Leydig stem cells and future therapies for hypogonadism

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Purpose of review
In this review, we outline the most recent advances in the development of Leydig stem cells (LSCs) and summarize the current and upcoming treatments for hypogonadism.

Recent findings
In-vitro and in-vivo studies show that inducing stem cells to differentiate into testosterone-producing adult Leydig cells is possible. In addition, LSCs can be grafted with Sertoli cells to increase testosterone levels *in vivo*. This therapy causes minimal effects on luteinizing hormone and follicle stimulating hormone levels. Novel therapies for hypogonadism include varying methods of testosterone delivery such as intranasal and oral agents, as well as novel selective estrogen and androgen receptor modulators.

Summary
LSC therapies provide an effective way of increasing testosterone levels without detrimentally affecting gonadotropin levels. Next steps in developing viable Leydig cell grafting options for the treatment of hypogonadism should include the assessment of efficacy and potency of current animal models in human trials. Recently, both intranasal and oral testosterone have been made available and shown promising results in treating hypogonadism while maintaining fertility. Enclomiphene citrate and selective androgen receptor modulators have been suggested as future therapies for hypogonadism; however, further studies assessing efficacy and adverse effects are needed.

Keywords
enclomiphene citrate, intranasal testosterone, Leydig stem cell therapy, oral testosterone, selective androgen receptor modulators

INTRODUCTION

Male hypogonadism, as defined by the Endocrine Society, is 'a clinical syndrome that results from failure of the testis to produce physiological levels of testosterone and a normal number of spermatozoa due to disruption of one or more levels of the hypothalamic–pituitary–testicular axis' [1]. A low testosterone state can impact both physical and mental health, leading to concurrent symptoms of decreased libido, erectile dysfunction, infertility, low energy, low mood, depression, gynecomastia, decreased lean muscle mass, increased body fat gain, and low-trauma fractures that are possibly due to osteoporosis [1]. In addition, low serum testosterone has been shown to impact cardiovascular health, metabolism, and longevity. The Endocrine Society recommends using both clinical evaluation of symptoms and consistently low serum total or free testosterone concentrations to diagnose hypogonadism [1]. Whereas the American Urological Association recommends a serum total testosterone cutoff of below 300 ng/dl, in conjunction with clinical symptoms [2]. Total serum testosterone measurements should be taken in the morning ideally before 10 a.m., when testosterone levels peak. Abnormal levels should be followed up with a confirmatory repeat measurement [1]. The exact prevalence of hypogonadism is unknown. Due to the difference in criteria used to define hypogonadism and the lack of validated standard to define the cutoffs of different age groups current epidemiological studies are not easily comparable. Both the

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KEY POINTS

- In-vitro studies have shown that both induction of stem cells into adult Leydig cells and direct harvesting of LSCs for grafting purposes are viable methods of increasing testosterone levels. Similarly, in-vivo rodent studies show that LSC harvesting and later grafting significantly increase testosterone levels with minimal effects on hypothalamic–pituitary–gonadal axis signaling.
- Both intranasal (Natesto) testosterone and oral testosterone undecanoate have been shown to treat symptomatic hypogonadism in men, with minimal side effects.
- Enclomiphene citrate has not shown any difference in side effect profile when compared with clomiphene citrate; however, it has been shown to preserve or improve spermatogenesis when compared with testosterone gels.
- Selective androgen receptor modulators are currently not being used to treat symptomatic male hypogonadism; however, their similarity to selective estrogen modulators could make them a potential future treatment for low testosterone.

Massachusetts Male Aging Study and the Boston Area Community Health Survey found the prevalence of male hypogonadism to be 5.6%, while the European Male Aging Study reported a prevalence of 2.1% [3]. All of the above studies found that as age increased, the prevalence of symptomatic male hypogonadism increased as well. A study by Lokeshwar *et al.* [4▪] showed that serum total testosterone in adolescent and young adult (AYA) males has been decreasing, with increasing BMI, over recent decades. Identification of hypogonadism in AYA is important as this population presents with few signs, low energy being the most common. Treatment choices should be thoroughly discussed with the patient as some may impact fertility, a detrimental adverse effect in AYA. The treatment for hypogonadism in older men is of equal importance, as the desire for fertility and children has been increasing in this population as well [5▪]. Testosterone replacement therapy (TRT) has been shown to increase hematocrit, blood pressure (BP), and atherosclerosis among other adverse effects, hence novel strategies for hypogonadism that maintain physiology with minimal disruption of homeostasis are needed. In this review, we will summarize the most recent data on the use of Leydig stem cells (LSCs) therapies to treat hypogonadism in men and will discuss other future novel treatments currently under investigation.

LEYDIG STEM CELL AUTOGRAFT

The nonpharmacological treatment of hypogonadism regimens remains an area with many unanswered questions. Until recently, LSC therapy had been relatively unexplored. Interest increased along with the possibility of avoiding the unintended negative effects that current TRTs are faced with such as increased visceral fat content, prostate size, hypertension, worsening obstructive sleep apnea, and increased risk of cardiovascular events. A concerning side effect for hypogonadal men receiving TRT and still desiring to maintain their fertility, is the treatment's suppressive effects on the hypothalamic–pituitary–gonadal (HPG) axis and associated sequelae. Namely, spermatogenesis is detrimentally affected, causing decreased fertility in men receiving exogenous testosterone. This can be attributed to the negative feedback testosterone has on the hypothalamus and pituitary leading to a decrease in pulsatile gonadotropin releasing hormone (GnRH) release, as well as lower levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH), both essential for appropriate signaling and maintenance of intratesticular testosterone necessary for spermatogenesis. Even though the short-term effects testosterone therapy has on spermatogenesis can be reversed, long-term TRT can be discouraging for men trying to have children. As such, LSC therapies may present a novel long-term treatment opportunity that could have little effect on the HPG axis and spermatogenesis.

IN-VITRO LEYDIG CELL INDUCTION

Efforts have been made to show that stem cells, both human and animal, can differentiate into testosterone producing Leydig cells. In one study, human bone marrow cells were cultured in vitro in an induction medium containing gonadotropins such as human chorionic gonadotropin (hCG), human menopause gonadotropin, LH, and FSH [6]. Immunohistochemistry showed an increased number of cells expressing 3-beta hydroxysteroid dehydrogenase, a protein commonly expressed by LSCs. Flow cytometry confirmed these results. Notably, a significant increase in testosterone production was found compared with controls, showing that testosterone producing Leydig cells can be induced from human stem cells through exogenous factors such as gonadotropins [6]. A similar study investigated the effects of an induced pluripotent cell-derived conditioned medium on immature Leydig cells (ILCs) [7]. ILCs were isolated from Sprague Dawley rats and seeded for treatment with the medium. The conditioned medium caused increased proliferation and decreased apoptosis when exposed to H_2O_2 [7]. In

addition, an increase in testosterone and steroidogenic enzyme synthesis were detected in ILCs cultured in the conditioned medium [7].

Limitations on the expansion of mesenchymal cells in culture led to the investigation of human-induced pluripotent cells as a method of treating hypogonadism [8]. Notably, the pluripotent cells were first induced to differentiate into early mesenchymal cells and then exposed to steroidogenic factor 1, desert hedgehog (DHH), hCG, and cyclic adenosine monophosphate for the final differentiation into Leydig-like cells (LLCs) [8]. These cells were shown to have similar steroidogenic pathways for testosterone biosynthesis as adult Leydig cells (ALCs) [8]. It was noted that exposure with DHH, which is secreted by Sertoli cells, was important in the induction of pluripotent cells into LLCs [8]. This suggests that Sertoli cells may provide necessary signals for the appropriate development of stem cells into ALCs [8]. These results show that grafting stem cells or stem cell-like cells and exposing them to exogenous factors could be a viable method of treating symptomatic hypogonadism in men.

ANIMAL STUDIES ON LEYDIG CELL GRAFTING

In-vivo models are necessary to assess the viability of autografting stem cells to induce Leydig cell differentiation. Huang *et al.* [9] showed that fibroblast cells extracted from the foreskin of patients with phimosis were able to be virally transfected with genes inducing Leydig cell transformation. Even though differentiation of fibroblasts into Leydig cells was carried out *in vitro*, the resulting Leydig cells were able to partially recover testosterone levels when grafted in castrated male rats [9]. This provides an important avenue to explore whether the differentiation of stem cells into Leydig cells should be carried out *in vitro* prior to grafting to increase testosterone levels. Chen *et al.* [10] used direct intratesticular injection of fibroblast growth factor 1 (FGF-1) into Sprague Dawley rats with ablated ALCs to assess the possibility of regeneration. It was found that FGF-1 treatment promoted LSC proliferation, but did not promote differentiation into ALCs [10]. In addition, treated rats were shown to have increased levels of serum testosterone with no significant effects on serum LH and FSH [10]. This suggests that the changes in serum testosterone were due to direct effects of FGF-1 on testicular tissue [10]. Arora *et al.* [11▪] proposed a different method of harvesting LSCs directly from seminiferous tubules in 6-week-old mice for later subcutaneous implantation in castrated adult mice to assess changes in testosterone levels. Serum testosterone levels in rats grafted with extracted LSCs were shown to have significantly increased compared with control [11▪]. Significantly, Sertoli and myoid cells grafted in conjunction with extracted LSCs were shown to be essential for graft viability [11▪]. Mice grafted with LSCs were shown to maintain significantly higher levels of FSH and LH in comparison with mice treated with exogenous testosterone, further supporting the idea that Leydig cell autografting can be a viable way of treating low testosterone levels with little HPG suppression [11▪]. A study involving Sertoli cells transplantation in mice to ameliorate azoospermia showed that Sertoli cells grafting was able to increase the number of spermatogonia, primary spermatocytes, and Leydig cells [12▪▪]. This strengthens the association of Sertoli cells and their positive effects on Leydig cell viability. Nevertheless, testosterone levels in Leydig cell grafts with or without Sertoli cell support remains to be explored in human models.

FUTURE THERAPIES

Direct TRT is available in many preparations, such as patches, topical gels, intramuscular injections, and subcutaneous pellets. These long-acting formulations have been shown to not only treat male hypogonadism, but also suppress the pulsatile release of GnRH from the hypothalamus. This suppression of the hypothalamic component of the HPG axis results in decreased intratesticular testosterone and inadequate spermatogenesis. Off-label treatments for hypogonadism include selective estrogen modulators (SERMs) like clomiphene citrate, aromatase inhibitors, and gonadotropins such as LH and FSH, as well as U.S. Food and Drug Administration (FDA)-approved treatments like hCG. These have all been shown to increase endogenous testosterone while maintaining spermatogenesis. All of the above therapies pose their fair share of burdens such as excessive dosing, pain with injections, invasiveness of pellets, and various other side effects. In addition to the ongoing research into the possibility of using autologous LSCs as a source of testosterone, various other avenues are being explored to maximize the available options for symptomatic hypogonadism. In this section, we discuss the current research and use of intranasal and oral testosterone, enclomiphene citrate, and selective androgen receptor modulators (SARMs) in the treatment of male hypogonadism.

INTRANASAL TESTOSTERONE

Intranasal testosterone was first approved by the FDA in 2014 for use in men with low testosterone.

Natesto (Acerus Pharmaceuticals, Mississauga, Canada) 4.5% testosterone nasal gel requires frequent dosing at 11 mg/dose, three times daily; however, due to its short acting properties, Natesto mimics physiological testosterone release better than other testosterone replacement modalities. Due to the high permeability of the nasal mucosa, intranasal testosterones display superior bioavailability while bypassing first-pass metabolism. In a recent single institute, open-label, single-arm trial, 60 men with symptomatic hypogonadism were treated with Natesto for 6 months [13▪,14]. Despite a slight decrease in serum gonadotropins, intranasal low-dose testosterone maintains LH and FSH levels within normal limits in the majority of men. In the trial, greater than 95% of men saw an increase in serum testosterone levels while maintaining spermatogenesis, in contrast to men treated with patches, gels, injections, or pellets. Men also reported improvement of symptoms of hypogonadism and erectile dysfunction, as well as quality of life. The most common side effects reported were nasal irritation and oligospermia, seen in 5% of men [13▪].

ORAL TESTOSTERONE UNDECANOATE

Jatenzo (Clarus Therapeutics, Northbrook, Illinois, USA), an oral testosterone undecanoate, was recently approved by the FDA in 2019. Oral testosterone agents have been available for over 50 years, but due to first-pass metabolism through the liver, the bioavailability and risk of hepatotoxicity were too great, and these agents were never approved in the USA. However, Jatenzo is a castor-oil based preparation of testosterone undecanoate designed to be taken twice daily (starting dose 237 mg) with a fatty meal and absorbed via intestinal lymphatics thereby avoiding first-pass metabolism through the liver. This allows for a greater safety profile compared with other oral agents. In a recent study, Jatenzo was able to restore serum testosterone levels to eugonadal levels in 87.3% of hypogonadal men, while providing significant clinical symptom relief [15▪▪]. A study by Chen, *et al.* [16▪▪] showed that the combination of Jatenzo with hCG normalized serum testosterone and promoted virilization without impacting spermatogenesis in hypogonadotropic hypogonadal men. Side effects are minimal and include heartburn, headache, decreases in HDL, and a slight increase in BP. With a half-life of only 29 h, oral testosterone undecanoate could be used to treat symptomatic male hypogonadism as well as maintain physiology, similar to what has been shown with intranasal testosterone.

ENCLOMIPHENE CITRATE

Enclomiphene citrate is a more potent trans-isomer of the SERM clomiphene citrate with similar antiestrogenic effects in the hypothalamus and pituitary. Phase 2 randomized clinical multicenter trials have shown enclomiphene citrate to increase serum testosterone and gonadotropin (LH and FSH) levels while preserving or improving spermatogenesis in both obese and nonobese men, when compared with testosterone gel [17]. As phase 3 studies continue, enclomiphene citrate remains non-FDA approved due to the lack of symptomatic benefits when compared with clomiphene citrate [18]. If approved by the FDA, it may be an oral alternative for treatment of hypogonadism in younger men desiring to maintain fertility.

SELECTIVE ANDROGEN RECEPTOR MODULATORS

SARMs are a conceptual analog to SERMs. They would offer good oral bioavailability, strong anabolic effects, small to no androgenic effects, and tissue specificity to reduce off-target effects. Currently, Enobosarm (GTx, Inc., Memphis, Tennessee, USA) is the only SARM in clinical trials. It has been tested in two double-blind placebo-controlled phase 3 clinical trials looking at its ability to prevent therapy-associated cachexia in men and women with nonsmall cell lung cancer (NSCLC). These trials showed promising increase to lean body mass and physical function versus placebo, and Enobosarm has been fast tracked by the FDA for treatment in NSCLC [19]. Side effects for Enobosarm include increases in hemoglobin and alanine aminotransferease, as well as decreases in HDL [20]. However, no virilization or hirsutism was seen in women, and no prostatism was seen in men [20]. The selectivity, as well as the ability to increase endogenous levels of gonadotropins, in turn increasing endogenous testosterone make SARMs a promising future therapy for male hypogonadism.

CONCLUSION

LSC therapies provide an opportunity to treat hypogonadism while avoiding the suppressive effects of exogenous testosterone. Many questions remain to be answered about the longitudinal effects that LSC transplantation may have. Further studies are needed to elucidate the most efficient methods of grafting and implantation that will provide an appropriate increase in testosterone. Recently, intranasal and oral testosterone have proven to treat hypogonadism in men who desire fertility. Enclomiphene citrate and SARMs could prove another avenue of treating symptomatic hypogonadism

without impacting spermatogenesis. Future efforts should focus on developing LSC models that can move forward into clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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