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ORIGINAL ARTICLE *Reproductive endocrinology*

Short-term testosterone use in female mice does not impair fertilizability of eggs: implications for the fertility care of transgender males

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STUDY QUESTION: Does testosterone use in females affect reproductive potential, particularly with regard to the production of fertilizable gametes?

SUMMARY ANSWER: Testosterone (T) injections given to post-pubertal female mice caused virilization and although the ovaries were smaller than controls they were still responsive and produced fertilizable eggs when superovulated.

WHAT IS KNOWN ALREADY: Studies to examine the effects of testosterone on reproductive potential in transgender males are lacking. Recently, a model was developed that simulates many aspects of testosterone use in transgender males in order to look at reproductive effects of testosterone in female mice. This study found masculinizing effects on the mice but did not find significant deficits on the number of ovarian follicles; however, effects of testosterone use on ovarian stimulation and fertilizability of oocytes were not investigated.

STUDY DESIGN, SIZE, DURATION: A total of 66, 6-week-old Hsd:NSA (CF-1) female mice and six Hsd:ICR (CD-1) male mice were used for this study. Mice were injected s.c. with 400 µg T or sesame oil once a week for 6 weeks and were either killed 1 week after the sixth injection (active exposure group), or 6–7 weeks after the final T injection (washout group).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Both active exposure and washout groups were further subdivided into three groups: unstimulated, equine CG (eCG)-stimulated or eCG/hCG-stimulated. eCG-stimulated mice were killed 44–48 h after eCG injection. eCG/hCG-stimulated mice were injected with eCG, followed 48 h later with hCG. Mice were killed ∼13–18 h after the hCG injection. Data collected included daily vaginal cytology, terminal testosterone levels, ovary weights and histology, number of oocytes/eggs collected in each group, and cleavage to the two-cell stage following IVF.

MAIN RESULTS AND THE ROLE OF CHANCE: Testosterone-treated mice had testosterone levels elevated to the level of male mice and ceased cycling. Ovaries were significantly smaller in testosterone-treated mice, but they contained normal cohorts of follicles and responded to gonadotrophin stimulation by ovulating similar numbers of eggs as controls, that fertilized and cleaved *in vitro*.

LIMITATIONS, REASONS FOR CAUTION: Mice were treated for only 6 weeks, whereas many transgender men use testosterone for many years before considering biological children, and developmental competence was not assessed. Importantly, a mouse system may not perfectly simulate human reproductive physiology.

WIDER IMPLICATIONS OF THE FINDINGS: The current standard of care for transgender men who desire biological children is to cease testosterone therapy prior to ovarian stimulation, but the necessity for stopping testosterone is not known. Our model demonstrates that it is possible for testosterone-suppressed ovaries to respond to gonadotrophic stimulation by producing and ovulating fertilizable eggs, thereby obviating the need for testosterone cessation prior to ovarian stimulation. In time, these results may provide insights for future clinical trials of fertility treatment options for transgender men.

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Introduction

Transgender males are individuals who were assigned female at birth but identify as males. Many, but not all, transgender males opt to undergo gender-affirming treatment, which can consist of surgery and/or hormone therapy (HT) by long-term administration of testosterone (Quinn *et al.*, 2017). HT improves gender dysphoria through testosterone-driven development and maintenance of desired male secondary sex characteristics; however, a potential adverse effect of testosterone exposure is a decrease in fertility.

A recent study by the Williams Institute (UCLA, Los Angeles, CA, USA) estimated that about 1.4 million individuals identify as transgender in the USA (Flores, 2016), and there are reports that approximately half of transgender adults desire biological children (Wierckx *et al.*, 2012; Moravek, 2019). The reproductive consequences of HT are still unclear, and both the World Professional Association for Transgender Health and the Endocrine Society recommend that all transgender males be counseled regarding options for fertility preservation before initiating testosterone therapy (Meyer, 2009; Hembree *et al.*, 2017). Transgender males may not consider fertility preservation to be important at the start of testosterone therapy, which can be initiated as early as 14 years old. In addition, ART centers have little experience in stimulation of peripubertal ovaries, or in performing transvaginal oocyte harvest in children. Owing to a variety of physiological and psychological barriers, ovarian stimulation and oocyte harvest is best avoided in children, if it can be safely postponed to adulthood. A recent study showed that only 2 of 72 (2.8%) young transgender individuals chose to utilize fertility preservation after counseling (Nahata *et al.*, 2019), which reflects a priority for HT initiation to attain features of their affirmed gender while avoiding the delay, invasiveness or costs of fertility preservation (Armuand *et al.*, 2017; Insogna *et al.*, 2020). The desire for biological children may arise later in life, after months or years of HT exposure.

The approach to fertility options in transgender males already taking HT therefore warrants more investigation. To date, studies to evaluate the impact of HT on reproductive potential for transgender males are lacking. The options for transgender males presenting for fertility preservation after HT are either surgical oophorectomy to collect ovarian tissue or surgical oocyte retrieval following ovarian stimulation (De Roo *et al.*, 2016; Neblett and Hipp, 2019). Methods for maturation and fertilization of oocytes collected directly from isolated ovarian tissue without hormonal stimulation, while improving, are still considered to be experimental (Yang and Chian, 2017) and to date, there have been no studies to examine this method for fertility preservation in transgender males. Accordingly, if a transgender male presents for fertility treatment now or in the near future and plans to have the pregnancy carried by a cis-female partner or gestational carrier, the best option is IVF after ovarian stimulation and oocyte retrieval. Owing to the unknown effects of high-level testosterone on ovarian response and oocyte quality, the current recommended practice before IVF is discontinuation of testosterone to allow the resumption of menses (Broughton and Omurtag, 2017; Adeleye *et al.*, 2019; Young *et al.*, 2019). While this treatment regimen can be effective, HT cessation for the purpose of fertility treatment has been reported to cause significant psychological distress in the form of gender dysphoria attributed to the gender-incongruous effects of testosterone withdrawal, estrogen exposure and menses (Armuand *et al.*, 2017). These negative consequences could lead to treatment avoidance even when fertility is desired.

Ovarian tissue taken from HT-exposed transgender males has demonstrated changes including a thickened cortex, stromal hyperplasia, an increased number of atretic follicles and increased cortical stiffness (Ikeda *et al.*, 2013; De Roo *et al.*, 2019). However, the ovarian tissue follicular pool is not diminished (Van Den Broecke *et al.*, 2001; De Roo *et al.*, 2016). Markers of ovarian reserve, including anti-Müllerian hormone and inhibin, are unchanged (Rodriguez-Wallberg *et al.*, 2014), and successful pregnancies have been reported after testosterone use (Light *et al.*, 2014). Case reports have been published of subjects successfully undergoing IVF after temporarily discontinuing testosterone therapy for 1–12 months, and healthy live births were reported (Broughton and Omurtag, 2017; Leung *et al.*, 2018; Adeleye *et al.*, 2019). These data suggest that the follicular pool and oocyte quality are preserved.

A study on a primary mouse model for HT in transgender males was recently published and found that ovaries from mice treated with testosterone for 6 weeks were generally normal, with the exception of some cyst-like late antral follicles (Kinnear *et al.*, 2019). The fertility potential in terms of ovarian response to gonadotrophins, oocyte integrity or fertilizability was not examined. There is very limited information about the necessity for cessation of testosterone therapy prior to ovarian stimulation, and no mouse models have addressed this problem. The aim of the present study was to build on the model established by Kinnear *et al.* (2019) to determine if reproductive potential could be evaluated following 6 weeks of testosterone injection in female mice with and without a period of testosterone cessation.

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 & 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. Testosterone cypionate (hereafter

referred to as 'T') was from Steraloids (Newport, RI, USA) and was prepared as an 8 mg/ml solution in sesame oil. Equine CG (eCG) was from Calbiochem (San Diego, CA, USA). The medium for oocyte collection was HEPES-buffered MEMα (Gibco 12000022, Thermo Fisher, Waltham, MA, USA) containing penicillin, streptomycin and polyvinyl alcohol (PVA), and 10 μM milrinone to prevent spontaneous meiotic maturation (Mehlmann *et al.*, 2019). For overnight oocyte maturation, oocytes were washed into bicarbonate-buffered MEMα (Mehlmann *et al.*, 2019) containing 5% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) without milrinone. For IVF, cumulus masses were collected in human tubal fluid medium (HTF; Cook Medical Inc. #K-RVFE) containing reduced glutathione. Sperm were capacitated in IVF medium (Mehlmann and Kline, 1994) containing 15 mg/ml Fraction V bovine serum albumin (BSA).

Experimental design

Six-week-old female CF-1 (Envigo, Indianapolis, IN, USA) and >8-week-old male CD-1 mice (Envigo) were used for all experiments. Three female mice were housed per cage in a temperature and light-controlled room on a 14L:10D light cycle. Male mice were housed individually.

Six-week-old female mice were lightly sedated with isoflurane and injected weekly, s.c., with 400 μg T or vehicle using 27-gauge needles. In the first set of experiments, mice were killed within 8 days after the sixth testosterone injection, when testosterone levels were high. These mice are referred to as the 'active exposure' group. In the second set of experiments, mice were killed 6–7 weeks after the sixth testosterone injection, when testosterone returned to basal levels. These mice are referred to as the 'washout' group. Both groups were subdivided into three more groups: mice that were not stimulated with gonadotrophins; mice that were stimulated with eCG only; and mice that were stimulated with eCG followed by hCG to induce ovulation.

For both the active exposure and washout groups, vaginal smears were performed to examine cyclicity. We analyzed the following: serum testosterone levels, ovary weights and histology, oocyte number prior to and after priming with eCG or eCG + hCG, structure of the meiotic spindle, and egg fertilizability. Control mice were injected with sesame oil and were treated in parallel with the testosterone-injected groups. All mice were euthanized by isoflurane overdose followed by cervical dislocation.

Vaginal cytology

For the active exposure group, daily vaginal smears were performed using standard methods (Goldman *et al.*, 2007) starting in the 5th week of testosterone treatments. For the washout group, daily vaginal smears started 1 week after the final testosterone injection. Staging of the estrous cycle was determined by the presence and distribution of leukocytes, cornified epithelial cells, and nucleated epithelial cells. Proestrus was identified by nucleated epithelium, estrus was identified by large cornified epithelial cells, metestrus was identified by leukocytes and large cornified epithelial cells, and diestrus was identified by the predominance of leukocytes in the presence of nucleated and cornified cells (Gaytan *et al.*, 2017). Clitoral size was visually assessed at the time of cytology.

Blood collection and hormone analysis

After the mice were killed, they were weighed and terminal blood was collected by cardiac puncture using a heparinized 18-gauge needle and syringe. Blood samples were kept on ice. Within 30 min of collection, samples were centrifuged at 4°C for 15 min (1000×*g*) and supernatants were stored at −80°C. Diethyl ether extraction was performed as directed by Cayman Chemical, with extracted samples stored at −20°C in Cayman ELISA buffer. Testosterone analysis was performed using an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. For comparison, we also collected blood from mature male mice that were used for IVF (see below). These males had been acclimated to the lab for at least 1 week prior to the experiment, were housed individually, and were not exposed to females prior to blood collection.

Ovarian histology

Ovaries were collected, most of the fat and oviducts were removed by dissection under a stereoscope, and they were then weighed. One ovary from each mouse was fixed in 10% formalin for 24–48 h, washed into PBS, then dehydrated in ethanol, embedded in paraffin, and 5 μm serial sections through the entire ovary were cut and processed by the Histology Core at UConn Health. Sections were stained with hematoxylin and eosin.

Total numbers of antral follicles and corpora lutea (CLs) in each ovary were counted. Antral follicles were defined as being ~250–320 μm in diameter with a clearly visible antral cavity and oocyte with two or more layers of granulosa cells. Preovulatory follicles were defined as being >320 μm in diameter. Each antral follicle was counted only when the oocyte was present and while scanning between adjacent sections to prevent duplicate counting. CLs were defined as discrete eosinophilic round structures. CLs were numbered as the sections were serially assessed through the entire ovary to prevent duplicate counting. Two of the investigators independently counted follicles.

Oocyte collection and immunofluorescence staining

Ovaries from unstimulated and eCG-primed mice were weighed and one ovary from each mouse was fixed for histological analysis while the contralateral ovary was used for oocyte collection. The ovary for oocyte collection was placed in HEPES-buffered MEMα containing milrinone and punctured using a 30-gauge needle. Oocytes were collected with a mouth pipet and counted. For IVM, oocytes were washed into bicarbonate-buffered MEMα without milrinone and were incubated overnight at 37°C in a humidified incubator containing 5% CO_2/95% air. IVM was confirmed by the disappearance of the nuclear envelope and the formation of first polar bodies using a stereoscope. Oocytes were fixed for 30–60 min at 37°C in 2% formaldehyde, 100 mM HEPES, 50 mM EGTA, 10 mM $MgSO_4$ and 0.2% Triton X-100, then were permeabilized in PBS containing 0.1% Triton X-100, and blocked for at least 15 min in PBS containing 3% BSA and 0.01% Triton X-100. Oocytes were incubated overnight at 4°C in primary antibody against tubulin (YL1/2; Serotec Inc., Raleigh, NC, USA) diluted to 10 μg/ml in blocking buffer. After washing in PBS-PVA, oocytes were incubated in Alexa488-conjugated secondary antibody

for 1 h at room temperature in the dark. Oocytes were washed in PBS-PVA containing 5 µM SYTOX Orange (ThermoFisher) to label chromosomes. Imaging for spindle integrity was performed using a Zeiss Pascal confocal microscope with a 40×, 1.2 NA water immersion objective (C-Apochromat; Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

IVF

Female mice were superovulated with 5 IU eCG, followed 48 h later with 5 IU hCG. Approximately 13–15 h later, ovaries and oviducts were removed and cumulus masses obtained by puncturing the swollen ampullae. Cumulus masses were incubated in 200 µl drops of HTF containing reduced glutathione for ~30 min prior to adding sperm. Sperm were collected from the epididymides of male mice by gently snipping with fine scissors into a 100 µl drop of capacitation medium and were capacitated for 1–2 h before adding 3–5 µl of the sperm suspension to the drops containing the eggs. The sperm and eggs were incubated together for 4 h, then were washed into 200 µl drops of HTF without glutathione. Fertilized eggs were incubated overnight in a humidified incubator containing 5% CO_2/95% air. The next day, two-cell embryos were counted.

Statistical analysis

Statistical analyses were performed using Prism 6.0 software for Windows, GraphPad Software, La Jolla, CA, USA (www.graphpad.com). Specific statistical tests for each experiment are indicated in the figure legends. $P < 0.05$ was considered to be significant.

Results

Testosterone cypionate elevates serum testosterone levels and induces virilization in female mice

To elevate serum testosterone, we injected 6-week-old female mice with testosterone cypionate, which is commonly used by transgender men to elevate T levels (Luthy *et al.*, 2017; Moravek *et al.*, 2020). T-injected mice showed signs of virilization, including distinct clitoromegaly and cessation of estrous cycles (Fig. 1A). All control-injected mice clearly cycled throughout the entire experiment, whereas all T-injected mice appeared to be in diestrus (Fig. 1B), which is consistent with what was observed previously (Kinnear *et al.*, 2019). One week after the sixth T injection (referred to herein as the 'active exposure' group), T-injected mice had elevated T levels compared to controls, with all values within the normal range reported for adult male mice (Coquelin and Desjardins, 1982) and also within the range we measured in males (Fig. 2). In one set of experiments, we did not kill females after the 6-week injection period; rather, the mice were kept for several weeks after cessation of injections (referred to herein as the 'washout' group). T levels declined to baseline levels within 5 weeks following the last injection (Fig. 2) and these 'washout mice' resumed cycling, as assessed by daily vaginal smears. Clitoromegaly was no longer apparent in these washout mice 5 weeks after the last injection (Fig. 1A). The weights of the mice at this time did not differ between T-treated and controls. T-treated and control mice in the active exposure group weighed 30.5 ± 0.8 g (SEM) versus 31 ± 1 g, respectively, whereas in the washout group T-treated and control mice weighed 33.9 ± 1 and 33.7 ± 0.8 g, respectively.

Ovaries from T-treated mice are smaller than control ovaries but contain normal complements of follicles and respond to stimulation by gonadotrophins

Currently, little is known about the effects of T treatment on the ability of ovaries to respond to gonadotropic stimulation. To investigate this, ovaries were collected from both the active exposure and washout groups that were unstimulated, stimulated with eCG, or superovulated with eCG and hCG. Testosterone treatment resulted in significantly lower ovary weights, whether or not mice were stimulated with gonadotrophins (Fig. 3A). Notably, the lower ovarian weights were still apparent in the washout group, which more closely mimics the standard of care for T-treated transgender males who wish to obtain functional eggs (Fig. 3B).

Histological analysis of unstimulated and eCG-stimulated ovaries from the active exposure groups (~12 weeks in age) showed similar follicle morphology and comparable numbers of follicles from T-treated mice with their respective controls, despite the overall smaller size of ovaries in T-treated mice (Fig. 4). Because the numbers of preantral and primary follicles have already been reported to be the same for control and T-treated ovaries (Kinnear *et al.*, 2019), we focused on counting antral follicles of various sizes. We analyzed follicles in detail from three mice per group, one ovary from each mouse. In the unstimulated group, T-treated and control ovaries contained similar numbers of antral follicles, as well as similar numbers of atretic follicles. Most of the atretic follicles were in the 250–320 µm size range, while there were almost no atretic follicles in the preovulatory size range (Supplementary Fig. S1). The major difference between T-treated, unstimulated ovaries and control ovaries was a significantly lower number of CLs in the T-treated group compared with controls (Fig. 4). The eCG-stimulated ovaries contained significantly more preovulatory follicles than the unstimulated ovaries in the T-treated mice and contained 0–1 atretic preovulatory follicles. Similar to unstimulated ovaries, ovaries from T-treated mice contained fewer CLs than controls. Most of the CLs present in the T-treated ovaries were likely to be from cycles that occurred prior to T treatment, as they were eosinophilic rather than basophilic (Gaytan *et al.*, 2017) and, in general, located deep within the ovary rather than at the periphery (Fig. 4). The one exception was a T-treated mouse that ovulated in response to the eCG injection; this mouse contained mostly basophilic CLs (Fig. 4A). We did not evaluate ovulated ovaries in detail with histology.

T-treated mice contain comparable numbers of meiotically competent oocytes and ovulate similar numbers of fertilizable, mature eggs as controls

To examine if the follicles from T-treated mice contain normal oocytes, we first collected immature oocytes from ovaries of unstimulated and eCG-stimulated mice, and then collected ovulated eggs from the groups that were injected with both eCG and hCG. In the active

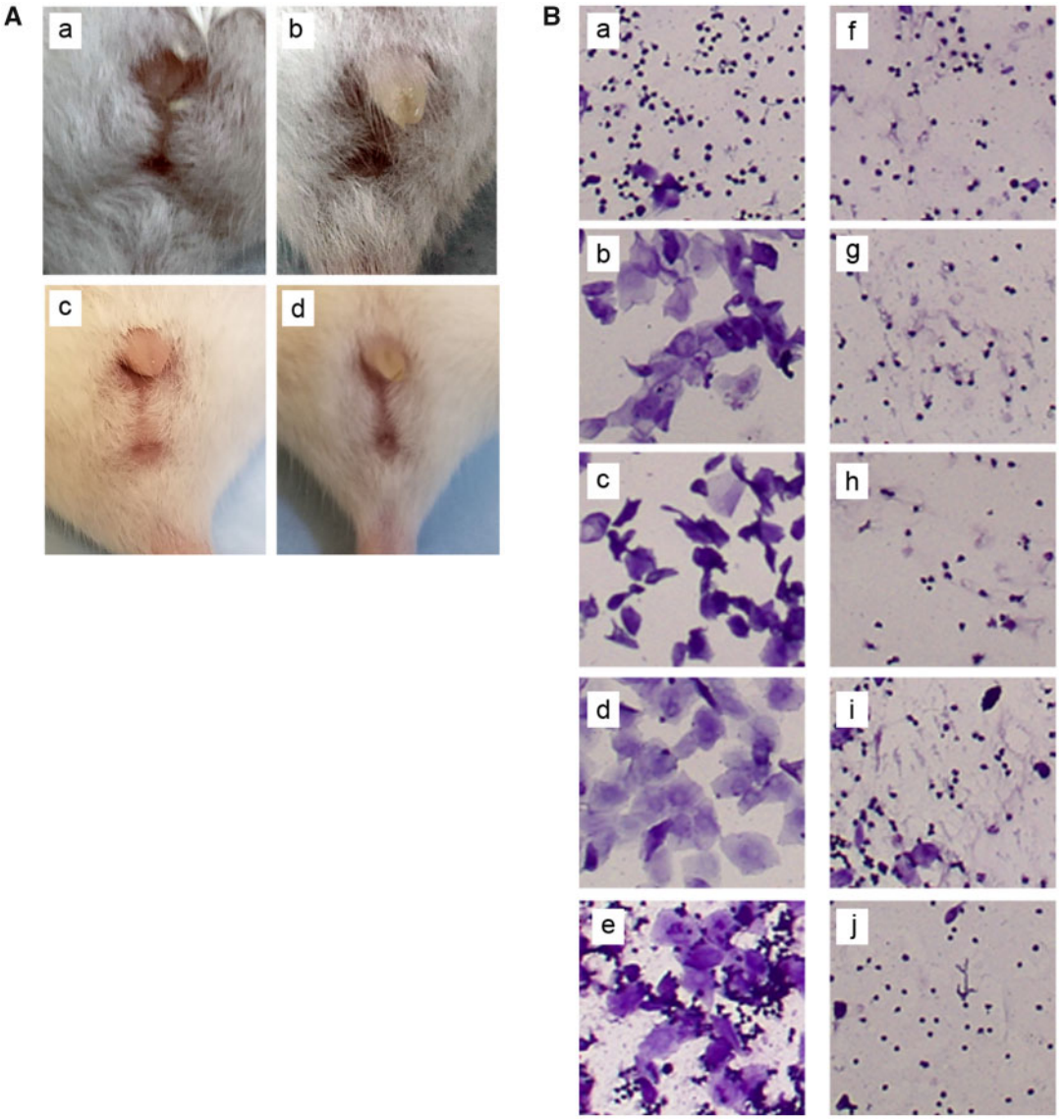

Figure 1. Testosterone cypionate treatment induces virilization in female mice. (**A**) Clitoromegaly was apparent in the active exposure group (mice killed 1 week after the sixth injection of testosterone cypionate (T)) (a = control; b = T-treated) but was no longer apparent in the washout group (mice killed 6–7 weeks after the sixth injection of T) (c = control; d = T-treated). (**B**) Vaginal smears from a cycling, control mouse (**a–e**) and a T-treated mouse (**f–j**). Smears were obtained during the fifth week of T injections. Shown here is five sequential days of a representative control and T-injected mouse. a = diestrus; b = diestrus into proestrus; c = proestrus; d = estrus; e = metestrus from a control mouse. f–j = T-injected mouse in diestrus.

exposure group, we recovered significantly more immature oocytes from the T-exposed ovaries than from their respective controls (Fig. 5A). In the washout group, we found no effect of T treatment on the number of oocytes or eggs (Fig. 5B). Both active exposure and washout groups contained similar numbers of ovulated eggs for T-treated and control mice (Fig. 5A and B). Immature oocytes from T-treated ovaries (active exposure and washout groups) underwent germinal vesicle breakdown, extruded first polar bodies, and formed morphologically normal meiotic spindles in culture (Fig. 5C and D). The proportion of oocytes with intact spindle structure versus poor spindle structure—characterized by degeneration or misaligned chromosomes—was similar among the groups (Fig. 5D). The diameters of eggs following IVM from the active exposure T group were not significantly different than controls (68.5 ± 3.0 (SD) vs 69.1 ± 2.7 (SD); $P = 0.17$).

We tested the fertilizability of ovulated eggs using IVF by evaluating the number of two-cell embryos that were observed 24 h after insemination. Overall, cleavage to the two-cell stage was comparable between T-treated and control mice and was similar to percentages of fertilized eggs obtained from the washout group (Fig. 6).

Discussion

Transgender men who have been undergoing testosterone therapy and who wish to obtain eggs for fertilization or freezing are generally stimulated with gonadotrophins. This is usually done following a period of T cessation that is sufficient for the menstrual cycle to resume (Broughton and Omurtag, 2017; Leung et al., 2018; Adeleye et al. 2019). There is strong evidence that transgender men who have taken T can produce viable, developmentally competent eggs after discontinuing its use (Light et al., 2014; Broughton and Omurtag, 2017; Leung et al., 2018; Adeleye et al., 2019), but to date, there are no publications regarding the quality and developmental capacity of eggs retrieved from transgender men who remain on HT. Here, we show that treating female mice with T weekly for 6 weeks does not impair the fertilizability of their eggs, and our results suggest that T treatment does not need to stop before gonadotrophic stimulation.

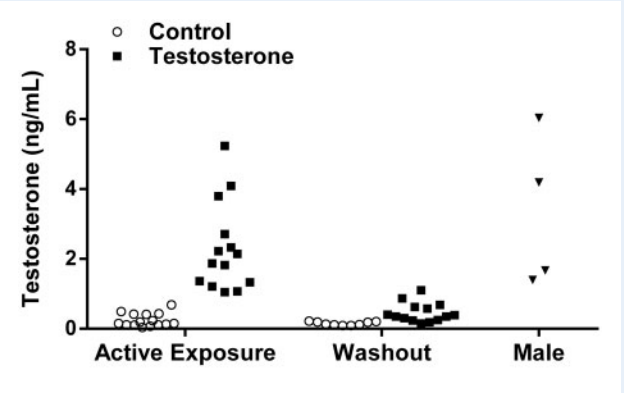

Figure 2. Injections of T transiently elevate serum testosterone levels to those of untreated adult males. Total testosterone levels were measured 1 week (active exposure) or 6 weeks (washout) after the sixth injection of T.

In a recent study investigating the effects of testosterone on female mice, Kinnear et al. (2019) injected testosterone enanthate twice weekly to maintain elevated testosterone levels. However, they did not compare testosterone levels in their treated female mice with the levels found in male mice. In the current study, we injected a closely related form of testosterone, testosterone cypionate, weekly. Our regimen resulted in elevated blood T levels that were ~5 times lower than those obtained by Kinnear et al. (2019), but that were comparable to those found in male mice. The dose we provided stopped the estrous cycle and caused significant clitoral growth, changes that are commonly observed in transgender males on HT (Unger, 2016). It was noteworthy that the clitoromegaly did not persist in the washout group, as there have been few case reports in humans to determine if adult exposure to androgens causes permanent clitoral growth (see Kathiresan et al., 2011; Sielert et al., 2013). Our mouse model may provide more insight into this occurrence. Careful measurements using a larger sample size would need to be done to determine definitively if clitoromegaly persists in mice following testosterone withdrawal.

T-treated mice had smaller ovaries than controls, which is likely due to the greatly reduced number of CLs in these mice. A recent study reported a complete absence of CLs in T-treated female mice (Kinnear et al., 2019). The finding that our T-treated mice had CLs at all was unexpected, as we used a concentration of T that was similar to the mid-range effective dose used by Kinnear et al. (2019). In general, the eosinophilic staining of CLs we observed in T-treated mice was consistent with residual rather than freshly ovulated CLs (Gaytan et al., 2017) and there were considerably fewer CLs in our T-treated mice than in controls, suggesting that the estrous cycle was indeed inhibited in our mice, as was also shown by vaginal cytology. Smaller ovaries were also apparent in T-treated mice in the washout group, which likewise contained significantly fewer CLs than controls,

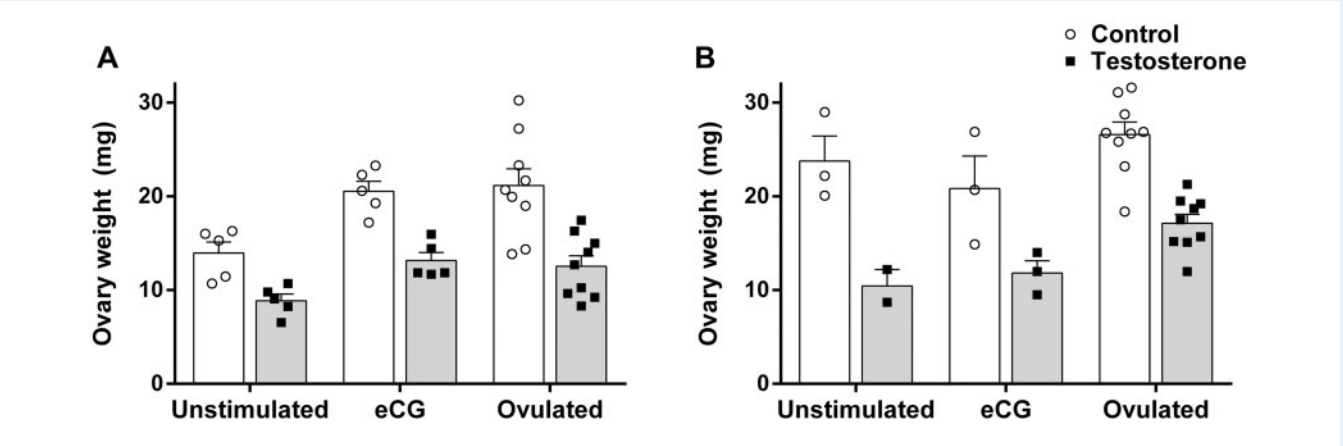

Figure 3. Ovaries from T-treated mice weigh less than control ovaries, regardless of treatment. (**A**) Ovary weights from mice in the active exposure group. (**B**) Ovary weights from mice in the washout group. Bars are mean ± SEM (n = 5 for the active exposure group and n = 3 for the washout group) and each point represents the weight of a single ovary. Significance was determined by two-way ANOVA followed by Bonferroni's multiple comparisons test ($P < 0.05$). All testosterone-treated ovaries weighed significantly less than controls, except for the unstimulated mice in the active exposure group. eCG, equineCG.

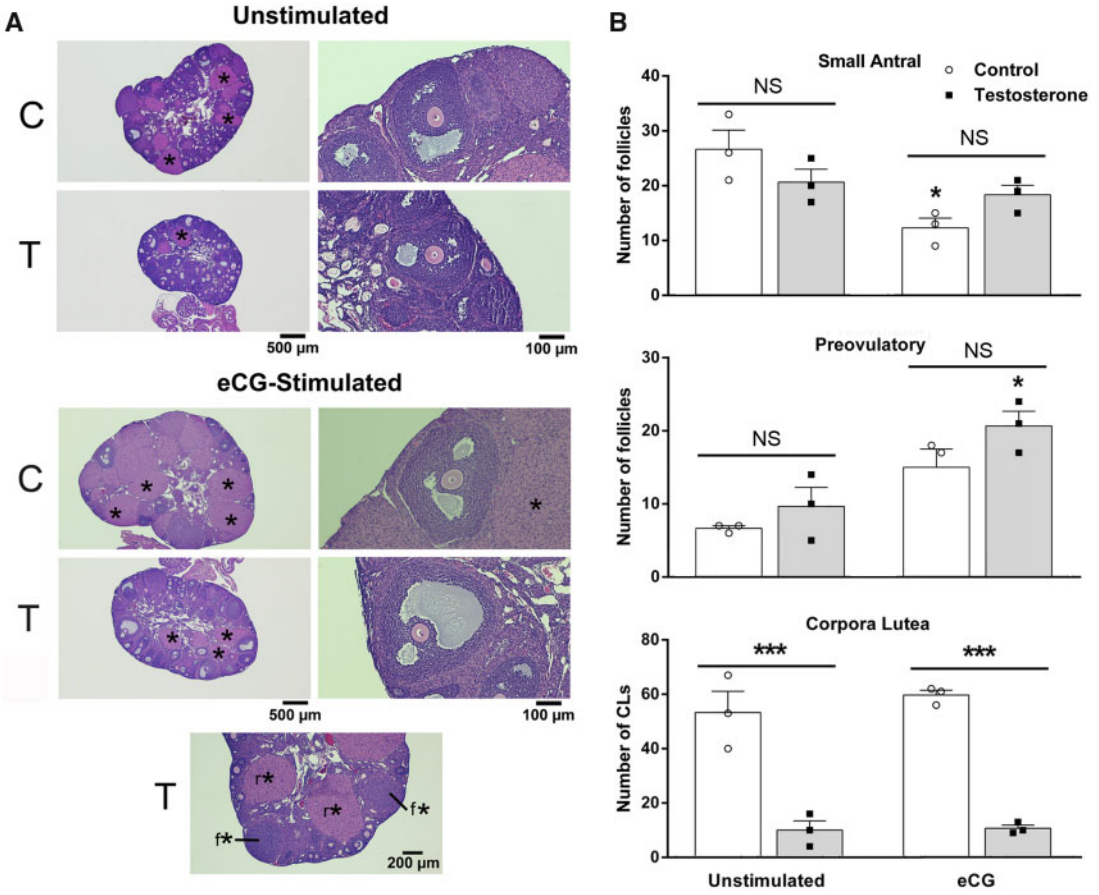

Figure 4. Ovaries from T-treated mice have normal complements of antral follicles but fewer corpora lutea. (**A**) Histology sections showing representative images through ovaries from unstimulated and eCG-stimulated control and T-treated mice from the active exposure group using 2×, 4× or 10× objectives. C = control; T = testosterone; *corpus luteum. The bottommost image is from a T-treated mouse that ovulated in response to eCG stimulation, showing fresh CLs (f*) and residual CLs (r*). (**B**) Quantification of follicles and CLs in unstimulated and eCG-stimulated control and T-treated mice. 'Small antral' follicles measured ∼250–320 µm in diameter and 'preovulatory' follicles measured >320 µm in diameter. Data are mean ± SEM (n = 3) and each point represents follicle/CL counts from a single ovary. A single asterisk in the small antral and preovulatory groups represents a significant difference between unstimulated and eCG-stimulated mice ($P < 0.05$). ***$P < 0.0001$; two-way ANOVA followed by Bonferroni's multiple comparisons test. NS = not significant.

suggesting that these mice had only recently begun cycling prior to ovary harvest and therefore had many fewer ovulations than controls.

Although they weighed less than controls, the ovaries from T-treated mice contained a complement of histologically normal antral follicles that were similar to controls. Unlike the study by Kinnear *et al.* (2019), who reported a higher incidence of late-stage atretic, cyst-like follicles, we only observed a single preovulatory atretic follicle in three out of six ovaries examined, which was not significantly different from controls. Rather, the majority of atretic antral follicles we observed came from follicles that were in the ∼250–320 µm diameter size range, not yet preovulatory, and the percentages were not different between control and T-treated ovaries. One of the T-treated mice unexpectedly ovulated in response to eCG stimulation. There is evidence that T induces the expression of FSH receptors in granulosa cells (Garcia-Velasco *et al.*, 2012; Sen *et al.*, 2014; Liu *et al.*, 2015), and if this occurred in our mice, then it is possible that the T-treated follicles were sensitized to eCG such that spontaneous ovulation occurred prior to the administration of hCG.

Ovaries from T-treated mice produced meiotically competent oocytes. Oocytes retrieved from unstimulated and eCG-stimulated, T-treated ovaries were able to mature to the metaphase II stage in culture, forming morphologically normal meiotic spindles. This is consistent with a descriptive study of human oocytes collected from the ovarian cortex of HT-exposed transgender males, in which oocyte meiotic spindle structure after IVM was found to be normal (Lierman *et al.*, 2017). In addition, T-treated mice in both the active exposure and washout groups ovulated similar numbers of eggs in response to eCG and hCG injection as controls. These eggs fertilized to the same extent as controls and cleaved to the two-cell stage. One exception in the active exposure group was a single T-treated mouse that only produced three poor-quality eggs, none of which fertilized. One hypothesis is that this mouse ovulated prematurely, as there was

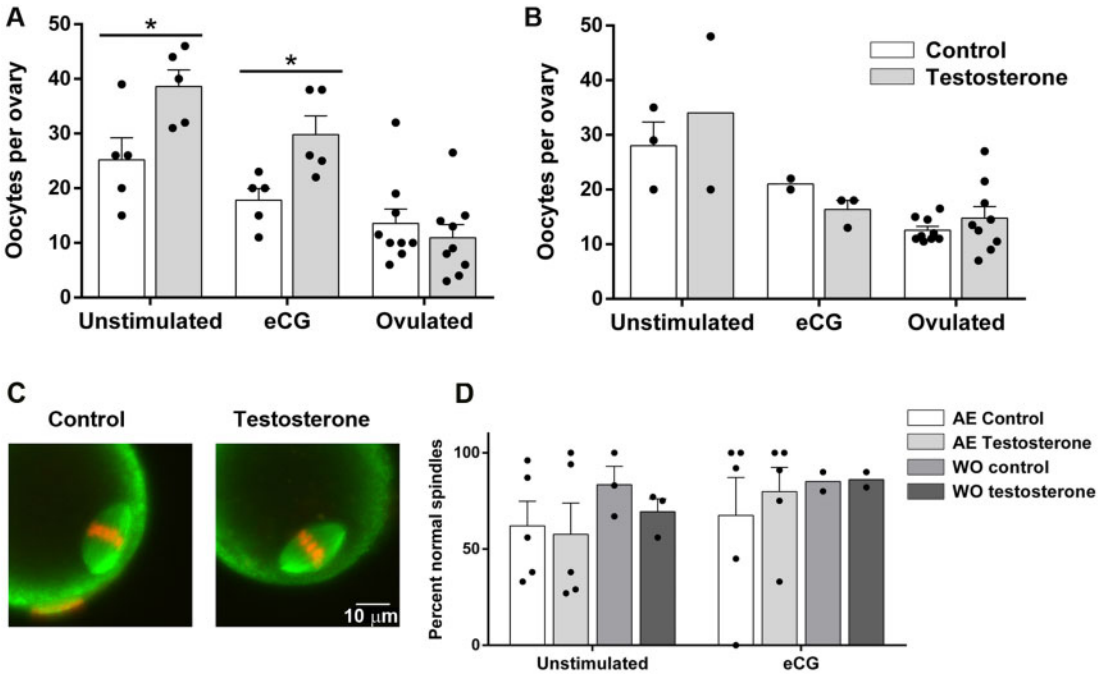

Figure 5. T-treated mice produce meiotically competent oocytes and ovulate comparable numbers of eggs as controls. (**A**, **B**) Numbers of oocytes and ovulated eggs recovered per ovary in the active exposure (A) and washout group (B). Bars are mean ± SEM (n = 5 experiments for the active exposure group and n = 3 for the washout group) and each point represents oocyte counts from an ovary from a single mouse; SEM was not calculated for groups with n = 2. *$P < 0.05$ was considered significant, as determined by two-way ANOVA followed by Bonferroni's multiple comparisons test. (**C**) Representative meiotic spindles from IVM, eCG-stimulated ovaries from the active exposure group. Green = tubulin; Red = DNA. (**D**) Percentage of eggs that formed normal meiotic spindles following IVM. Bars are mean ± SEM (n = 5 experiments for the active exposure group and n = 3 for the washout group) and each point represents a single mouse. SEM was not calculated for groups with n = 2. AE, active exposure; WO, washout.

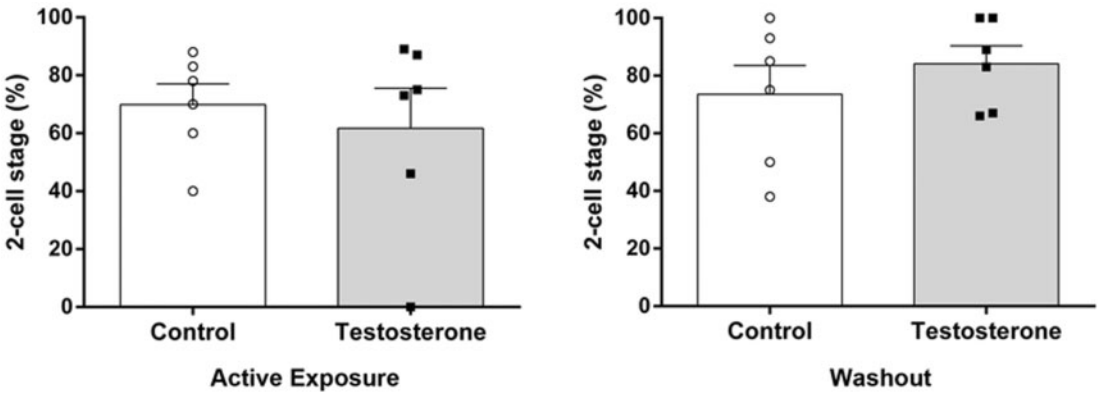

Figure 6. Eggs from T-treated mice are fertilizable. Completed fertilization rates, defined as the number of two-cell embryos per number of inseminated eggs, in control versus T-treated mice in both active exposure (**A**) and washout (**B**) groups. n = 6 and each dot represents data from a single mouse.

some histological evidence in a different T-treated mouse of premature ovulation after eCG. There was insufficient data to fully explore this isolated scenario, and the other T-treated mice produced fertilizable eggs. One limitation of the current study is that developmental competence of fertilized eggs was not assessed: technical issues prevented us from following fertilized eggs through to blastocyst development. The ultimate test of developmental competence would be to transplant embryos into host females to evaluate the formation of pups. This experiment was beyond the scope of the current study, but one that we are interested in pursuing in the future.

In conclusion, we provide evidence showing that female mice produce normal, fertilizable eggs after testosterone exposure, whether T levels are low after a washout period or high during active exposure. One limitation in our study could be that we only exposed mice to T for 6 weeks. While this length of exposure was sufficient to produce phenotypes characteristic of human transgender males exposed to T, it may not completely mimic the human situation, in which many transgender males seeking fertility treatment have been on HT for several years. Further studies that expose mice to T for longer periods of time would help to confirm that its effects are not detrimental to the reproductive process. Despite this concern, our results provide promising data that could help influence the treatment options for transgender men seeking fertility treatment. If testosterone has no detrimental impact on ovarian function, transgender males will have greater flexibility in making reproductive decisions. It is important to note, however, that if a transgender male plans to become pregnant by spontaneous pregnancy or use of ART, testosterone must be discontinued owing to its teratogenic effects (De Roo *et al.*, 2016). Our study suggests that the current practice of T cessation prior to ovarian stimulation and surgical oocyte retrieval may not be necessary when the transgender male does not plan to carry the pregnancy at that time, and could potentially help serve as the basis for human trials to examine this current clinical practice.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

C.B.B. designed the study, acquired and interpreted the data, and helped draft the article. T.F.U. contributed to the study design, data acquisition, and article review. L.L. contributed to data acquisition and article review. L.M.M. designed the study, contributed to data acquisition and interpretation, and helped draft the article.

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Conflict of interest

The authors have no competing interests.

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