

Serum LH correlates highly with intra-testicular steroid levels in normal men

Running title: Intra-testicular hormones in normal men

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35 **Abstract**

Background: Sex steroids are essential for spermatogenesis; however, normal intra-testicular concentrations of these hormones in man have not been extensively studied. To improve our understanding of intra-testicular hormone concentrations, we performed bilateral testicular aspirations in a group of normal men, determined sex steroid concentrations within each testicle, and compared these levels to serum hormone concentrations.

Methods: Ten healthy human subjects ages 20-49 underwent bilateral testicular aspirations. Intra-testicular hormone concentrations of testosterone, dihydrotestosterone (DHT) and estradiol were measured using liquid chromatography-tandem mass spectrometry.

Results: Intra-testicular testosterone concentrations ranged from 119-1251 ng/mL, with a mean of 635 ± 368 ng/mL. Intra-testicular estradiol ranged from 0.41-3.9 ng/mL, with a mean of 2.4 ± 1.3 ng/mL. Intra-testicular DHT ranged from 1.1-7.9 ng/mL, with a mean of 3.5 ± 3.2 ng/mL. Intra-testicular testosterone and estradiol concentrations correlated highly with serum LH ( $r=0.87$  and  $r=0.70$  respectively,  $p<0.01$ ). Intra-testicular testosterone correlated highly with serum testosterone. Moreover, a significant correlation between the right and left testes was observed for testosterone ( $r=0.82$ ,  $p=0.003$ ), but not for estradiol or DHT.

Conclusions: Intra-testicular hormone concentrations can be safely assessed by testicular aspiration. Intra-testicular testosterone and estradiol correlate highly with serum LH concentrations, and variation in serum LH accounts for most of the variation in intra-testicular testosterone between men. In addition, intra-testicular testosterone is highly correlated between testes in a given individual. Direct measurement of intra-testicular testosterone will improve our understanding of the relationship between intra-testicular sex steroids and spermatogenesis, and may have implications for the development of male hormonal contraception.

## Introduction

Intra-testicular androgens have a critical role in supporting spermatogenesis. In rodents, reductions of up to 75% of the intra-testicular testosterone concentration are still compatible with normal levels of spermatogenesis; however, below that level sperm maturation is not detected (Ahmad, et al., 1973, Cunningham and Huckins, 1979, Zirkin, et al., 1989). In men, whether a minimum concentration of intra-testicular steroids is necessary to support spermatogenesis has not been determined. Residual androgens in the testis, despite a paucity of luteinizing hormone (LH), appear to play a role in supporting spermatogenesis in some mouse models (Zhang, et al., 2004). It has been proposed that such “gonadotropin-independent” intra-testicular steroidogenesis could play a role in supporting residual spermatogenesis in men who do not achieve azoospermia during male contraceptive treatment (Amory, et al., 2006, Anderson, et al., 1996, Jarow and Zirkin, 2005, McLachlan, et al., 2002). A greater understanding of the normal intra-testicular hormonal milieu and the relationship between intra-testicular hormone levels and gonadotropins in normal, healthy men could facilitate such investigations.

Understanding the hormonal requirements necessary to support spermatogenesis in men has been difficult. Until recently, methods for measuring intra-testicular hormone concentrations in men required testis tissue obtained by testicular biopsy or at the time of orchidectomy (Marie, et al., 2001, Morse, et al., 1973, Takahashi, et al., 1982). These methods are considered to be too invasive for assessment of normal, healthy men, therefore prior studies were in infertile men. In 2001, Jarow and colleagues demonstrated that fine needle tissue aspiration of the testes could be used to quantify intra-testicular hormone concentrations (Jarow, et al., 2001). This procedure used to obtain intra-testicular fluid for quantification of steroids by radioimmunoassay was further refined with measurement of testicular aspiration fluid steroid hormone levels by mass spectrometry (Zhao, et al., 2004); however, comparison with contemporaneously measured serum hormone concentrations and between the testes in a given individual was performed only for testosterone. In this study, we performed testicular aspirations in a group of normal men to better

understand the relationship between intra-testicular concentrations of testosterone and its active metabolites, as well as the relationship between intra-testicular sex steroids and circulating gonadotropins (LH and follicle stimulating hormone (FSH)) and sex steroid levels in normal men.

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## **Subjects and Methods**

### **Subjects**

Healthy men, aged 18-50 years old were recruited for this study. Subjects had to have a normal general medical and reproductive history and physical examination, including a testicular exam with measurements of testis volume by Prader orchidometer, normal serum gonadotropins and testosterone levels, and normal seminal fluid analysis based on the World Health Organization criteria with sperm concentration > 20 million/mL, > 50% motility, and > 15% normal morphology (WHO, 1999). Exclusion criteria included men in poor general health, abnormal blood test results, active alcohol or drug abuse, history of testicular or scrotal surgery, infertility, chronic pain syndrome, use of steroids, testosterone, or medications which might interfere with androgen metabolism including ketoconazole, glucocorticoids, known bleeding disorder, or use of medications which may affect bleeding time (such as ongoing aspirin or warfarin use).

Prior to the aspiration procedure, each subject's vital signs were taken and a blood sample was drawn for assessment of serum hormone levels (15-20 minutes prior to testicular aspiration). After local anesthesia administration at the spermatic cord with 1% buffered lidocaine, a 19-gauge needle was used to perform bilateral testicular aspirations as previously described (Coviello, et al., 2005, Jarow, et al., 2001). Subjects were evaluated one week following the procedure, and one month later a follow-up semen analysis was performed.

The institutional review board of the University of Washington approved this study protocol prior to study initiation (National Clinical Trial 00756561). Informed consent was obtained from all subjects prior to screening evaluation.

## Measurements

115 Testicular fluid samples were placed immediately on ice, and centrifuged at 300 x g.  
Supernatant fluid was stored at -70°C. We measured right and left testicular fluid samples for  
intra-testicular testosterone (IT-T), intra-testicular dihydrotestosterone (IT-DHT), and intra-  
testicular estradiol (IT-E2) by liquid chromatography-tandem mass spectrometry on a Waters  
Aquity UPLC coupled with a Micromass Premiere-XE tandem quadrupole mass spectrometer  
120 (Waters Corp., Milford, MA, USA) as described previously (Kalhorn, et al., 2007). For IT-E2,  
human serum samples (100µL) or intra-testicular fluid samples were diluted with water to a final  
volume of 0.5mL. The samples were left at room temperature for 1 hour at which time 4.0 mL t-  
butyldimethyl ether (TBDME) was added. The tubes were sealed, extracted on a horizontal  
shaker, centrifuged and flash frozen on dry ice. The top organic phase was decanted into a  
125 conical screw top tube and evaporated to dryness under nitrogen. Additional TBDME (150µL)  
was added, the tubes vortexed, and the solvent again removed under nitrogen. The residue was  
dissolved in 40µL 100mM pH 10.5 sodium carbonate followed by the addition of 40µL  
1.0mg/mL dansyl chloride in Acetonitrile. The tubes were sealed and heated at 60 degrees for  
five minutes. The tubes were centrifuged and the supernatant removed for analysis. Samples  
130 were analyzed in triplicate and injected twice.

The lower limit of quantification for all three sex steroid assays is 0.04 pg/ml.  
The intra- and inter-assay coefficient of variation for testosterone is 3.5% and 7.7% respectively,  
and for DHT is 3.5% and 6.3%. The intra-assay coefficient of variation for estradiol was 14%  
using human serum. Serum LH and FSH were quantified by immunofluorometric assay (Page, et  
135 al., 2007). The intra-assay coefficient of variation for LH is 5.6%, inter-assay coefficient is  
13.9%. For FSH, the intra- and inter-assay coefficient of variation is 3.0% and 5.0% respectively.  
All samples for all subjects were batched and measured in one assay.

## Statistical analysis

140 Due to non-normal distributions, all hormone concentrations were natural-log-transformed prior  
to analysis using parametric statistics. Correlations between serum hormone levels and intra-  
testicular hormones, and between testosterone and other steroid hormones, were performed using  
the Pearson technique. Statistical analyses were performed using STATA version 10.0  
(StataCorporation, College Park, TX). For all analyses, a p-value of <0.05 was considered  
145 significant.

## Results

Thirteen men were screened for the study and ten met inclusion criteria and completed all  
study procedures. Of the three men who were excluded from the study, two men were excluded  
150 for abnormal semen parameters and one was excluded for abnormal liver function tests.  
Testicular aspiration samples ranged in volume from 0.5 $\mu$ l to 50  $\mu$ l, with a mean volume of 8.5 $\mu$ l.  
All subjects tolerated the procedure well with pain during the procedure ranging from 1-5 on a 10  
point scale. There were no serious adverse events. On follow-up exam, two out of ten patients  
had mild bruising noted at the site of the lidocaine injection bilaterally.

155 Subject characteristics are shown in Table 1. Six subjects were Caucasian, two Asian,  
one African-American, and one Native Hawaiian. The range of IT-T was 119-1251 ng/mL, with  
a median (interquartile range (IQ)) of 486 (429,897) ng/mL. The range of IT-DHT was 1.1-7.9  
ng/mL, with a median (IQ range) of 3.7 (1.1, 4.7) ng/mL. The range of IT-E2 was 0.4-3.9 ng/mL,  
with a median (IQ range) of 2.7 (1.3, 2.4) ng/mL (Table 2). Serum testosterone measured at the  
160 time of the testicular aspiration ranged from 1.4-8.2 ng/mL, with a median (IQ range) of 3.0 (2.3,  
3.9) ng/mL. Serum DHT ranged from 82–525 pg/mL, with a median (IQ range) of 200 (120,

240) pg/mL. Serum estradiol ranged from 14-33 pg/mL, with a median (IQ range) of 25 (19, 29) pg/mL.

To investigate the relationship between serum gonadotropins and intra-testicular steroid concentrations that could contribute to the wide variability in intra-testicular hormone levels observed, especially in testosterone, we looked for correlations between gonadotropins and intra-testicular hormones. Serum LH, drawn approximately 15-20 minutes prior to the testicular aspiration, correlated strongly with IT-T ( $r=0.87$ ,  $p=0.001$ ) (Figure 1a) and IT-E2 ( $r=0.70$ ,  $p=0.025$ ) (Figure 1b), but not with IT-DHT ( $r=0.25$ ,  $p=0.5$ ) (data not shown). Serum FSH also correlated with IT-T ( $r=0.70$ ,  $p=0.024$ ) (Figure 1c), but not for IT-E2 ( $r=0.50$ ,  $p=0.13$ ) (Figure 1d) or IT-DHT ( $r=0.09$ ,  $p=0.82$ ) (data not shown).

We also evaluated the relationship between intra-testicular and serum steroid concentrations. IT-T was significantly correlated with serum testosterone,  $r=0.67$ ,  $p=0.03$  (Figure 2a). However, IT-DHT did not correlate with serum DHT,  $r=0.44$ ,  $p=0.20$  (Figure 2b). IT-E2 displayed a borderline correlation with serum estradiol,  $r=0.60$ ,  $p=0.06$  (Figure 2c). The ratio of intra-testicular to serum hormone concentrations is presented in Table 2.

When examining the relationship between IT-T and its metabolites, we discovered that IT-T concentrations correlated highly with IT-E2 ( $r=0.79$ ,  $p<0.01$ ), but not with IT-DHT ( $r=0.57$ ,  $p=0.83$ ). IT-T concentrations were highly correlated between the right and left testes ( $r=0.82$ ,  $p=0.003$ ) (Figure 3a). There was no correlation between IT-DHT in the right and left testes ( $r=0.03$ ,  $p=0.93$ ) (Figure 3b). There was a weak, non-significant correlation for IT-E2 between the right and left testes ( $r=0.50$ ,  $p=0.14$ ) (Figure 3c). Serum testosterone correlated with serum DHT ( $r=0.63$ ,  $p=0.05$ ), but not serum estradiol ( $r=0.31$ ,  $p=0.40$ ).

## 185 Discussion

We have used intra-testicular fine needle aspiration coupled with liquid chromatography-tandem mass spectrometry to determine the intra-testicular concentrations of testosterone, DHT and estradiol in healthy normal men. We found a strong correlation between LH and both IT-T and IT-E2, as well as a strong correlation between IT-T and IT-E2, though not between IT-T and IT-DHT. We found that serum T also correlates strongly with IT-T, though does not for serum E2 and IT-E2. Interestingly, we found no correlation between serum and IT-DHT. We also found a strong correlation for IT-T between the two testes.

Our results regarding intra-testicular testosterone concentrations are comparable to previous reports using this technique (Zhao et al, 2004) and add to the small body of data in this regard. We used the same minimally-invasive percutaneous testicular aspiration technique under local anesthesia to obtain intra-testicular fluid. However, we measured simultaneous serum steroid hormone concentrations in addition to intra-testicular fluid. We also used a LC/MS/MS assay which allows for a more sensitive and specific assay, in comparison to most previous data using radioimmunoassay (Coviello, et al., 2004, Jarow, et al., 2001, Matthiesson, et al., 2005, McLachlan, et al., 2002). Earlier reports of intra-testicular concentrations used extracts from testis tissue obtained by biopsy or at orchidectomy, but primarily evaluated infertile men or men with prostate cancer, and used general anesthesia which can alter hormone concentrations (Marie, Galeraud-Denis and Carreau, 2001, Morse, et al., 1973, Takahashi, et al., 1982). While our IT-T concentrations measured by LC/MS/MS are similar to those reported by Zhao and colleagues (Zhao, et al., 2004), our IT-E2 and IT-DHT values are somewhat lower. Increased sample size in future studies may narrow this apparent discrepancy although we cannot rule out that differences in assay methodology might contribute to these differences.

While the range of IT-T levels was quite broad, this variation seems to be explained by variation in LH and may reflect a pulsatile concentration of IT-T, similar to pulsatility in serum testosterone levels (Baker, et al., 1975). The strong correlation between serum LH and intra-

testicular testosterone and estradiol illustrates that intra-testicular testosterone and estradiol likely vary with LH pulses. Further evidence for this comes from the observation that such a relationship between IT-T and gonadotropins does not exist in men who have undergone prolonged gonadotropin suppression using exogenous testosterone in combination with a progestin (Page, et al., 2007).

The significant correlations between IT-T and serum FSH, and IT-E2 and serum FSH, were partially due to the presence of one subject with the highest IT-T and FSH. When this subject was omitted from these analyses, the correlations were no longer significant, implying that these correlations may be due to chance. In any case, it is clear that this relationship is not as strong as that between IT-T and LH and IT-E2 and LH. This is likely due to the known stimulatory role of LH on testosterone biosynthesis in Leydig cells, whereas FSH is not known to play a role in this process. It seems possible that the observed correlations are more likely due to co-secretion of FSH and LH from the pituitary rather than any direct effect of FSH on IT-T. Similarly, the significant correlation between serum T and IT-T is due to the presence of a signal outlier (not the same subject as omitted above) as removal of this subject reduced the p-value from 0.03 to 0.07. This further illustrates the need for additional study of these relationships in a larger sample of men.

We also examined the relationship between intra-testicular and contemporaneous serum hormone concentrations. Intra-testicular hormone concentrations are significantly higher than serum concentrations, as previously shown in both mice and humans (Jarow, et al., 2001, Turner, et al., 1984). Interestingly, the intra-testicular to serum ratio of testosterone and estradiol are nearly 200 and 100 respectively, but serum DHT is only about 15 times higher than IT-DHT. This suggests that DHT is primarily formed at peripheral sites rather than within the testes. This is compatible with studies of  $5\alpha$ -reductase expression which demonstrate high levels of expression in the skin, gut, kidney and prostate and only modest expression in the male reproductive tract (Thigpen, et al., 1993). Indeed, DHT may not be critical for spermatogenesis

since chronic inhibition of  $5\alpha$ -reductase has a minor impact on sperm concentrations in most men (Amory, et al., 2007) and does not appear to augment sperm suppression when added to other male hormonal contraceptive agents (Kinniburgh, et al., 2001).

240           Intra-testicular concentrations of testosterone are highly correlated between the right and left testes. The correlation for estradiol between the two testes did not quite reach statistical significance, likely due to our small sample size. Interestingly, there was no correlation between DHT levels in the right and left testes in this small sample of normal men. Many factors could contribute to this finding, including possible differential geographic expression of the enzyme  $5\alpha$ -  
245           reductase that metabolizes testosterone into DHT (Mahony, et al., 1998). Blood flow in the right and left testes is also known to be unequal due to variations in the anatomy of the blood supply to each testis (Fritjofsson, et al., 1969) which might theoretically impact non-testicular derived sources of DHT within each testicle

          There are several weaknesses with our study. In particular, the small sample size  
250           prevents us from having adequate statistical significance to define normative ranges for intra-testicular hormone concentrations. However, the strengths of this study include our ability to use a highly sensitive LC/MS/MS assay in normal men and the use of a minimally-invasive technique that allows the subjects to avoid general anesthesia which can alter serum steroid concentrations. Future studies using the same assay will allow us to develop a larger cohort of normal men from  
255           which to calculate a normal range for intra-testicular hormones. Serum hormone concentrations also fluctuate with circadian rhythms (Plymate, et al., 1989), yet we did not attempt to time our aspirations with identified LH pulses which may also alter intra-testicular hormone concentrations.

          Measurement of intra-testicular steroid hormone concentrations will provide essential  
260           information for future studies of the hormonal regulation of spermatogenesis. Our findings have implications for the treatment of male infertility and male contraceptive development. For

example, the relationship between intra-testicular hormones and infertility has not been explored systematically in large numbers of infertile compared to normal men, despite the known central contribution of testosterone to spermatogenesis. The use of the technique presented here, which  
265 allows for repeat assessment of intra-testicular hormones in an individual, combined with sensitive assay techniques using LC/MS/MS, will allow us to explore the relationship between intra-testicular steroid hormone concentrations and spermatogenesis in future cross-sectional and interventional studies. These findings also have implications for the development of male hormonal contraception. Male hormonal contraception uses exogenously administered androgens  
270 and progestins to suppress hypothalamic release of GnRH and pituitary release of gonadotropins (LH and FSH). This suppresses endogenous production of testosterone, and subsequently spermatogenesis, while providing systemic androgens to maintain activity at peripheral sites and prevent symptomatic hypogonadism. While rates of azoospermia with use of both androgens and progestins reach 90-95% (Page, et al., 2008), we have little understanding of why the remaining  
275 5-10% of men fail to achieve azoospermia. Animal models suggest that gonadotropin independent androgen production may support residual spermatogenesis in the setting of gonadotropin ablation (Zhang, et al., 2004). Alternatively, high dose exogenous androgens used in such regimens could diffuse into the testes and support spermatogenesis. These hypotheses have been difficult to test in humans, but could be examined using our technique of testicular  
280 aspiration in men during trials of experimental male hormonal contraceptives.

In summary, our study has confirmed and extended earlier work demonstrating that testosterone, DHT, and estradiol can be measured by a minimally-invasive, percutaneous, fine-needle aspiration technique of the testes, and that these concentrations are much higher than those in the serum. In addition, we have demonstrated that serum LH correlates very strongly with  
285 intra-testicular testosterone and estradiol, accounting for some of the wide range of normal values and providing evidence for the importance of gonadotropins in the regulation of intra-testicular steroid concentration. A strong correlation also exists between testosterone concentrations in the

right and left testes, indicating that a unilateral aspiration technique is likely sufficient to determine intra-testicular testosterone and estradiol concentrations. Future studies will include larger populations to establish a normal hormone range for intra-testicular hormone concentrations. This paradigm, coupled with hormone manipulation, will allow us to identify the critical relationships and thresholds for intra-testicular hormones to support spermatogenesis in men.

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Tables:

Table 1: Subject Characteristics (median, IQ range) (n=10)

Age (yr)	23 (21,32)
BMI (kg/m <sup>2</sup> )	25.9 (23.4, 28.7)
Sperm concentration (millions/mL)	75 (28, 133)
LH (IU/L)	3.7 (2.1, 5.4)
FSH (IU/L)	1.8 (1.4, 2.5)
Serum testosterone (ng/mL)	3.0 (2.3, 3.9)
Serum DHT (ng/mL)	0.20 (0.12, 0.24)
Serum E2 (ng/mL)	0.025 (0.019, 0.029)
Testis volume – right (mL)	25 (20, 25)
Testis volume – left (mL)	25 (15, 25)

Table 2: Intra-testicular hormone concentrations (average between right and left testis for each subject) and serum hormone concentrations in 10 normal men (all values are medians (IQ range)).

Hormone	Intra-testicular Concentration (ng/mL)	Serum concentration (ng/mL)	Ratio of Intra-testicular:Serum concentration
Testosterone	486 (429, 897)	3.0 (2.3, 3.9)	181 (128, 233)
DHT	3.7 (1.1, 4.7)	0.20 (0.12, 0.24)	15 (9, 24)
E2	2.7 (1.3, 2.4)	0.025 (0.019, 0.029)	88 (68, 141)

Figure 1: The relationship between serum LH and intra-testicular testosterone (A), LH and intra-testicular estradiol (B), FSH and intra-testicular testosterone (C), and FSH and intra-testicular estradiol (D) in 10 normal men undergoing testicular aspiration.

Figure 1A:

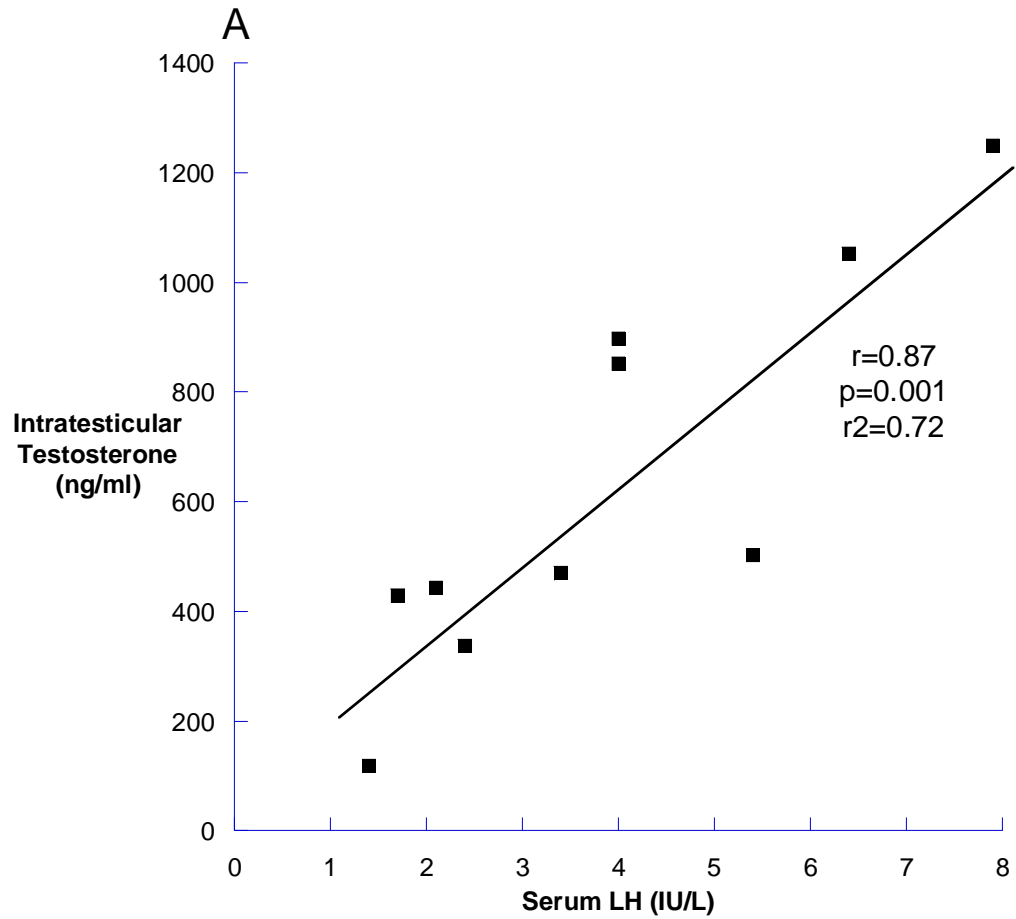


Figure 1B:

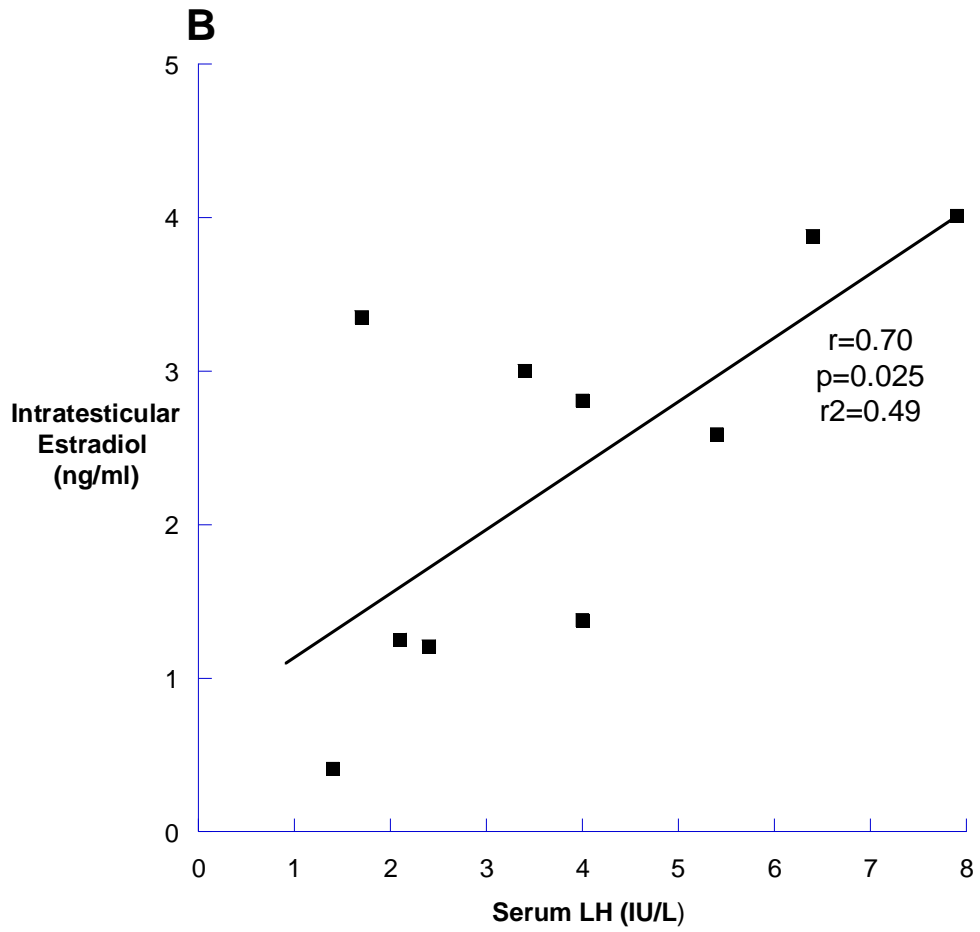


Figure 1C:

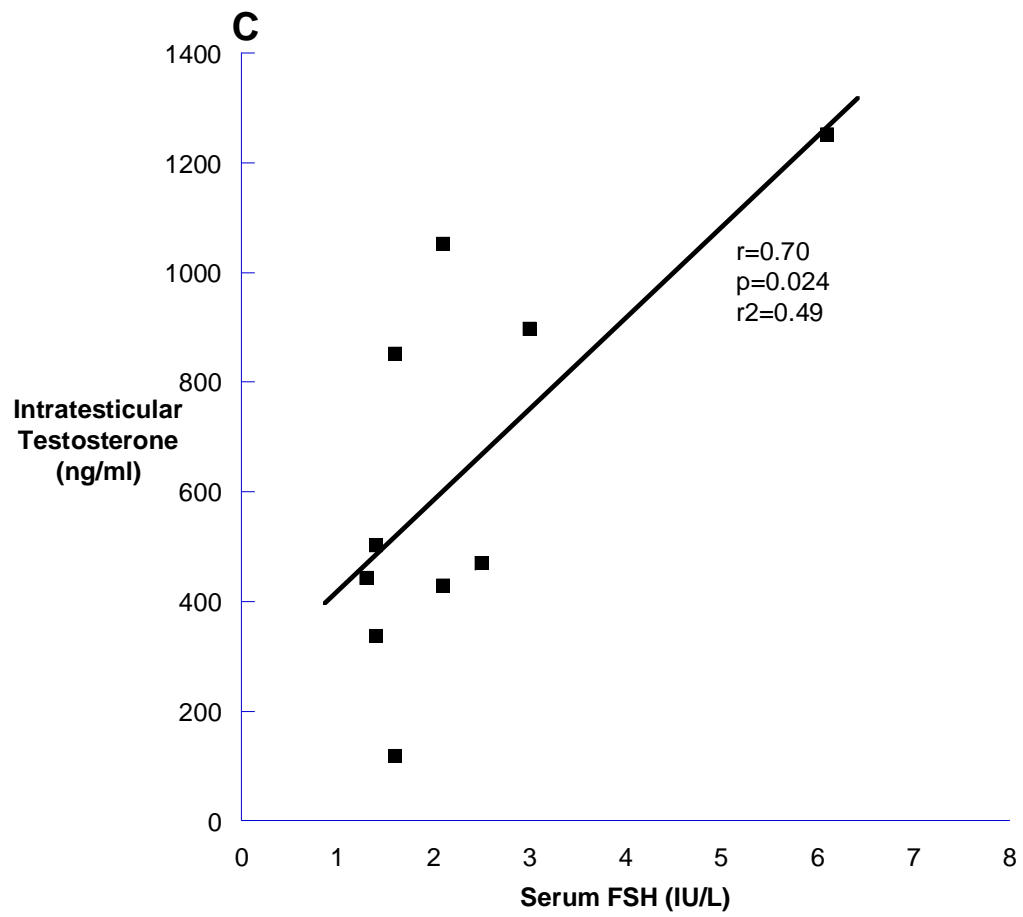


Figure 1D:

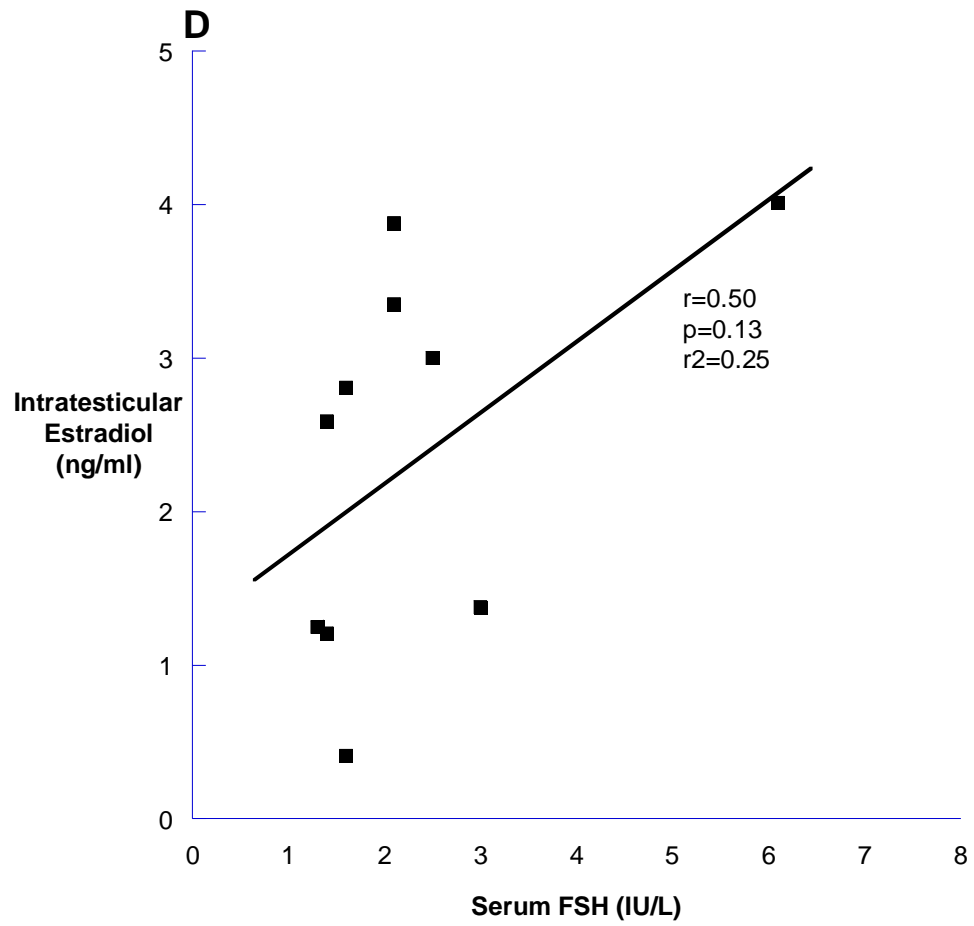


Figure 2: The relationship between intra-testicular testosterone and serum testosterone (A), intra-testicular DHT and serum DHT (B), and intra-testicular estradiol and serum estradiol (C) in 10 normal men undergoing testicular aspiration.

Figure 2A:

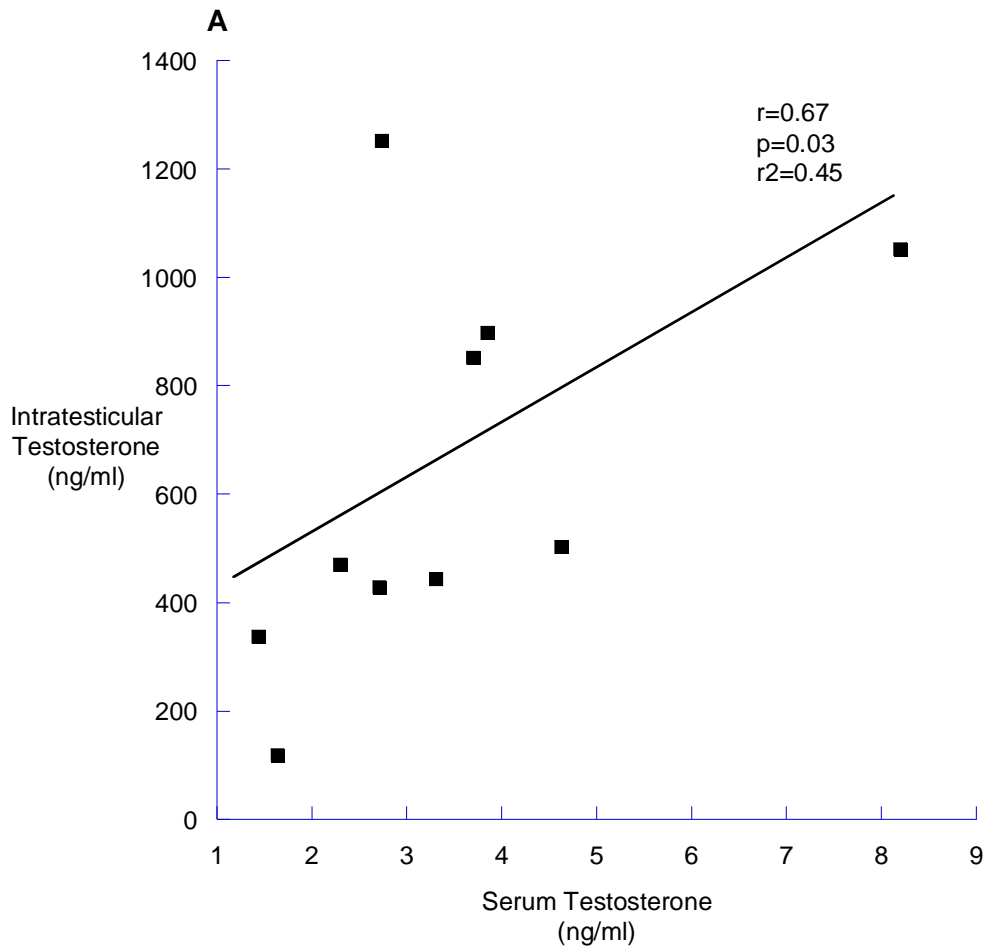


Figure 2B:

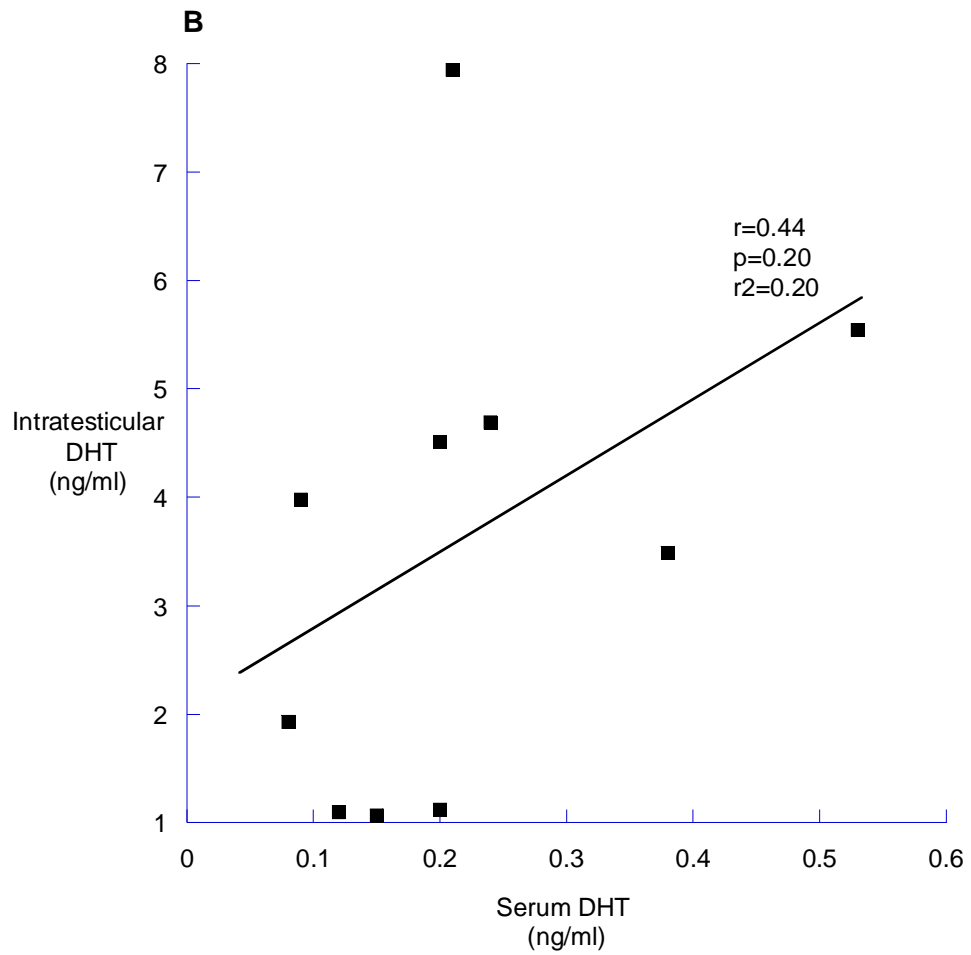


Figure 2C:

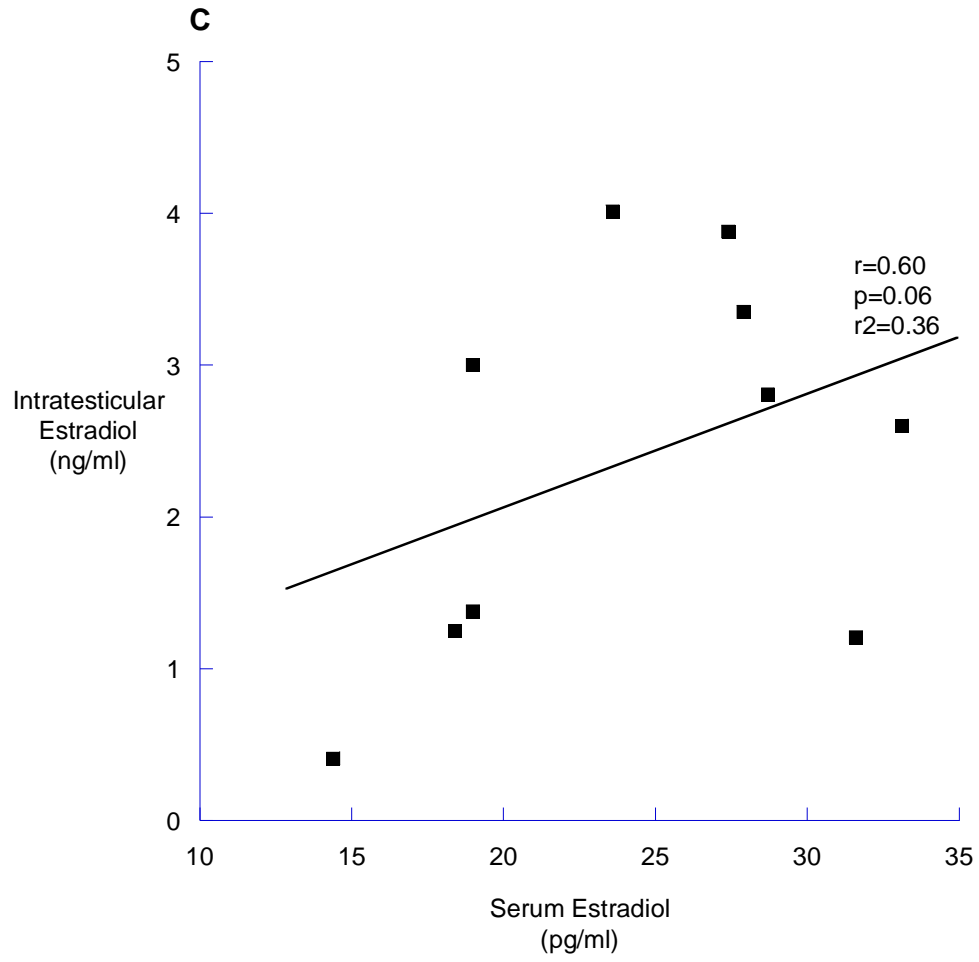


Figure 3: The relationship between right and left testes of intra-testicular testosterone (A), intra-testicular DHT (B), and intra-testicular estradiol (C) in 10 normal men undergoing testicular aspiration.

Figure 3A:

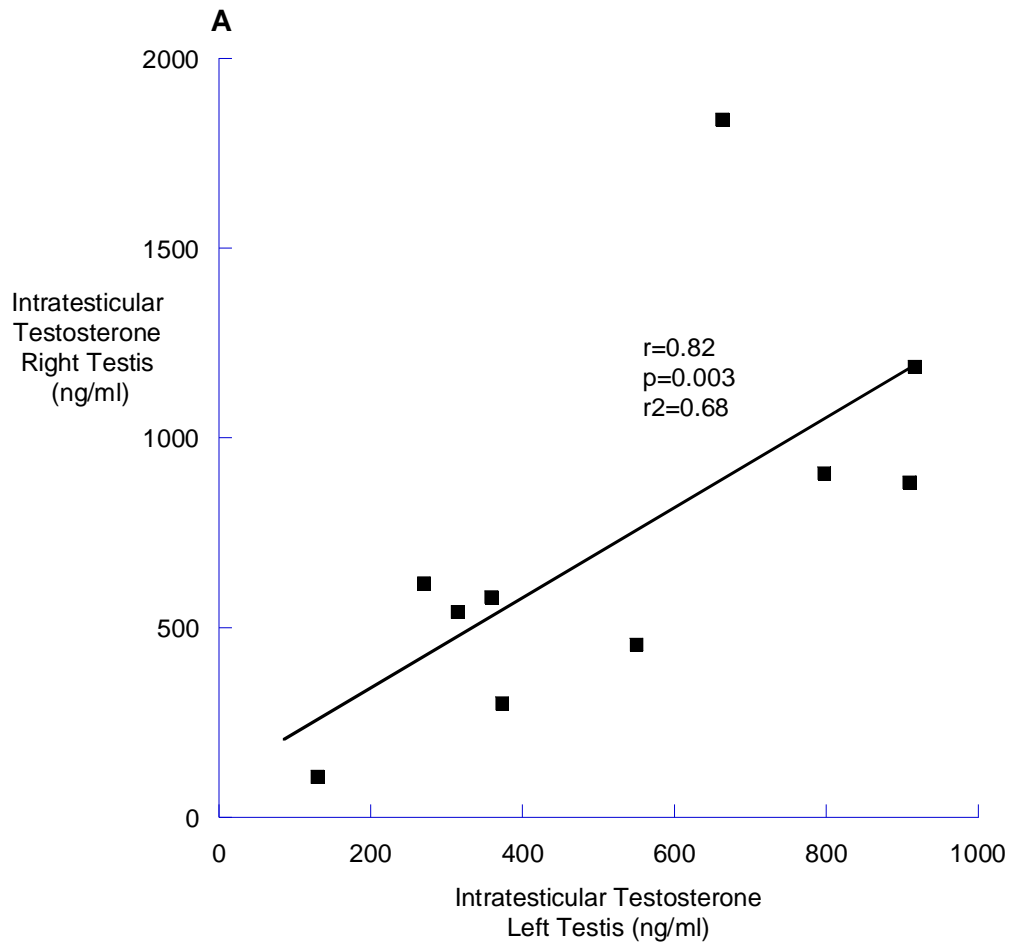


Figure 3B:

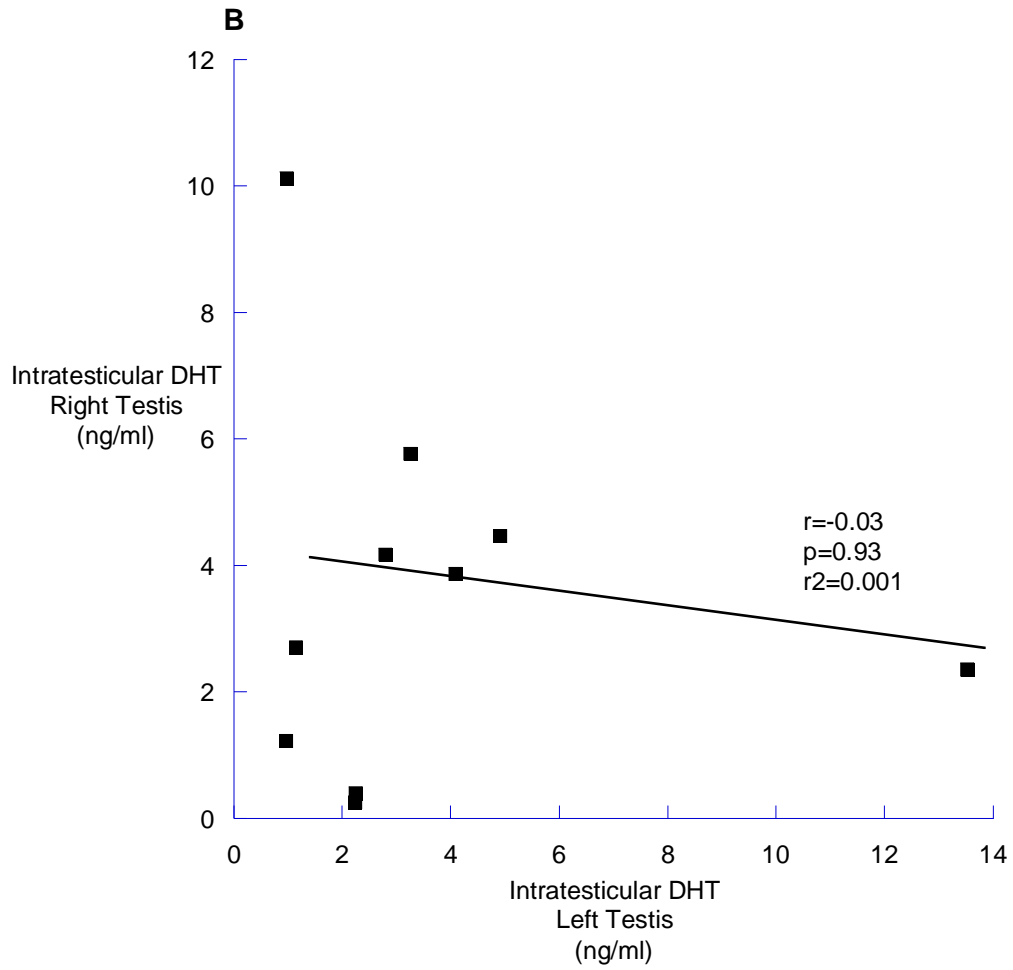


Figure 3C:

