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ESTROGEN METABOLITES IN EQUINE OVARIAN FOLLICLES: GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATIONS IN RELATION TO FOLLICULAR ULTRASTRUCTURE AND PROGESTIN CONTENT

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Summary—Equine follicular fluid was aspirated at various developmental stages (viable, preovulatory and atretic) determined by ultrastructural study. Estrogens and progestins were analyzed by gas chromatography–mass spectrometry associated with stable isotope dilution. Progesterone and 17-hydroxyprogestrone were the principal progestins of the preovulatory and viable follicles. Among the catechol estrogens, 2-hydroxy-estradiol was particularly abundant in the preovulatory follicle and its definitive identification was made by the scan of a full mass spectrum.

INTRODUCTION

The intrafollicular metabolism of E_2-17β was first described by Short[1] who identified 6α-OHE_2 in follicular fluid (FF) of the mare and noted the existence of an estrogen-like fraction representing about 20% of total estrogens [2]. The possibility of E_1 and E_2 metabolism in the rhesus monkey was suggested by Channing and Coudert[3] and although these authors did not identify any metabolites, they estimated their overall proportion to be about 30% of total estrogens. In the human, Smith[4] demonstrated first the presence of E_3 and we have shown recently that estrogen 2-, 4-, 6- or 16-hydroxylation occurs in preovulatory follicles of women treated with clomiphene and hMG + hCG [5].

The present work was designed to determine estrogen and progestin levels by gas chromatography–mass spectrometry (GC–MS) in FF of the mare at different stages of follicular growth and atresia.

EXPERIMENTAL

Follicular fluid

Ovaries were obtained from the local slaughterhouse within 30 min after death of the animal. The FF of large Graafian follicles (2–75 ml) was aspirated by syringe and frozen at −20°C until analysis. Preovulatory follicles were obtained from 3 mares whose follicular growth was followed manually and by echography. At the end of maturation, an ovary was excised from one mare and a follicle dissected out for structural study. Preovulatory FF was aspirated from the two remaining mares *in vivo*. without anesthesia and with the animal standing [2].

Ultrastructural study

Fragments of the follicular wall were fixed in 2.5% (v/v) glutaraldehyde in a sodium cacodylate buffer 0.1 M, pH 7.4, for 2 h at 4°C [6], rinsed in the same buffer containing 0.2 M sucrose and postfixed for 2 h at 4°C in 1% osmium tetroxide. After dehydration and embedding in Epon 812, semi-thin (1 μm) sections were stained with 1% toluidine blue in 1% sodium borate solution. Thin sections were collected on bare 200 mesh grids and stained with uranyl acetate and lead citrate [7]. The grids were examined with a Siemens Elmiscop 1 A or 102 electron microscope.

Analytical methods

Estrogens

Qualitative and quantitative analyses of trimethylsilyl ethers were performed by GC–MS associated with stable isotope dilution according to procedures outlined previously [5].

Progestins

Unlabelled steroids. 5P, 17-OH5P, P, 17-OHP, 20α-DHP and 20β-DHP were obtained from Makor Chemicals Ltd (Jersualem, Israel), purified by crystallization and checked for analytical purity by GC.

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Abbreviations and trivial names of steroids: 5P = Pregnenolone; 17-OH5P = 17-hydroxy-pregnenolone; P = progesterone; 17-OHP = 17-hydroxy-progesterone; 20α-DHP = 20α-dihydroprogesterone; 20β-DHP = 20β-dihydroprogesterone; E_1 = estrone; E_2-17β = estradiol-17β; E_2-17α = estradiol-17α; E_3 = estriol; 2-OHE_1 = 2-hydroxy-estrone; 4-OHE_1 = 4-hydroxy-estrone; 2-$MeOE_1$ = 2-methoxy-estrone; 2-OHE_2 = 2-hydroxy-estradiol-17β; 4-OHE_2 = 4-hydroxy-estradiol-17β; 2-$MeOE_2$ = 2-methoxy-estradiol-17β; 6α-OHE_2 = 6α-hydroxy-estradiol-17β; 6β-OHE_2 = 6β-hydroxy-estradiol-17β

Stable isotope labelled steroids. [3,4-$^{13}C_2$]P was obtained from ORIS-CEA (Gif-sur-Yvette, France). 17-Hydroxy-[1α,2α,$^2H_2$]P, 20α-dihydro-[1α,2α,$^2H_2$]P, [21,21,21-$^2H_3$]5P and 17-hydroxy-[21,21,21-$^2H_3$]5P were synthesized as described by Dehennin *et al.* [8]. Deuterated 20α-DHP was used as internal standard for quantitative determination of both 20α-DHP and 20β-DHP.

Extraction and liquid chromatography. A volume (0.1 ml) of FF was spiked with appropriate amounts of ^{13}C-labelled or deuterated analogues (amounts were close to those of the native compounds) and equilibrated for at least 1 h at room temperature. The mixture was diluted up to 0.5 ml and applied to columns (80 × 10 mm) of Extrelut (Merck, Darmstadt, F.R.G.), allowed to soak for 20 min and extracted with 5 ml dichloromethane. 5P and P were isolated on a column (120 × 4 mm) of Sephadex LH 20 (Pharmacia, Uppsala, Sweden) swollen in *n*-hexane, by elution in the first 3 ml *n*-hexane. Similar columns of Sephadex LH 20 swollen in dichloromethane were used for the chromatography of 17-OH5P, 17-OHP and 20-DHP, which were eluted in 1.5 ml dichloromethane after initial discarding of 0.8 ml.

Derivatization. Trimethylsilyl ethers (TMS), *t*-butyldimethylsilyl ethers (TBDMS), *O*-methyloximes (MO), *O*-ethyloximes (EO), *O*-trimethylsilyloximes (TMSO) were prepared as described previously [8]. 5P was analyzed as the 3β-TBDMS-20-TMSO derivative made by sequential derivatization at C-3 and C-20.

Qualitative and quantitative analyses. Identification criteria and quantitative estimations under appropriate GC–MS conditions were similar to those described previously for estrogens [9].

RESULTS

Follicular ultrastructure

The follicles were classified according to the ultrastructural findings into 2 stages of viable follicle growth, 1 preovulatory stage and 2 stages of atresia.

Viable follicle stage I. The granulosa cells were oval or polygonal and presented desmosome cellular junctions. The nucleus occupied almost the entire cell. The cells of the theca interna were small, dark, very irregular and separated from one another by numerous connective fibers, ground substance and a few vessels.

Viable follicle stage II. There were several layers of well developed granulosa cells connected by desmosomes and gap-junctions. The mitochondria had lamellar cristae and the endoplastic reticulum was essentially rough. The theca interna achieved maximal development and was composed of hypertrophic cells presenting numerous lipid droplets, smooth reticulum and mitochondria with vesicular cristae. The intercellular spaces were reduced and contained numerous capillaries. Sinusoidal vessels could be observed adjacent to the theca which they had begun to invade.

Preovulatory follicle. The granulosa cells were well developed and dissociated, showing numerous gap junctions. The number of desmosomes had dropped abruptly. The cells were most frequently luteinized, with a significant development of the smooth endoplasmic reticulum, osmiophilic lipid droplets and typical mitochondria with vesicular cristae. The basal membrane presented a thin and discontinuous structure. The theca interna showed a decrease in cell number with signs of degeneration.

Atretic follicle stage I. The granulosa was composed of several layers of cells which were most often dissociated and vacuolated with pyknotic nuclei and degenerating cellular organelles. The cells closest to the antrum were the first to show alterations, but the basal membrane was intact. A fibrous-like anhistic space appeared beneath the basal membrane as the theca cells became deformed and began to autolyze.

Atretic follicle stage II. The granulosa consisted of

Table 1. Estrogen and progestin derivatives: retention indices ($I_{240}^{OV\text{-}73}$), characteristic mass-to-charge (*m/z*) values of ions found in authentic and follicular fluid compound and used for quantitative determinations

Parent steroid	Derivatives	$I_{240}^{OV\text{-}73}$	*m/z* Values of characteristic ions	
			Unlabelled	Labelled
5P	3-TBDMS-20-TMSO	3191	460 (M^+-57)	464
17-OH5P	3,17-TMS-20-MO	2904	474 (M^+-31)	477
P	3,20-$(EO)_2$	3037	400 (M^+), 355	402
17-OHP	3,20-$(MO)_2$-17-TMS	2977	460 (M^+), 429	431
20α-DHP	3-EO-20-TMS	3002	431 (M^+)	433
20β-DHP	3-EO-20-TMS	2982	431 (M^+)	433
E_1	3-TMS	2672	342 (M^+)	346
E_2-17α	3,17-$(TMS)_2$	2675	416 (M^+)	418
E_2-17β	3,17-$(TMS)_2$	2715	416 (M^+)	420
E_3	3,16,17-$(TMS)_3$	2927	504 (M^+)	507
2-OHE_1	2,3-$(TMS)_2$	2816	430 (M^+)	434
4-OHE_1	3,4-$(TMS)_2$	2849	430 (M^+)	434
2-$MeOE_1$	3-TMS	2800	372 (M^+)	374
2-OHE_2	2,3,17-$(TMS)_3$	2858	504 (M^+)	506
4.OHE_2	3,4,17-$(TMS)_3$	2896	504 (M^+)	506
2-$MeOE_2$	3,17-$(TMS)_2$	2847	446 (M^+)	449
6α-OHE_2	3,6,17-$(TMS)_3$	2849	414 (M^+-90)	417
6β-OHE_2	3,6,17-$(TMS)_3$	2807	414 (M^+-90)	417

Table 2. Estrogen and progestin levels (ng/ml) found in various follicles aspirated *post mortem*

	V1	V2	V3	V4	V5	PO1	A1	A2	A3	A4
5P	2.2	3.0	—	1.5	3.5	1.7	3.7	3.5	7.9	3.1
P	25	19	85	30	41	269	117	14	61	11
17-OHP	126	56	99	246	251	361	34	32	18	11
20α-DHP	2.7	3.1	8.4	2.0	2.8	16	6.8	3.5	19	1.1
20β-DHP	0.44	—	1.9	—	0.36	4.0	—	—	0.46	0.50
E_1	49	52	344	229	223	145	43	81	46	49
E_2-17α	3.2	1.3	1.9	3.5	1.5	2.2	3.0	3.1	3.3	3.2
E_2-17β	386	962	1996	3154	3554	2331	350	575	60	143
E_3	1.4	1.0	6.2	6.4	5.4	1.9	1.1	3.0	0.10	0.93
2-OHE_1	0.24	0.16	1.6	0.86	0.77	0.48	0.34	0.42	0.10	0.11
4-OHE_1	0.30	0.09	0.28	0.31	0.35	0.70	0.09	0.14	0.03	0.05
2-$MeOE_1$	1.5	0.79	8.6	6.2	6.1	2.2	2.7	9.9	1.9	2.2
2-OHE_2	3.7	1.4	2.3	6.0	7.2	15	0.52	0.95	0.51	0.58
4-OHE_2	0.45	1.8	0.91	0.66	1.3	1.2	0.16	0.22	0.03	0.09
2-$MeOE_2$	79	47	37	130	247	73	31	69	1.9	42
6α-OHE_2	306	95	355	679	650	261	54	300	12	76
6β-OHE_2	2.0	2.4	8.8	6.6	8.8	5.3	3.2	1.8	0.39	1.1

V1 and V2 are viable follicles, stage I; V3, V4 and V5 are viable follicles, stage II; PO1 is a preovulatory follicle; A1 and A2 are atretic follicles, stage 1; A3 and A4 are atretic follicles, stage II.

Table 3. Levels (ng/ml) of the principal steroids found in two preovulatory follicles aspirated *in vivo*

	P	17-OHP	E_2-17β	2-OHE_2	4-OHE_2	2-MeO_2
PO2	217	118	3780	56	0.6	310
PO3	10	140	2691	250	3.2	180

only one layer of degenerate and spindle-shaped cells. A dense fibrous zone replaced the theca interna under the basal membrane which was continuous and folded.

Steroid determinations

Our feasibility study on human follicular estrogen analysis by GC–MS associated with stable isotope dilution [5] allowed us to apply this technique to the equine species, with the same criteria for identification and quantitative estimation.

The GC–MS properties of estrogen and progestin derivatives are listed in Table 1. Quantification of estrogen and progestin in relation to the stages of follicular viability or atresia are given in Table 2. Steroid concentrations of the follicles aspirated *in vivo* are shown separately on Table 3. 2-OHE_2, isolated from one of these follicles containing as much as 250 ng/ml of FF, could be identified by scanning the entire mass spectrum of the TMS derivative (Fig. 1).

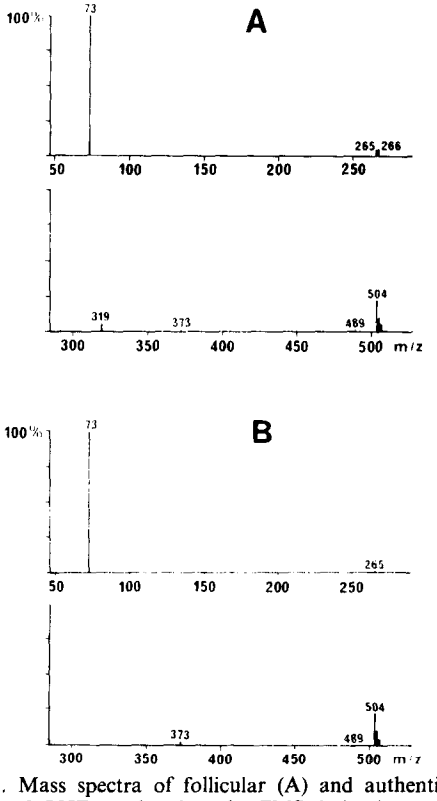

Fig. 1. Mass spectra of follicular (A) and authentic (B) 2-OHE_2 analysed as the TMS derivative.

DISCUSSION

Follicular ultrastructure

The ultrastructural characteristics of the follicles in our study correspond to those described in other mammals [10]. Our findings were also in agreement with the results of Bjersing and YoungLai[11] and Kenney *et al.*[12] obtained in the mare. However, the latter authors described viable follicles as a single category whereas we have subdivided it into 2 growth stages and one preovulatory stage.

Steroids

Especially in the mare, most authors define the developmental stage of the follicle according to its volume, size or vascularisation and not to its cellular and histological characteristics, thus was it often difficult to compare our results with those reported in the literature. Moreover, steroids were not assayed individually in the work of Bjersing and YoungLai[11] and in that of Kenney *et al.*[12].

Progestins. 5P concentrations determined by GC–MS were markedly lower, at all developmental stages, than those reported in the human [13] and in the pig [14]. The high 5P levels of the human follicle

(about 10 μg/ml) increase 2- to 3-fold during the late follicular phase and they may be related to the high cholesterol concentrations [15]. YoungLai was able to detect 5P in corpus luteum but not in FF of the mare [16].

17-OH5P was undetectable at a detection limit of 0.1 ng/ml and this confirms the predominance of the 4-ene metabolic pathway in the horse [17].

P concentration in the growing follicle was low and did not appear to increase during the maturation process. Levels rose abruptly during the preovulatory stage, although up to a limited extent (5–10-fold). Our top levels matched those of Bjersing and YoungLai[11], but these authors observed their maximal concentrations during the proestrus phase. The preovulatory intrafollicular increase of P and the concomitant peripheral rise have been described in other mammals, in whom P concentrations rise up to the μg range [15]. This increase thus seems less important in the mare, although Bjersing and YoungLai[11] have suggested that preovulatory luteinization is particularly marked in the equine follicle.

Our 17-OHP levels were similar to those reported by YoungLai[16] but unlike P, 17-OHP concentration rose during maturation. Similarly to P, peak levels of 17-OHP were reached in the preovulatory follicle, which were about double those of P up to the preovulatory phase and lower than those of P in the atretric follicles.

20α-DHP and 20β-DHP have been demonstrated in FF of the pig [14], but 20α-DHP only was identified by Short [18] in the equine corpus luteum. In this study, it seemed that the concentration of these isomers was independent of the developmental stages of the follicles.

A marked elevation of P and 17-OHP was thus observed in the preovulatory follicle. This increase was concomitant on the one hand, with the luteinization of the granulosa cells as evidenced by the appearance of a smooth endoplasmic reticulum and mitochondria with vesicular cristae typically associated with steroid production, and on the other hand, with the onset of thecal regression essentially marked by a decrease in cell number. The transformation of the granulosa cells would thus indeed seem responsible for the increase of progestin levels which precedes ovulation. P and 17-OHP levels then progressively decreased during atresia when the granulosa and the theca layer regressed.

Ginther[17] has suggested a pathway of steroid biosynthesis in the growing equine follicle in which steroid biosynthesis is performed from acetate to P by the granulosa cells and then from P to E_2 by the theca cells. At the end of growth or at the beginning of atresia, thecal regression apparently blocks steroid biosynthesis at the stage of P. However, the ultrastructural characteristics of the granulosa cells at growth phases I and II, as well as the low levels of P present in the FF during growth, did not seem to confirm this hypothesis which is based essentially on the presence of high Δ_5-3β-hydroxysteroid dehydrogenase activity in granulosa cells, and its absence in theca cells [20].

Estrogens. At the end of maturation or at the beginning of atresia, estrogen metabolites (total estrogens—E_2–E_1) represented about 20% of the total estrogens and this confirms the original evaluation of Short[1]. The proportion was about 12% in the preovulatory follicle, but rose to 40–50% in follicles at the beginning of growth or at the end of atresia. These metabolites represent only about 2% of total estrogens in human preovulatory FF [5]. The concentration of E_1 metabolites appeared to be independent of developmental stage. All the other estrogens except 2-OHE_2, reached at growth stage II peak levels which were in the range of those observed in women, although plasma concentrations of E_2 in the mare were lower (15 pg/ml) than those in the human (50–60 pg/ml). This low E_2 level may perhaps be linked with the absence of testosterone-estradiol binding globulin (TEBG) in the mare [21]. The concentration of these estrogens in the preovulatory FF was lower than that observed in viable stage II and a similar drop of E_2 occurs also in other mammals [15]. *In vitro* studies have revealed that isolated equine granulosa cells show marked aromatization capacity [22] but maximal follicular aromatization is obtained with a combination of theca and granulosa cells [23]. Our results indicate that during the preovulatory phase when the estrogens decreased, granulosa cells were already partially luteinized and regression of theca cells was clearly visible. The decreased production of each of these cell types, or both, would thus also account for the drop of total estrogen.

The low E_2-17α levels compared to those of E_2-17β indicate a very weak aromatization of 17-epitestosterone, which is present at a high level in equine FF [24]. This weak conversion to E_2-17α may result from aromatase inhibition by E_2-17β [25]. The possible conversion of E_1 to E_2-17α must also be low.

6α-OHE_2 and 2-$MeOE_2$ were the most abundant metabolites at all maturation stages and their respective levels appeared to correlate with those of E_2-17β ($r = 0.80$ for 6α-OHE_2 and 0.71 for 2-$MeOE_2$, $P < 0.05$, $n = 10$).

2-OHE_2 was the only estrogen rising markedly in the preovulatory follicle. This singular behaviour prompted us to measure this catechol estrogen in two other preovulatory follicles which were aspirated *in vivo*. 2-OHE_2 levels in both were elevated and higher than in the follicle aspirated *post mortem*, with the highest concentration being observed in a follicle with the lowest P level. Due to the lack of ultrastructural data for these follicles, we did not have enough information for a clear definition of their characteristics and a comparison with the follicles aspirated *in vitro*. The variability of P levels in the FF of follicles diagnosed as preovulatory by palpation nonetheless

suggests that the luteinization phase, just before ovulation, must be brief.

Catechol estrogens are described here for the first time in mare FF and this corroborates an earlier study where we have indicated in particular the presence of 2-OHE_2 at a considerably lower level (350 pg/ml) in preovulatory follicles of women treated with clomiphene and hMG + hCG [5]. Although the physiological role of catechol estrogens is uncertain [26], they are known to have an affinity for E_2 receptors, but this does not allow an interpretation of the high level of 2-OHE_2 in the preovulatory follicle of the mare.

On basis of the data discussed in this paper, it can be concluded that P and 17-OHP are the principal progestins of the preovulatory mare follicle and that estrogen metabolism by 2- or 6-hydroxylation is predominant as well as further conversion by catechol-*O*-methyltransferase.

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