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Effects of pharmacologically induced Leydig cell testosterone production on intratesticular testosterone and spermatogenesis†

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Abstract

The Leydig cells of the mammalian testis produce testosterone (T) in response to luteinizing hormone (LH). In rats and men with reduced serum T levels, T replacement therapy (TRT) will raise T levels, but typically with suppressive effects on sperm formation. The rate-determining step in T formation is the translocation of cholesterol to the inner mitochondrial membrane, mediated by protein–protein interactions of cytosolic and outer mitochondrial membrane proteins. Among the involved proteins is cholesterol-binding translocator protein (TSPO) (18 kDa TSPO). We hypothesized that in contrast to TRT, the administration of the TSPO agonist *N*,*N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27), by stimulating the ability of the Leydig cells to produce T, would result in the elevation of serum T levels while maintaining intratesticular T concentration and therefore without suppression of spermatogenesis. Age-related reductions in both serum and intratesticular T levels were seen in old Brown Norway rats. Both exogenous T and FGIN-1-27 increased serum T levels. With exogenous T, serum LH and Leydig cell T formation were suppressed, and intratesticular T was reduced to below the concentration required to maintain spermatogenesis quantitatively. In contrast, FGIN-1-27 stimulated Leydig cell T formation, resulting in increased serum T without reductions in intratesticular T concentrations or in testicular sperm numbers. FGIN-1-27 also significantly increased serum and intratesticular T levels in rats made LH-deficient by treatment with the gonadotropin-releasing hormone antagonist cetrorelix. These results point to a possible approach to increasing serum T without negative effects on spermatogenesis, based upon stimulating T production by the Leydig cells themselves rather than administering T exogenously.

Summary Sentence

The TSPO agonist FGIN-1-27 stimulates the ability of the Leydig cells producing testosterone and results in elevating serum and intratesticular testosterone without negative effect on spermatogenesis.

Key words: Leydig cells, TSPO, testosterone, spermatogenesis

Introduction

The Leydig cells are testosterone (T)-producing cells of the mammalian testis. T is required to initiate and subsequently support spermatogenesis [1]. Leydig cells are stimulated to produce T by luteinizing hormone (LH) released from the anterior pituitary in response to gonadotropin-releasing hormone (GnRH). LH binds to and activates G protein-coupled receptors, resulting in the activation of adenylyl cyclase and the stimulation of cyclic adenosine monophosphate (cAMP) [2, 3]. cAMP stimulates the translocation of cholesterol from intracellular stores through the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), the site of cytochrome P450 side-chain cleavage enzyme (CYP11A1). CYP11A1 converts cholesterol to pregnenolone. Pregnenolone then is converted to T by a series of reactions in the smooth endoplasmic reticulum [4–6].

The rate-determining step in T formation is the translocation of cholesterol to the IMM [7]. Numerous studies have reported that this process is mediated by protein–protein interactions of cytosolic and OMM proteins. Among these proteins are LH-inducible STAR, the mitochondrial translocator protein (TSPO) (18 kDa TSPO), voltage dependent anion channel 1, and 14-3-3ε, the latter serving as a negative regulator of steroidogenesis) [8–10]. TSPO has high affinity for cholesterol, which binds TSPO at its cholesterol recognition/interaction amino acid consensus (CRAC) motif [11–13]. Targeted disruption/removal of the Leydig cell *Tspo* gene was shown to arrest cholesterol transport and steroid formation [14], suggesting an important role for TSPO in steroidogenesis. Although TSPO's role in steroidogenesis has been questioned by studies reporting that knocking out *Tspo* had no effect on steroidogenesis [15, 16], recent studies using global and cre-mediated *Tspo* conditional knockout in steroidogenic cells reported loss of hormone-stimulated steroidogenesis and changes in lipid distribution in Leydig cells [17–20]. These studies provide further evidence that TSPO in fact is involved in mediating steroid hormone formation. Additionally, the administration of TSPO-specific drug ligands has been shown to induce the translocation of cholesterol from the outer to the IMM and to stimulate steroid formation in Leydig cells and other steroidogenic cells [21–24].

Low serum T, or hypogonadism, is reported to affect 4 to 5 million men in the U.S. [25–27]. This condition primarily affects older men, but many younger men as well [28]. Insufficient serum T levels are accompanied by symptoms that include decreased muscle mass, increased weight gain, reduced bone density, cognitive changes, erectile dysfunction, increased fatigue, and low libido [29, 30]. In many men, hypogonadism is not the result of inadequate serum LH levels (secondary hypogonadism), but rather results from the reduced sensitivity of the Leydig cells to LH (primary hypogonadism) [31]. There is considerable interest in therapies to combat the decline in T production and thus the side effects of hypogonadism. Exogenous T administration (T replacement therapy [TRT]) typically is used to elevate serum T levels and thereby treat the symptoms of hypogonadism [30]. This approach has benefits but also is reported to have negative effects on the cardiovascular system and possibly on the prostate [32–41]. Moreover, although exogenous T administration results in increased serum T levels, in many men, it also results in diminished sperm count. Indeed, there are ongoing clinical trials in which T administration is being tested for contraceptive purposes [42–47]. Thus, TRT is less than an ideal treatment for men who wish to father children.

Several TSPO-specific drug ligands have been shown to stimulate cholesterol import into the mitochondria of MA-10 mouse tumor cells and primary Leydig cells in vitro and in this way to increase steroid formation by the cells. TSPO ligands also have been shown to result in elevated serum T levels when administered in vivo [8, 22, 48, 49]. Blocking the CRAC domain of TSPO was shown to block *N*,*N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27)-induced T production by Leydig cells both in vitro and in vivo [8, 22]. The exact mechanism remains uncertain, however, a recent study reported at least transient increases in serum LH as well as in serum T in response to TSPO drug ligand, thus raising the possibility that this approach might increase Leydig cell T production not only by stimulating cholesterol translocation but also by increasing LH synthesis or release [49]. Although the exact mechanism by which TSPO drug ligands function remains uncertain, these previous studies suggest that TSPO, and perhaps other cytosolic/outer membrane proteins, may serve as therapeutic targets to elevate serum T levels in cases of hypogonadism. Such an approach would be particularly valuable if it was shown that elevating serum T by stimulating the Leydig cells to produce T would not result in reducing intratesticular T concentration and therefore would not have negative effects on spermatogenesis.

In the studies presented herein, we compared the in vivo effects of TRT versus administering the high-affinity TSPO drug ligand FGIN-1-27 on serum T levels, Leydig cell T formation, and intratesticular T concentration in relationship to the quantitative maintenance of spermatogenesis.

Materials and methods

Reagents

FGIN-1-27, Percoll, HEPES, sodium-bicarbonate, penicillin–streptomycin, dimethylsulfoxide (DMSO), and cetrorelix acetate were obtained from Sigma-Aldrich (St Louis, Missouri, MO). Type III collagenase was from Worthington Biochemical Corporation (Lakewood, NJ). M-199 medium was from ThermoFisher Scientific (Waltham, MA). Bovine serum albumin (BSA) was from ICN Biomedicals, Inc (Aurora, OH). Gentamicin was from Quality Biological (Gaithersburg, MD). [1,2,6,7,16,17-^{3}H(N)]-testosterone (115.3 Ci/mmol) was from Perkin-Elmer Life Sciences, Inc. (Boston, MA). T antibody was from MP Biomedical (Solon, OH). Bovine LH (USDA-bLH-B-6) was obtained by the US Department of Agriculture Animal Hormone Program (Beltsville, MD).

Animals

Young (3-months-old) and aged (21-months-old) Brown Norway rats were supplied by the National Institute on Aging, NIH. The

rats were housed in the animal facilities of the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) in controlled light (14-h light, 10-h dark), at a temperature of 22 °C, and with free access to food and water. All animal handling and care were based on protocols approved by the institutional Animal Care and Use Committee of Johns Hopkins University.

FGIN-1-27 administration versus TRT; effects on Leydig cell T production and on serum and intratesticular T concentrations

Young ($N = 6$) and old ($N = 6$) rats received FGIN-1-27 (1 mg/kg body weight dissolved in 10% DMSO in PBS) via daily ip injection for 10 days [22]. Control rats ($N = 6$) were injected daily for 10 days with vehicle. In some studies, the effects of FGIN-1-27 on T production in vivo were assessed after blocking LH secretion with centrorelix, a GnRH antagonist [8]. For the latter analyses, young rats ($N = 6$) were injected with cetrorelix (0.5 mg/animal dissolved in 10% methanol). One day later, rats began daily ip injections of FGIN-1-27 along with cetrorelix. On day 14, T was measured in duplicate samples of serum and intratesticular fluid (IF) by radioimmunoassay (RIA), according to methods described previously [50]. The assay sensitivity was 10 pg/tube, with intra-assay and interassay coefficients of variation of 11.2 and 9.6%, respectively. For studies of the effects of exogenous T administration, young and old rats ($N = 6$/group) were administrated subdermal 2 cm T-containing or empty silastic implants for 10 days [51].

Primary Leydig cells were isolated by established procedures [52]. In brief, rats were euthanized by decapitation, and the testes were immediately placed in cold dissociation buffer (M-199 medium with 2.1 g/L HEPES, 2.2 g/L sodium bicarbonate, 1.0 g/L BSA, 25 mg/L trypsin inhibitor, pH 7.4). The testicular artery was cannulated, and testes were perfused with type III collagenase (1 mg/mL) in dissociation buffer to clear testicular blood. The testes then were placed in dissociation buffer containing collagenase (0.25 mg/mL) at 34 °C and shaken at 90–100 rpm for 30 min. Digested testes were passed through a 100-µm nylon mesh to remove the tissue clumps, and the interstitial cells were pelleted by centrifugation at 1500 rpm for 5 min. Leydig cells were purified by Percoll gradient separation at 15 000 rpm, 4 °C for 1 h. The final purity of the Leydig cells, determined by staining the cells for 3beta-hydroxysteroid dehydrogenase activity, was consistently approximately 90%. To culture Leydig cells, isolated cells were resuspended in M-199 culture medium, and the cells (5×10^5) were added to a 24-well culture plate and cultured for 2 h in the absence or presence of LH (1 ng/mL) at 34 °C in 5% CO_2 and 95% air [53]. After 2 h incubation, the media were collected and stored at −80 °C for T measurement by RIA.

Serum and IF were collected for T measurement by RIA, as described above. IF was collected as follows: The tunica albuginea was incised at one pole, and testes were centrifuged at low speed ($50 \times g$; 15 min; 4 °C) to drain interstitial fluid. The seminiferous tubules were then extruded through the hub of a syringe, and the preparation was centrifuged ($6000 \times g$; 15 min; 4 °C) to collect seminiferous tubule fluid (STF) as a supernatant above the collapsed seminiferous tubules [43]. Immediately after collection, STF was snap-frozen in liquid nitrogen and subsequently stored frozen at −80 °C before assay for T. LH concentration in serum was determined using rat LH ELISA kits (Abnova Corp., Taipei, Taiwan) following the manufacturer's instructions. The intra- and interassay variabilities were 5.4 and 5.17% coefficient of variation. The detection range of the assay was 0–50 ng/mL.

T and spermatogenesis maintenance

To determine the T concentration required to maintain spermatogenesis, rats ($N = 6$/group) received subdermal T-containing implants of 1, 2, 3, 6, 12, or 24 cm lengths. After 8 weeks, rats were euthanized by decapitation, trunk blood was collected, and serum was collected and stored frozen at −80 °C for subsequent determination of T by RIA. STF was collected from one testis per rat as above, snap-frozen in liquid nitrogen, and subsequently stored frozen at −80 °C before assay for T. The contralateral testis from each rat was used for determining the numbers of advanced spermatids per testis by the hemocytometric counting of testicular homogenates under phase contrast microscopy [47].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments. For multiple group comparisons, one-way ANOVA followed by a Duncan's post hoc test were performed by GradPad Prism software ver. 4.02 (GraphPad Inc., La Jolla, CA). Values were considered significantly different at $P < 0.05$.

Results

The mean serum T level was significantly lower in old as compared to young Brown Norway rats (Figure 1A). The mean intratesticular T level in aged rats, approximately 20–25 ng/mL, also was significantly lower, by about 35–40%, than in young rats (Figure 1B). Leydig cells isolated from old rats produced significantly less T than Leydig cells isolated from young rats whether under basal conditions (without LH, Figure 1C) or in response to LH (Figure 1D). Interestingly, the efficiency of LH stimulation was comparable in Leydig cells from young vs old rats. These results suggest that reduced serum and intratesticular T levels in old rats results from reduced T production by the Leydig cells.

To determine the effects on serum and intratesticular T levels of administering T to hypogonadal old rats, 2 cm T-containing silastic capsules were implanted subcutaneously. Control rats received implants containing vehicle only. After a 10-day period, T concentrations were measured in serum and IFs. A significant (4-fold) increase was seen in T concentration in the serum of T-implanted old rats compared to the controls (Figure 2A). In contrast, there was a significant, approximate 50%, decrease in intratesticular T in rats that received the implants, with the level below 10 ng/mL (Figure 2B).

We next examined the effect of administering the TSPO ligand FGIN-1-27 on serum and intratesticular T levels. FGIN-1-27 (1 mg/kg body weight) was administered to aged rats via daily ip injection for 10 days. Control rats received vehicle. A significant increase in serum T concentration was seen in FGIN-1-27-treated old rats (Figure 3A). In striking contrast to the reduction in intratesticular T observed following the administration of exogenous T (Figure 2B), FGIN-1-27 induced a significant, 1.5-fold, increase in the IF T concentration compared to controls (Figure 3B). Consistent with this, Leydig cells isolated from old rats that had received FGIN-1-27 produced significantly higher levels of T in response to LH than cells isolated from rats that had been administered exogenous T (Figure 3C).

We hypothesized that the differences in Leydig cell T production and intratesticular T concentration seen in response to T versus FGIN-1-27 might result from differential effects on serum LH levels. As seen in Figure 4, T administration resulted in significant reduction

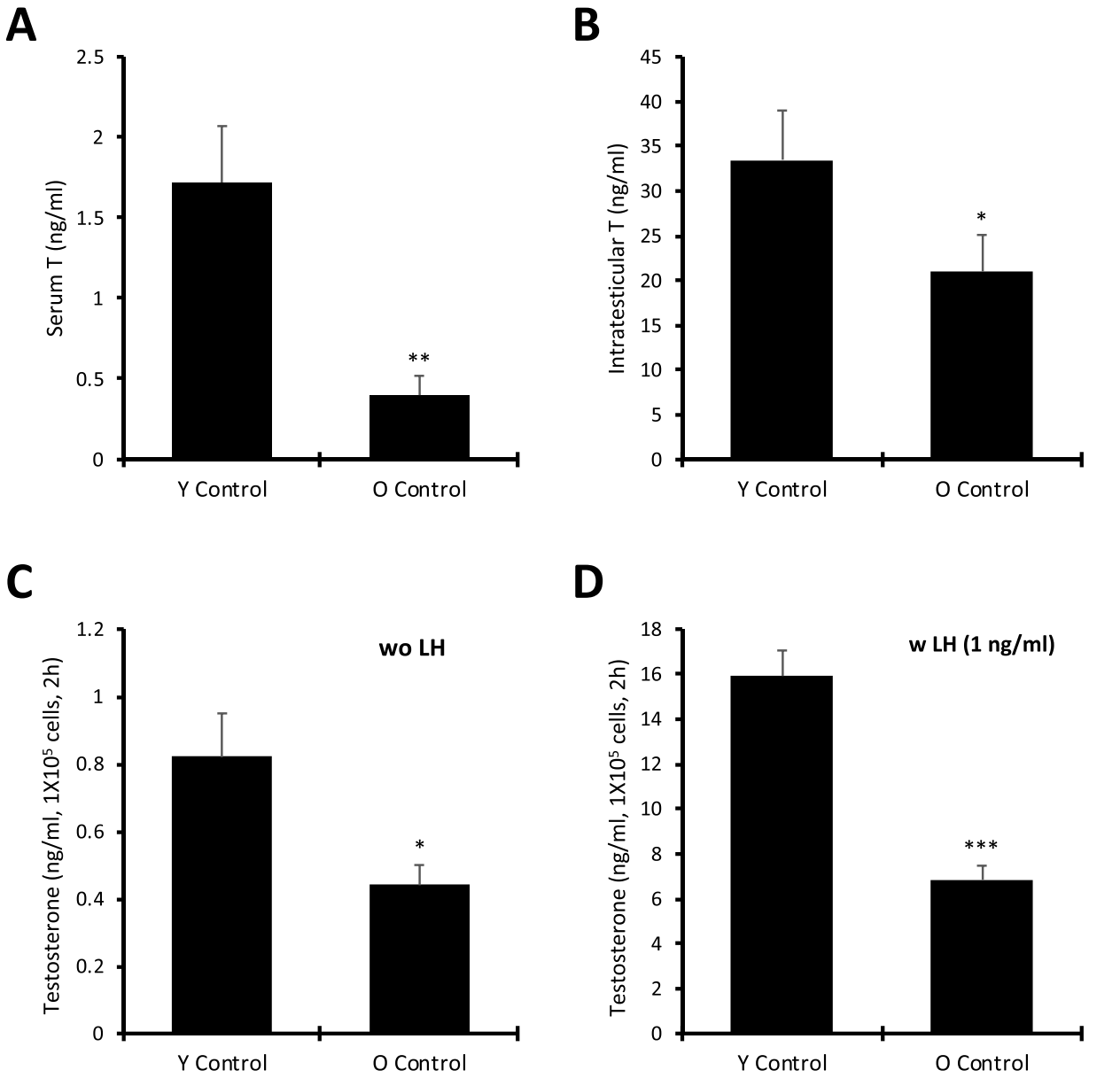

Figure 1. Serum (A) and intratesticular (B) T concentrations in young versus old control rats; and T produced by Leydig cells isolated from young and old control rats and cultured without (C) or with (D) LH. In each case, at least three independent experiments were performed (mean ± SEM). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of LH levels from controls. In contrast, the mean serum LH level in rats administered FGIN-1-27 for 10 days was comparable to that of controls (Figure 4). These results suggested that the increased T produced by the Leydig cells of FGIN-1-27-treated rats might be the result of stimulation of the cells by LH, stimulation by FGIN-1-27, or both. To address this, we asked whether FGIN-1-27 alone, in the absence of LH, would result in increased serum and intratesticular T concentrations. Rats were administered cetrorelix, a GnRH antagonist, to inhibit LH, and then received cetrorelix alone or in combination with FGIN-1-27 daily for 13 days. Cetrorelix administration resulted in reductions in LH (Figure 5A), as well as reductions in serum (Figure 5B) and intratesticular T (Figure 5C). Administering FGIN-1-27 together with cetrorelix did not stimulate LH production (Figure 5A) but did significantly increase both serum and intratesticular T (Figure 5B and C). These increases were not as dramatic as after FGIN-1-27 treatment alone, in the absence of cetrorelix (Figure 3A and B).

In light of the significant, quantitative differences in intratesticular T concentrations resulting from exogenous T versus FGIN-1-27 administration, we addressed the quantitative relationship between intratesticular T concentration and numbers of advanced spermatids. To this end, T-containing silastic implants of increasing sizes were implanted in young rats. After 8 weeks, T concentrations in IF were determined by RIA (Figure 6A), and the numbers of advanced spermatids per testis were determined by hemocytometric counting of advanced spermatids in testicular homogenates (Figure 6B). The intratesticular T concentration and sperm count in control rats were 31.2 ± 2.5 ng/mL and $214.9 \pm 4.5 \times 10^6$ homogenization-resistant advanced spermatids/testis, respectively. In rats that received 2 cm implants for 8 weeks, the intratesticular T concentration

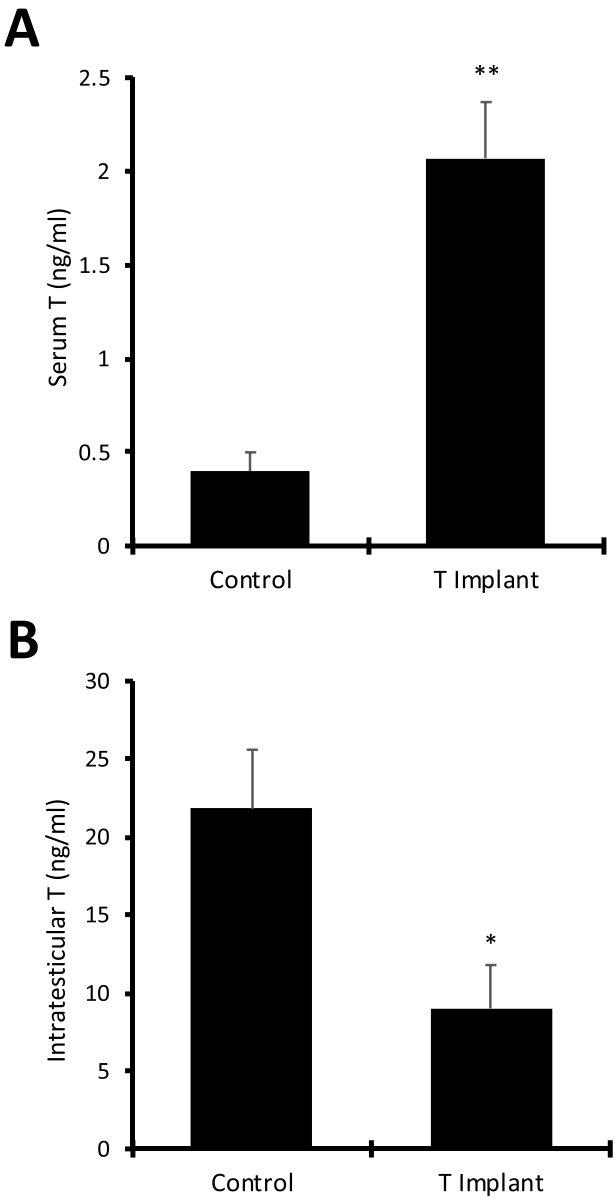

Figure 2. Serum (A) and intratesticular (B) T concentrations in old rats administered an empty 2 cm silastic implant (control) or a 2 cm T-containing implant (T implant). In each case, at least three independent experiments were performed (mean ± SEM). $^{*}P < 0.05$, $^{**}P < 0.01$.

(7.5 ± 0.6 ng/mL) and sperm count (34.1 ± 16.0 × 10^6/testis) were reduced significantly from controls to their lowest values. Increases in intratesticular T concentrations and advanced spermatid numbers were seen with increased implant lengths from 3 to 24 cm. Comparison of Figure 6A and B shows that advanced spermatid numbers were reduced significantly at intratesticular T concentrations of 10 ng/mL and below. Notably, the administration of T resulted in an intratesticular T concentration below 10 ng/mL (Figure 2B) and that after FGIN-1-27 was well above this concentration (Figure 3B).

Discussion

Male fertility requires T concentrations within the seminiferous tubules that are sufficiently high to maintain spermatogenesis [1].

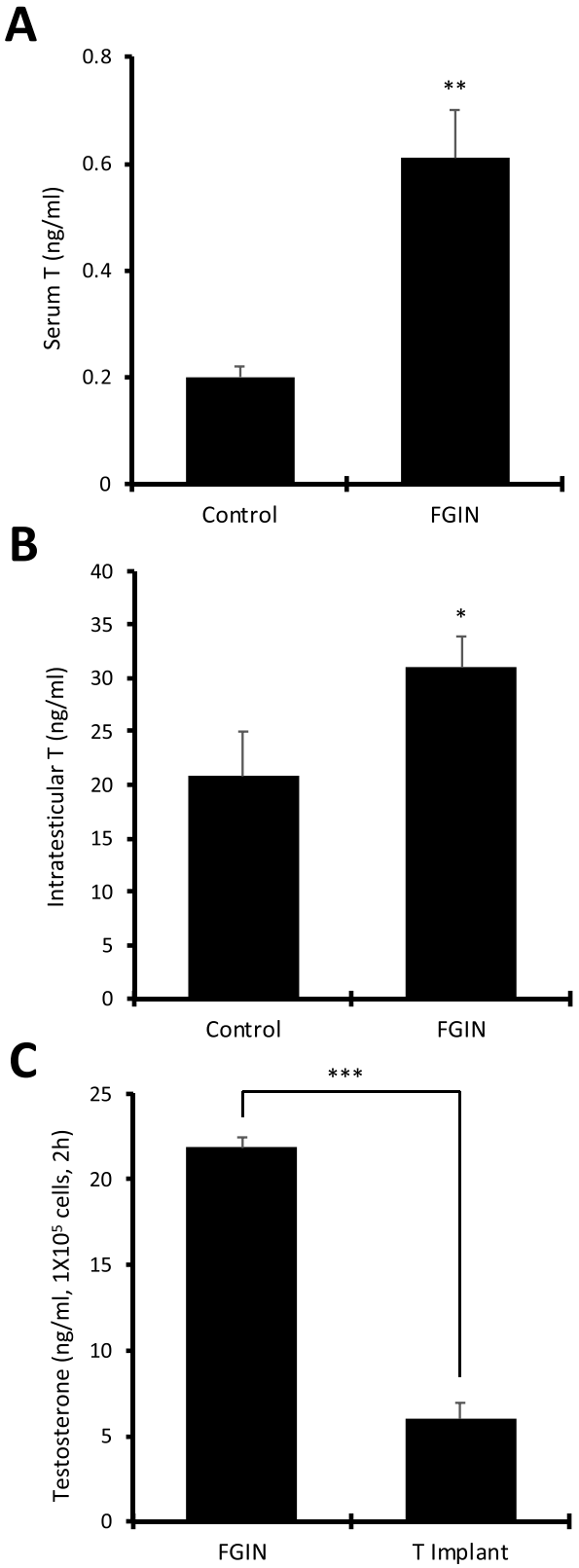

Figure 3. Serum (A) and intratesticular (B) T concentrations in old control rats and old rats administrated FGIN-1-27 (1 mg/kg body weight) via daily ip injection for 10 days, (C) T produced by Leydig cells isolated from old rats administered FGIN-1-27 or T-containing silastic implant. In each case, at least three independent experiments were performed (mean ± SEM). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

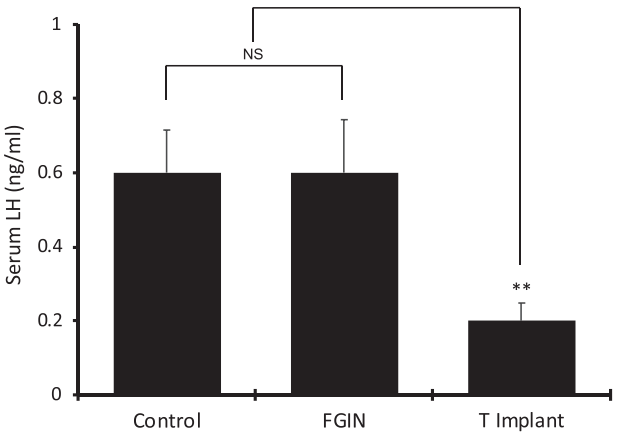

Figure 4. Serum LH levels in old control rats, and old rats administered FGIN or T. In each case, at least three independent experiments were performed (mean ± SEM). NS, nonsignificant, $^{**}P < 0.01$.

In man and rat, the intratesticular T concentration is far higher than its concentration in the peripheral circulation [36, 54]. In both, intratesticular T can be reduced substantially without an effect on spermatogenesis. However, there is a minimally required concentration below which there will be reduced advanced spermatid numbers. Infertility will result if the intratesticular concentration becomes low enough [47, 55, 56].

It has been known for some time that in men and rats, the minimally required T concentration is considerably higher than the serum T concentration. Much of the T within the seminiferous tubules of man and rat (but not mouse) [57, 58] is bound by androgen-binding protein and therefore is unavailable for binding to the Sertoli cell androgen receptors [59]. Whether or not this explains the intratesticular T concentration required for the quantitative maintenance of spermatogenesis, however, remains unanswered. Indeed, previous studies reported that the T concentration required to maintain or restore spermatogenesis exceeds the intratesticular androgen receptor concentration [60].

The results of the basic studies described above are relevant to the treatment of men with hypogonadism. Although of the 4–5 million men in the United States affected by hypogonadism, many may not be concerned about their fertility (25–27), there also are many men of reproductive age who are hypogonadal (28). Thus, approximately 15% of men who are among the 15% of couples who seek infertility-related medical appointments have been reported to be hypogonadal [61, 62]. Other young men who also may present with hypogonadism include those with sickle cell disease, spinal cord injury, orchitis, genital injury, or testicular torsion, or after surgery, chemotherapy or irradiation [28, 63–66]. The therapy of choice for most hypogonadal men is exogenous T administration (TRT). It has been reported that 12% of men in the United States who have initiated TRT treatment are between the ages of 18 and 39 years [67]. Although this treatment does result in increased serum T levels, in many men, it also results in diminished sperm count. Indeed, there are ongoing clinical trials in which T administration is being tested for contraceptive purposes [42–47]. Thus, TRT is less than an ideal treatment for men who wish to father children. Therapies that are capable of elevating serum T levels without negative effects on spermatogenesis clearly would be desirable.

Knowledge of the steps and mechanisms involved in T formation has made it possible to use pharmacological means to restore the ability of the hypofunctional Leydig cells of aging, hypogonadal

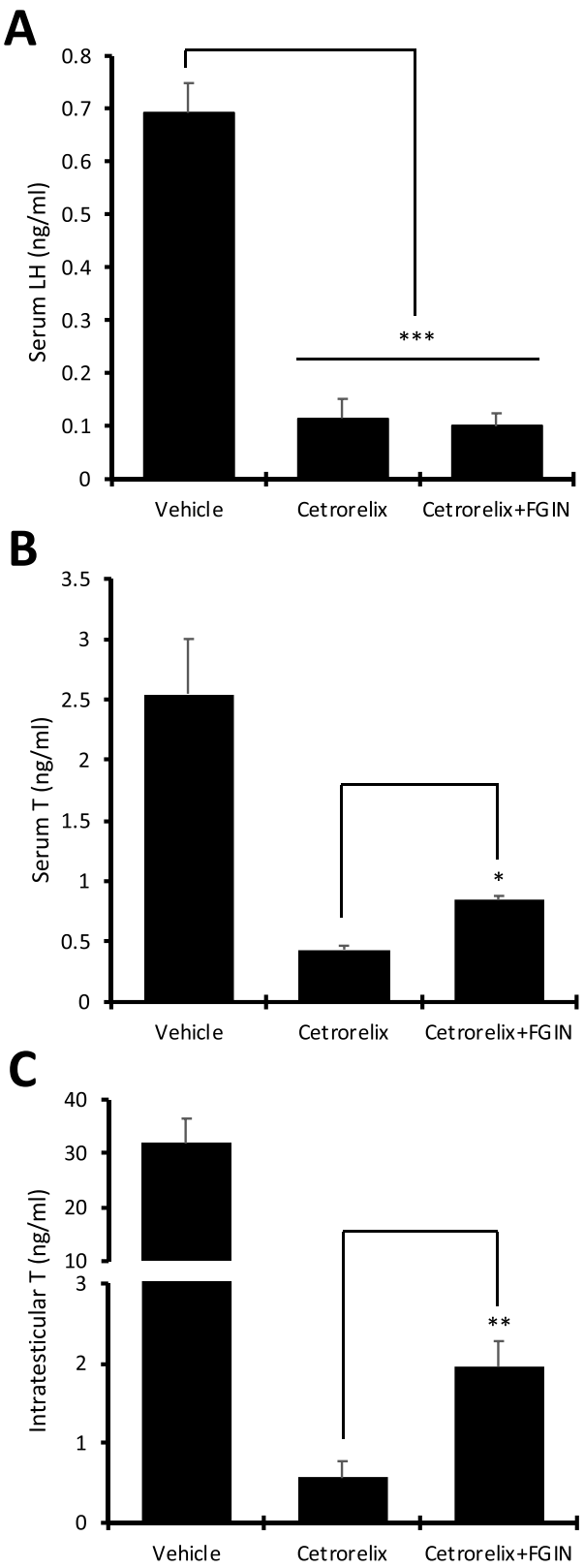

Figure 5. Serum LH (A), serum T (B), and intratesticular T (C) concentrations in young rats administrated cetrorelix (0.5 mg/animal/day, 14 days) alone or in combination with FGIN-1-27. In each case, at least three independent experiments were performed (mean ± SEM). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

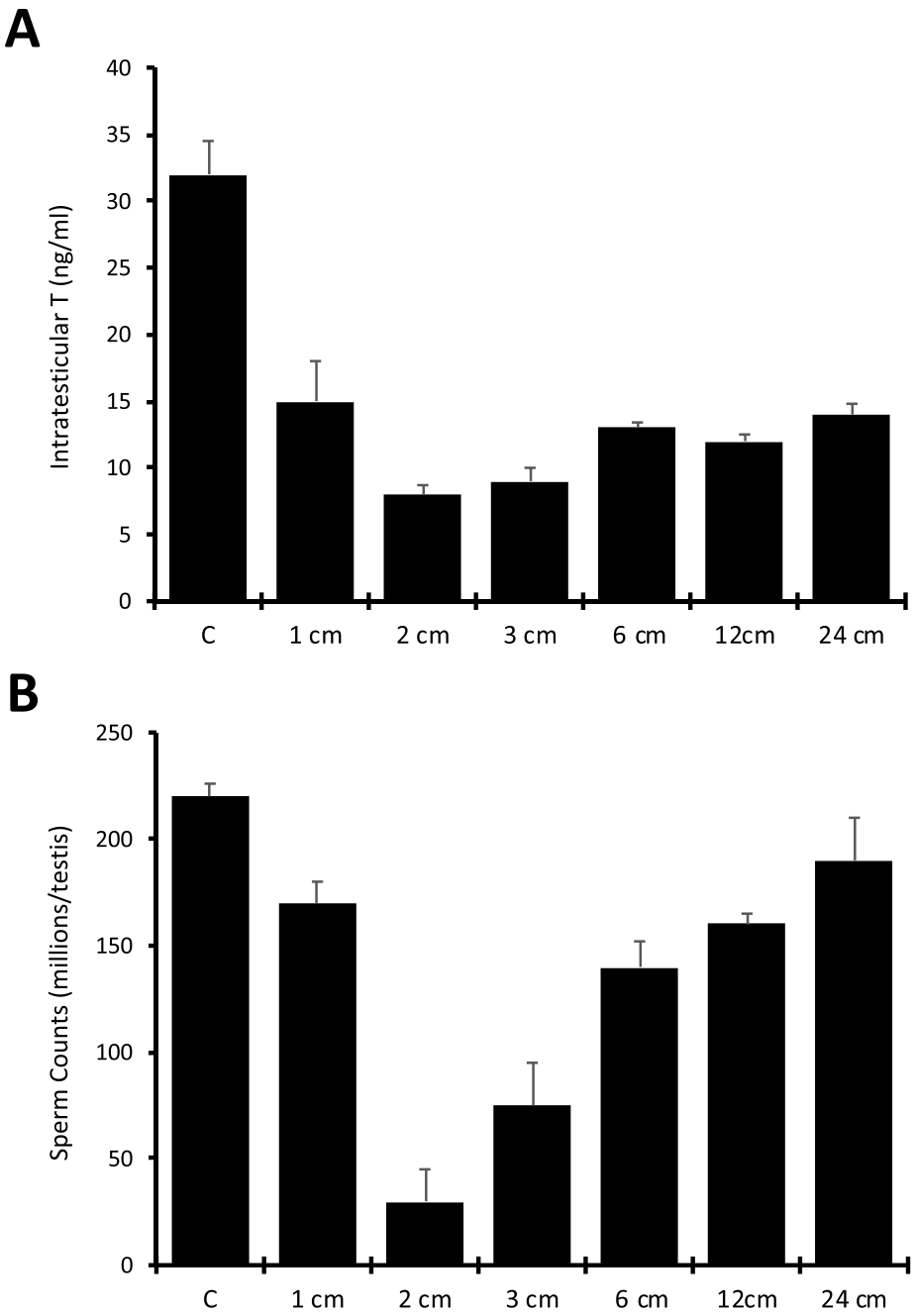

Figure 6. Intratesticular T concentration (A) and advanced spermatid numbers (sperm count, B) in young rats administered T implants of 1–24 cm total lengths for 8 weeks. Control rats (denoted C) received empty implants.

Brown Norway rats to produce higher levels of T and thus elevate serum T levels [68]. TSPO, which comprises 2% of OMM proteins, has been widely reported to be involved in the translocation of cholesterol to the IMM [11], the rate-determining step in steroid formation. We hypothesized herein that administering FGIN-1-27 to hypogonadal aged Brown Norway rats, by stimulating the Leydig cells themselves to produce T, would increase serum T to eugonadal levels, but that, in contrast to TRT, would do so without diminishing intratesticular T levels and therefore without suppressing spermatogenesis. The approach was based on previous animal and cell studies that had shown that T production can be stimulated by specific high affinity TSPO drug ligands [22, 69–71] and by clinical studies showing that TSPO-activating ligands are able to increase neurosteroid formation in men [24, 72, 73]. We found that both FGIN-1-27 and T administration significantly increased serum T levels in hypogonadal rats, to levels found in young rats. However, whereas T administration resulted in significant reductions both in the ability of the Leydig cells to produce T and in intratesticular T concentration, FGIN-1-27 administration increased the ability of the Leydig cells to produce T and increased intratesticular T concentration.

In untreated control rats, T is produced in pulses in response to LH pulses that occur as serum T diminishes. Administering exogenous T results in constant serum T levels and thus would be expected to have negative feedback effects on LH, resulting in the

suppression of Leydig cell T production. FGIN-1-27 administration results in increased T production by the Leydig cells, but as in control rats, mean serum LH levels changed little. It is not known whether, as in controls, the Leydig cells of FGIN-1-27 treated animals respond to the LH that is present and therefore T production is pulsatile, Alternatively, as we reported recently, FGIN 1-27 might act both by increasing cholesterol translocation into the mitochondria and by increasing LH synthesis and/or release (49). The results of studies with cetrorelix were consistent with the ability of FGIN-1-27 to act to increase T even in the absence of LH. Cetrorelix blocks GnRH so that there is little or no LH release. FGIN-1-27 was found to elevate serum and intratesticular T levels in the cetrorelix-treated rats despite the absence of LH. This is in agreement with previous data by Aghazadeh et al. [8]. However, the increases were well below the response to FGIN-1-27 treatment of rats that had not received cetrorelix. We conclude from these studies that there may be additive or synergistic effects between LH and FGIN in the case of FGIN administration to otherwise untreated rats.

FGIN-1-27 administration increased intratesticular T to levels above those seen in controls, whereas administering T resulted in intratesticular T levels reduced to below 10 ng/mL. As seen in Figure 6, the 10 ng/mL concentration represents a T concentration below that required to sustain advanced spermatid production quantitatively in the Brown Norway rat testis. These results suggest that in contrast to elevating serum T levels by administering T, targeting TSPO with specific drug ligands might provide a means by which to increase serum T levels without negative effect on spermatogenesis [68]. Moreover, TSPO is not the only Leydig cell protein involved in cholesterol translocation that might be targeted for enhancing serum T levels in hypogonadal individuals. For example, 14-3-3ε is a negative regulator of T production [8, 74]. If negative regulation could be removed in Leydig cells, the cells would produce more T, resulting in increased serum and intratesticular T. Taken together, these data suggest that the possibility of a clinical approach to increasing serum T levels in hypogonadal individuals that would not affect intratesticular T concentration and therefore would not affect, or might even enhance, spermatogenesis. Such an approach would be compatible with the desire to father children.

Conflicts of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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