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CONTROLLED DELIVERY OF TESTOSTERONE PROPIONATE SUPPRESSES FERTILITY IN TREATED FEMALES AND INDUCES PRENATAL ANDROGENIZATION IN FEMALE OFFSPRING WITHOUT PHENOTYPIC MASCULINIZATION

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ABSTRACT

Four experiments were conducted on the controlled delivery of testosterone propionate in cattle and sheep. Blood testosterone concentrations were more consistent across time when silicone implants were used for delivery than when compressed pellets were used for delivery. Heifers with high testosterone concentrations were infertile. Female offspring born to heifers exposed to a consistent delivery of testosterone propionate, beginning before and continuing throughout gestation, had normal female phenotype. Prenatally androgenized females were at least as fertile as untreated heifers. Combined, the four experiments demonstrate that 1) testosterone suppresses fertility of treated females, and 2) phenotypic masculinization and sterility of female offspring can be avoided when androgens are delivered prenatally by controlled release implants regardless of stage of gestation when treated.

INTRODUCTION

Researchers are continually seeking methods to enhance meat animal growth and feed efficiency. In

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particular, enhancement of growth and feed efficiency of female cattle and sheep are desired since males, whether intact or castrate, exhibit faster rates of growth which are characterized by more protein and less fat accretion than that of females. One method that has been developed is prenatal treatment with androgens: prenatal androgenization.

Prenatal androgenization involves exposing in utero offspring to androgens during the "critical period" of sexual differentiation. The brain controls a variety of functions, including behavior, body weight, and hypothalamic/pituitary hormone secretions, that differ in males and females (9, 10, 17). During the "critical period" the brain sexually differentiates and male phenotype develops as a result of male androgens produced by the developing testes. Even genotypic males differentiate into phenotypic females if the males testes are not present or are ineffective (23). Exposing in utero female offspring to male androgens will induce varying degrees of differentiation from complete phenotypic masculinization (including the presence of a penis and an empty scrotal sac; 1, 19) to no phenotypic masculinization (3, 4, 6, 7). Growth rate and feed efficiency are enhanced by about 15 % (3, 4, 6, 7).

Two basic delivery procedures have been used to accomplish prenatal androgenization: injection or implantation of androgens. In general, offspring resulting when injections were administered early in gestation developed male phenotypic characteristics and had growth characteristics similar to males (16, 19). In one study (11), however, male phenotypic characteristics were not obtained but neither were improved growth characteristics. In fact, the average daily gain of the androgenized heifers from birth to weaning was less than for the control heifers (11). Implants were used to deliver the androgens in other studies. In all of those studies (3, 4, 6, 7), except one (1), the female offspring had female phenotype and enhanced growth characteristics. The exception, Clarke et al. (1), used compressed pellets to deliver testosterone as compared to the other studies that used silicone implants. Therefore, in all reported studies outside our laboratory (3, 4, 6, 7), enhanced growth characteristics were obtained in female offspring when they displayed male phenotype.

There are several factors that are potentially critical to prenatal androgenization: 1) dosage of androgen, 2) androgen used, 3) timing of administration (including when initiated and duration), and 4) delivery profile of the androgen administered. The following four experiments (in support with data in the literature) were conducted to demonstrate that a controlled delivery of androgen prenatally induces improved growth characteristics of female offspring without changes in phenotype. In addition, the effect of testosterone on the fertility of treated adult females was evaluated.

MATERIALS AND METHODS

Experiment 1. Six non-pregnant mature (non-growing) ewes were used for this study. Three testosterone compressed pellets were manufactured with a hand press with testosterone (mean = 1.001 g/pellet; C.V. = 1.03 %). Three capsule type silicone implants (10 cm in length) were manufactured with testosterone propionate (approximately 1.5 g of testosterone propionate per implant; C.V. = 0.75 %). Three ewes were implanted with one compressed pellet each in the neck (1) and three ewes were implanted with one silicone implant each in the axilla (3, 7). All implants were left *in situ* for 50 days. After removal, the implants were dried under heat (40°) for 72 hours and weighed to determine hormone loss *in vivo*.

Immediately before implantation (time 0), 1, 2, and 4 hours, and 1, 3, 7, 10, 14, 17, 21, and 50 days after implantation blood samples were collected for testosterone determination via a validated enzyme immunoassay (13).

The hypothesis of experiment 1 was that blood testosterone concentrations in animals administered androgens from silicone implants would be more consistent than in animals administered androgens from compressed pellets. Therefore, this could be a factor causing the differences observed in animals prenatally androgenized with these two procedures.

Experiment 2. Forty-eight (growing) yearling crossbred beef heifers were randomly assigned to four groups: 1) untreated controls, 2) heifers implanted with Synovex® H [compressed pellets containing testosterone propionate; one on day 0 and another on day 84], 3) heifers implanted with testosterone propionate silicone implants [one 15 cm

implant on day 0 and another on day 84], and 4) heifers implanted with two 15 cm implants on day 0. Synovex® H implants were placed subcutaneously on the convex surface of the ear and testosterone propionate/silicone implants were implanted subcutaneously behind the shoulder and over the dorsal aspect of the rib cage. Blood samples were collected via jugular venipuncture prior to treatment (day 0) and on days 28, 56, 84, 112, 140, 156 (day of implant removal), and on day 158. Concentrations of progesterone and testosterone were determined with validated enzyme immunoassays (13).

This study was conducted to determine the blood testosterone concentrations of heifers administered testosterone propionate via silicone implants or by compressed pellets and to determine if our hypothesis, that high concentrations of testosterone administration would suppress female fertility, was correct.

Experiment 3. Twenty-five crossbred beef heifers, approximately 15 months of age, were randomly assigned to treated (n = 13) or control (n = 12) groups. The treated heifers were subcutaneously implanted with four capsule type testosterone propionate implants (each 15 cm in length). The implants were placed behind the shoulder and over the dorsal aspect of the rib cage. Three days after the treated heifers were implanted, all females were exposed to a single fertile bull for 75 days. On days 36 and 102 after the beginning of the breeding season, serum samples were collected for the determination of testosterone concentrations using validated enzyme immunoassay (13). Blood samples collected on day 105 were also assayed for progesterone concentrations using a validated enzyme immunoassay (13). Approximately 3 weeks before the onset of the calving season the testosterone propionate implants were removed.

At calving the number, genotype, and phenotype of the resulting offspring were recorded. All female offspring were maintained. At approximately 13 months of age the female offspring (both treated and untreated) were administered Syncro-Mate B® to determine cyclicity (from blood progesterone concentrations [≥ 1.5 ng/ml] on day 13 after implant removal). At approximately 15 months of age the heifers were again administered Syncro-Mate B® and were artificially inseminated subsequent to synchronization. Females were observed for estrus over the next 30 days and were bred by artificial insemination subsequent to estrus. On day 30, females were exposed to

a fertile bull for the remainder of the 65 day breeding season. Pregnancy status was determined per rectum 45 days after the end of the breeding season.

The hypothesis of experiment 3 was that testosterone propionate exposure in a controlled and consistent manner would induce prenatal androgenization without complete masculinization regardless of how early in prenatal development the androgens were administered.

Experiment 4. Crossbred beef females (n = 235) were randomly assigned to treated or control groups 30 days after the end of a 60 day breeding season. Treated females were subcutaneously implanted with four capsule type testosterone propionate implants (each 15 cm in length). Implants were removed approximately 3 weeks before the onset of the calving season.

The resulting offspring (trial 1) of the testosterone propionate treated cows (n = 50) and of the untreated cows (n = 66) were weaned from their dams at approximately 7 months of age and were retained as replacement heifers. At approximately 12 to 14 months of the age the heifers were synchronized with Syncro-Mate B® (14). Heifers were artificially inseminated approximately 47 hours after norgestomet implant removal. Heifers that had subsequent estrus were bred either artificially or naturally for a 70 day breeding season. Pregnancy was determined per rectum 63 and 153 days after the timed breeding.

A second group (trial 2) of 71 cross-bred heifers (41 controls and 30 prenatally androgenized) were evaluated for fertility. At approximately 12 to 14 months of the age the heifers were synchronized with Syncro-Mate B® (14). Heifers were artificially inseminated approximately 47 hours after norgestomet implant removal. Forty-five days after the timed insemination pregnancy was determined per rectum. No additional data were collected from these heifers.

The hypothesis of experiment 4 was that offspring born to prenatal androgenization (with testosterone administered in a controlled and consistent manner) would have normal postnatal reproductive function.

Implants and Implantation. The silicone implants were made from medical grade silicone tubing[c] and testosterone

[c] Dow Corning Corporation, Midland, MI.

propionate[d] as previously described (12, 15). The medical grade silicone tubing had an internal diameter of .635 cm and an external diameter of .953 cm. After manufacturing the implants, they were rinsed with absolute ethanol and dried. They were then coated with a lyophilized antibiotic (Naxcel)[e].

The implants were surgically implanted without anesthesia with a scalpel, a hemostat to separate the skin from the subcutaneous tissue, and a suture to close the wound. The implantation area was cleaned and disinfected both immediately before and after implanting. Implants were surgically removed with a scalpel and a hemostat after cleaning and disinfecting the area.

Testosterone propionate was used in the silicone implants because of previous data (2) that demonstrated that about four times more testosterone propionate than testosterone diffused through silicone in a given period of time. Testosterone propionate is rapidly converted to native testosterone shortly after diffusion from the implant (21).

Blood Collection. Blood was collected using 10 cc syringes and 18 gauge needles 3.81 cm long. After collection, the blood was stored in glass culture tubes until centrifugation which was done within 6 hours after collection (24). Serum was harvested after centrifugation and stored in plastic vials at -20°C until it was assayed for progesterone and/or testosterone concentrations.

Syncro-Mate B®. Syncro-Mate B®[f] consists of implantation of a 6 mg norgestomet implant and injection of 5 mg of estradiol valerate and 3 mg of norgestomet on the same day. The implant is placed subcutaneously on the convex surface of one ear and left in situ for 9 days. Females are bred at a fixed time, 47 to 52 hours, after implant removal.

Synovex® H. Synovex® H[g] implants are compressed pellets that contain 200 mg of testosterone propionate and 20 mg of estradiol benzoate. They are used as anabolic implants for feedlot heifers.

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[d] Sigma Chemical Company, St. Louis, MO.
[e] The Upjohn Company, Kalamazoo, MI.
[f] Sanofi Animal Health, Overland Park, KS.
[g] Syntex Animal Health, Des Moines, IA.

Data Analysis. Qualitative data were analyzed by Chi-square analysis and quantitative data were analyzed by analysis of variance (22).

RESULTS AND DISCUSSION

Experiment 1. The testosterone pellets released 357.8 mg of testosterone. The testosterone propionate/silicone implants secreted 444.2 mg of testosterone propionate. This is equivalent to 372.9 mg of testosterone which is within 4.0 % of the quantity of testosterone release from the testosterone pellets.

Testosterone concentrations across time are illustrated in figure 1. There was a burst release detected for the ewes implanted with the pellets which was not detected for the ewes implanted with the testosterone propionate/silicone implants. Blood testosterone concentrations for both implantation methods were within the same general range although blood testosterone concentrations for the ewes implanted with testosterone propionate/silicone implants were higher than for the ewes implanted with testosterone pellets during most of the study period (mean testosterone concentrations on days 1 to 50 of 3.1 ng/ml and 4.7 ng/ml for pellet and silicone groups, respectively). Although testosterone concentrations increased more rapidly for ewes implanted with a testosterone pellet, the increase for the ewes implanted with silicone implants was also relatively rapid (concentrations were one-half of the day one sample 2 hours after implantation [hour +2 mean = 1.8 ng/ml]). Blood testosterone concentrations were less consistent for the testosterone pellet implanted ewes than for the silicone implanted ewes (see figure 1).

Although no controlled behavior tests were conducted, the three ewes administered the testosterone pellets became very aggressive during the study period. The three ewes administered the testosterone propionate/silicone implants did not display this aggressive/fighting behavior. We have previously demonstrated that an injection (and the resulting spike in testosterone concentrations) superimposed on the constant delivery of testosterone induced male sexual behavior (15, 18, 20). The spike in combination with the continuous release of the testosterone from the pellet may have been the cause of the aggressive/fighting behavior.

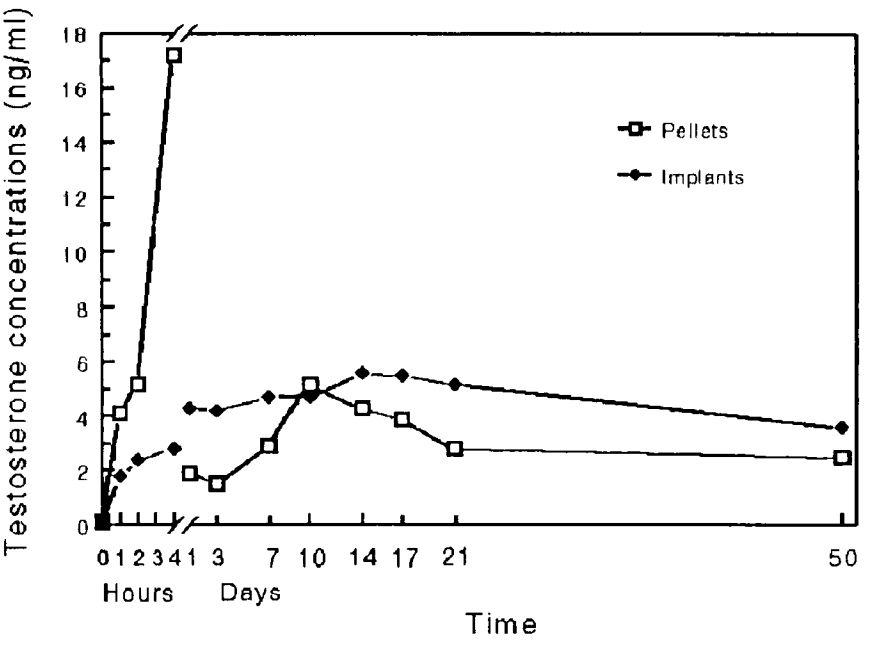

Figure 1. Blood testosterone concentrations in ewes administered testosterone compressed pellets or testosterone propionate silicone implants.

Experiment 2. Mean concentrations of testosterone are illustrated in figure 2. Testosterone concentrations, as expected, were greater when heifers were implanted with twice as many silicone implants and testosterone concentrations gradually declined with time. This decline was expected for two reasons: 1) heifers were increasing in weight therefore less hormone was being administered per kg body weight, and 2) less crystalline hormone was within the implant because of secretion and therefore less internal implant surface area to the testosterone propionate was available for diffusion. Testosterone concentrations fell to pre-treatment concentrations within 2 days after silicone implant removal. Synovex® H compressed pellet implants elevated testosterone concentrations but concentrations were four times more variable among animals within sampling days.

As reported in table 1, more heifers administered the two 15 cm implants at the onset of the study remained

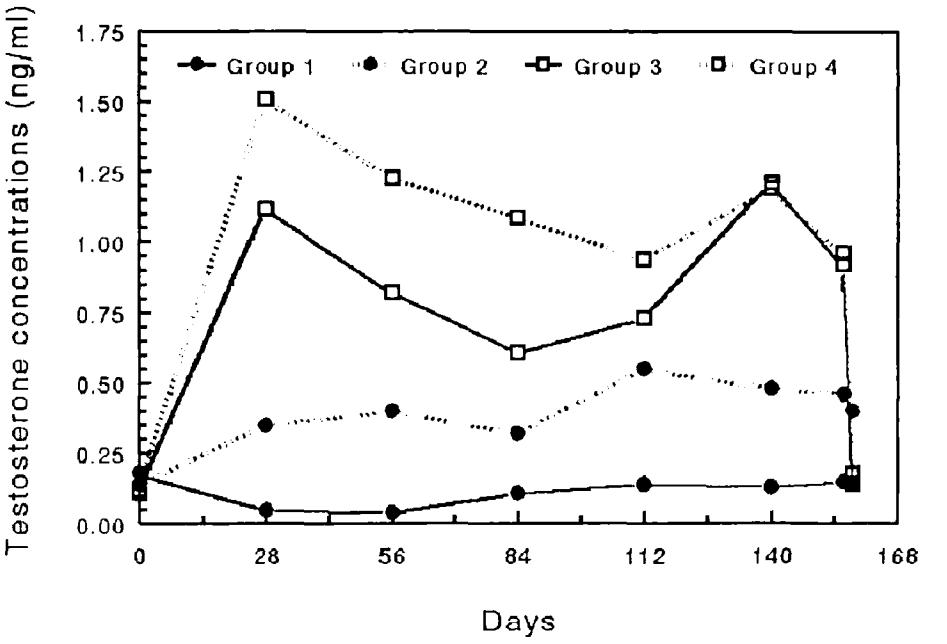

Figure 2. Blood testosterone concentrations in untreated heifers (controls-group 1), heifers administered testosterone propionate compressed pellets (one implant on day 0 and another on day 84-group 2), heifers implanted with testosterone propionate/silicone implants (one 15 cm implant on day 0 and another on day 84-group 3; two 15 cm implants on day 0-group 4).

TABLE 1. Effect of Testosterone Propionate Implants on Ovarian Cyclicity.

Group	Anovulatory	Days to First Increase in Progesterone > 1.5 ng/ml[c]
Untreated	2/12[a,b] (17 %)	100.0± 17.7[d]
Synovex® H	1/12[a] (8 %)	74.1± 11.5
TP[e](1+1 15 cm implants)[f]	1/12[a] (8 %)	91.1± 14.3
TP (2 15 cm implants)	6/12[b] (50 %)	90.9± 22.6

[a,b] Values with different superscripts differ (P < .05).
[c] Only for heifers that became ovulatory.
[d] Standard error.
[e] Testosterone propionate.
[f] Heifers received 1 15 cm implant on day 0 and a second 15 cm implant on day 84).

anovulatory during the study. This suppression on fertility was not detected in the other groups. The testosterone propionate and Synovex® H implants have previously been demonstrated to have anabolic effects and feed efficiency effects in feedlot heifers (8).

Experiment 3. As expected, the testosterone propionate implants were effective in elevating testosterone concentrations for an extended period of time (table 2). Fewer ($P < .01$) testosterone propionate implanted heifers became pregnant during the breeding season than control heifers (table 2). Progesterone concentrations were non-statistically ($P > .10$) reduced in testosterone propionate treated heifers. The majority of the testosterone propionate treated heifers that did not become pregnant (89 %; table 2) had progesterone concentrations suggesting that the treatment suppressed ovarian cyclicity. Heifers in both groups that became pregnant calved at similar times during the calving season. Closer examination of the testosterone concentration in the heifers that became pregnant vs those that did not become pregnant revealed an effect ($P < .05$) of testosterone concentrations. Testosterone concentrations on day 39 in heifers that became pregnant (2.97 ng/ml) were lower ($P < .05$) than in heifers that did not become pregnant (4.19 ng/ml).

Testosterone propionate treatment had no effect on the sex of the resulting offspring. At 13 months of age all heifer offspring, treated and untreated, were cyclic and 6 of the 7 heifer offspring became pregnant (table 2). This absence of male phenotype in the heifers was an important finding since these heifers were exposed to a constant amount of testosterone beginning at conception. Therefore, exposure to a controlled release of testosterone did not induce male phenotype. In this experiment there was no question that testosterone was present throughout the "critical period."

Experiment 4. Results (summarized in table 3) demonstrate that prenatal androgenization clearly had no adverse affects on reproductive function. In fact the first service synchronized pregnancy rate was higher ($P < .05$) for the prenatally androgenized heifers than for the control heifers. The fertility during the entire breeding season was similar between untreated and prenatally androgenized females. The increased fertility detected for the first synchronized breeding may have been caused by a hastening of puberty in the prenatally

TABLE 2. Effect of Testosterone Propionate on Blood Testosterone and Progesterone Concentrations, Genotype, and Pregnancy Rate of Treated Females and on Reproductive Function of Female Offspring.

Item	Control	Treated
Testosterone Concentrations:		
day 39 post-implantation	0.15 ng/ml	3.82 ng/ml
day 105 post-implantation	0.18 ng/ml	3.47 ng/ml
Progesterone:		
levels in pregnant heifers	6.95 ng/ml	4.97 ng/ml
number of non-pregnant heifers < 1.5 ng/ml	1/ 1	8/ 9 (89%)
Total Pregnancy Rate (%)	11/12[a] (92%)	4/13[a] (31%)
Mean Day of Birth for Offspring	Feb. 10	Feb. 17
Offspring Genotype:		
males	7	1
females	4	3
Offspring:		
Ovarian Cyclicity Status	4/4 (100%)	3/3 (100%)
Total Pregnancy Rate	4/4 (100%)	2/3 (67%)

[a] Values differ ($P < .05$).

TABLE 3. Effect of Prenatal Androgenization[a] on Reproductive Performance of Beef Heifers

Trial	Control		Treated	
First Service Synchronized Pregnancy Rate[b]				
1	21/ 65	(32%)	23/50	(46%)
2	14/ 41	(34%)	16/30	(53%)
Combined	35/106[c]	(33%)	39/80[c]	(49%)
Total Pregnancy Rate				
1	47/ 66[d]	(71%)	40/50	(80%)

[a] Treated heifers were administered testosterone propionate prenatally.

[b] Pregnancy rate to the Syncro-Mate B® synchronized first service.

[c] Values differ ($P < .05$).

[d] One heifer lost her Syncro-Mate B® implant and was not used for the first service synchronized pregnancy rate but was included in the total pregnancy rate.

androgenized females. This may be an indirect effect, however, since it is well established that the major controlling factor for the onset of puberty in ruminants is weight. We have previously demonstrated that prenatally androgenized females grow more rapidly (4, 6). However, other causes, such as direct effects, may be involved.

The purpose of these studies was first and foremost to demonstrate that controlled delivery of testosterone was necessary to evoke prenatal androgenization without phenotypic masculinization. Previously, all prenatally androgenized genotypic females that grew more rapidly were phenotypically male (11, 16, 19). All studies in which testosterone propionate was administered via implants produced phenotypic females that had enhanced growth and carcass characteristics (3, 4, 6, 7) with one exception (1). Clarke et al. (1) administered testosterone via compressed tablets and the resulting female offspring were phenotypically male. The cause of this variation, as demonstrated herein, is that compressed pellets do not exhibit the controlled delivery profile as silicone implants. When compressed pellets were administered, a short term spike, similar to a peak from an injection, resulted. Hence, implantation of the compressed pellets provided therapy similar to injection therapy. Therefore, the only method to produce prenatally androgenized female offspring without masculinization is to administer the androgen such that peaks and valleys in blood concentrations are avoided, i.e. controlled delivery.

Other factors, besides delivery profile, mentioned in the introduction included dosage, timing, and androgen used. DeHaan et al. (5) used a synthetic androgen and obtained poor results. Therefore, currently it is suggested that only testosterone or testosterone esters be used for prenatal androgenization. More research is needed to determine the minimal and maximal dosage. However, since phenotypic masculinization is obtained with injections and compressed pellets, higher doses used, even in a controlled delivery format, will likely cause phenotypic masculinization. Time of initiation and duration of therapy are important factors. Based on the literature, we conclude that therapy should be initiated by about day 30 to 60 in sheep and by day 40 to 80 in cattle and be continued for approximately three weeks or more.

Other findings in these studies were 1) that prenatal androgenized heifers have a higher fertility and 2) that a controlled delivery of testosterone propionate will cause sterility in the treated heifers if the dosage is sufficiently elevated. We have not previously seen a sterility effect in treated cows (4, 6) because we have only treated pregnant cows. The enhanced fertility rates in the prenatal androgenized heifers is another advantage of prenatally androgenizing female offspring (both those intended for the feedlot and those used as replacement heifers).

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