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Androgens in the Bovine Fetus and Dam[1] (38567)

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The role of mammalian fetal gonads in the development of the reproductive system has been investigated in rats, rabbits, sheep, and horses (1–4). In the bovine fetus, serum testosterone was greatest at about 180 days of gestation and declined progressively toward the last month of gestation (5, 6). Testicular androgens could be detected in sheep as early as 30 days gestation (3). Castration early in fetal life suppressed the development of masculine features (7) and increased the pituitary basophiles, suggesting a functional fetal relationship exists between the gonads and the hypothalamic–pituitary axis (8). Hence, it appears that sexual differentiation and reproductive development depends on fetal sex steroids. The objective of this research was to study the effects of fetal sex and age on androgens in bovine fetuses and their dams.

Materials and Methods. Male and female fetuses from pregnant Holstein heifers at 90, 180 or 260 days were exposed by hysterotomy (9). Jugular blood was obtained from the cows at the start of surgery, averaging 1 hr before uterine and fetal samples were collected. For 180- and 260-day fetuses, 40 ml blood were aspirated from one umbilical artery and from one umbilical vein of each fetus. Blood was obtained from the thoracic aorta in 90-day fetuses, since insufficient blood could be obtained from umbilical vessels. Concurrently, blood samples were drawn from a uterine artery and a uterine vein of the cows. After the blood samples had been collected, the fetus was excised, and one testis from each 180- and 260-day male was stored at −20°. The testes were homogenized with a Teflon-glass grinder in physiological saline for quantification of androgens.

Radioimmunoassay (RIA) of Androstenedione and Testosterone. The RIA procedures used to quantify androstenedione and testosterone were modifications of the RIA for progesterone described in detail by Louis *et al.* (10). These two androgens were extracted from serum aliquants similar to the described procedure for progesterone. The androgen antibodies[3, 4] were diluted in 0.1% Knox gelatin in 0.1 *M* phosphate-buffered saline (PBS), pH 7.1, instead of 1:400 normal rabbit serum. Free tritated steroid[5] was separated from antibody-bound steroid using dextran-coated charcoal[6] (carbon decolorizing neutral Norit).

Separation of the steroids for assay validation. The steroids were separated by column chromatography (LH-20 Sephadex) using two consecutive solvent systems. Firstly, the benzene:hexane extracts of steroids were placed on LH-20 columns and eluted with chloroform:ethanol (98:2). Testosterone and androstenedione were isolated in the fraction between 15 and 22 ml of effluent. Secondly, the 15–22 ml fraction was evaporated, dissolved and placed on an LH-20

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[3] The rabbit antiandrostenedione (No. 866) and antitestosterone (No. 667) were supplied by Dr. G. D. Niswender, Colorado State University.

[4] The antitestosterone was diluted 1:3000 and the antiandrostenedione was diluted 1:1000.

[5] For the testosterone assay 30,000 dpm of 1,2,6,7^{3}H-testosterone and for the androstenedione assay 20,000 dpm of 1,2^{3}H-androstenedione was used.

[6] For the testosterone assay 1.0 ml (0.25% neutral norit and 0.025% dextran 150; in distilled water) and for the androstenedione assay, 0.5 ml (0.5% neutral norit and 1% dextran T70; in distilled water) was used.

column; then eluted with chloroform:heptane:ethanol (200:200:1, saturated with water) to separate androstenedione from testosterone. The second solvent system separated androstenedione (15–25 ml fraction) from testosterone (40–50 ml fraction). The tubes containing the highest radioactivity representing androstenedione or testosterone were quantified by RIA and compared with the results of RIA following benzene:hexane extraction without chromatography.

The data presented in this paper were statistically analyzed by analysis of variance. Factors considered were fetal sex, fetal age and source of blood for separate analysis of fetal and maternal data. Then a statistical comparison was made of fetal versus maternal data.

Results. Assay validation. Extraction efficiency of ^{3}H-testosterone averaged 83.0 ± 0.2% (SE), n = 240 and 87.5 ± 0.5%, n = 48 for ^{3}H-androstenedione. The sensitivity of the assays was less than 0.025 ng of testosterone or androstenedione, a quantity which displaced approximately 8% of the ^{3}H-androgen bound to the antibody. The specificity of the testosterone antibody was described by Ismail, Niswender and Midgley (11) and by Smith and Hafs (12). Specificity of the androstenedione antibody was tested by adding to assay tubes 0.1, 1, 10 or 100 ng of the steroids shown in Table I.

TABLE I. RELATIVE ACTIVITY[a] OF SELECTED STEROIDS IN THE RADIOIMMUNOASSAY FOR ANDROSTENEDIONE.

Hormone	Relative activity
Androstenedione (4-androstene-3,17, dione)	1.0
Testosterone (4-androstene-17β-ol-3-one)	0.214
Dihydrotestosterone (5α-androstan-17β-ol-3-one)	0.0086
Dehydroepiandrosterone (5-androstene-3β-ol-17-one)	0.0082
Estrone (1,3,5(10)-estratriene-3-ol-17-one)	0.0084
Estradiol-17β (1,3,5(10)-estratriene-3,17β-diol)	0.1069
Cholesterol (5-cholesten-3β-ol)	<0.0030[b]
Corticosterone (4-pregnene-11β,21-diol-3,20-dione)	<0.0030[b]
Cortisol (4-pregnene-11β,17α,21-triol-3,20-dione)	<0.0030[b]
Progesterone (4-pregnene-3,20-dione)	<0.0030[b]

[a] Relative activity = ng of androstenedione at 50%/ng of steroid at 50%.

[b] One hundred ng was the maximum amount of the steroid tested, therefore 100 was used for the denominator in the above fraction although 50% depression was not achieved.

As another means of validating the androstenedione and testosterone RIA's in fetal sera, we isolated testosterone and androstenedione in eight extracts of fetal serum samples and one steer serum on LH-20 Sephadex columns. The mean values determined by RIA for either androstenedione or testosterone after solvent extracts without chromatography did not differ significantly from mean values obtained by RIA after LH-20 chromatography (1.2 ± 0.5 vs 0.9 ± 0.3 ng/ml and 0.08 ± 0.02 vs 0.05 ± 0.01 ng/ml for testosterone and androstenedione, respectively).

The testosterone and androstenedione data presented in this paper were quantified in benzene:hexane extracts of serum without chromatographic separation.

Fetal and maternal androgens. The concentration of testosterone (μg/g of fetal testes) decreased ($P < 0.05$) from 0.96 ± 0.29 at 180 days to 0.39 ± 0.13 at 260 days. Comparable concentrations of androstenedione were 0.35 ± 0.19 and 0.50 ± 0.28 μg/g at 180 and 260 days. The testes from the 90-day fetuses were used for *in vitro* studies and testicular androgens were not quantified.

Serum testosterone concentrations were significantly ($P < 0.01$) higher in male fetuses than females (Table II). In addition serum testosterone was highest ($P < 0.01$) at 90 days for both males and females. Testosterone concentrations were similar in the umbilical artery and vein samples. Serum testosterone decreased ($P < 0.01$) from 2.76 ng/ml in 90-day males to an average of 0.60 and 0.21 ng/ml at 180 and 260 days gestation, respectively. In females serum testosterone decreased from 0.22 ng/ml at 90 days to barely detectable concentrations at 260 days of gestation.

TABLE II. SERUM TESTOSTERONE AND ANROSTENEDIONE CONCENTRATIONS IN BOVINE FETUSES.[a]

Hormone	Sex	Umbilical vessel	Days gestation 90[b]	180	260
			(ng/ml)		
Testosterone	Male	Artery	2.76 ± 0.48	0.71 ± 0.16	0.16 ± 0.06
		Vein	—	0.50 ± 0.04	0.26 ± 0.07
	Female	Artery	0.22 ± 0.09	0.07 ± 0.02	0.06 ± 0.03
		Vein	—	0.10 ± 0.03	0.02 ± 0.01
Androstenedione	Male	Artery	2.68 ± 1.11	1.01 ± 0.51	0.52 ± 0.10
		Vein	—	2.20 ± 0.94	1.15 ± 0.47
	Female	Artery	1.86 ± 0.51	0.55 ± 0.06	0.21 ± 0.05
		Vein	—	0.93 ± 0.28	0.44 ± 0.10

[a] Values are Means ± SE (n = 5–7).
[b] Samples by cardiac puncture for 90-day fetuses.

TABLE III. SERUM CONCENTRATIONS OF TESTOSTERONE AND ANDROSTENEDIONE IN THE UTERINE SERUM FROM PREGNANT HEIFERS.[a]

Hormone	Sex of fetus	Uterine vessel	Days gestation 90	180	260
			(ng/ml)		
Testosterone	Male	Artery	0.35 ± 0.05	0.59 ± 0.06	0.74 ± 0.10
		Vein	0.51 ± 0.03	0.86 ± 0.07	0.82 ± 0.11
	Female	Artery	0.13 ± 0.02	0.28 ± 0.12	0.37 ± 0.10
		Vein	0.14 ± 0.02	0.30 ± 0.08	0.31 ± 0.10
Androstenedione	Male	Artery	0.25 ± 0.13	1.00 ± 0.21	1.39 ± 0.35
		Vein	2.02 ± 0.48	3.96 ± 1.00	3.20 ± 0.78
	Female	Artery	1.00 ± 0.13	0.85 ± 0.14	1.15 ± 0.19
		Vein	4.55 ± 1.73	2.82 ± 1.14	2.35 ± 0.56

[a] Values are Means ± SE (n = 5–7).

Similar to serum testosterone, male fetuses had nearly two-fold higher serum androstenedione than females throughout gestation, and fetal androstenedione decreased ($P < 0.05$) as gestation advanced for both males and females (Table II). However, in contrast to serum testosterone, the umbilical vein androstenedione concentrations were greater than those in the umbilical artery in both males and females at 180 and 260 days of gestation.

Testosterone concentrations were similar in serum from the uterine artery and vein of the pregnant heifers (Table III).

Contrary to fetal concentrations testosterone in maternal blood increased ($P < 0.01$) as gestation advanced regardless of sex of the fetus. Heifers with male fetuses had greater ($P < 0.05$) serum testosterone in samples from the uterine vessels during gestation than heifers with female fetuses.

In contrast to testosterone, androstenedione was markedly greater ($P < 0.01$) in the uterine vein than in the uterine artery in pregnant heifers regardless of the sex of the fetus (Table III). Serum androstenedione did not differ in cows with male or female fetuses.

As in the case of fetal androstenedione, maternal venous blood leaving the placenta contained significantly ($P < 0.01$) greater androstenedione concentrations than arterial blood supplying the placenta.

Jugular samples from the pregnant heifers (Table IV) contained concentrations of serum testosterone similar to those from uterine vessel serum shown in Table III. Heifers with male fetuses also had greater

TABLE IV. TESTOSTERONE AND ANDROSTENEDIONE CONCENTRATIONS IN JUGULAR SERUM OF PREGNANT HEIFERS[a]

Hormone	Sex of fetus	Days gestation		
		90	180	260
		(ng/ml)		
Testosterone	Male	0.28 ± 0.04	0.60 ± 0.06	0.46 ± 0.12
	Female	0.05 ± 0.01	0.39 ± 0.19	0.36 ± 0.19
Androstenedione	Male	0.31 ± 0.16	0.65 ± 0.17	1.02 ± 0.13
	Female	0.70 ± 0.07	0.77 ± 0.23	0.73 ± 0.11

[a] Values are Means ± SE (n = 5–7).

testosterone in jugular sera than heifers with female fetuses. Androstenedione in jugular sera from pregnant heifers were similar regardless of fetal sex (Table IV), and were also similar to the androstenedione concentrations in sera from the uterine artery (Table III).

Discussion. Hormonal secretions by the fetal testis appear to be required for normal development of the male reproductive tract (13). Early work with rabbits verified that not only were the fetal testes required for differentiation of the male reproductive tract but fetal pituitary gonadotropins were required to stimulate androgen production by fetal testes (7). More recently androgens have been quantified in testes from fetal calves (14), fetal sheep (3), fetal rats (1), fetal monkeys (15) and fetal humans (16). The fetal testicular androgen concentrations reported here agree with those reported by Struck *et al.* (14) for cattle and by Attal (3) for sheep.

In contrast to testicular testosterone, androstenedione concentrations increased with fetal age in agreement with data by Struck *et al.* (14). The decrease in testosterone–androstenedione ratio in the fetal testes from 180 to 260 days may indicate that (a) more testosterone is being metabolized to androstenedione, or (b) testosterone synthesis is reduced as birth approaches.

Although both serum androgens were significantly higher in males than females, the difference between males and females was much larger for testosterone than androstenedione. Therefore testosterone may be the androgen responsible for masculinization of the male reproductive tract. The low concentrations of testosterone found in the sera from female fetuses may be from peripheral conversion of androstenedione to testosterone as suggested by Baird (17).

Previously, we reported (9) that LH concentrations in fetal sera were more than 50% lower for males than females at 90 days of gestation. At that time we speculated that LH concentrations in males were suppressed by fetal androgens. This hypothesis now has added support because fetal testosterone was tenfold higher in males than females. A similar inverse relationship between LH and testosterone in males and females was also evident at 180 days gestation. Possibly fetal male androgens may be associated with sexual differentiation of the hypothalamus as early as the first trimester in the bovine fetus. In addition when the 180-day pituitaries from these fetuses were removed and incubated *in vitro*, male pituitaries synthesized threefold more LH than those from females (18), suggesting that removing the pituitary from the influence of circulating androgens increased fetal pituitary LH synthesis. However, androgen suppression of LH synthesis by fetal bovine pituitaries has not been tested directly.

Androstenedione quantified in the fetal sera may have been synthesized by the placenta, because the concentrations in the umbilical vein were twofold greater than those found in the umbilical artery and threefold greater in the uterine venous blood than in the uterine arterial samples. Resko (15) previously reported that androstenedione concentrations were greater in uterine venous than systemic blood from pregnant monkeys. The possibility exists that androstenedione is a product of steroid metabolism by the placenta. For example, when

Milewich and Axelrod (19) incubated microsomes from baboon placenta with ^{3}H-testosterone they found that androstenedione was the major metabolic product.

Serum testosterone concentrations were greater in cows with male than female fetuses throughout gestation in samples from uterine and jugular blood, in agreement with data from pregnant monkeys (15). However, similar work involving human subjects (20, 21) did not show significant differences in testosterone concentration in maternal plasma as a function of sex of the fetus. This species difference suggests that androgens produced by the fetal testes may be transferred to the maternal system directly, or they may indirectly cause increased placental or adrenal testosterone synthesis in some pregnant mammals but not others.

Summary. Umbilical arterial and venous blood, and fetal testes were taken from 38 bovine fetuses at 90, 180 or 260 days of gestation. Concurrently blood also was taken from the jugular, and from the uterine artery and vein of the dams. Testosterone and androstenedione were determined by radioimmunoassays. Fetal testicular homogenates had 0.96 and 0.35 μg/g of testosterone and 0.39 and 0.50 μg/g of androstenedione at 180 and 260 days of gestation, respectively. Males had five to tenfold more serum testosterone and about twofold more androstenedione than female fetuses at each trimester of gestation. Male fetal blood testosterone decreased ($P < 0.01$) from 2.7 to 0.3 ng/ml between 90 and 260 days of gestation. But, maternal testosterone and androstenedione increased ($P < 0.05$) during gestation in cows with males, but not in cows with female fetuses. Testosterone was higher ($P < 0.05$) in cows carrying males than in cows with female fetuses. Androstenedione was higher in blood leaving the placenta on both the maternal and on the fetal sides suggesting placental synthesis of androstenedione.

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