Endocrinology, 2023, **164**, 1–10
https://doi.org/10.1210/endocr/bqad145
Advance access publication 28 September 2023
Research Article

Puberty Suppression Followed by Testosterone Therapy Does Not Impair Reproductive Potential in Female Mice

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Abstract

More adolescents are coming out as transgender each year and are put on puberty blockers to suppress natal puberty, which is then followed by cross-hormone treatment to achieve puberty of the desired gender. Studies to examine the effects of puberty suppression and virilizing therapy on future reproductive potential among transgender males are lacking. This study used a translational murine in vitro fertilization model to examine the effects of female puberty suppression with depot leuprolide acetate (LA), followed by virilizing therapy with testosterone cypionate (T), on embryologic and pregnancy outcomes. LA effectively inhibited puberty when mice were treated beginning at 3 weeks of age. LA treatment was associated with higher mouse weight but lower ovarian weight. LA-treated mice ovulated developmentally competent eggs in response to gonadotropin administration, albeit at a higher dose than controls. Ovaries from mice treated with LA and T produced oocytes that had morphologically normal meiotic spindles after in vitro maturation and responded to gonadotropin stimulation. Eggs from mice treated with LA and T were fertilizable and produced developmentally competent embryos that led to births of fertile pups. These results suggest that fertility may not be impaired after puberty suppression and cross-hormone therapy for transgender males.

Key Words: transgender, fertility, leuprolide acetate, testosterone, mouse model, ovary

Individuals who are transgender identify with the gender opposite of the sex they were assigned at birth [1-3]. Among adolescents, a recent study of almost 81 000 students in grades 9 to 11 found that 2.7% identify as transgender or gender nonconforming [4]. Sixty-eight percent of these individuals were assigned female at birth and are referred to as transgender males [5].

Many transgender males opt to undergo some form of gender-affirming treatment [3, 6]. This often involves administration of testosterone, which provides desired secondary sex characteristics such as lowered voice and facial hair growth and also has positive effects on mood and behavioral health [2, 7]. Prepubertal transgender males have the option of using puberty blockers to avoid the development of incongruous female secondary sex characteristics [8, 9]. Puberty blockers such as the gonadotropin releasing hormone (GnRH) agonist leuprolide acetate inhibit the pulsatile secretion of GnRH that heralds puberty and has the effect of blocking estrogen production, which leads to unwanted secondary sex characteristics such as breast development [10]. When ready to begin the physical transition to male, the patient begins testosterone therapy to develop male secondary sex characteristics.

A possible negative consequence of prolonged hormone therapy, however, is a decrease in fertility potential. Human studies have noted changes in the structure of ovarian tissue, including follicular atresia as well as stromal and cortical hyperplasia, among transgender men utilizing testosterone therapy [11, 12]. At the follicular level, high levels of endogenous or exogenous androgens can inhibit follicular growth, especially among mature antral follicles [13, 14]. Ovaries in female mice treated with testosterone weigh significantly less than those of control mice [15], though they contain normal complements of follicles at all stages [15, 16].

It has been estimated that approximately half of transgender adults desire biological children [17, 18], although they may not desire to carry a baby themselves but rather to obtain eggs that will be fertilized and carried by a partner or surrogate [19]. The long-term consequences of testosterone on fertility in transgender males are still unclear, and both the World Professional Association for Transgender Health and the Endocrine Society recommend that all transgender and gender-nonconforming patients be counseled regarding options for fertility preservation before initiating transition [1, 2]. However, use of fertility preservation techniques among young transgender individuals is low [20], reflecting the fact that among transgender youth, beginning their gender transition with hormones typically takes priority over the costly, invasive, and time-consuming process of fertility preservation [21]. Therefore, the problem of fertility preservation in transgender males on hormone therapy warrants more investigation, given that few large studies have investigated outcomes of these individuals desiring to cryopreserve oocytes

Received: 14 December 2022. **Editorial Decision:** 21 September 2023. **Corrected and Typeset:** 3 October 2023

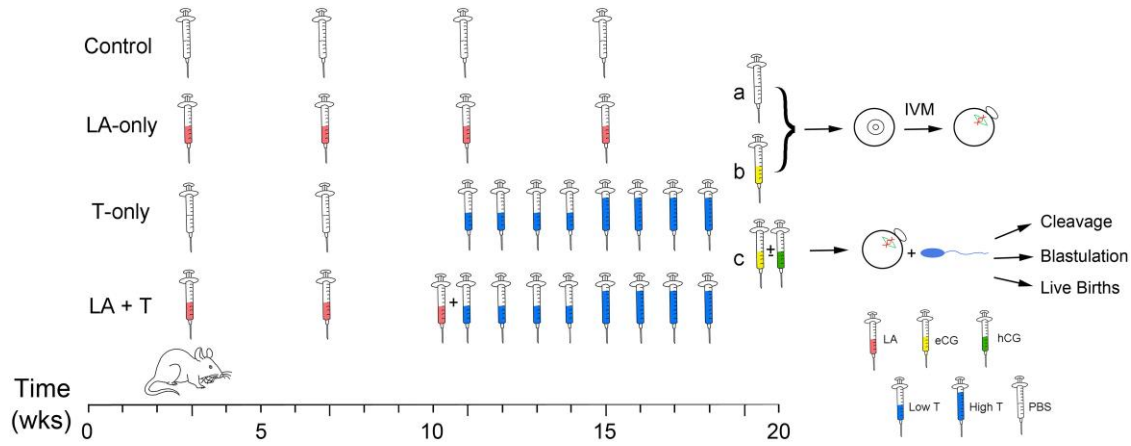

Figure 1. Overview of experimental design. (A) Mice injected with PBS; (B) mice injected with eCG only; (C) mice injected with eCG and hCG. Abbreviations: eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin; IVM, in vitro maturation.

after years of exposure to hormones. Particularly, the impact on future fertility potential of administering drugs to block puberty followed by testosterone therapy, which bypasses female puberty altogether, is completely unknown.

In this study, we utilized a mouse model to investigate the effects of bypassing female puberty using puberty blockers followed by the induction of virilization with testosterone on ovarian function and embryologic outcomes.

Materials and Methods

Ethical Approval

Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences 1996) and were approved by the Institutional Animal Care and Use Committee at UConn Health (protocol number 101977-0122).

Media and Reagents

All chemicals were purchased from Millipore Sigma (St. Louis, MO, USA) unless otherwise indicated. Leuprolide acetate (LA; 3.75 mg 1-month depot suspension) was from AbbVie Inc. (North Chicago, IL, USA) [22]. Depot LA was reconstituted as directed, aliquoted, frozen on liquid nitrogen, and stored at −80°C. LA was thawed on the day of use and diluted further with PBS. Testosterone cypionate (T; Steraloids, Newport, RI, USA) was prepared as a low-dose (4 mg/mL; 200 µg dose) and a high-dose (8 mg/mL; 400 µg dose) solution in sesame oil. Equine chorionic gonadotropin (eCG) was from Calbiochem (San Diego, CA, USA) or from ProSpec (East Brunswick, NJ, USA).

Experimental Design

Female CF1 (Envigo, Indianapolis, IN, USA) and male B6SJLF1 mice (Jackson Labs, Bar Harbor, ME, USA) were used for experiments, and female CD-1 mice (Charles River, Raleigh, NC, USA) were used as surrogates for embryo transfers. These strains are commonly used for studies of fertilization. Up to 5 female CF1 mice were housed per cage in a temperature- and light-controlled room on a 12 light:12 dark light cycle. Male mice were housed in pairs. CD-1 female surrogates were housed individually.

An outline of the experimental design is shown in Fig. 1. Three-week-old female mice were injected intraperitoneally with either depot LA (100 µg in 100 µL PBS) or vehicle for pubertal suppression. This dose is approximately 3 times higher by weight than that given to prepubertal transgender children to suppress puberty [9] and in preliminary experiments was determined to suppress puberty for extended periods in mice (data not shown).

Mice were injected with LA or PBS every 4 weeks for a total of 3 doses. Concurrently with the third dose of LA or PBS, low-dose T (200 µg) or vehicle (sesame oil) were injected weekly for 4 weeks, followed by 4 weekly injections of high-dose T (400 µg) or vehicle to simulate virilizing therapy. The concentrations and doses of T administered were chosen based on previously published studies utilizing transgender mouse models [15, 16]. For the T injections, mice were lightly sedated with isoflurane and injected subcutaneously using 27-gauge needles. For mice receiving LA therapy only, a fourth injection of LA was given 4 weeks after the third dose of LA to continue pubertal suppression; these mice were not exposed to T and therefore could undergo puberty while other mice were undergoing 8 weeks of T therapy without continued pubertal suppression with LA. This protocol was implemented to simulate conditions under which transgender males generally are treated with puberty-suppressing agents and subsequent virilizing agents. To compare our results with previous reports [15, 16], 1 group of mice was injected weekly with testosterone only (no LA), beginning when the mice were 11 weeks old.

Superovulation and in vitro fertilization (IVF) were performed approximately 5 days after the last injection of high-dose T, when these mice were ~19 weeks old. Mice were then subdivided into 3 more groups: mice that were not stimulated with gonadotropins, mice that were stimulated with eCG only, and mice that were stimulated with eCG followed by human chorionic gonadotropin (hCG) to induce ovulation (Fig. 1).

All mice were euthanized by isoflurane overdose followed by cervical dislocation. The following markers of response to treatment and ovarian stimulation were analyzed: serum testosterone and estradiol-17β (E2) levels, ovary weights and histology, structure of the meiotic spindle following in vitro oocyte maturation, total numbers of ovulated eggs, cleavage

rates, live birth rates, and fertility of the pups. Here "oocytes" are defined as prophase-arrested immature cells, while "eggs" are defined as mature cells at the metaphase II stage that had extruded a polar body.

Vaginal Cytology

Daily vaginal smears were performed to evaluate whether mice were undergoing estrous cycles. Smears were conducted on a subset of mice after the first week of high-dose T and were continued for 3 weeks. Vaginal smears were performed using standard methods [23, 24].

Blood Collection and Hormone Analysis

After the mice were sacrificed, they were weighed and terminal blood was collected by cardiac puncture using heparinized 25-gauge needles and syringes. For comparison to male levels of T, blood was also collected from mature male mice that were used for IVF (see later discussion). These males had been acclimated to the animal care facility for at least 1 week prior to the experiment, were housed in pairs, and were not exposed to females prior to blood collection. Blood samples were kept on ice. Within 30 minutes of collection, samples were centrifuged at 4°C for 15 minutes (1000 × g) and supernatants were stored at −80°C. Extraction of steroids was carried out with methanol as directed by Cayman Chemical's technical support, and extracted samples were stored at −80°C in ELISA buffer.

Total testosterone was measured using an ELISA kit from Cayman Chemical (Ann Arbor, MI, USA; catalog no. 582701, RRID:AB_2895148) according to the manufacturer's instructions. Samples were analyzed twice in 2 separate batches, and the mean of each value was reported. This assay is selective for testosterone but also interacts with high affinity to 19-nortestosterone (140%) and with less affinity to other testosterone derivatives (27% with 5α-dihydrotestosterone, 19% with 5β-dihydrotestosterone, 5% with methyl testosterone, 4% with androstenedione, and 2% with 11-keto testosterone, as well as trace amounts with other testosterone metabolites). Importantly, there is <0.01% cross-reactivity with E2 (manufacturer's product information sheet). The sensitivity was 6 pg/mL, and intra- and inter-assay coefficients of variation at the midpoint concentration were 6%, and 7.2%, respectively.

E2 was measured using an ELISA kit from Cayman Chemical (catalog no. 501890, RRID:AB_2832924) according to the manufacturer's instructions. This assay is selective for E2 with less cross-reactivity to metabolites [2.5% methoxyestradiol, 2.3% estradiol 3-(β-D-glucuronide); 1.4% estrone, 1.3% 2-hydroxyestradiol, 1% estriol, and <1% of others, and 0.03% cross-reactivity to testosterone; manufacturer's product information sheet]. The sensitivity was 20 pg/mL, and intra- and inter-assay coefficients of variation at the midpoint concentration were 12% and 7%, respectively.

Ovarian Histology

Ovaries from unstimulated and eCG-primed mice were collected; the adipose and oviducts were removed by dissection under a stereoscope and weighed. The smaller ovary from each mouse was used for ovarian histology while the larger ovary was used for oocyte collection. The ovary for histology was fixed in 10% formalin for 24 to 48 hours, washed in PBS, dehydrated in 70% ethanol, and embedded in paraffin. Serial 5 μm-thick sections through the entire ovary were stained with hematoxylin and eosin and were imaged using either an Aperio GT 450 slide scanner (Leica Biosystems, Danvers, MA, USA) or a 3DHISTECH Pannoramic MIDI II Automatic Digital Slide Scanner. Histology was performed by the Histology Core at UConn Health or by Ichor Life Sciences, Inc. (Lafayette, NY, USA). Figures were prepared using Adobe Photoshop.

Total numbers of corpora lutea (CL) were counted independently by 2 investigators and these counts were averaged together. Counts were similar for the 2 investigators. CL were defined as discrete eosinophilic round structures. CL were numbered as the sections were serially assessed through the entire ovary to prevent duplicate counting.

Oocyte Collection and Immunofluorescence Staining

The ovary for oocyte collection was placed in HEPES-buffered MEMα [Gibco 12000022, Thermo Fisher Scientific, Waltham, MA, USA [25]] containing 10 μM milrinone to prevent spontaneous oocyte maturation and was punctured using a 30-gauge needle. Oocytes were collected, and cumulus cells were mechanically stripped using a small-bore glass pipet. For in vitro maturation, oocytes were washed into bicarbonate-buffered MEMα [25] containing 5% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and were incubated overnight at 37°C in a humidified incubator containing 5% CO_2/95% air. Completion of oocyte maturation was confirmed by the disappearance of the nuclear envelope and the formation of first polar bodies using a stereoscope. For immunofluorescence, in vitro-matured eggs were fixed, extracted, and stained as previously described [15]. Eggs were imaged with a Zeiss Pascal confocal microscope with a 40×, 1.2 NA water immersion objective (C-Apochromat; Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

IVF and Embryo Transfer

Mice were superovulated by priming with 5 to 10 IU eCG, followed 48 hours later with 5 to 10 IU hCG. Approximately 13 to 15 hours later, cumulus masses were obtained from the oviducts by puncturing the swollen ampullae. Cumulus masses were incubated in 200 μL drops of human tubal fluid (Cook Medical Inc., no. K-RVFE) containing 250 μM reduced glutathione for ~30 minutes prior to adding sperm. Sperm were collected from the epididymides of a single male mouse by gently snipping with fine scissors into a 100 μL drop of IVF medium [26] containing 15 mg/mL Fraction V BSA (Calbiochem) and were capacitated for 1 to 2 hours before adding 2 to 3 μL of the sperm suspension to the drops containing the eggs. The sperm and eggs were incubated together for 4 hours, then were washed into 200 μL drops of human tubal fluid without glutathione. Eggs that were fragmented were discarded and were not counted in the total number of eggs per mouse. Fertilized eggs were cultured overnight in a humidified incubator at 37°C with 5% CO_2, and O_2 adjusted to 5%. The next day, 2-cell embryos were counted, transferred to KSOM medium (MR-101-D, Sigma), and cultured for 3 days in the low (5%) O_2 incubator. On day 4 after IVF, blastocysts were noted. Blastocysts were defined as embryos with a distinct cavity, inner cell mass, and trophectoderm. Cleavage rates were calculated as the number of 2-cell embryos divided by the total number of ovulated eggs × 100.

In a total of 3 experiments, cleavage-stage embryos were transferred to pseudopregnant females. Up to 15 2-cell embryos were transferred into each oviduct of pseudopregnant CD-1 females for up to 30 embryos total per surrogate dam. Dams were examined to confirm pregnancy 2 weeks after embryo transfer, and dams delivered 3 weeks after embryo transfer. Live birth rate, calculated as the number of live born pups divided by the number of embryos transferred × 100, as well as male to female ratios, were calculated. The pups were housed with the surrogate dam for approximately 3 weeks until they were weaned and then were transferred to cages in which up to 5 mice were housed per cage. To evaluate the fertility of the pups, siblings were mated and live births were counted.

Statistical Analysis

Statistical analyses were performed using Prism 6.0 software for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). All data were continuous, and error bars represent the SEM. One-way ANOVA was used to determine differences between 3 or more groups, with multiple comparisons analyzed using Tukey's multiple comparisons test. $P < .05$ was considered to be statistically significant.

Results

LA Suppresses Puberty in Female Mice

We first confirmed that LA at a dose of 100 µg every 4 weeks inhibits puberty in female mice by analyzing daily vaginal smears initiated after the second LA dose was given, when mice were 9 weeks old. Mice started on LA at 3 weeks of age displayed persistent cornified cells and/or leukocytes in the vaginal epithelium at 7 to 8 weeks of age, indicating that these mice were not cycling (data not shown) [27]. Ovaries from 9-week-old mice treated with 2 monthly doses of LA also lacked CL as determined by ovarian histology [Supplementary Fig. S1A [28]]. The ovaries from LA-treated mice appeared similar to the ovaries of untreated 3-week-old female control mice [Supplementary Fig. S1B [28]]. Vaginal smears indicated that control mice were cycling, and CL were also observed in histological sections [Supplementary Fig. S1C [28]]. As CL typically remain for up to 8 days after ovulation in the mouse [24], these results suggest that LA-injected mice were not cycling for at least 8 days prior to evaluation.

Mice treated with T using a low-dose/high-dose regimen (see *Methods* and Fig. 1) had elevated T levels compared to controls at the termination of the experiment, with levels typically between 0 and 1 ng/mL among untreated mice and between 1 and 6 ng/mL among treated mice. These T levels were within the normal range measured in male mice in our study, 0 to 20 ng/mL, which is consistent with previous reports in the literature (Fig. 2) [29]. It should be noted that because we measured T levels at the termination of the experiment, and ~1 week after the last dose of T was injected, these values are in the trough phase, assuming that testosterone is gradually metabolized. In transgender males, the peak T concentration occurs 1 to 3 days after T injection, and many clinicians prefer to test T levels at the trough when making clinical decisions due to more consistent measurements [30].

Control mice that were not treated with LA or T had estrous cycles based on vaginal cytology, and their ovaries contained numerous CL (Fig. 3A, 3B, and 3F). Mice treated with LA or LA + T did not appear to cycle as determined by the lack of proestrus/estrus on vaginal smears (Fig. 3A). However, 2 out of 3 of the ovaries from LA-only mice and 5 out of 9 of the ovaries from LA + T-treated mice that were examined histologically showed the presence of a few CL (Fig. 3C and 3F). Mice injected with T but not LA showed a persistent mix of cornified cells and leukocytes on vaginal smears, consistent with previous findings [15, 16]. However, 25% (2/8) of these mice showed the presence of large, nucleated cells indicative of proestrus, suggesting they could be cycling. Correspondingly, the ovaries from mice showing nucleated cells on vaginal smears had high numbers of CL on histology, which is not in accordance with previous reports (Fig. 3D and 3F; see *Discussion*). Overall, our results clearly demonstrate that LA significantly inhibits ovary growth and suggest that LA effectively suppresses puberty in female mice.

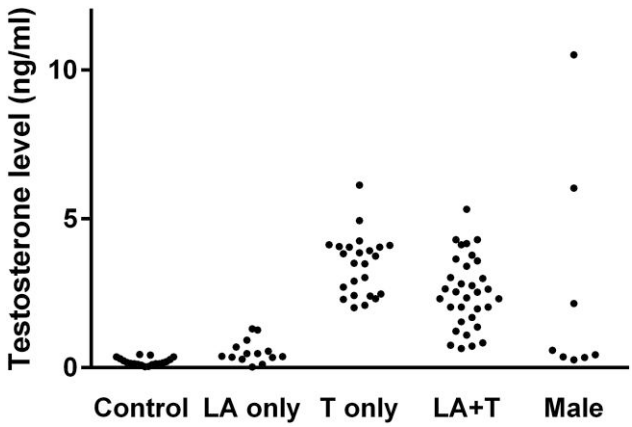

Figure 2. Testosterone levels in untreated mice, mice treated with testosterone cypionate, and males.

Pubertally Suppressed Female Mice Weigh More, But Ovaries are Smaller, Than Control Mice

Mice treated with LA weighed slightly but significantly more than control mice of the same age (Fig. 4A), which is consistent with previous reports of mice treated with GnRH agonists [31]. This higher mouse weight was maintained regardless of T exposure (Fig. 4A). Despite the larger size of the mice exposed to LA, ovaries from LA-treated mice were significantly smaller than ovaries from controls. This discrepancy in ovarian weight associated with LA exposure was true regardless of concomitant exposure to T (Fig. 4B). Ovaries from LA- and LA + T-treated mice weighed approximately 2 to 3 times less than that of control mice (Fig. 4B). Mice treated with T only had ovaries comparable in size to controls [Fig. 4B(i)]. However, when the mice were stimulated with eCG, their ovarian weight did not increase as much as controls [Fig. 4B(ii)]. Ovaries from LA- and LA + T-treated mice approximately doubled in weight when mice were stimulated with eCG (Fig. 4B). Similar results were obtained when presenting the data as a ratio of ovarian weight to body weight (Fig. 4C). The ovaries from LA-treated mice were subjectively more fragile and prone to deterioration with manipulation in all groups.

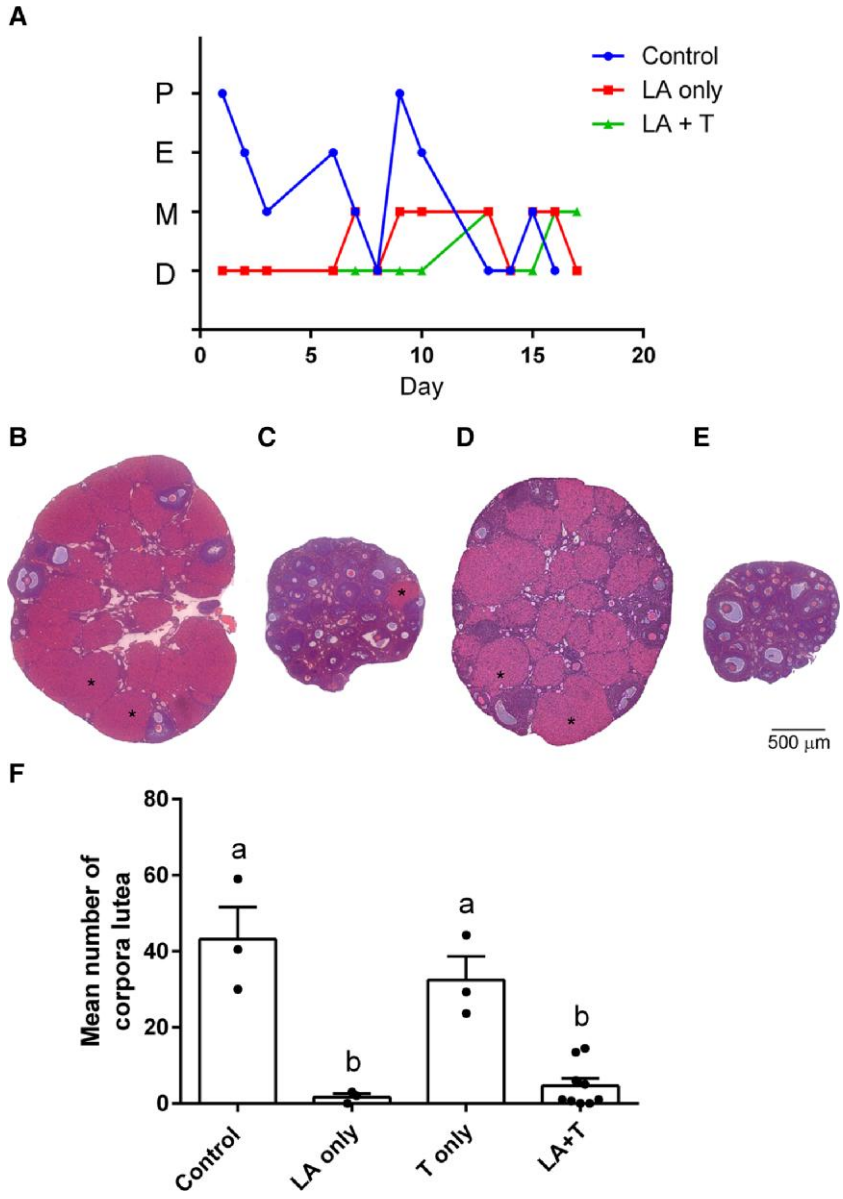

Figure 3. Mice treated with LA and T ovulate significantly less than mice treated with T alone. (A) Sample estrous cycles from a control (circles), LA-only (squares), and LA + T (triangles) mouse. Note the absence of a proestrus surge in the LA-treated mice. (B)-(E) Ovarian histology from 19-week-old mice that were (B) untreated, (C) treated with LA only, (D) treated with T only, and (E) treated with LA + T. Examples of CL are indicated by asterisks. (F) Mean number of CL within ovaries. Bars with different letters are significantly different ($P < 0.05$).
Abbreviations: CL, corpora lutea; LA, leuprolide acetate; T, testosterone cypionate.

Ovaries From Mice Treated With LA and T Produce Oocytes That Have Morphologically Normal Meiotic Spindles After In Vitro Maturation and Respond to Gonadotropin Stimulation

To examine if ovaries from T- or LA + T-treated mice produce viable oocytes, we examined their ability to mature in vitro. Oocytes from mice that were treated with T or with LA + T produced mature metaphase II eggs that formed morphologically normal spindles after in vitro maturation (Fig. 5A). Normal spindles were defined as being bipolar with chromosomes aligned on a metaphase plate, whereas abnormal spindles lacked a bipolar structure or had misaligned chromosomes (Fig. 5B). Unstimulated, LA + T-treated mice had a significantly lower normal meiotic spindle rate compared to eCG-stimulated mice exposed to LA and T, suggesting that eCG may rescue follicles that would otherwise be atretic.

To examine ovarian responsiveness to gonadotropin stimulation in LA-treated mice, we measured E2 levels and the ability to ovulate. In preliminary experiments using 9-week-old control and LA-only mice, E2 levels in both unstimulated, LA-treated, and control mice were similar and approximately doubled in response to stimulation with 5 IU eCG (Fig. 6A), demonstrating that ovaries from LA-treated mice can respond to gonadotropins. To examine ovulation, we first tried stimulating these 9-week-old mice with 5 IU eCG and 5 IU hCG. We found that while LA-treated mice ovulated similar numbers of mature eggs as controls when stimulated with 5 IU eCG/5 IU hCG, only 10 out of 16 mice were responders (62.5%). When mice were injected with 10 IU eCG/10 IU hCG, 83% (5/6) responded

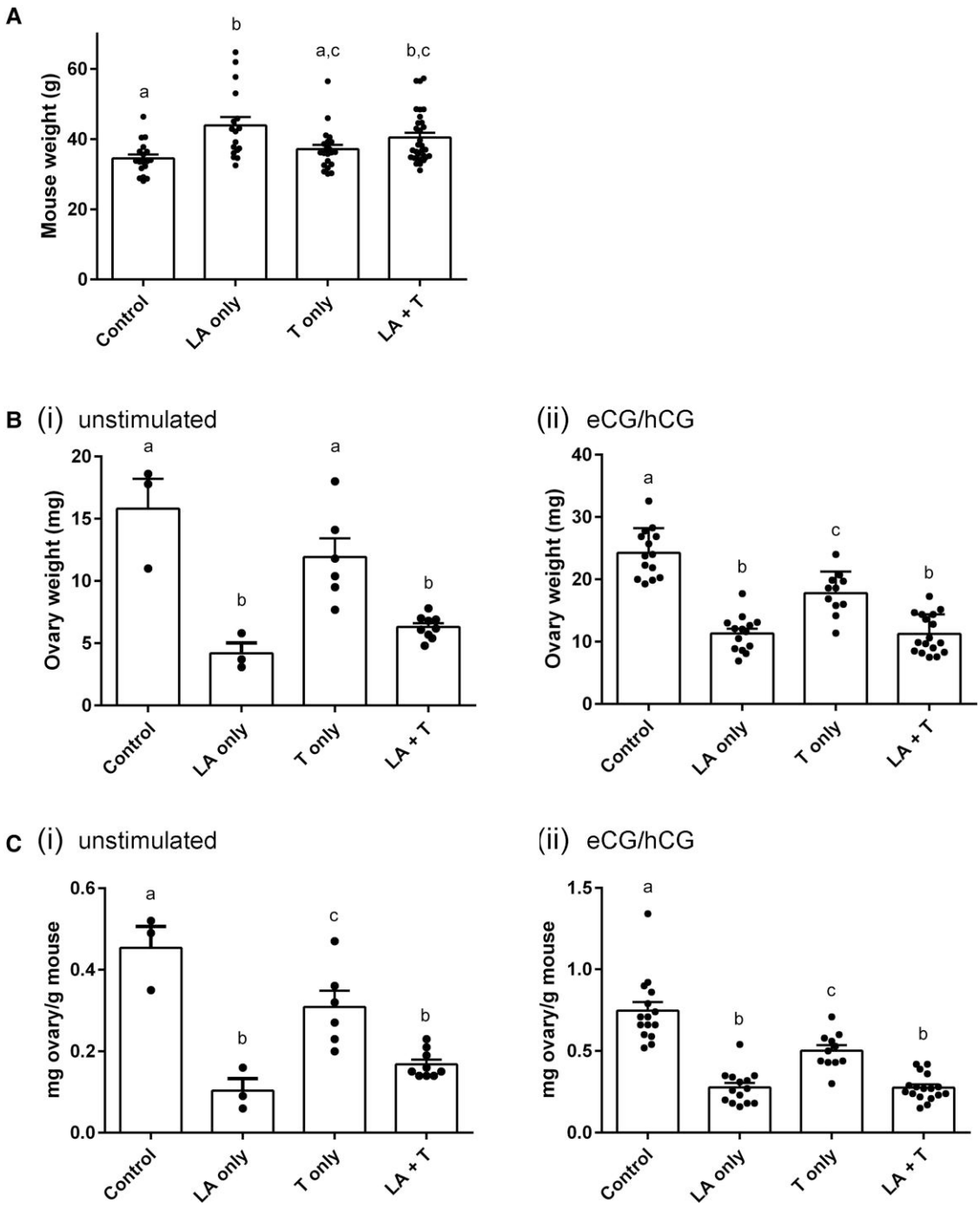

Figure 4. LA-treated mice weigh more but have smaller ovaries than age-matched controls. (A) Mouse weights. (B) Ovarian weights from mice (i) unstimulated or (ii) stimulated with eCG and hCG. (C) Ratio of ovary:mouse weights in mice that were (i) unstimulated or (ii) stimulated with eCG and hCG. Each data point represents 1 mouse or ovary. Bars with different letters are significantly different ($P < 0.05$).
Abbreviations: eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin; LA, leuprolide acetate.

(Fig. 6B). The number of eggs ovulated was variable, but there was no difference between LA-treated mice and controls.

Basal E2 levels in 19-week-old, control or LA-only mice were similar to those in 9-week-old mice. However, there were no statistically significant increases in E2 levels when these mice were stimulated with 10 IU eCG (Fig. 6C). E2 levels in the unstimulated, T-treated mice were similar to those of LA-only mice that were stimulated with eCG (Fig. 6C). All LA-only and T-only mice ovulated, while 86% (12/14) of mice treated with both LA and T ovulated in response to 10 IU eCG/10 IU hCG. The absolute number of eggs retrieved per mouse was highly variable, but there were no significant differences in egg yield between control, LA only, T only, and LA + T groups (Fig. 6D).

Eggs From Female Mice Treated With LA and T Are Fertilizable and Produce Developmentally Competent Embryos and Fertile Pups

To examine if eggs obtained from LA + T-treated mice were developmentally competent, superovulated eggs were inseminated

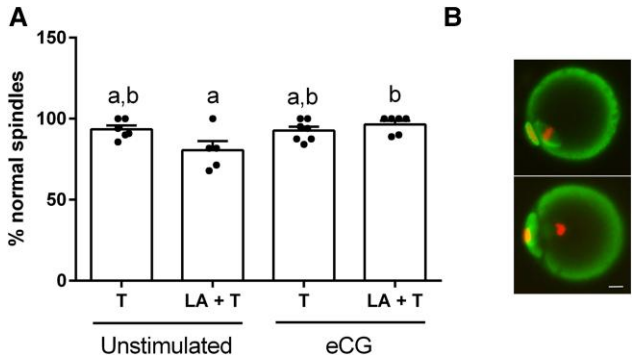

Figure 5. Oocytes from mice treated with LA and T undergo IVM normally. (A) Mean rates of normal meiotic spindle formation among unstimulated and stimulated mice that were treated with T only or with LA + T. Each data point represents spindle counts from a single mouse. Bars with different letters are significantly different ($P < 0.05$). (B) Representative normal (top panel) meiotic spindle and abnormal (bottom panel) meiotic spindle from IVM with visible polar bodies. DNA = orange, and α-tubulin = green. Bar = 10 μm.
Abbreviations: IVM, in vitro maturation; LA, leuprolide acetate; T, testosterone cypionate.

and embryo formation and development rates were assessed. Eggs from LA-only, T-only, and LA + T-treated mice had similar cleavage rates as controls (Fig 7A), produced normal-appearing blastocysts capable of hatching (Fig. 7B), and had similar live birth rates when embryos were transferred into pseudopregnant hosts (Fig. 7C). The male to female ratios of the pups derived from these experiments were similar (Fig. 7D). When sibling pups were mated, all of the mating pairs reproduced with comparable numbers of pups (Fig. 7E); therefore, offspring from mice whose female parent was exposed to LA, T, or both LA and T were found to be fertile without any reproductive issues.

Discussion

As more adolescent transgender males seek to bypass normal female puberty, it is important to have a thorough understanding of possible ramifications for future fertility. There have been essentially no human studies on the effects of puberty suppression followed by testosterone administration. In this study, we found that administration of the puberty-blocking agent, LA, followed by T, to female mice did not impair subsequent reproduction. Indeed, ovarian function and embryologic outcomes were similar among female mice treated with LA in combination with T compared to controls. This is the first prospective study to our knowledge to report fertility outcomes after treatment with pubertal suppression and testosterone therapy among female mice. Our results also extend previous studies [15, 16] examining the effects of T on reproduction in adult female mice.

LA treatment by itself effectively suppressed puberty for at least 3 months when administered to 3-week-old mice. Mice did not cycle as assessed by vaginal cytology, and their ovaries contained very few corpora lutea. Unexpectedly, mice treated with T but not LA had a substantial number of CL in their ovaries. Two recent studies reported the use of mouse models to study the effects of T treatment on female mice [15, 16]. These studies showed that postpubertal female mice treated with T stopped cycling, as determined by persistent diestrus on vaginal smears. One of these prior studies showed a complete absence of CL in mice treated with T [16], whereas the other study showed a greatly reduced number of CL in the T-treated groups [15]. Differences in the current study are that (1) our mice were not treated with T until they were 11 weeks old, and (2) they were only given a low dose of T for the first 4 weeks. The mice were most likely cycling by the time T administration began. It is also possible that mice treated with T alone were able to cycle during the first 4 weeks of T treatment, when the dose of T administered was relatively low. Previous studies have reported that mouse CL disappear after 8 days [24]. However, multiple studies have shown that androgens administered to rodents prevent the decline in serum progesterone implicated in luteal cell apoptosis, and androgens themselves may directly inhibit apoptosis of luteal cells as well [32]. Given these observations, our results may reflect one of two possible scenarios: administration of T in our mice may not have caused ovulation to cease, or it may have caused cessation of ovulation while also preventing CL regression.

Regardless, the presence of CL in the T-treated mice reinforces the fact that T administration in humans, while causing vaginal and endometrial atrophy, may not necessarily be an effective contraceptive, and transgender males on T therapy can continue to ovulate, which is supported by multiple reports in the literature [33–35]. Much of the existing data regarding infrequent ovulation associated with excess testosterone is from patients with polycystic ovarian syndrome (PCOS) or other causes of hirsutism or virilization. However, some of the conclusions drawn from PCOS-related data can be applied to those taking exogenous T despite the differences between these populations, including the higher androgen levels among individuals on exogenous hormones and more complex metabolic changes among those with PCOS [36, 37].

Although vaginal cytology observations suggested that LA-treated mice did not cycle, their ovaries were responsive to gonadotropins. Mice treated with LA only had elevated E2 levels in response to eCG and ovulated eggs after a subsequent injection of hCG. These mice appeared to be less sensitive to gonadotropins, as fewer mice responded to 5 IU eCG/5 IU hCG than to 10 IU eCG/10 IU hCG, but most mice responded to 10 IU of gonadotropins. This is similar to the response in humans, as prepubertal children and adolescents undergoing stimulation have been reported to have a higher response to stimulation with higher doses of gonadotropins for a longer period of time [38, 39].

Vaginal cytology also suggested that LA- and T-treated mice did not cycle. However, 2 out of 3 of LA-only ovaries and 6 out of 9 LA + T ovaries that were examined histologically showed the presence of CL, indicating that these mice did ovulate. The average number of CL that were observed from these ovulating mice (2.5 for LA only and 7 for LA + T) was significantly lower than the average number of CL that were counted in the control (43) and T-only-treated ovaries (32). The most likely explanation for the higher number of CL in the LA + T group compared to LA only is that higher levels of circulating E2 as a result of peripheral conversion of T were sufficient to trigger an LH surge in some of the mice. The E2 levels that were measured at the end of the experiment in all T-treated mice were above the threshold at which E2 levels become stimulatory for LH [40, 41]. It should be noted that LA downregulates GnRH receptors that are required to stimulate an LH surge. However, the mice utilized in these experiments were started on low-dose T at the same time they were given their last injection of LA. It is likely

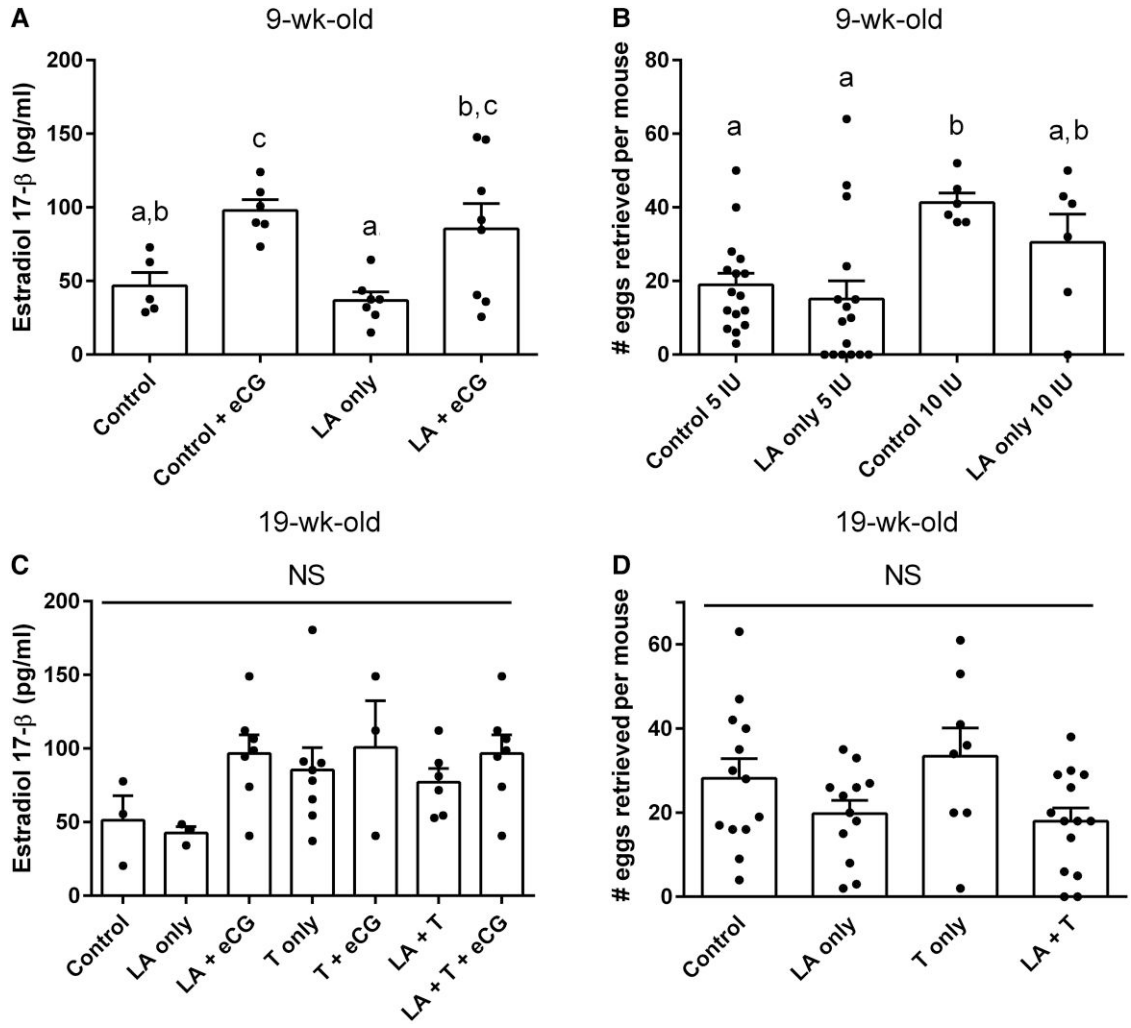

Figure 6. Mice exposed to LA and T respond to gonadotropins. (A) Estradiol-17β levels in response to 5 IU eCG in control and LA-treated 9-week-old mice. Bars with different letters are significantly different ($P < 0.05$). (B) Number of ovulated eggs retrieved per mouse by dose of eCG and hCG among 9-week-old control and LA-treated mice. Bars with different letters are significantly different ($P < 0.05$). (C) Estradiol-17β levels in 19-week-old control, LA-treated, T-treated, or LA- and T-treated mice before or after stimulation with 10 IU each of eCG and hCG. (D) Number of ovulated eggs retrieved per mouse among 19-week-old mice primed with 10 IU eCG and 10 IU hCG. There were no significant differences among any groups. In all cases, each data point represents a single mouse.
Abbreviations: eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin; LA, leuprolide acetate; NS, not significant; T, testosterone cypionate.

that GnRH receptors slowly returned as the effects of LA diminished, such that the elevated levels of E2 were able to stimulate an LH surge that resulted in ovulation. The ovaries of the mice that ovulated weighed the same as anovulatory ovaries, and the observation that they produced fewer CLs than controls suggested that puberty in these mice was indeed suppressed.

LA and T treatment did not seem to affect the development of meiotically competent oocytes. Oocytes collected from the ovaries of LA- and T-treated mice matured to the metaphase II stage in vitro, producing morphologically normal meiotic spindles. While the overall rate of normal spindle production was high among all groups examined, spindle development in mice treated with LA, T, and eCG was significantly higher than LA- and T-treated mice that were not given eCG. This could be due to a higher proportion of atretic follicles in the unstimulated group. A higher rate of atretic follicles among T-treated individuals has been previously reported in both humans and mice [11, 16]. The atresia and low rate of normal spindles in the mice exposed to LA and T may be overcome by stimulation with eCG, which allows for recruitment of a larger number of follicles including those that would have become atretic without eCG rescue. Previous studies have shown that despite the atresia of follicles exposed to T, the size of the ovarian follicular pool is not significantly diminished based on the numbers of antral follicles seen in all groups [15, 16, 42, 43].

The ultimate test of egg quality after puberty suppression and cross-hormone therapy is the ability to produce viable offspring. Indeed, LA- and T-treated mice ovulated eggs that were fertilizable, developed to blastocysts in culture, and completed gestation comparable to control mice after embryo transfer to foster females. The offspring that resulted from IVF were fertile after mating with siblings, demonstrating no fertility defects in either male or female pups.

In conclusion, LA, which is routinely used for puberty suppression in adolescent transgender males, effectively inhibits puberty in female mice. Pubertally suppressed mice treated

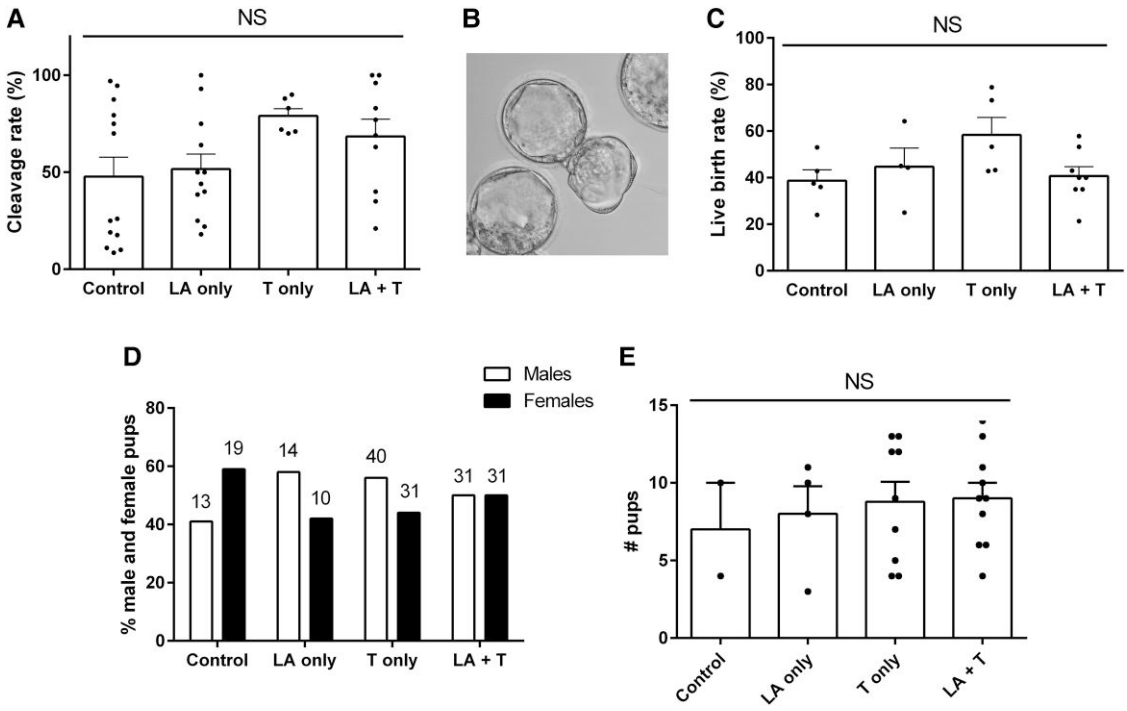

Figure 7. Eggs from mice treated with LA and T are fertilizable and developmentally competent. (A) Mean cleavage rate per mouse. (B) Representative image of a hatching blastocyst surrounded by other blastocysts from a mouse treated with both LA and T. Imaged at 400× magnification. (C) Mean live birth rate per mouse, defined as the number of pups divided by the number of embryos transferred × 100. (D) Ratios of male and female pups resulting from IVF. Total number of pups is indicated above each bar. (E) Number of pups resulting from sibling matings. In all cases, each data point represents a single mouse.
Abbreviations: IVF, in vitro fertilization; LA, leuprolide acetate; NS, not significant ($P < 0.05$); T, testosterone cypionate.

with T stop cycling, but their ovaries remain sensitive to gonadotropins and produce meiotically and developmentally competent eggs capable of producing fertile offspring. These results are the first to demonstrate that fertility is not impaired in mice after puberty suppression and cross-hormone therapy, in which natal puberty is avoided entirely. Though this study is an exciting first step, it must be noted that some aspects of the mouse model may not translate well to humans, in which many transgender males use hormone therapy for several years prior to seeking fertility treatment. Therefore, more long-term studies are warranted. Regardless, the results provide an optimistic outlook for fertility preservation in transgender adolescents who may not decide until years after hormone therapy has begun whether they would like to have biological children.

Acknowledgments

The authors thank Drs. Daniel Grow, Chantal Bartels, and Laurinda Jaffe for their support, advice, and thoughtful suggestions on the manuscript. The authors would like to acknowledge the Center for Advanced Reproductive Services and Village Fertility Pharmacy for their generous donation of depot leuprolide acetate for this study. We also thank Zhifang Hao at the histology core at UConn Health and Hanna Colegrove at Ichor Life Sciences, Inc. for their help with ovarian histology and Drs. Melinda Sanders and Mingfu Yu in the pathology department at UConn Health for their collaboration on taking photographs for this manuscript.

Funding

This study was funded by the Reproductive Endocrinology and Infertility fellowship program through UConn Health Graduate Medical Education.

Disclosures

The authors have no competing interests to disclose.

Data Availability Statement

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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