

Hypogonadotropic Hypogonadism in Obese Young Males

Obesity in men is associated with hypogonadotropic hypogonadism (HH). 25% of obese men have subnormal testosterone (T) concentrations (1, 2). Men with type 2 diabetes and metabolic syndrome also have a high prevalence of HH (3). The prevalence of subnormal T concentrations is directly proportional to the insulin resistance in these men (4). This association is observed at all ages, including young men and obese adolescents (5). We have recently shown that T concentrations of adolescent boys at completion of puberty (Tanner stage 5) are only half of those in lean boys (6, 7). 40% of the obese boys have free testosterone (FT) concentrations lower than the 5th percentile of those in lean boys (6). The gonadotropin concentrations in these boys with subnormal T concentrations were not high, hence they had HH.

The cause of obesity related HH is not well understood. Since T and androstenedione in the male can be converted to estradiol and estrone respectively through the action of aromatase in the mesenchymal cells and pre-adipocytes of adipose tissue, it has been suggested that excessive estrogen secretion due to aromatase activity in the obese may potentially suppress the hypothalamic secretion of gonadotropin releasing hormone (GnRH) (2, 8). It therefore follows that the estradiol concentrations in men with HH and obesity should be elevated to account for the suppression of gonadotropin secretion. However, we have shown that this widely believed presumption is not true (9, 10). Estradiol concentrations are lower in males with HH as compared to eugonadal obese men. This is true in men at all ages (adolescents, middle-age and elderly) (6, 9, 11). Thus, other factors associated with obesity likely account for the HH. It is now known that kisspeptin, a hypothalamic neuropeptide encoded by the *KISS1* gene, and the presence of kisspeptin receptors on the GnRH neurons (G protein-coupled receptor 54) is obligate for the release of GnRH (12, 13). Kisspeptin neurons express both leptin and insulin receptors, thus possibly accumulating evidence of metabolic health and translating it into reproductive health. Leptin appears to serve as a signal of energy reserves to regulate the hypothalamo-pituitary-gonadal axis in relation to nutritional status (14). It is possible that leptin resistance in neurons may contribute to the pathogenesis of hypogonadotropism seen in obesity. Direct evidence in humans supporting or disproving this reasoning is, however, lacking. Interestingly, the selective deletion of the insulin receptor from neurons leads to a reduction in LH and T concentrations (15). In addition, these animals have atrophic seminiferous tubules with markedly impaired or absent spermatogenesis. Incubation of hypothalamic neurons with insulin results in the facilitation of secretion of GnRH (16, 17). Thus, insulin action and insulin responsiveness in the brain are necessary for the maintenance of the functional integrity of the hypothalamo-hypophyseal-gonadal axis. A combination of leptin and insulin resistance may play a role in the pathogenesis of these defects.

Obese men with HH have more subcutaneous and visceral fat and are more insulin resistant than eugonadal obese men (4). Similarly, T concentrations in obese adolescent boys are inversely related to insulin resistance and C-reactive protein (CRP) concentrations, independent of obesity (6). We have recently shown that T replacement in middle aged men decreases fat mass, increases lean body mass and increases insulin sensitivity (4). The expression of insulin signaling genes (IR- β , IRS-1, AKT-2, and GLUT4) in adipose tissue was significantly lower in men with HH and was upregulated after T treatment. T replacement also decreased free fatty acids (FFA), CRP, Interleukin (IL)-1 β and tumor necrosis factor (TNF)- α concentrations. In addition, T therapy suppressed the mRNA expression of proinflammatory mediators that inhibit insulin signaling: suppressor of cytokine signaling (SOCS)-3, inhibitor of nuclear factor kappa-B kinase (IKK)- β , and phosphatase and tensin homolog (PTEN) in mononuclear cells (MNC). However, T therapy is not ideal for long term use in those who desire fertility since it decreases spermatogenesis (18). Therefore alternatives need to be explored for younger men. GnRH and human chorionic gonadotropin (hCG) are both effective in increasing T concentrations and increasing spermatogenesis. GnRH has to be infused continuously by an external pump while hCG therapy has to be injected subcutaneously or intramuscularly 2-3 times a week. Both are expensive for chronic long term treatment. Aromatase inhibition can suppress estradiol concentrations and increase T concentrations. However, the sharp fall in estradiol concentrations may not be ideal since males depend upon estradiol for the maintenance of their bone density and strength. Aromatase inhibitors decrease bone mineral density in elderly men (19). Clomiphene citrate is a mixture of two diastereoisomers, (cis)zuclomiphene citrate (38%) and (trans)enclomiphene citrate (62%). Zuclomiphene is thought to be an estrogen agonist in high doses (44). By contrast, enclomiphene citrate is primarily responsible for causing an increase in FSH and LH in studies of male subjects by estrogen antagonism. Enclomiphene binds potently to estrogen receptor while zuclomiphene is not believed to be active at physiological concentrations. Clomiphene is available as an oral preparation and is an estrogen antagonist at hypothalamic level. It can thus interfere with the negative feedback of estradiol. It is effective in increasing T concentrations, while maintaining spermatogenesis (18, 20). By inhibiting the normal estrogen mediated feedback, enclomiphene has been shown to increase T concentrations in obese men with HH, even though they do not have elevated estradiol concentrations (18). Clomiphene increases bone density and does not affect lipid profile (21). In a randomized placebo controlled cross over trial, Pelusi et al randomized 24 obese men (age range 35-55 years, mean 47 years) with diabetes or prediabetes and low total testosterone (<300 ng/dl) to clomiphene 25mg or placebo capsules daily (22). There were two periods of treatment (3 months each) separated by a washout of 6 weeks. Treatment with clomiphene doubled the calculated free testosterone concentrations (7.5 ng/dl to 15 ng/dl). LH,

FSH and free estradiol also doubled. Bendre et al noted similar results in a retrospective chart review of obese young men (18-21 years) with low total testosterone (<350 ng/dl) after treatment with clomiphene(23). Clomiphene is not FDA approved for treatment of hypogonadism, but it may be an attractive and inexpensive alternative to T therapy in young men who are interested in fertility.

Some (but not all) studies have shown that BMI is inversely related to sperm counts, sperm morphology and sperm motility(24-29). Data are most consistent about the impact of morbid obesity on spermatogenesis. In a meta-analysis of 21 studies, BMI>40kg/m² was associated with a doubling of risk of oligospermia (<40 million spermatozoa per ejaculate) or azoospermia (30). Weight loss in obese males increases sperm count (31). Obese males have evidence of decreased testicular function, as shown by lower concentrations of insulin like factor 3 (sensitive marker of Leydig cell function) and inhibin B (marker of Sertoli cell function) (32-34). A longitudinal study of testicular function across childhood has shown that weight gain during childhood is associated with lower testicular volume, serum T and inhibin B concentrations at the age of 21 years(35).

We **hypothesize** that 1) obese young males with HH (18-~~45~~30 years of age) will show evidence of decreased Leydig and Sertoli cell function, decreased insulin sensitivity and increased inflammation; and 2) Clomiphene treatment will reverse these abnormalities.

Aim 1.1: To compare the serum concentrations of insulin like factor 3 (sensitive marker of leydig cell function) and inhibin B (marker of Sertoli cell function) in obese males with and without HH and in lean males.

Aim 1.2: To compare the insulin resistance (measured by homeostatic model assessment-insulin resistance (HOMA-IR) in obese males with and without HH and in lean males.

Aim 1.3: To compare FFA, TNF- α , IL-1 β and CRP concentrations, and mRNA expression of SOCS-3, IKK- β , and PTEN in MNC in obese males with and without HH and in lean males.

Aim 2.1: To measure the change in serum concentrations of T (**primary endpoint**), insulin like factor 3 and inhibin B after treatment with clomiphene tablets or placebo for 12 weeks in obese males with HH.

Aim 2.2: To measure the change in waist circumference and HOMA-IR in obese males with HH after treatment with clomiphene or placebo for 12 weeks in obese males with HH.

Aim 2.3: To measure FFA, TNF- α , IL-1 β and CRP concentrations, and mRNA expression of SOCS-3, IKK- β , and PTEN in MNC in obese males with HH after treatment with clomiphene or placebo for 12 weeks in obese males with HH.

Significance

The prevalence of obesity in adolescents has seen a steep rise in the last few decades. 20% of all adolescent boys are now considered obese (BMI $\geq$ 95th percentile)(36). However, the consequences of the lower FT at completion of puberty in obese boys are not known. Males are supposed to achieve their “peak T concentrations” at puberty. Thereafter, there is a decline of FT at the rate of 2% per year for the rest of life (37). It is possible that HH in obese young males with HH have more insulin resistance, inflammation and possibly increased predisposition to diabetes and cardiovascular diseases. They may also have deficient spermatogenesis and decreased fertility. It is the intent of this project to understand the effects of having HH in obese young men on testicular function, insulin sensitivity and inflammation. This study will also generate preliminary efficacy data on a therapeutic option (clomiphene) for this condition. The results will inform the design of future trials and provide a comparison for other treatment modalities to increase T concentrations in young males with HH.

APPROACH

The study will be conducted at the division of Endocrinology, Saint Louis University. Young males between the ages of 18-~~45~~30 years will be recruited for the study. A total of 90 males will be recruited: 30 obese males (defined as $\geq$ 30 kg/m²) with HH, 30 obese males with normal FT concentrations and 30 lean males (<25 kg/m²) with normal FT concentrations.

Hypogonadal males: 30 male subjects with HH will be recruited into the study. HH will be defined as subnormal FT concentrations along with normal or low LH concentrations ($\leq$ 10 IU/L). We will define subnormal FT as <6.5 ng/dl. Subjects with subnormal FT and high LH (>10 IU/L) will not be recruited into the study. Obese subjects will be randomized to receive clomiphene or placebo (15 subjects each) thrice a week for 12 weeks.

Eugonadal men: 30 obese and 30 lean males with normal FT ($\geq$ 6.5 ng/dl) will also be recruited in the study to serve as comparison groups. They will not be treated clomiphene.

INCLUSION CRITERIA FOR THE STUDY: Males with age 18-~~45~~30 years inclusive will be recruited. Subjects with type 2 diabetes on anti-hyperglycemic drugs (including insulin) will be allowed as long as they are on stable doses of these compounds for at least 6 weeks. The doses of anti-hyperglycemic can be changed if the subject has hypoglycemia or hyperglycemia. A record of all the changes in the doses of the anti-diabetic drugs will be kept.

EXCLUSION CRITERIA FOR THE STUDY: 1) Use of androgens, clomiphene, hCG, aromatase inhibitors or over the counter health supplements which contain androgens currently or in the past 6 months; 3) Hematocrit > 50%; 4) Congestive heart failure; 5) currently suffering from depression; 6) type 1 diabetes; 7) Hepatic disease (transaminase > 3 times normal) or cirrhosis, or a history of liver dysfunction; 8) Renal impairment (eGFR < 30 ml/min/1.73m²); 9) HIV or Hepatitis C positive status; 10) Participation in any other concurrent clinical trial; 11) currently suffering from foot ulcer, significant periodontal disease or any other chronic infectious condition 12) known allergy to clomiphene 13) uncontrolled adrenal or thyroid dysfunction 14) presence of an organic intracranial lesion e.g. pituitary tumor 15) history of cataracts 16) history of venous thromboembolic disease (i.e. deep vein thrombosis or pulmonary embolism).

STUDY DESIGN:-

Screening Day (part of study): All subjects will have completed the following procedures prior to participating in the study:-

1) Medical History; 2) Physical Exam: testicular volume will be measured by an orchidometer (38); 3) Informed consent; 4) Baseline lab (30 ml) draw to measure CBC, comprehensive metabolic panel (CMP that includes liver and kidney function tests), total and free T, SHBG, LH and FSH. All labs will be drawn in the fasting state in the morning before 10am. All laboratory samples will be tested in the Endocrine laboratory in Doisy Hall Room 204.

Study visits for males with normal FT: The participants will come fasting and have blood drawn for research labs (insulin, glucose, inflammation). They will then be discharged from the study.

Study visits for obese males with HH: These subjects will enter a double blind randomized placebo controlled trial. Subjects who qualify and consent to take part in the study will be assigned a number by a computerized random number generation program and will be randomized (1:1) to receive either clomiphene or placebo. An unblinded research nurse and pharmacist will administer study product, all other investigators and the study participants will be blinded. This number will be used as the study subject ID. A master list of subject name and study ID will be kept under lock and key to protect patient identifiers. Only the assigned research nurse will have access to the randomization list.

1. Visit day 0: The participants will come fasting and have blood drawn for research labs (insulin, glucose, CRP; 45 ml). Waist circumference will be measured. They will be given a supply of clomiphene or placebo pills containing cellulose. Clomiphene citrate is available as 50 mg tablets and has a half life of 5-7 days. The starting dose of clomiphene will be 25mg on Monday, Wednesday and Friday mornings. All subjects will be given a pill container that will have separate boxes for different days of the week.

2. Visits to adjust study drug (week 4 and week 8): A blood sample (30 ml) will be collected at weeks 4 and 8 to measure CBC, CMP and FT concentrations. The time frame for follow-ups will be within a week of the designated follow up time. Dose of clomiphene will be adjusted to keep FT between 12 and 16 ng/dL (mid-normal for healthy young men). If FT is <12 ng/dl, then the dose of CC will be increased by 25mg/week. If FT is >16 ng/dl, then the dose will be reduced by 25mg/week. The blood samples for dose adjustment will be taken on Wednesday or Friday. Patients in both clomiphene and placebo groups will be told that their tablet quantities may vary during the study. If the number of pills of a patient in the clomiphene group is changed, a similar change will be made in the number of placebo tablets in the next patient in placebo group.

3. Week 12: The participants will come fasting and have blood drawn for research labs, CBC, CMP, SHBG, LH, FSH total and free T (45 ml). Waist circumference and testicular size will be measured. Gynecomastia will be assessed. Subjects will then be discharged from the study.

The following study termination criteria will be applied:-

- Adverse events considered by the PI to be associated with the use of the study drug
- Suspected thromboembolic event, including pulmonary embolism. Subjects who have a thromboembolic event ruled out may reinstate treatment
- Hemoglobin of 18 g/dL or higher, or a hematocrit of 54% or higher
- Visual changes which may be prolonged and possibly irreversible. Subjects will be advised to stop drug and be referred to an ophthalmologist.
- The subject requests to be withdrawn from the study

Laboratory measurements:-

Measurement in research laboratory: Insulin concentrations will be determined using an ELISA kit from Diagnostic Systems Laboratories Inc. (Webster, TX). Glucose levels will be measured in plasma by YSI 2300 STAT Plus glucose

analyzer (Yellow Springs, Ohio). HOMA-IR will be calculated from fasting insulin and glucose level using the formula: [fasting insulin (mU/l) X fasting glucose (mmol/l)]/22.5. TNF- α , IL-1 β and CRP concentrations will be measured by ELISA. FFA will be measured by a colorimetric assay. MNC will be isolated by Ficoll-hypaque method.

Quantification of mRNA expression of SOCS-3, IKK- β , and PTEN will be done by RT-PCR as previously published (4, 39, 40). Insulin like factor 3 and inhibin B will be measured by ELISA.

Measurements in commercial laboratory: T is largely bound to SHBG (44%) and albumin (54%). Hence, total T does not reflect the bio-availability of T at the cellular level (41). Obesity is associated with lower SHBG concentrations. It is therefore important to measure FT concentrations in obese males to get an accurate assessment of gonadal status. Reliable assays for FT were not widely available until recently. In our study, we will measure T by the gold standard method, liquid chromatography tandem mass spectrometry (LC-MS/MS) (42). FT will be separated by equilibrium dialysis. SHBG, LH and FSH will be measured by immunoassays. CBC and CMP will be measured by well established clinical assays. The assays will be performed by Quest Diagnostics. We have previously published many studies in which measurements of serum T on our study subjects were done by Quest Diagnostics (1, 3, 5, 6, 9).

Data Analysis: The focus of the proposed research is to evaluate the efficacy of clomiphene treatment on T concentrations in young obese post-pubertal males. Transformations of the data in order to meet statistical assumptions may be considered. The results will be computed as mean $\pm$ standard deviation (SD). The primary endpoint of the study is to detect a difference in FT concentrations after treatment with clomiphene for 12 weeks. There are no prior studies analyzing the effect of clomiphene in young males with HH. However, studies in middle aged men with obesity related HH and use of clomiphene or its isomer, enclomiphene have shown an increase in FT concentrations by 150%(21, 43). Change in FT concentrations following clomiphene or placebo for 12 weeks will be compared among the groups by unpaired *t*-test. Conservatively estimating a difference in FT concentrations of 50%, a sample size of 15 patients per treatment group (assuming a drop-out rate of 20%) should provide adequate power($\beta = 0.8$) to detect a significant difference ($\alpha = 0.05$), provided the standard deviation of the residuals is not greater than 40%. Thus 30 obese subjects with HH (15 patients each in clomiphene and placebo groups) will be sufficient. For baseline comparisons with men without HH, we will recruit 30 eugonadal obese and 30 lean males. Thus there will be 90 subjects in the study.

Secondary End Points: The secondary endpoints for the study will be comparison of the relative change from baseline in HOMA-IR and inflammatory mediators after clomiphene or placebo. These parameters will also be compared between obese and lean males at baseline. We have previously shown that obese men with HH have 36% higher insulin resistance than eugonadal men. Estimating a difference in HOMA-IR of 20%, a sample size of 30 patients per treatment group should provide adequate power($\beta = 0.8$) to detect a significant difference ($\alpha = 0.05$), provided the standard deviation of the residuals is not greater than 25%.

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