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Serum INSL3 is highly correlated with intratesticular testosterone in normal men with acute, experimental gonadotropin deficiency stimulated with low-dose hCG: a randomized-controlled trial

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Abstract

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Objective—To study the potential role for using serum biomarkers including insulin-like factor 3 (INSL3), 17-hydroxyprogesterone (17-OHP), anti-Müllerian hormone (AMH) and inhibin B (INHB) as correlates of intratesticular testosterone (IT-T) concentrations in men.

Design—Prospective, randomized-controlled trial

Setting—University-based medical center

Patients—37 healthy men ages 18–50

Interventions—All men received the gonadotropin-releasing hormone antagonist, acyline, plus very low doses of human chorionic gonadotropin (hCG) (0 IU, 15 IU, 60 IU or 125 IU) subcutaneously every other day or 7.5 grams testosterone gel daily (7.5 mg delivered). IT-T concentrations obtained by percutaneous testicular aspiration with simultaneous serum protein and steroid concentrations were measured at baseline and after 10 days of treatment.

Main Outcome Measures—Intratesticular and serum hormone and gonadotropin concentrations

Results—Following 10 days of gonadotropin suppression, serum INSL3 decreased by over 90% and correlated highly with IT-T concentrations. In contrast, serum INHB, AMH and 17-OHP did not correlate with IT-T. Serum INSL3 increased with the dose of hCG administered and returned to baseline after treatment.

Conclusions—Serum INSL3 correlates highly with IT-T and serum testosterone concentrations during acute gonadotropin suppression in men. HCG stimulates dose-dependent increases in INSL3 and IT-T in healthy men and might be a useful biomarker of IT-T concentration in some clinical settings.

Clinicaltrials.gov NCT# 00839319

Keywords

Androgens; INSL3; Male Infertility; Gonadotropins; Intratesticular Testosterone

Introduction

Male factor infertility accounts for twenty percent of infertile couples and in as many as 60% of these cases the underlying cause of their infertility is unknown (1). Androgens play a vital role in spermatogenesis. Testosterone (T), secreted by testicular Leydig cells upon stimulation by luteinizing hormone (LH), is present in very high concentrations within the testes, 100–1000 fold higher than the concentrations found in the circulation (2–7). Infertile men with low gonadotropins, and in some cases men with idiopathic infertility, can be treated with exogenous gonadotropins in an effort to initiate or optimize spermatogenesis. Human chorionic gonadotropin (hCG), which stimulates Leydig cells similarly to LH, is used to increase testosterone production in the testes prior to the addition of follicle-stimulating hormone (FSH). In men with hypogonadotropic hypogonadism, the dose of hCG is often adjusted to normalize serum T, yet the intratesticular testosterone (IT-T) concentration is one of the keys to inducing spermatogenesis. In some men, normal serum T concentrations can be achieved by administering hCG without normalizing IT-T concentrations or stimulating spermatogenesis (3, 8, 9), raising the possibility that serum T may not be an accurate marker of IT-T. In addition, given that gonadotropin therapy to induce spermatogenesis often requires several years to achieve conception (10), identifying serum markers to potentially predict response to therapy would benefit infertility patients. Unfortunately, measurement of intratesticular androgens is invasive, requiring either percutaneous testicular aspiration or surgical testicular biopsy, so IT-T concentrations are

not often evaluated in infertile men. A serum biomarker of IT-T might be useful in the diagnosis, prognosis and treatment monitoring of some men with infertility.

Insulin-like factor 3 (INSL3), anti-Müllerian hormone (AMH), inhibin B (INHB) and the testosterone precursor 17-hydroxyprogesterone (17-OHP) are candidate substances, quantifiable in serum, and have the potential to serve as serum correlates of IT-T. Similar to testosterone, INSL3 is a protein produced by Leydig cells and is known to play a key role in testicular descent during fetal development (11, 12). INSL3 expression increases during pubertal development (13) and is abundant in serum of adult men (14). Although the role of INSL3 in adults remains unclear, some studies suggest that serum concentrations of INSL3 are lower in men with infertility, even those with normal serum testosterone concentrations (15). INSL3 production is clearly regulated by LH, although it has yet to be fully determined whether this regulation is direct or via IT-T (13, 16). While testosterone levels often vary widely in a given individual over the course of a day (17), INSL3 appears to be constitutively expressed in normal adult men and declines with age (18). Therefore, serum INSL3 has the potential to reflect steady state IT-T levels without the confounding pulsatility of serum LH and T (19, 20).

AMH and INHB are glycoproteins secreted by Sertoli cells. AMH plays a key role in male embryos to trigger Müllerian duct regression (21). Although AMH is present in high concentrations in adult men, its functional role is unclear (22). The glycoprotein INHB, also secreted by Sertoli cells, correlates strongly with sperm count, concentration and testicular volume in adult men (23). INHB largely functions to regulate the release of FSH (24–26). Both INHB and AMH have been reported to be lower in men with non-obstructive azoospermia (27) although attempts to use INHB and AMH as predictors for the success of infertility treatment in azoospermic men have not been successful to date (28, 29).

In addition to these testicular products, previous studies have suggested that serum concentrations of the testosterone precursor 17-hydroxyprogesterone (17-OHP) reflect IT-T when normal concentrations of serum testosterone are maintained (30). Approximately 70% of circulating 17-OHP is thought to be of testicular origin, and similar to T, production of 17-OHP is stimulated by LH and hCG (31, 32). Whether or not serum 17-OHP concentrations are a good biomarker for IT-T when IT-T concentrations are very low has not been investigated to date.

To investigate the relationship between serum markers of testicular function and IT-T, we evaluated serum INSL3, AMH, INHB and 17-OHP in healthy men undergoing experimental gonadotropin suppression (3). In conjunction with the administration of a potent gonadotropin releasing hormone (GnRH) antagonist that effectively suppresses gonadotropins for 2 weeks, acyline (33), subjects were randomized to receive low doses of hCG to create a continuum of IT-T concentrations across the experimental groups (3). Based upon their regulation by LH, we hypothesized that serum 17-OHP and INSL3 would be highly correlated with IT-T in the setting of low-dose LH (hCG) stimulation, while AMH and INHB concentrations, which are stimulated primarily by FSH, would not change.

Subjects and Methods

Subjects

The study design has been reported previously (3). In brief, we enrolled 40 healthy men, aged 18–50 years old, with normal serum gonadotropins, serum testosterone concentrations, and normal seminal fluid analyses. Men were excluded if they were in poor general health or had abnormal blood test results, active skin conditions that would prevent the use of testosterone gel, active alcohol or drug abuse, history of testicular or scrotal surgery,

infertility, chronic pain syndrome, use of steroids, testosterone, or medications that might affect androgen metabolism including ketoconazole, glucocorticoids, known bleeding disorder, or if they were using anticoagulant medications such as aspirin or warfarin. Thirty-seven men completed all study procedures. A blood sample for the assessment of serum hormones and proteins and a unilateral testicular fine needle aspiration were performed on Day 1 (2, 34). We then administered acyline (NeoMPS, San Diego, CA), a GnRH antagonist, 300 mcg/kg subcutaneously to all subjects. On the same day, subjects then received their first dose of hCG (Pregnyl, Organon, Roseland, NJ) or 1% testosterone gel (Androgel, Solvay, Marietta, GA) based on treatment group randomization (groups 1–5): placebo hCG (normal saline), 15 IU hCG, 60 IU hCG, 125 IU hCG SC, or 1% testosterone gel 7.5gm daily (resulting in 7.5 mg absorbed daily). Administration of hCG/placebo continued every other day for 5 doses in Groups 1–4. Group 5 applied the testosterone gel to the skin daily for 10 days. A blood sample was collected on Day 7 to assess for effective suppression of gonadotropins as indicated by a serum LH concentration below 1.2 IU/L. On Day 10 subjects underwent a second testicular fine needle aspiration of the contralateral testis and a blood sample for protein and hormone quantification was collected. A follow-up visit on day 40 was performed to ensure the testicular examination, serum and semen parameters had all returned to normal. The University of Washington Institutional Review Board approved the study and all subjects provided written, informed consent prior to study procedures. The study was registered in advance on clinicaltrials.gov as NCT# 00839319.

Measurements

Testicular fluid samples were immediately placed on ice, centrifuged at $300 \times g$ to remove any aspirated cells; the supernatant fluid was decanted and stored at -70°C . Serum was stored at -20°C . Testicular fluid and serum samples were assayed simultaneously for testosterone by liquid chromatography-tandem mass spectrometry on a Waters Aquity UPLC coupled with a Micromass Premiere-XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) as previously reported (3). Serum LH, FSH, and hCG concentrations were quantified by immunofluorometric assay as reported previously (3).

Serum samples were shipped to Copenhagen, Denmark, on dry ice for INSL3 analysis and were in transit for less than 48 hours. Serum concentrations of INSL3 were measured using a time-resolved fluorescence immunoassay with a detection limit of 0.05 ng/ml and inter- and intra-assay coefficients of variation of 11.3 and 8.0%, respectively. Development and evaluation of this assay has been described in detail previously (20).

Serum INHB and AMH were both measured by enzyme-linked immunosorbent assay (Kamaya Biomedical Company, Seattle, WA, USA). The lower limit of detection for INHB was 31 pg/ml, and the inter- and intra-assay coefficients of variation were 7% and 8.5% respectively. The lower limit of detection for the AMH assay was 0.15 ng/ml, and the inter-assay coefficient of variation was 8%. Serum 17-OHP was quantified using a radioimmunoassay (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) with a lower limit of detection of 0.1 ng/ml. The inter- and intra-assay coefficients of variation were 11% and 6.7% respectively for low pools, and 8.5% and 3.5% for high pools. All samples for all subjects were batched and measured in a single assay.

Statistical analysis

The primary endpoint of the study was the differences in intratesticular testosterone concentration between groups. Eight subjects per group conferred an 80% power to detect a difference of greater than 8 ng/ml with a variance of 20% between the groups at an alpha of 0.05 when adjusted for multiple comparisons. Due to non-normality, the data are expressed as medians and 25th and 75th percentiles. Six subjects (one each in groups 1 and 2 and two

each in groups 3 and 4) had evidence of incomplete gonadotropin suppression with serum LH values above 1.2 IU/L on day 7. These subjects were excluded from further analysis as their serum and intratesticular hormones were confounded by normal serum concentrations of LH (3). Therefore, analysis of baseline and end-of-treatment hormone concentrations was performed on the thirty-one subjects who suppressed serum LH below the lower limit of the normal range by day 7. Comparisons of hormone concentrations between groups were performed in a non-parametric fashion using Kruskal-Wallis analysis of variance with a Wilcoxon rank-sum post-hoc test. Comparisons between baseline and end-of-treatment hormone concentrations within a group were performed using a non-parametric Wilcoxon sign-rank (paired) test. Correlations between serum and intratesticular hormones concentrations were performed using the non-parametric Spearman technique and graphed using best linear fit lines. No corrections were made for multiple comparisons. All statistical analyses were performed using STATA version 10.0 (College Park, TX, USA). For all comparisons, an alpha of < 0.05 was considered significant.

Results

Baseline Hormone Concentrations

A complete description of the subjects screened and enrolled in this study, including the complete inclusion and exclusion criteria, has been previously reported (3). There were no serious adverse events during this study and the testicular aspiration procedure was well tolerated. Testicular aspirate volumes were similar at baseline and after 10 days of treatment with hCG or testosterone, with a median volume of 10 μ l.

The subject's baseline characteristics and serum hormones are reported in Table 1. Serum gonadotropins and androgens as well as intratesticular hormone data have been previously reported (3, 35). There were no statistically significant differences in any of the measurements between the treatment groups at baseline. Baseline hormone concentrations did not correlate with age, race or BMI (data not shown).

Baseline INSL3 was normally distributed for the 31 subjects. Neither baseline IT-T nor serum T correlated significantly with baseline INSL3 ($r=0.31$, $p=0.09$ and $r=0.19$, $p=0.3$, respectively), but serum LH did correlate significantly with INSL3 at baseline (Figure 1). Re-analysis of the data after removal of outliers in the data did not change the results. As presented previously, serum T also did not correlate with IT-T at baseline (3). INHB did not correlate with serum testosterone, FSH, or LH, but had a negative correlation with IT-T at baseline ($r=-0.4$, $p<0.05$). Neither AMH nor 17-OHP correlated significantly with serum T, IT-T, FSH, or LH at baseline (data not shown).

Post-treatment Hormone Concentrations

Serum INSL3 decreased by over 90% compared to baseline with the administration of the GnRH antagonist acyline by itself and in combination with testosterone gel and was not significantly different after treatment between these two groups (Table 1, Figure 2). As reported previously, serum LH decreased by nearly 95% from baseline (median of 3.4 (2.6, 4.9) IU/L to 0.19 (0.1, 0.5) IU/L), and serum FSH decreased by 89% from baseline (median of 2.4 (1.5, 3.0) IU/L to 0.27 (0.2, 0.4) IU/L), with acyline administration alone ($p<0.001$ for both comparisons) (3). INSL3 decreased by 80% from baseline ($p<0.05$) in the group receiving 15 IU hCG, but did not differ significantly from post-treatment concentrations of the acyline alone group or acyline plus testosterone gel group, both of which also decreased significantly from baseline ($p<0.05$ compared to baseline for acyline alone and acyline plus testosterone gel). INSL3 decreased by 70% from baseline ($p<0.05$) in the group receiving 60 IU hCG, and was significantly different from all other treatment groups ($p<0.01$). The

INSL3 concentration in the group receiving the highest hCG dose, 125 IU, did not change significantly from baseline ($p=0.22$), but the post-treatment INSL3 concentration in the 125 IU group differed significantly from all other treatment groups ($p<0.01$).

Suppression of INSL3 production with acyline, then stimulation of INSL3 with low doses of hCG, established a dose-response relationship between hCG dose and serum INSL3 concentrations (Figure 2). In addition, after 10 days of hCG treatment, serum INSL3 correlated highly with IT-T, serum T, and serum hCG (Figure 3A–C). After treatment with HCG, serum INSL3 and IT-T correlated strongly ($r=0.79$, $p<0.001$) in a pattern similar to the significant correlation of serum T and IT-T after suppression with a GnRH analog ($r=0.86$, $p<0.001$ (3)).

Serum INHB, AMH and 17-OHP concentrations did not change significantly from baseline after gonadotropin suppression followed by 10 days of hCG or testosterone therapy (Table 1). As compared to baseline, post-treatment serum INHB concentrations did not correlate significantly with IT-T ($r=-0.3$, $p=0.07$). In addition, neither AMH nor 17-OHP concentrations correlated with IT-T after treatment ($r=-0.2$, $p=0.2$ and $r=0.2$, $p=0.2$, respectively). A negative correlation between serum INHB and FSH approached significance after 10 days of hCG treatment ($r=-0.4$, $p=0.07$), but serum AMH did not correlate with FSH after treatment ($r=0.2$, $p=0.4$).

Discussion

In this placebo-controlled, randomized clinical trial we have shown that serum INSL3 concentrations in normal men decrease dramatically with acute gonadotropin suppression and increase in a dose-response relationship with low-dose hCG stimulation, correlating highly with IT-T and serum T concentrations. In contrast, serum INHB, AMH, and 17-OHP did not significantly change with acute gonadotropin suppression nor hCG stimulation, and did not correlate with IT-T. Our results expand upon previous observations by Bay and colleagues examining the effect of high-dose gonadotropin stimulation on INSL3 concentrations (19, 20). The prior data illustrate that INSL3 appears to be maximally produced at baseline in normal men and does not increase with gonadotropin stimulation (20). In infertile men with hypogonadotropic hypogonadism, high hCG doses are capable of stimulating otherwise suppressed INSL3 production (19). Here, use of gonadotropin suppression with a GnRH antagonist, acyline, coupled with very low-dose hCG stimulation (to mimic LH stimulation), demonstrates a dose-response relationship between hCG/LH stimulation and INSL3 concentrations in a low-gonadotropin environment.

Because serum INSL3 correlates highly with IT-T concentrations, serum INSL3 might be useful as a serum biomarker for the effect of hCG therapy and LH-like stimulation of Leydig cells. Studies evaluating INSL3 concentrations in hypogonadotropic hypogonadal men treated with hCG versus testosterone therapy have suggested that INSL3 production and secretion is regulated by LH, rather than IT-T (20). Our data cannot help to distinguish whether LH or IT-T stimulates INSL3 production, since both correlate similarly with INSL3 in this study.

During hCG therapy in normal men with experimentally-induced hypogonadotropic hypogonadism, serum T also correlates highly with IT-T (3), but men may achieve a normal serum T concentration while still having an IT-T concentration below the normal range. In addition, men receiving exogenous testosterone therapy may have a normal serum T, but significantly suppressed IT-T, suggesting that perhaps an alternative serum biomarker for IT-T could be useful. As serum INSL3 is a protein, it may be a more stable marker of IT-T than serum testosterone in infertile men being treated with hCG who are intermittently

compliant with their injections. In addition, a marker of IT-T in men receiving exogenous testosterone may be useful in understanding suppression of spermatogenesis while using a male hormonal contraceptive regimen.

INHB did not change significantly with acute gonadotropin suppression or hCG stimulation. INHB has been identified as a product of Sertoli cells and as a clinical marker of spermatogenesis (24, 26–29). In the normal male, FSH receptors are found only on Sertoli cells (36), and FSH stimulates INHB production. INHB inhibits FSH secretion from pituitary gonadotropes via negative feedback (26). Based on prior data in male hormonal contraceptive trials, we anticipated that serum INHB concentrations would significantly decrease as FSH levels fell in all treatment groups, and serum INHB production would not be stimulated by hCG stimulation (37). The lack of INHB suppression in our study is likely related to the brief duration of gonadotropin suppression and less suppression of FSH (89%) as compared to LH (95%) (3).

In contrast to INHB, AMH is primarily produced by prepubertal Sertoli cells in response to FSH stimulation (38). AMH subsequently declines with rising LH stimulation and appears to be suppressed by high intratesticular androgen concentrations (39). While AMH concentrations might be expected to rise in settings of low IT-T, the lack of FSH stimulation in our experimental model may explain the lack of rise in AMH concentrations. Alternatively, the AMH results were highly variable between subjects, raising doubt about the reliability of the assay used for these measurements.

In addition, our study did not show a correlation between 17-OHP, a testosterone precursor, and IT-T in the setting of acute gonadotropin suppression or in response to low dose hCG stimulation. A previous study done by Amory *et al.* using testosterone enanthate for gonadotropin suppression and treating with higher doses of hCG suggested that 17-OHP may serve as a useful correlate for IT-T in men receiving gonadotropin therapy for infertility (30). However, when comparing the lowest hCG dose in that study, 125 IU every other day, to the group receiving gonadotropin suppression alone, the 17-OHP concentration was the same. Therefore, it seems possible that serum 17-OHP correlates with IT-T in the presence of normal or near-normal hCG stimulation but not with low-dose hCG stimulation.

One limitation of this study is our limited sample size. Correlations of several of the hormones with one another approached, but did not attain statistical significance in our study. As the study was powered to determine the differences in intratesticular testosterone between dose groups of hCG, it may have lacked the necessary power to identify all significant associations between the various hormones. Future, larger studies designed with adequate power to examine these relationships in men with infertility will be needed to clarify the relative utility of these serum markers for IT-T during hCG therapy. A second possible limitation is the use of normal men in this study, not infertile men. Future studies investigating the best serum biomarker for IT-T during hCG therapy should be conducted in infertile men, as the ability to extrapolate the findings discussed here may not reflect the associations observed in infertile men.

In summary, we demonstrated an acute decrease in serum INSL3 concentrations in response to gonadotropin suppression, and a dose-response relationship between INSL3 and IT-T concentrations using low-dose hCG stimulation in normal men. We have also shown that serum INHB, AMH and 17-OHP do not correlate significantly with IT-T. This work may have relevance in the use of serum INSL3 as a potential marker for IT-T concentrations. While IT-T concentrations vary significantly among fertile men and reflect LH pulsatility (2), both the minimal and the “ideal” concentration of IT-T for optimal spermatogenesis remain unknown. However, given that spermatogenesis depends upon extremely high

concentrations of IT-T and serum T concentrations do not accurately reflect IT-T concentrations in some settings (for example, in men on therapeutic testosterone replacement), the use of INSL3 as an alternative serum marker for IT-T concentrations might allow for more accurate monitoring of hCG therapy in infertile men. Whether or not INSL3 is a superior marker to serum T as a reflection of Leydig cell function during therapy for infertility will require a future, larger study in infertile men.

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Figure 1. Baseline correlation between serum INSL3 and serum LH for all 31 subjects. The line shown represents the best linear fit.

Figure 2.

Box plot of serum INSL3 concentration in gonadotropin-suppressed subjects at baseline (n=31) and on day 10 by treatment group (n=6 for the 0 IU hCG group, n=7 for the 15 IU hCG group, n=5 for the 60 IU hCG group, and 125 IU hCG group).

* p<0.05 compared to baseline, 60 IU hCG group, and 125 IU hCG group. Dotted lines and gray shading represent the normal range for serum INSL3.

Figure 3.

Figure 3A–C. Correlations between post-treatment serum INSL3 for all 23 subjects receiving hCG therapy intratesticular testosterone (A), serum testosterone (B), and serum hCG (C). Dotted lines represent the lower limit of the normal range. Lines shown represent the best linear fit.

Table 1

Baseline characteristics, and baseline and post-treatment serum hormones of 31 participants by treatment group (median (25th, 75th percentiles)).

	0 IU hCG (N=6)	15 IU hCG (N=7)	60 IU hCG (N=5)	125 IU hCG (N=5)	Testosterone gel (N=8)	All subjects (N=31)
Age (yr)	21 (20, 26)	25 (20,29)	22 (20, 24)	22 (21, 26)	22 (20, 24)	22 (20,26)
BMI (kg/m ²)	24.8 (23.6, 26.3)	24.1 (23.2, 26.7)	24.9 (21.2, 26.3)	25.8 (22.9, 26)	23.7 (21.1, 25.4)	24.1 (22.9,26.3)
Baseline Hormones						
Serum Testosterone (ng/ml)	3.7 (3.2, 4.8)	4.3 (3.3, 6.0)	4.1 (3.6, 4.3)	4.8 (4.1, 5.3)	4.3 (3.9, 5.2)	4.2 (3.4, 4.9)
Serum INSL3 (ng/ml)	0.9 (0.7,1.1)	1.0 (0.7,1.5)	1.1 (1.0,1.2)	0.9 (0.8,1.3)	1.1 (1.1,1.3)	1.1 (0.8,1.2)
Serum Inhibin-B (pg/ml)	86.5 (78,124)	109 (96,165)	136 (135,180)	117 (104,142)	123 (88,147)	114 (100,151)
Serum Anti-mullerian hormone (ng/ml)	5.8 (2.1,9.1)	2.5 (0.6,28)	2.7 (2.4,3.9)	1.0 (0.8,1.6)	2.5 (0.6,12)	2.5 (0.9,6.8)
Serum 17-hydroxyprogesterone (ng/ml)	4.7 (3.8,7.8)	4.9 (4.2,6.5)	5.9 (5.1,7.6)	4.3 (4.4,5)	4.6 (3.7,5.4)	4.9 (3.9,6.5)
Intratesticular Testosterone (ng/ml)	991 (717, 1098)	694 (486, 967)	521 (501, 690)	1002 (659, 1132)	839 (437, 970)	717 (501, 1002)
Post-Treatment Hormones						
Serum Testosterone (ng/ml)	0.13 (0.11, 0.19)	0.3 (0.11, 0.34)	0.86 (0.31, 1.01)	2.36 (1.79, 3.44)	5.08 (4.11, 6.97)	
Serum INSL3 (ng/ml)	0.07 (0.06,0.17)	0.18 (0.14,0.26)	0.29 (0.28,0.44)	0.84 (0.64,1.04)	0.1 (0.06,0.17)	
Serum Inhibin-B (pg/ml)	96 (81,106)	114 (103,157)	147 (134,175)	138 (98,139)	138 (93,172)	
Serum Anti-mullerian hormone (ng/ml)	6.4 (2.24)	4.2 (1.1,33)	3.6 (2.8,4)	2.0 (0.9,2.1)	5.9 (2.2,16.7)	
Serum 17-hydroxyprogesterone (ng/ml)	3.4 (1.8,4.3)	2.4 (1.6,4.3)	2.8 (2.8,5.6)	3.2 (2.7,5.3)	2.3 (1.4,3.2)	
Intratesticular Testosterone (ng/ml)	21.6 (11.4, 63.8)	38.9 (22.6, 73.8)	91.2 (39.8, 702)	282.3 (256.0, 357.5)	20.9 (9.7, 75.5)	