peaks was used for quantitative comparisons of the products formed (as % of total radioactivity). Identification of the radiolabeled metabolites was based on their co-elution with authentic reference steroids run as internal standards or in separate chromatograms.

Statistical analysis

Results are expressed as means ± s.d. Data were analyzed by Student's *t*-test. Differences were considered significant when *P* < 0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada, the Grayson-Jockey Club Research Foundation, Equine Guelph, and by the Ontario Ministry of Agriculture, Food and Rural Affairs.

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Received 8 July 2009

First decision 29 July 2009

Accepted 25 August 2009