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Fetal Programming: Prenatal Testosterone Treatment Causes Intrauterine Growth Retardation, Reduces Ovarian Reserve and Increases Ovarian Follicular Recruitment

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Exposure to testosterone (T) during d 30–90 of fetal life results in low-birth-weight offspring, hypergonadotropism, multifollicular ovaries, and early cessation of cyclicity. The multifollicular phenotype may result from failure of follicles to regress and consequent follicular persistence or, alternatively, increased follicular recruitment. We tested the hypothesis that prenatal exposure to excess T causes intrauterine growth retardation and increases ovarian follicular recruitment. Time-mated pregnant ewes were treated with 100 mg T propionate in cottonseed oil or vehicle twice weekly from d 30–90 of gestation. Ewes were euthanized near term, from d 139–141 of gestation (term is 147 d). After determining fetal measures and organ weights, ovaries were removed from fetuses of control and T-treated dams, and follicular distribution in each ovary was determined by morphometric quantification. Total number and percentage distribution of the various classes of follicles (primordial, primary, preantral, and antral follicles) were compared between treatment groups. Prenatally T-treated female fetuses were smaller in size, had an increased head circumference to fetal weight ratio ($P < 0.01$), increased adrenal to fetal weight ratio ($P < 0.05$), decreased number of follicles ($P < 0.05$), a decrease in percentage of primordial follicles ($P < 0.001$), and a corresponding increase in the remaining classes of follicles ($P < 0.05$). Ovarian findings support decreased ovarian reserve and enhanced follicular recruitment, potential contributors of early reproductive failure. The extent to which metabolic changes associated with intrauterine growth retardation contribute toward altered trajectory of ovarian folliculogenesis remains to be determined. (*Endocrinology* 146: 3185–3193, 2005)

FOLLICULOGENESIS, the developmental progression of an ovarian follicle from the primordial to the preovulatory state, is a key reproductive event in the female (1). In most mammals, folliculogenesis begins before birth and continues throughout reproductive life. Generally, the lifetime quota of follicles in the female is established at birth (2, 3), although recent evidence suggests the existence of proliferative germ cells capable of oocyte/follicle production in the postnatal mammalian ovary (4). The number of primordial follicles, which constitute the ovarian reserve at birth, the rate of replenishment during postnatal life (4), and the rate at which follicles are recruited dictate the functional ovarian life span of an individual (2–7). Many patients with galactosemia originating from a deficiency of the enzyme galactose-1-phosphate uridyltransferase show ovarian failure because of a decrease in initial follicle number (8). In contrast, fetuses with a single X chromosome, such as in Turner's syndrome, develop normal ovaries with the normal endowment of primordial follicles but undergo ovarian failure because of an accelerated rate of follicular recruitment (9). The rate at which germ cells and primordial follicles diminish is high in women undergoing radiotherapy suggestive of increased atresia (10). *In utero* exposure to irradiation also causes primordial oocyte loss in squirrel monkeys, mice, and rats (11). Cytotoxic therapies also lead to ovarian failure in women by disrupting follicular maturation and causing germ cell loss (11, 12). Autoimmune diseases such as Addison's lead to ovarian failure by involving autoantibodies that target steroid-producing cells (13, 14).

From a developmental perspective, in addition to genetic susceptibility, there are several threats to the normal ontogeny of fetal organ differentiation. These include poor nutrition (15–17), disease states (18), unintended exposure to steroids via contraceptive pills (19, 20), and exposure to endocrine-disrupting chemicals during early stages of fetal development (21–23). A developing fetus undergoes specific adaptations to changes in the intrauterine environment depending on the nature, timing, and intensity of extra- and intrauterine challenges. In the reproductive context, exposure of fetuses to adverse conditions at times when reproductive organs are differentiating may not only retard fetal growth, but also induce faulty or delayed developmental programming of reproductive tissues. In this regard, it is of interest that adolescent girls of Spanish origin born small for gestational age, suggestive of intrauterine growth retarda-

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Abbreviations: ER, Estrogen receptor; GDF, growth differentiation factor; IUGR, intrauterine growth retardation; PCOS, polycystic ovary syndrome; T, testosterone.

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tion (IUGR), were found to be at risk for anovulation, hyperinsulinism, subclinical hyperandrogenism, dyslipidemia, and central adiposity (24, 25), attributes of women with polycystic ovarian syndrome (PCOS) (26, 27). Paradoxically, young women born small for gestational age also had reduced ovarian volume and increased FSH levels (28), features not seen in women with PCOS. Two other studies have failed to associate low birth weight with subsequent appearance of PCOS in adult women (29, 30).

Fetal exposure to excess testosterone (T) leads to postnatal reproductive failure in females of many species (31–34). In sheep, prenatal exposure to T not only results in low birth weight and postnatal catch-up growth (35), but also culminates in reproductive deficits manifested as neuroendocrine defects (36–40), multifollicular ovarian development (41), and early reproductive failure (33, 34). Although development of multifollicular ovaries (41), the most conspicuous effect of prenatal T treatment in females, may explain, at least in part, the decrease in fertility observed in these animals (33, 34), the mechanisms regulating development of this aberrant ovarian phenotype are not well understood. Multifollicular ovaries in prenatally T-treated females may be the result of increased follicular persistence and/or recruitment. Evidence exists in support of a contributory role for follicular persistence in the development of the multifollicular phenotype in women with PCOS (42), the ovarian phenotype the prenatally T-treated female sheep mimic. On the contrary, studies that addressed follicular recruitment in women with PCOS have provided conflicting results; only one (43) of three studies (43–45) demonstrated increased recruitment. Considering that 1) small-for-gestational-age babies develop features of women with PCOS during adolescence and 2) offspring of T-treated sheep have low birth weight and develop attributes of women with PCOS, including multifollicular ovarian morphology, it is conceivable that fetuses of T-treated sheep will exhibit IUGR and display disrupted folliculogenesis. This study was designed to test this hypothesis.

Materials and Methods

Breeding and prenatal treatment

Two- to 3-yr-old Suffolk ewes were purchased locally and moved to a nearby United States Department of Agriculture-inspected and University of Michigan Department of Laboratory Animal Medicine-approved farm for breeding. The University Animal Care and Use Committee approved all procedures involving animals. Ewes were blocked by location of purchase and then randomly assigned to treatment. Starting 2–3 wk before and continuing until the time of breeding, ewes were group-fed daily with 0.5 kg shelled corn and 1.0–1.5 kg alfalfa hay per ewe to increase energy balance. Day of mating was determined by visual confirmation of a paint mark left by an intact ram on the hindquarter of bred ewes. After breeding, all ewes were maintained on pasture under natural photoperiod and supplemented with 1.25 kg alfalfa/brome mix hay per ewe. Ewe weights before mating averaged 80.6 ± 4.0 and 81.0 ± 3.2 kg (mean ± SEM) for control and T-treated groups, respectively. Beginning on d 30 of gestation, pregnant ewes were injected twice weekly in the musculature of the right shoulder with 100 mg T propionate (Sigma-Aldrich Corp., St. Louis, MO) suspended in cottonseed oil (Sigma-Aldrich) until d 90 of gestation. The dose and mode of T administration was chosen to reflect the large body of data available documenting postnatal reproductive disruptions (32, 34–39). Control ewes received vehicle alone.

Fetal measures

On d 140 ± 1 of gestation (term, 147 d), after euthanasia with a barbiturate overdose (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI), fetuses from control and T-treated dams were removed. A total of nine control and nine T-treated dams were euthanized to procure the desired number of dams with female fetuses. A total of 10 and 11 female fetuses were obtained from six control and seven T-treated dams, respectively. Accidental loss of both ovaries from a fetus from a T-treated dam (singleton pregnancy) resulted in six dams per treatment group for ovarian studies. At the time of euthanasia, the control and T-treated dams weighed 91.1 ± 4.2 and 92.7 ± 3.1 kg, respectively. Three control and two T-treated dams yielded only male fetuses. A total of six control males and nine prenatally T-treated male fetuses were also obtained from four control and seven T-treated dams. Hypothalami, pituitary, and testes from male fetuses were harvested and frozen for future studies. Fetal weights and growth measures of all fetuses were obtained, although only female fetuses were analyzed (males were not analyzed because of the small number of control dams with male fetuses). Growth measures included shoulder height (bottom of hoof to top of the withers), chest and head circumference, and crown-rump length (from the highest midpoint on the top of the head to the base of the tail). Organ wet weights (adrenal, kidney, spleen, liver, ovary, and uterus) were also recorded. All ovaries were snap frozen in a 2-methylbutane/dry-ice bath and stored at −80 C. The left femur of female fetuses from five control and six T-treated dams (collection of femurs from the fetuses of one control and one T-treated dam were inadvertently missed) was dissected free, stripped of excess muscle and connective tissue, and stored at −80 C until a later date. The femurs were then autoclaved for 10 min to facilitate the removal of all remaining excess tissue and allowed to dry at room temperature for 2 d before recording the weight, diameter, and length. The diameter of each femur was taken at the narrowest point on the shaft (see Fig. 2). The length of the femur was defined as the distance from the trochanteric fossa to the intercondyloid fossa.

Ovarian morphometry

Two sets of two adjacent 5-μm serial sections taken 480 μm apart were cut from each ovary (approximately one third and two thirds of the way through the ovary) at −20 C using a cryostat (Reichert-Jung 2800 Frigocut, Cambridge Instruments GmbH, NuBloch, W. Germany). Sections were mounted onto microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA), dried, and stored at −80 C until analysis. Frozen sections were thawed directly on a slide warmer at 45 C and fixed for 10 min in Bouin's fixative at room temperature. Sections were subjected to routine hematoxylin and eosin staining protocol and mounted with DPX (distyrene, tricresyl phosphate, and xylene) before morphometric evaluation of follicle number and oocyte diameter (46). Morphometric analysis of the percentage of follicles in different classes of development and oocyte diameter were performed using OpenLab (Improvision, Inc., London, UK) image analysis software as previously described (47). To identify follicles in different size classes, the follicle classification proposed by McNatty *et al.* (48) was used. Briefly, because the sections of the sheep ovary were large, each section was divided optically into nonoverlapping, multiple fields of vision. Follicles in various classes [primordial (fattened granulosa cells), primary (fewer than one complete layer of cuboidal granulosa cells), small preantral (with fewer than five layers of cuboidal granulosa cells), large preantral (with greater than five layers of cuboidal granulosa cells), and antral (with an antral cavity)] showing an oocyte with a nucleolus were counted in all fields for the entire section to avoid duplicate counting of the same follicle, and the protocol was repeated for remaining sections to obtain total number of follicles in the four sections of each ovary. This number was corrected for number of sections and section thickness using previously established criteria (4). Finally, the percentage of follicles in different classes was calculated relative to the total number of follicles. Oocyte diameter was computed from five randomly selected control and prenatally T-treated fetuses; only one fetus was chosen from a dam. Average oocyte diameter, expressed in micrometers, was determined from two perpendicular estimates of each oocyte showing a nucleolus.

Statistical analysis

The number of offspring born and sex distribution did not differ between groups. For all analyses, dam was considered the experimental unit, and data from fetuses from the same dam were averaged. Analyses of body measurements involved fetuses from six control and seven T-treated dams and ovarian measures from fetuses of six control and six T-treated dams. Data for body measurements (body weight, shoulder height, chest and head circumference, left femur measures, and ratio of head circumference to fetal weight), organ weights (ratio of adrenal, kidney, spleen, liver, ovary, and uterus to fetal weight), and total number of follicles (counted in the four sections as well as corrected for ovarian volume) were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). For these analyses, dam was considered a random variable. The observations for total number of follicles were independent, and the statistical model considered variation as a result of treatment only. The percentage of follicles and oocyte diameter for follicles in each size classification were analyzed as separate dependent variables in a repeated-measures model using the MIXED procedure of SAS. The statistical model for percentage of follicles in each size classification considered variation as a result of treatment, follicle type, and the follicle by treatment interaction term. In all cases, observations on the same ewe were permitted to have correlated random errors when fitting the linear model.

Results

Growth measures

Prenatal T treatment from d 30–90 of gestation resulted in a reduction of fetal weight (Fig. 1) near term ($P < 0.01$). In addition to the observed decrease in fetal weight, prenatal T treatment also led to a reduction in head and chest circumferences ($P < 0.01$). Differences were also observed in fetal height and crown-rump length ($P < 0.05$). A reduction in fetal growth for the prenatally T-treated group was also reflected as an increase in head circumference/fetal weight ratio ($P < 0.01$) and reduced growth of the left femur (Fig. 2).

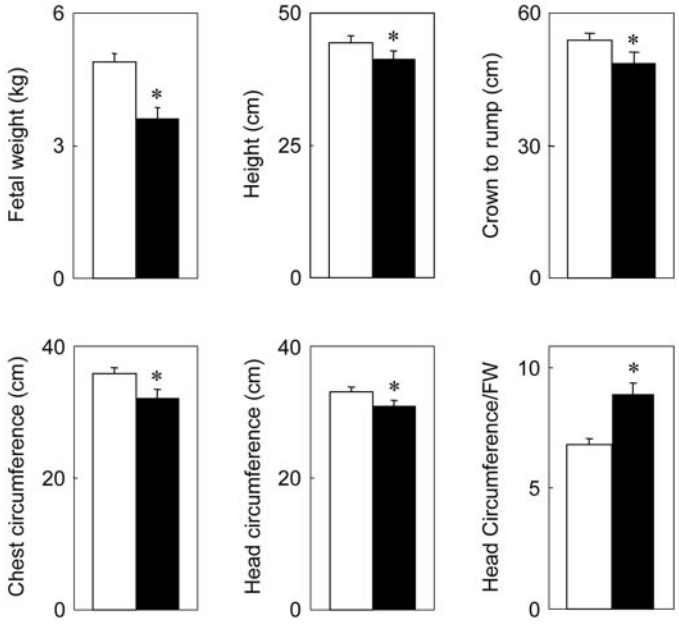

FIG. 1. Effect of prenatal T treatment (□, control; ■, prenatal T) from d 30–90 of gestation on the weight, height, and chest and head circumference (mean ± SE) of female fetuses at 140 ± 1 d of a 147-d gestation. *Asterisks* denote significant differences between control and prenatally T-treated females ($P < 0.05$).

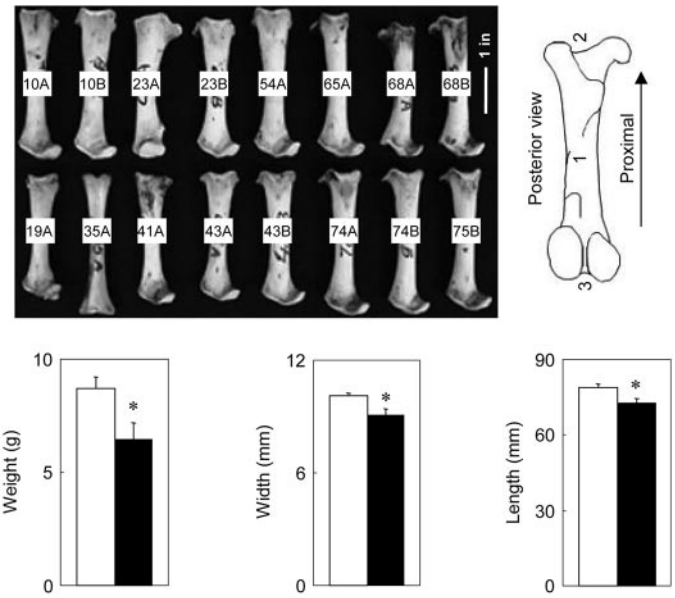

FIG. 2. Effect of prenatal T treatment (□, control; ■, prenatal T) from d 30–90 of gestation on the size (weight, diameter, and length) of the left femur of fetuses at 140 ± 1 d of gestation. Left femurs of female fetuses of six T-treated (*bottom*) and five control (*top*) dams are shown in the *top* panel (*scale bar*, 1 in). *Top right* shows schematic representation of a femur and the measures taken: 1, width; 2, trochanteric fossa; and 3, intercondyloid fossa. Distance between 2 and 3 denotes length. The *bottom* shows mean weight, width, and length of the femur. *Asterisks* denote significant differences ($P < 0.05$).

All measures (weight, diameter, and length) taken from the left femur were decreased ($P < 0.05$).

Organ weights

To evaluate the effects of prenatal T treatment on organ weight adjusted for variations in fetal weight, data were analyzed and are expressed as organ weight to fetal weight ratios (Fig. 3). The results showed a proportionate decrease in the weight of kidney, liver, spleen, and uterus in prenatally T-treated fetuses. In contrast, T treatment increased organ to fetal weight ratio for the adrenal (0.11 ± 0.013 *vs.* 0.172 ± 0.016; $P < 0.02$). Uterine and ovarian weights of prenatally T-treated females appeared to differ, but this difference did not achieve statistical significance ($P = 0.15$ and $P = 0.12$ for ovaries and uteri, respectively).

Ovarian measures

Photomicrographs depicting representative ovarian follicular distribution in two control and two prenatally T-treated fetuses are shown in Figs. 4 and 5. Ovaries of control females were observed to contain predominantly primordial and primary follicles (Fig. 4) and very few antral follicles (Fig. 5). In stark contrast to this picture of control ovaries, the ovaries from prenatally T-treated fetuses had multiple preantral and antral follicles (Fig. 5). These observed relationships, characterized by the presence of more developed follicles in T-treated compared with control fetuses, are reinforced by the summary statistics describing the distribution of follicles shown in Fig. 6. Although there were no differences in ovarian weight (Fig. 3), the total number of follicles per fetus was

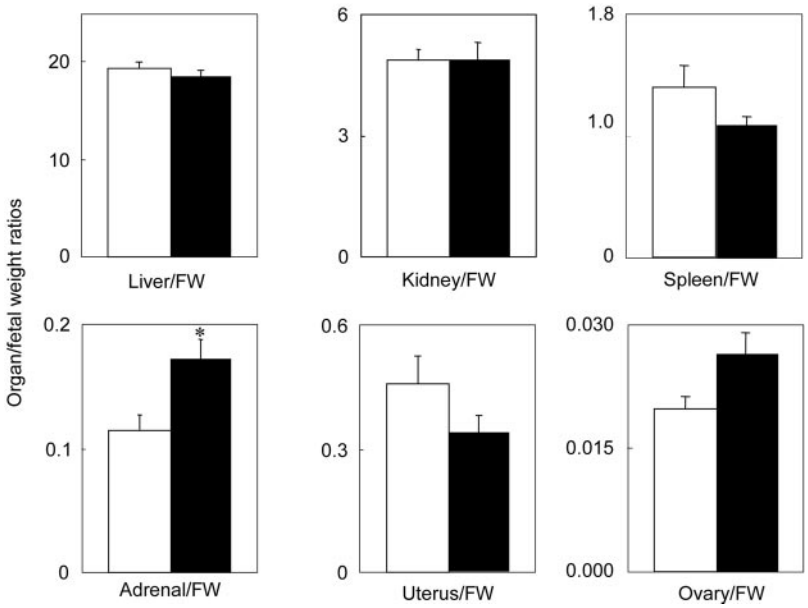

FIG. 3. Effect of prenatal T treatment (□, control; ■, prenatal T) from d 30–90 of gestation on organ weight/fetal weight (FW) ratio (mean ± SE) of female fetuses at 140 ± 1 d of a 147-d gestation. *Asterisks* denote significant differences from control females ($P < 0.01$).

reduced in prenatally T-treated females ($P < 0.05$) (Fig. 6, *left*). This reduction in ovarian reserve also held when the analysis was restricted to total follicles counted in the four sections (control *vs.* T-treated; $P < 0.05$). Ovaries from prenatally T-treated fetuses had a decreased ($P < 0.05$) percentage of primordial follicles when compared with controls. The observed decrease in the relative proportion of primordial follicles documented in ovaries of prenatally T-treated fetuses was offset by an increase in the percentage of primary, small preantral, preantral, and antral follicles (Fig. 6). The sum total of primary, small preantral, preantral, and antral follicles was greater in prenatally T-treated fetuses compared with controls ($P < 0.05$).

The oocyte diameter of both the primordial and primary follicles was greater in prenatally T-treated fetuses ($P < 0.05$) (Fig. 7). Once the follicles reached the small preantral size, no difference in oocyte diameter was observed. Additionally, no difference in oocyte diameter was noted for follicles classified as small preantral, preantral, or antral.

Discussion

Our findings demonstrate that prenatal exposure of sheep to T from d 30–90 of gestation results in 1) IUGR, 2) increased head circumference to fetal weight ratio, 3) increased adrenal to fetal weight ratio, and 4) a decrease in the relative proportion of primordial follicles that is associated with a complementary increase in the relative proportion of ovarian follicles in other size classes. Such differences in ovarian follicular distribution, observed in the face of reduced ovarian reserve in prenatally T-treated fetuses, suggest that prenatal exposure to excess T accelerates follicular recruitment. The implications of altered ovarian programming by prenatal T excess as it relates to 1) development of multifollicular morphology (41) and early cessation of cyclicity (33, 34), 2) the relative roles of androgen and estrogen in mediating ovarian programming, 3) IUGR, 4) etiology of ovarian disruptions characteristic of women with PCOS and women exhibiting premature ovarian failure, and 5) the threat to reproductive health posed by inappropriate prenatal exposure to steroid hormones, steroidal xenobiotics, or environmental steroids are discussed below.

Findings from this study clearly document that prenatal T treatment causes both quantitative and structural alterations in the ovary by d 140 of gestation that culminate in reduced ovarian reserve and the presence of a greater number of developmentally advanced ovarian follicles containing larger oocytes. With respect to the reduced ovarian reserve, it can be postulated that if the enhanced follicular recruitment seen at d 140 of gestation in prenatally T-treated fetuses continues throughout postnatal life and there is no compensatory increase in the rate of postnatal replenishment of follicles (4), then early follicular depletion and cessation of cyclicity would be the end result. Our studies (34) and those of others (33) found that prenatal T treatment culminates in early reproductive failure. Although the relative contributions of neuroendocrine (36–40) and ovarian defects (41) in mediating early reproductive failure remain to be determined, increased follicular recruitment may contribute, at least in part, to the development of the multifollicular phenotype of the prenatally T-treated females.

It is unclear whether the intrinsic differences in ovarian follicular distribution documented between control and prenatally T-treated fetuses evolved, in part, as a result of differences in gonadotropic input or are the result of direct programming at the ovarian level by either the androgenic actions of T or the estrogenic actions of its aromatized product. Machinery is in place for direct androgen and estrogen action at the ovarian level. Aromatase mRNA is expressed in the fetal ovary beginning on d 32–35 of gestation (49), as well as in the placenta, thereby providing a means by which T can be converted to estrogen (50, 51). Steroid-responsive cells are present within the sheep ovary as early as d 30–40 of fetal life (52). Androgen receptor mRNA is detectable in stromal cells of the medulla of the ovary on d 55 with the signal intensifying by d 75 of fetal life (McNatty, K., personal communication). Estrogen receptor (ER) β mRNA and protein

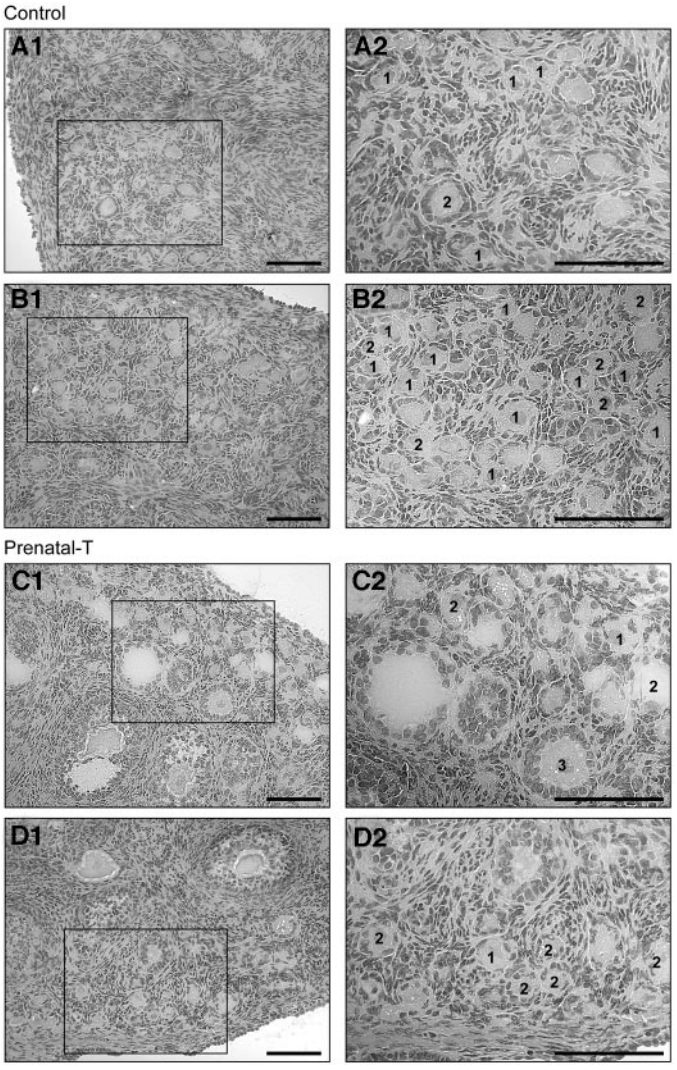

FIG. 4. Morphological features of cortical regions of ovaries from two representative control and two prenatally T-treated fetal sheep ovaries at 140 d of gestation. Ovaries from prenatally T-treated fetuses had more developmentally advanced follicles, whereas ovaries from control fetuses contained mostly nongrowing follicles. Frozen sections stained with hematoxylin and eosin are shown. The *right column* shows an enlarged (×200) view of the square overlays in the adjacent pictomicrographs in the *left column*. *Scale bars*, 100 μm. Only follicles showing an oocyte nucleolus were counted and are designated with a number: 1, primordial follicle (fattened granulosa cells); 2, primary follicles (fewer than one complete layer of cuboidal granulosa cells); 3, small preantral follicles (with fewer than five layers of cuboidal granulosa cells).

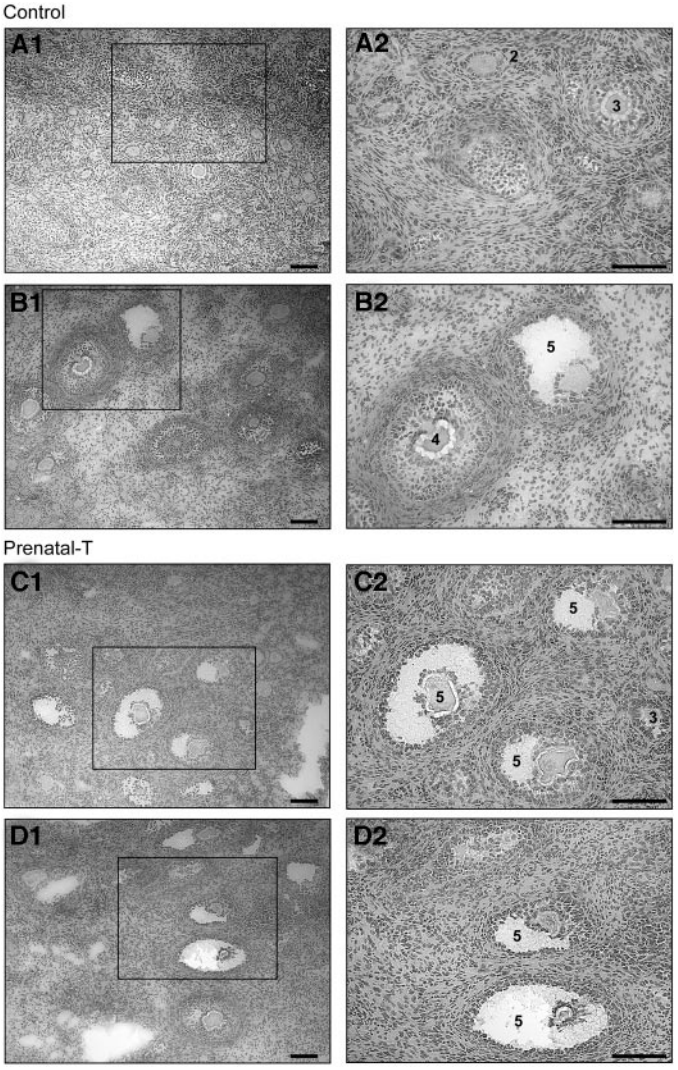

FIG. 5. Morphological features of inner regions of ovaries at 140 d of gestation from the same control and prenatally T-treated fetal sheep from Fig. 4. Frozen sections stained with hematoxylin and eosin are shown. The *right column* is enlarged (×400) to enhance viewing of the square overlays located in the adjacent pictomicrographs in the *left column*. Ovaries from prenatally T-treated fetuses contained more antral follicles. *Scale bars*, 100 μm. Only follicles showing an oocyte nucleolus are designated with a number: 2, primary follicles (fewer than one complete layer of cuboidal granulosa cells); 3, small preantral follicles (with no fewer than two layers but fewer than five layers of cuboidal granulosa cells); 4, large preantral (with no fewer than five layers of cuboidal granulosa cells); 5, antral (with an antral cavity).

are expressed in oocytes and granulosa cells of newly formed follicles on d 75 of fetal life (52). ERα protein is expressed as early as d 30 of fetal life on the ovarian surface epithelium and in cells entering the ovigenous cords that consist of oogonia-pregranulosa complexes (52). Based on ER, androgen receptor, and aromatase localization patterns and the time frame of prenatal T treatment, T and/or estrogen has the potential to act directly at the ovarian level beginning as early as d 30–40 of fetal life. Furthermore, because LH receptors are expressed on the theca cells at the time of preantral follicular differentiation and FSH receptors on granulosa cells during antral follicular development (53, 54), increased transition of primordial to primary follicles, as documented here, is likely to be facilitated by direct effects at the ovarian level rather than through changes in gonadotropin levels.

In terms of which aspects of ovarian differentiation are facilitated via androgenic or estrogenic effects of T, the absence of multifollicular morphology in prenatally dihydrotestosterone-treated sheep (41) suggests that expression patterns of genes responsible for enhanced follicular growth to the antral stage are likely to be programmed by estrogen after its conversion from T. Antral follicles of prenatal T- but not dihydrotestosterone-treated females do express higher

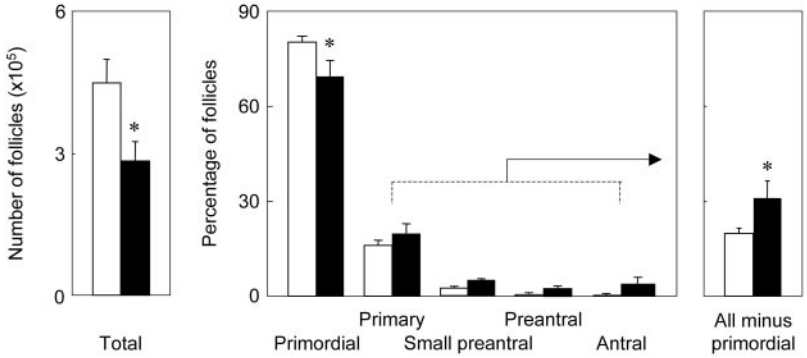

FIG. 6. Effect of T treatment from d 30–90 of gestation on the estimated total number of follicles in the ovary and distribution of follicles in fetal ovine ovaries at 140 d of gestation. Each *bar* represents a mean ± SEM. *Asterisks* indicate significant differences ($P < 0.05$).

levels of ERβ mRNA during postnatal life (Padmanabhan, V., and H. Jansen, unpublished data). On the other hand, which of these two steroids are involved in the programming of increased recruitment of primary from primordial follicles is unclear. Androgens are believed to facilitate early follicular differentiation (55). Studies in subhuman primates showed that androgens promote differentiation of primordial to primary follicles and implicated oocyte-derived IGF-I in this activation (56). Paradoxically, advanced follicular differentiation also occurs in mice treated prenatally with diethylstilbestrol (57), suggestive of estrogenic action. Irrespective of the steroid mediary, the target of ovarian programming is likely to involve changes in expression patterns of oocyte-derived factors. Interestingly, oocytes of primordial and primary follicles of prenatally T-treated females were larger in size compared with those of controls. Progression to the antral stage suggests that oocyte-derived factors such as growth differentiation factor (GDF)-9 and bone morphogenetic protein-15 are not limiting, because follicles in GDF-9 and bone morphogenetic protein-15 null mice fail to progress developmentally beyond the primary follicle stage (58). Because 1) Kit-ligand stimulates oocyte development (59, 60), 2) T in the presence of FSH maintains Kit-ligand expression in granulosa cells by overriding negative effects of oocytes on Kit ligand production (59), and 3) the marked increase in oocyte diameter that occurs in GDF-9 null mice has been attributed to increased production of Kit-ligand (61), one possibility is that *in utero* exposure to high levels of T stimulates oocyte growth by augmenting Kit-ligand production. Future studies measuring Kit-ligand expression in ovaries of prenatally T-treated females will help address this issue. Recent studies also implicate involvement of insulin in the transition of developmentally arrested primordial follicles to growing primary follicles (62). Furthermore, because formation of an antrum represents the transition from intrafollicular control of folliculogenesis to a combination of intrafollicular and endocrine regulation facilitated via acquisition of FSH receptors by granulosa cells (1), the role played by FSH in preventing atresia of recruited follicles and induction of the multifollicular ovarian phenotype described here remains to be determined.

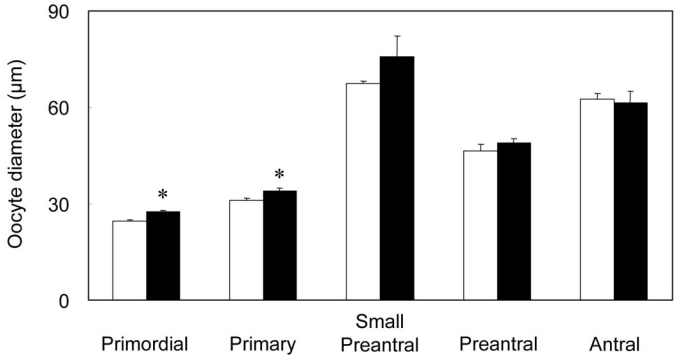

FIG. 7. Effect of T treatment from d 30–90 of gestation on oocyte diameter for follicles in fetal ovine ovaries at 140 d of gestation. Each *bar* represents a mean ± SEM. *Asterisks* indicate significant differences ($P < 0.05$).

Enhanced follicular recruitment may also stem from altered metabolic status associated with prenatal T-induced IUGR. Data presented here provide unequivocal evidence of IUGR induced by administration of T to pregnant sheep. This condition was manifested at the level of reduced fetal weight and increased head to fetal weight ratio. Although not the focus of this project, the potential for androgens to affect uteroplacental functionality either directly or indirectly, as androgen metabolites, must be considered as one possible source of differential growth effects described here. Selective changes in organ weight owing to extremes of gestational conditions that could affect nutrient delivery and/or partitioning *in utero* are well documented in several mammalian models of growth retardation. In this regard, it is not unusual for some organs, such as the brain, to be spared at the expense of visceral organs (63). In addition, several lines of evidence suggest that androgens support or stimulate neurogenesis, neuron survival, and even angiogenesis in the central nervous system (64–66). Thus, prenatal T treatment may have stimulated brain development and/or attenuated neuronal loss, thus contributing to a brain-sparing effect and the increased head to fetal weight ratio.

The increase in adrenal to fetal weight ratio can be interpreted to suggest that the developmental trajectory of the adrenal has undergone alterations in response to growth retardation programmed by excess T exposure. To what extent the enlarged adrenal activates the stress hormones and has an impact on ovarian development is unclear. For instance, gestational stress induced by handling of vixens reduces fetal ovarian weight and alters ovarian estradiol production (67). Whether the exaggerated cortisol responses of sheep fetuses subjected to maternal undernutrition has an impact on ovarian development is unknown (68).

Reduced ovarian reserve and increased follicular recruitment in the d 140 prenatally T-treated fetuses also represents

an altered developmental trajectory. Sheep are precocious in that follicular differentiation is completed during fetal life. By d 90–100 of fetal life, the number of primordial follicles in the fetal ovary corresponds to the lifetime maximum number of follicles that will form in the ovary (53, 54, 69). Our estimates of total number of follicles in control and prenatally T-treated fetuses suggest that the ovarian reserve is reduced in prenatally T-treated females. A decline in the proportion of primordial follicles and a corresponding increase in proportion of other classes of follicles in the d 140 prenatally T-treated fetuses suggest that increased follicular recruitment contributes in part to this decline. The extent to which this is a function of enhanced recruitment as opposed to reduced initial ovarian follicular reserve is unclear. Morphometric analyses of fetal ovaries at earlier time points will be required to address this question.

In assessing ovarian reserve, we calculated the total number of follicles from four sections per ovary and applied a correction factor to account for the number of 5-μm sections. The total length of the two ovaries based on the number of sections obtained from each ovary averaged 2290 $\pm$ 74 and 2362 $\pm$ 149 μm in control and prenatally T-treated fetuses, respectively. Considering that there were no differences in total weight of the two ovaries, and the location from which sections were taken was standardized across ovaries, the difference in ovarian reserve is likely to be real, although the absolute value obtained for total number of follicles may be overestimated. In the Booroola ewe, the total follicular complement is estimated to be approximately 100,000 by d 130 of fetal development (70). Investigators have counted follicles in cortical biopsies (43), defined areas of a subset of sections (71), or, alternatively, all follicles in a subset of sections and then applied different correction factors to arrive at absolute numbers (72). As recently highlighted by Tilly (72), all of these approaches are likely to yield accurate qualitative information, although absolute estimates will vary. In determining ovarian reserve and follicular distribution, the dam was used as an experimental unit because there were no treatment differences in number of offspring born or male/female distribution in twin pregnancies. Although the impact of male neighbors on sexual development of the female fetus has been reported in rats (73), earlier studies in sheep found no effects of twin pregnancy or sex distribution on body weight or ovarian characteristics of female fetuses obtained during early or mid-gestation (74).

In view of the similarities in reproductive characteristics of adult, prenatally T-treated female sheep with women diagnosed with PCOS (25, 26) and congenital adrenal hyperplasia (18), increased ovarian follicular recruitment documented here in fetal sheep exposed to excess T during d 30–90 of gestation suggests that the multifollicular phenotype observed in these reproductive disorders may stem, in part, from enhanced follicular recruitment. There is conflicting evidence in support of this premise. A reciprocal increase and decrease in the proportions of primary and primordial follicles, respectively, in ovaries of women with PCOS was reported in one study (43), although other studies can be interpreted to refute these findings (44, 45). Such differences may relate to the heterogeneity of PCOS women studied or, alternatively, to the site and extent of ovarian tissue biopsy used.

In conjunction with epidemiological and experimental studies linking low birth weight to diseases of adulthood, results of this study along with earlier studies, which document postnatal disruptions in reproductive function (33, 34, 36–40), suggest that fetal adaptations necessary to overcome growth retardation may prove to be detrimental to both reproductive performance and health. Our findings bring to the forefront threats to reproductive health of offspring posed by unintended or inappropriate exposure of pregnant women to excess steroids, naturally occurring steroidal compounds, and/or steroidal xenobiotics that can be encountered in the environment. Such exposures may stem from disease states (18), failed contraception, and continued exposure to contraceptive steroids (19, 20), use of anabolic steroids or inadvertent exposure to environmental compounds with estrogenic or androgenic activity (21–23). A cordocentesis study of 114 pregnancies in humans found that fetal serum T levels around mid-gestation (19–25 wk) were elevated to levels similar to those in the normal male fetal range in approximately four of 10 female fetuses sampled (75). These observations raise the possibility that such differences in the fetal steroid milieu may be responsible for marked differences in timing of cessation of fertility among human females. More recent studies have documented measurable levels of bisphenol-A, an estrogenic environmental endocrine disruptor, in amniotic fluid (76).

In summary, findings from this study clearly document that prenatal T treatment leads to IUGR and an alteration in developmental trajectory of ovarian follicle populations. The extent to which metabolic and hormonal responses associated with growth retardation contribute to the altered developmental trajectory of ovarian follicles as opposed to direct ovarian programming by steroids remains to be determined.

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