Fetal–maternal gradients of steroid hormones in the nine-banded armadillo (*Dasypus novemcinctus*)*

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Summary. Gonadal and adrenal steroid hormone levels in plasma were measured in pregnant, non-pregnant and fetal armadillos. Progesterone demonstrated the largest placental gradient with levels 6 to 10-fold higher in the fetus. Testosterone levels were higher in male and lower in female fetuses, when compared to the respective maternal serum values. Dehydroepiandrosterone, and cortisol were consistently high in maternal as compared to matched fetal serum samples whereas the reverse was true for androstenedione. Tissue incubations with labelled pregnenolone indicated the major source of progesterone production to be the fetal adrenal whereas the fetal testes demonstrated a major conversion product consistent with dehydroepiandrosterone.

Introduction

The nine-banded armadillo (*Dasypus novemcinctus*) has been of interest to reproductive biologists because of its unique characteristics of delay of implantation (Newman & Patterson, 1909) and polyembryony (Patterson, 1912). In the wild, the female armadillo has an annual cycle consisting of ovulation of a single ovum during the summer months followed by a 3–4-month delay of implantation with parturition of four monozygotic young in the spring (Hamlett, 1932). The discovery that the armadillo is a suitable host for *Mycobacterium leprae* (Storrs, Walsh, Burchfield & Binford, 1974; Convit & Pinardi, 1974) has promoted additional interest in the reproductive physiology of armadillos in order to improve breeding in captivity which has been the major problem in adapting this potentially useful animal to the laboratory.

It has also been suggested that the fetal nine-banded armadillo may be a possible experimental model for the primate feto–placental unit due to the presence of a fetal adrenal zone which involutes after birth like that of human and non-human primates (Moser & Benirschke, 1962). Although little is known about hormone changes in armadillos during pregnancy and the contribution of the fetal adrenal glands, the fetal armadillo adrenals appear to be steroidogenically active (Bloch & Benirschke, 1965). The precursors necessary for oestriol synthesis, dehydroepiandrosterone and 16-hydroxy-dehydroepiandrosterone, are present in fetal armadillo plasma, but although placental aromatase was found in early and late gestation, placental urinary and plasma oestrogens were very low (Brinck-Johnsen, Benirschke & Brinck-Johnsen, 1967; Brinck-Johnsen, 1970).

The present study was carried out (1) to compare plasma steroid concentrations in free-ranging and captive female armadillos in an attempt to demonstrate changes associated with sterility in captivity, and (2) to characterize further the armadillo feto–placental unit for comparison with that of other animals.

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Materials and Methods

Animals

Free-ranging armadillos were shot during daylight hours in Florida and Georgia and heart blood was immediately obtained along with adrenals and reproductive tracts. All animals were evaluated for maturity based upon body weight, secondary sex characteristics, and histological examination of gonadal tissues.

Captive armadillos were acquired from Texas, Florida and Georgia and maintained outside on a diet of milk, raw meat, wheat bran, and hard-boiled eggs. All animals were housed together in an enclosed 9 × 9 m concrete pen containing three 1 × 1 × 0.6 m community nest boxes filled with straw and a 1.8 × 1.8 m dirt litter box. The animals were anaesthetized with ketamine (Ketaset, 100 mg i.m.; Bristol Laboratories, Syracuse, New York) for blood sampling which was performed between 10:00 and 14:00 h by venepuncture of a superficial femoral vein with syringes containing EDTA (approx. 3.0 mg/ml whole blood). The blood was centrifuged and the plasma was stored at -15°C until used.

Captive pregnant armadillos, inseminated in the wild and at different gestational ages, were killed by exsanguination under ketamine anaesthesia, weighed, and the maternal heart blood allowed to clot (10 min at 25°C). The fetuses were then exposed by a midline ventral incision through the uterus, and, before weighing each fetus, the umbilical cord was cut 2 cm from the fetuses and 1–3 ml blood were milked from each fetus and allowed to clot. Resultant maternal and fetal serum samples were stored at -15°C until used. Mean fetal weights from the pregnancies of Armadillos 62, 86, 64, 21 and 46 were 38.3, 44.3, 54.6, 69.8 and 70.8 g, respectively (term fetal weight ~100 g). Maternal and fetal adrenals, fetal gonads and placentae were removed and weighed. A section of each was fixed for histology in Bouin's solution and the remaining tissues were frozen in liquid nitrogen for later use in tissue incubation studies.

Hormone assays

Progesterone, 17-hydroxyprogesterone, androstenedione, testosterone, dehydroepiandrosterone and androstenediol. These steroids were separated on one of the two following column systems: celite:propylene glycol (2:1 w/v), a modified method of Anderson, Lasley, Hopper & Yen (1976), or celite:ethylene glycol (2:1 w/v). After a 1 ml iso-octane rinse the following steroids were eluted from the propylene glycol column: 2.5 ml iso-octane, progesterone; 4 ml 8% ethyl acetate in iso-octane, androstenedione; 2.5 ml 8% ethyl acetate, rinse; 1 ml 8% ethyl acetate, 3 ml 15% ethyl acetate, dehydroepiandrosterone; 4 ml 24% ethyl acetate, testosterone. The remaining steroids were eluted from the ethylene glycol column in the following order: 5 ml iso-octane, pregnenolone and androstenedione; 5 ml iso-octane, dehydroepiandrosterone; 1 ml cyclohexane:benzene (95:5 v/v), rinse; 5 ml cyclohexane:benzene (95:5 v/v), 17 α -hydroxyprogesterone; 1 ml cyclohexane:benzene (95:5 v/v), rinse; 5 ml 30% ethyl acetate, androstenediol. Methodological loss was monitored by adding 1000 c.p.m. of the tritium label of each steroid to be measured to 0.01–0.5 ml serum before ether extraction and celite column chromatography.

Progesterone and testosterone concentrations were assayed as described by Czekala, Hodges, Lasley & Gause (1979). 17 α -Hydroxyprogesterone, androstenedione and androstenediol concentrations were evaluated similarly by combining 10 000 c.p.m. of the appropriate tritiated steroid and respective antiserum (17 α -hydroxyprogesterone-3-oxime-BSA, Endocrine Science P17-43; androstenedione-3-oxime-HSA, Abraham S-1557 No. 2; testosterone-3-oxime-HSA, Abraham S-741 No. 7, which has a 30% cross-reactivity with androstenediol). The dehydroepiandrosterone assay used 20 000 c.p.m. [³H]dehydroepiandrosterone (sp. act. 58.6 Ci/mmol; New England Nuclear, Boston, Massachusetts) and a dehydroepiandrosterone 3 β -monohemisuccinate-HSA antiserum (Abraham

S-1502 No. 7). The inter-assay coefficients of variation for the progesterone, 17 α -hydroxyprogesterone, androstenedione, testosterone and dehydroepiandrosterone assays were 18, 12, 15, 5 and 11%, respectively. All assays demonstrated a sensitivity between 10 and 25 pg/tube.

Co-chromatography of the 2.5 ml celite progesterone fraction was performed on 5 maternal and 5 fetal serum samples by monitoring the tritiated and immunoreactive steroid concentrations in each of the five 0.5 ml eluates. Co-chromatography on high pressure liquid chromatography was performed as previously described by Czekala *et al.* (1979). In all cases, a single parallel peak of the radioactive and immunoreactive progesterone was observed. All other steroids separated on the celite columns were assumed to be contaminant-free since the determined values were reasonably low.

Corticosterone and cortisol. These steroids were separated on celite columns as described by Manilimos & Abraham (1975) after extraction with dichloromethane (Mallinckrodt, St Louis, Missouri). Cortisol concentrations were determined by combining 10 000 c.p.m. [3 H]cortisol (sp. act. 80.6 Ci/mmol; New England Nuclear) and cortisol antiserum (F-21-hemisuccinate-BSA, Hopper No. F39), while the corticosterone assay used 6000 c.p.m. [3 H]corticosterone (sp. act. 82.0 Ci/mmol; New England Nuclear) and the cortisol antiserum which has a 20% cross-reactivity with corticosterone.

Other procedures

Incubations. Adrenal, gonadal and placental tissues were thawed slowly at 4°C and kept on ice throughout the preparatory procedure. Each tissue was weighed, minced and placed in a plastic vial containing 2 ml medium 199 (Gibco, Grand Island, New York) and [3 H]pregnenolone (sp. act. 17.2 Ci/mmol; New England Nuclear; 29 000 c.p.m./incubation vial). The incubation was carried out in a 37°C shaker bath under 95% O $_2$ and 5% CO $_2$ for 3 h, stopped by snap-freezing in methanol:solid CO $_2$ and kept frozen until extracted three times with 8 ml ether. The steroids were separated and assayed in the same manner as the serum samples, with monitoring of the radioactive conversion products and immunoreactive steroids in each fraction to observe bulk flow of the steroids. The amount of label which remained at pregnenolone and the amount that was converted to androstenedione and testosterone could not be assessed because these steroids were inseparable on the 17 α -hydroxyprogesterone column system used.

Recrystallization. For each steroid 10 mg were added to the radioactive sample and repeatedly recrystallized until the specific activity in the crystal and mother liquor were identical for two consecutive recrystallizations. The solvent systems used were methanol:water, ethyl acetate:iso-octane and ethanol:water with the second solvent added to the point of initial crystal formation at room temperature. Reduction of the temperature to 10°C overnight promoted the completion of crystallization.

Luteinizing hormone assay. Concentrations of biologically active luteinizing hormone (LH) were measured in maternal and fetal serum and placental homogenates by the effect *in vitro* on testosterone secretion by rat interstitial cells (Dufau, Pock, Neubauer & Catt, 1976).

Histology. The tissues fixed in Bouin's fluid were processed routinely into paraffin wax and 7 μ m sections were examined histologically after haematoxylin and eosin staining.

Statistical analysis. A Student's group *t* test was used for all comparisons.

Results

As shown in Table 1 captive armadillos had higher 17 α -hydroxyprogesterone levels (with and without CL) and higher progesterone values (no CL) than did those killed in the wild. The

differences in the concentrations of the other four hormones were not significant ($0.05 < P < 0.1$). The hormone concentrations in late gestation were generally similar to those of free-ranging and captive animals (Table 1).

Table 1. Circulating concentrations of steroid hormones in pregnant armadillos with corpora lutea (+CL) compared to non-pregnant levels in females without corpora lutea (–CL)

Hormone (ng/ml)	±CL	Maternal		Mid- to late gestation	Fetal
		Free-ranging	Captive		
Progesterone	+	129 ± 60.0 (6)	92.5 ± 23.4 (10)	60–90	480–920
	–	0.83 ± 0.13 (4)	*8.5 ± 2.7 (3)		
17 α -Hydroxyprogesterone	+	0.68 ± 0.1 (6)	†5.18 ± 1.2 (10)	1.8–13	2.4–14.4
	–	0.45 ± 0.1 (4)	†4.4 ± 1.4 (3)		
Androstenedione	+	0.54 ± 0.07 (6)	1.03 ± 0.15 (10)	0.75–3.4	1.1–5.5
	–	0.48 ± 0.03 (4)	0.45 ± 0.15 (3)		
Testosterone	+	1.53 ± 0.30 (6)	1.45 ± 0.29 (10)	0.4–1.4	0.2, 0.3 (♀) 1.7–3.9 (♂)
	–	1.65 ± 0.33 (4)	1.08 ± 0.35 (3)		
Dehydroepiandrosterone	+	7.31 ± 1.37 (6)	14.0 ± 2.3 (10)	4–22	3.5–13.0
	–	6.52 ± 1.64 (4)	6.13 ± 2.2 (3)		
Androstenediol	+	3.66 ± 0.92 (6)	2.32 ± 0.37 (10)	1.0–2.8	0.8–1.2
	–	4.07 ± 0.80 (4)	1.7 ± 0.37 (3)		

Concentrations of the same steroids are shown for serum pools of the 5 sets of quadruplets shown in Text-fig. 1. Values represent the mean ± s.e.m. with the no. of observations shown in parentheses.

Compared with values for wild armadillos: * $P < 0.02$; † $P < 0.001$.

Progesterone and androstenedione were consistently elevated in the fetal serum, and fetal testosterone concentrations appeared to be influenced by fetal sex (Text-fig. 1). In contrast, dehydroepiandrosterone, androstenediol, corticosterone and cortisol were generally higher in the maternal serum. 17 α -Hydroxyprogesterone values were higher in the fetal than maternal circulation in 2 of the 5 animals studied.

Progesterone levels were 6- to 10-fold higher in the fetuses than in the mothers. There were major differences in the conversion products of the various tissues incubated *in vitro*.

[³H]Progesterone was the major radioactive conversion product of the added [³H]pregnenolone in both fetal adrenal gland incubations with 54 and 50% of the [³H]pregnenolone converted to [³H]progesterone in Incubations 1 and 2, respectively. The [³H]progesterone was recrystallized to constant specific activity in each case. Progesterone was also the principal immunoreactive steroid in the fetal adrenal gland incubations as measured by radioimmunoassay (32 and 20 ng/mg tissue, respectively) and confirmed by mass spectrometry.

The maternal adrenals also converted a significant amount of the [³H]pregnenolone to [³H]progesterone, 45 and 53% in Incubations 1 and 2, respectively, which was recrystallized to

Text-fig. 1. Concentrations of steroid hormone in maternal and pooled fetal plasma of armadillos. ■, Maternal; □, fetal.

constant specific activity. Immunoreactive progesterone (11.7 and 15 ng/mg tissue) and 17 α -hydroxyprogesterone (6.5 and 7.3 ng/mg tissue) were found in substantial concentrations.

Although radioactive conversion products of cortisol and corticosterone were undetectable, high concentrations of immunoreactive cortisol (31.1 ng/mg tissue) and corticosterone (30.6 ng/mg tissue) were found in the maternal adrenal. Immunoreactive cortisol (2 ng/mg tissue) and corticosterone (0.8 ng/mg tissue) were also present in the fetal adrenals.

In the fetal testicular incubations 78% of the [3 H]pregnenolone was converted to [3 H]dehydroepiandrosterone with confirmation by recrystallization to constant specific activity (Text-fig. 2a). Dehydroepiandrosterone (14.4 ng/mg tissue) and testosterone (2.2 ng/mg tissue) were the major immunoreactive androgens.

In contrast, the fetal ovaries and the placentae appeared steroidogenically inactive: 83% of the label remained at [3 H]pregnenolone in the ovarian incubation, with 91 and 79% remaining in the two placental incubations (Text-fig. 2b). Traces of immunoreactive progesterone were found in the ovaries (0.4 ng/mg tissue) and placentae (0.3 and 1.3 ng/mg tissue) but other steroids were undetectable. In addition to appearing steroidogenically inactive, the placenta was virtually devoid of sulphatase activity when dehydroepiandrosterone sulphate was used as substrate.

Although a parallel dose-response curve was obtained for biologically active LH between a

Text-fig. 2. Radiolabelled (shaded bar) and immunoreactive (open bar) products of short-term incubations in the presence of radiolabelled pregnenolone of tissues from (a) male and (b) female fetuses. Solvents for discontinuous elution from celite columns are shown on the abscissa: IO, iso-octane; EA, ethyl acetate. P_o , progesterone; P_e , pregnenolone; A, androstenedione; 17OH P_o , 17 α -hydroxyprogesterone; T, testosterone; DHEA, dehydroepiandrosterone; B, corticosterone; F, cortisol.

crude armadillo pituitary extract and the WHO (69/104) LH urinary standard, there was no testosterone response to any concentration of the crude placental homogenates (Text-fig. 3). LH in the placental homogenates was at or below the limit of sensitivity of the assay (16 ng/mg protein), but values in maternal serum (41 ± 2.5 ng/ml, $n = 3$) and fetal male (131 ± 5 ng/ml, $n = 4$) and fetal female (161 ± 15 ng/ml, $n = 4$) serum were high.

Histologically, the fetal testes showed an abundance (~40%) of rounded, eosinophilic interstitial cells, while no steroidogenically active cell components were found in the fetal ovaries.

Discussion

Although a comprehensive study of the reproductive cycle of the male armadillo has been reported (Czekala *et al.*, 1979), there has been no comparable hormonal study of the female armadillo. The present results confirm the previous observations that non-pregnant female armadillos, which are infertile in captivity, have higher circulating levels of progesterone and 17 α -hydroxyprogesterone than do free-ranging armadillos. Dexamethasone suppression studies

Text-fig. 3. Testosterone production by dispersed rat Leydig cells in the presence of increasing amounts of armadillo fetal pituitary or placental homogenate.

by Czekala *et al.* (1979) in captive female armadillos indicate the high progesterone levels to be of adrenal origin, since progesterone concentrations greatly reduced by such treatment were restored to pretreatment levels by ACTH. In addition, ovariectomy did not affect the high progesterone levels (Lasley, Czekala, Nakakura, Amara & Benirschke, 1978). It is not known whether the high progesterone concentrations block ovulation directly or whether the adrenal dysfunction is a parallel effect of a primary hypothalamic or pituitary defect, but total glucocorticoid measurements (Lasley *et al.*, 1978) are not consistent with abnormal pituitary function.

The seemingly inappropriate adrenal production of progesterone in captive females may account for the persistent viability of the blastocyst and the occurrence of implantation after ovariectomy (Buchanan, Enders & Talmage, 1956; Enders & Buchanan, 1959). This is consistent with the concept, based on previous reports, that the corpus luteum of the delay period produces substantial amounts of progesterone which supports the viability of the blastocyst before implantation (Labhsetwar & Enders, 1968; Peppler & Stone, 1976). Since the source of circulating progesterone was not determined in these earlier studies, it is possible that an adrenal component was included. That progesterone rises and is continuously present throughout armadillo pregnancy is shown by progesterone values of 60–90 ng/ml in mid- to late pregnancy. More importantly, however, progesterone appears to be the primary adrenal steroid of the fetal armadillo rather than dehydroepiandrosterone which is the major secretory product of the fetal primate adrenal. This finding is supported by the 6- to 10-fold progesterone concentration gradients observed from the fetal to the maternal circulation, the high endogenous concentration in the fetal adrenals and the in-vitro conversion of [^3H]pregnenolone to [^3H]progesterone by the fetal adrenals. The validity of the progesterone results is confirmed by mass spectrometry, the recrystallization of the [^3H]progesterone to constant specific activity and the outcome of the co-chromatography of the serum progesterone fraction. Although the maternal adrenals also contained a substantial amount of progesterone, corticosterone and cortisol concentrations were approximately twice as high. Therefore, the lack of labelled corticosterone and cortisol in the adrenal incubation was unexpected but may have been due to the high concentration of endogenous progesterone and its possible dilution effect on the labelled precursor at this step.

A substantial concentration of dehydroepiandrosterone (3.5–13 ng/ml) in the fetal

circulation explains an earlier report by Brinck-Johnsen *et al.* (1967) that dehydroepiandrosterone may be a secretory product of the fetal armadillo adrenal gland. However, the finding that dehydroepiandrosterone is higher in the maternal circulation than in that of the fetus and that the concentration in the fetal adrenal tissue is low indicates that dehydroepiandrosterone is unlikely to be a major fetal adrenal steroid in this species. Rather, it appears that in the male fetus dehydroepiandrosterone may be a gonadal product as it is in the horse fetus (Raeside, 1976), because the [^3H]pregnenolone was converted to [^3H]dehydroepiandrosterone by the fetal testis and a substantial endogenous gonadal dehydroepiandrosterone concentration was found. These data, together with the histological evidence of Leydig cell abundance and higher circulating levels of testosterone in the male fetus, indicate that the armadillo fetal testis is steroidogenically active. In contrast, the fetal armadillo ovary appears quiescent both histologically and steroidogenically.

The LH results may explain the preferential stimulation of the fetal testis and also the nature of the trophic factor involved. The undetectable levels of placental LH and the higher levels in fetal serum relative to maternal serum suggest that the fetal pituitary is the source of LH which in turn stimulates the fetal testis to produce androgens. The lack of ovarian stimulation could be due to a lack of follicle-stimulating hormone, although this was not measured in this study.

The relative steroidogenic inactivity of the placenta in the tissue incubations and the lower concentration of progesterone when compared to the fetal adrenal suggest that armadillo represents a primitive endocrine system in which the fetal adrenal may preserve the pregnancy directly through the production of relatively large quantities of progesterone. However, the fetal rhesus monkey adrenal gland also appears to secrete a significant amount of progesterone early in gestation (Jaffe *et al.*, 1981). The undetectable levels of bioactive LH, the low steroidogenic capacity and lack of sulphatase activity in the placenta of the armadillo support the concept that little if any endocrine role is provided by the trophoblast in this species.

Irrespective of a haemochorial nature of primate and armadillo placenta, the similarity of the histology of the fetal adrenal cortical compartment and their parallel involution *post partum*, the fact that the endocrine performance of these tissues differs widely indicates that caution should be exercised when morphological criteria are used to predict secretory function of reproductive tissues.

Although these present data indicate that the nine-banded armadillo is not an appropriate model for the primate feto-placental unit, the apparent simplicity of its endocrine mechanisms may be advantageous in more basic studies. The lack of placental steroid conversion and LH activity point directly to the fetus as providing its own control during the pregnancy.

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