Journal of Andrology, Vol. 26, No. 3, May/June 2005

Bioactivity of Androgens Within the Testes and Serum of Normal Men

JONATHAN P. JAROW,*† WILLIAM W. WRIGHT,† TERRY R. BROWN,† XIAOHUA YAN,*† AND BARRY R. ZIRKIN†

*From the *Department of Urology, Johns Hopkins University School of Medicine, Baltimore, Maryland; and the †Division of Reproductive Biology, Department of Biochemistry and Molecular Biology, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, Maryland.*

ABSTRACT: Little is known about how human spermatogenesis is regulated, so it is not surprising that there have been few breakthroughs in the treatment of male infertility resulting from abnormalities of spermatogenesis. Testosterone is the predominant intratesticular steroid in both the rat and man. Previous studies have shown that the testosterone concentration within the rat testis that is required for the quantitative maintenance of spermatogenesis is far higher than the total testosterone concentration in rat blood, indicating that much of the testosterone within the testis might be biologically inactive. In contrast to the rat, little is known about the androgen requirements for human spermatogenesis, in part because, until recently, a minimally invasive method suitable for obtaining intratesticular fluids from the human testis has not been available. Percutaneous aspiration now makes it feasible to do so. A major objective of the present study was to assay the bioactive androgen concentration within the testes of normal, fertile men. Percutaneous aspiration was used to obtain intratesticular fluid from such men, and we adapted a highly sensitive recombinant protein mammalian cell-based bioassay to measure androgen bioactivity. Total intratesticular testosterone concentration, which we define as immunoreactive testosterone as measured by radioimmunoassay, was well in excess of that in serum (1236 ± 86 nM vs 11.7 ± 0.7 nM). The concentration of bioactive androgens within the normal human testis was found to be about two thirds that of the total testosterone concentration. Interestingly, the concentration of the major, known binding proteins for testosterone within the testis, serum hormone-binding globulin (SHBG)/ABP (52.4 ± 9.7 nM), was insufficient to account for the difference between total testosterone and bioactive androgens. This indicates that, in addition to its binding to SHBG/ABP, androgens may also be bound by unknown molecules, and that this contributes to reducing androgen bioactivity. These observations could have relevance for understanding the relationship between spermatogenesis and intratesticular androgens in normal men and in men diagnosed with infertility.

Key words: Intratesticular testosterone, infertility, human.
J Androl 2005;26:343–348

Approximately 20% of couples have difficulty conceiving, with a male factor the sole or contributory cause in more than 50% of these couples (Thonneau et al, 1991). Among the many known causes of male infertility, at least some conditions, including varicocele and idiopathic infertility, are potentially correctable with therapy (Jarow, 1997). Although there have been significant advances in the understanding and treatment of female infertility, unfortunately there has been little progress in the development of treatment modalities for male infertility. Rather, assisted reproductive technologies are used to allow subfertile men, and even men who previously were labeled as ''sterile,'' to initiate conception without any attempt to improve their underlying medical conditions and thus their fertility status (Palermo et al, 1996).

Testosterone is the predominant intratesticular steroid in the testes of both the rat (Turner et al, 1984) and man (Morse et al, 1973; Huhtaniemi et al, 1987; Jarow et al, 2001; McLachlan et al, 2002). The concentration of the biologically active metabolite of testosterone, 5-dihydrotestosterone (DHT), is 5% that of testosterone in the rat testis (Turner et al, 1984) and 2% that of testosterone in the human testis (McLachlan et al, 2002). Prior studies in the rat have shown that the normal total intratesticular testosterone concentration, 50–70 ng/mL, is significantly higher than the 20 ng/mL concentration shown experimentally to be required for the quantitative maintenance of spermatogenesis (Zirkin et al, 1989) or for its quantitative restoration in rats that had been rendered azoospermic by testosterone administration or active immunization against GnRH (Awoniyi et al, 1989). The requisite threshold of 20 ng/mL, although less than 50% of the testosterone concentration that is normally present within the rat testis, nonetheless is 10-fold higher than the total testosterone concentration in normal rat serum (2 ng/mL).

Supported by grant HD-44258 from the National Institute of Child Health and Human Development, National Institutes of Health.

Correspondence to: Dr Jonathan P. Jarow, Department of Clinical Chemistry, University of Helsinki, 601 North Caroline St, Baltimore, MD 21287 (e-mail: jjarow@jhmi.edu).

Received for publication June 20, 2004; accepted for publication December 7, 2004.

DOI: 10.2164/jandrol.04100

In part, the explanation for the need for relatively high concentrations of testosterone in the rat testis may be that at least some of the testosterone is bound to proteins in the seminiferous tubular fluid and therefore that only a fraction of the total testosterone is bioactive (Roberts and Zirkin, 1993).

Until recently, a major problem in conducting comparable studies on the hormonal regulation of human spermatogenesis has been the lack of minimally invasive methods suitable for repeatedly obtaining intratesticular fluids from the human testis. We recently reported the development of such a method, percutaneous aspiration of the human testis (Jarow et al, 2001). Using this method, we found that, as in the rat, the mean total testosterone concentration within the human testis, about 600 ng/mL, is well in excess of the average testosterone concentration in serum (5 ng/mL). We originally reported that DHT concentration in the human testis was below the limit of detection by routine radioimmunoassay (RIA) procedures (Jarow et al, 2001). Subsequently, using more sensitive liquid chromatography tandem mass spectrometry (LC/MS/MS), we found that the concentration of DHT in human testicular fluid is about 2% that of testosterone (unpublished data). Our results are consistent with previous findings by McLachlan and associates (McLachlan et al, 2002), who were able to measure both testosterone and DHT by RIA, since they had larger volume samples obtained by testicular biopsy.

The serum hormone-binding globulin (SHBG)/ABP that is present in intratesticular fluid is thought to bind androgens and thereby to reduce androgen bioactivity. However, there is virtually no information on the percentage of free and/or biologically active androgens within the testis. Herein we report on the adaptation of a recently described recombinant protein mammalian cell bioassay (Raivio et al, 2001) that is sensitive enough to be used for the measurement of bioactive androgen concentrations in the small volumes of testicular fluids collected by percutaneous aspiration from normal, fertile men.

Materials and Methods

Patient Population

Percutaneous testicular aspiration (Jarow et al, 2001) was used to obtain intratesticular fluid from 22 healthy, fertile, adult men undergoing vasectomy. Testicular aspirates were transferred to the laboratory on ice and centrifuged at 300 × *g* at 4°C, and the supernatants were stored at −80°C until assayed. Peripheral blood serum samples were simultaneously obtained from these men, and the serum was stored at −80°C. All men donated 3 separate semen samples before their vasectomy. Informed consent was obtained from all subjects. The Joint Committee on Clinical Investigation of Johns Hopkins University approved this study.

Immunoassays

Immunoreactive testosterone concentrations were measured in testicular fluid aspirates and in blood serum by RIA in duplicate samples, as previously described in detail (Khan et al, 1982; Chen et al, 1994). The sensitivity of the RIA for testosterone was 10 pg/tube, with interassay and intra-assay coefficients of variation of 11.2% and 9.6%, respectively. SHBG and testicular fluid SHBG/ABP were measured using a commercially available kit (Diagnostic Systems Laboratories Inc, Webster, Tex). The RIA used to measure SHBG does not distinguish between ABP and SHBG. According to the manufacturer, the sensitivity of the SHBG/ABP assay is 3 nmol/L; inter- and intra-assay coefficients of variation (CVs) were less than 12% and 5%, respectively. All samples were assayed simultaneously and run in duplicate.

Mammalian Cell Recombinant Protein Interaction Bioassay for Androgens

We adapted the mammalian cell recombinant protein interaction bioassay of Ravio and co-workers (Raivio et al, 2001) to measure bioactive androgen concentrations in serum and testicular fluid. Major advantages of this assay are its specificity and the fact that it can be used to assay bioactive androgen concentrations in small-volume samples. The bioactivity assays were conducted using COS-7 monkey kidney cells grown in Dalbecco's Modified Eagle Medium (DMEM) culture media. The media contained 10% fetal bovine serum (FBS) on day 1 to allow growth and attachment of cells following plating; on day 2, the cells were washed with serum-free media and subsequently maintained in serum-free DMEM media throughout DNA transfection in the presence of lipofectamine (see below). Following transfection, the serum-free media was replaced with media containing 5% dextran-coated charcoal-stripped FBS. On day 3, fresh media containing 5% stripped FBS was added to each well. The final volume of the culture media was 250 μL/well, inclusive of the sample volume. Samples, including testicular fluid and serum, were added to the wells. Testosterone used for the standard curve was 0, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 nM. The standard curve was consistently linear, with an $R^2 = 0.99$ in a typical run.

To perform the assay, the COS-7 cells were transfected with plasmids to express recombinant proteins of the N-terminus of the androgen receptor (AR) fused to the VP16 activation domain, the C-terminal ligand binding domain of AR fused to the Gal4 DNA-binding domain, the AR coactivator ARIP3, and a firefly luciferase reporter under the regulation of 5 Gal4 binding sites (pG5-LUC). In brief, the specific binding of androgen alters the conformation of the AR ligand-binding domain fusion protein to promote its interaction with the AR N-terminal domain fusion protein. The binding of this activated complex of recombinant proteins, one of which contains the GAL4 DNA-binding domain, to one or more of the GAL4 binding sites of the luciferase reporter gene, activates expression of the firefly luciferase enzyme. The sensitivity of the assay for androgen-mediated receptor transactivation is further enhanced by the coexpression of the androgen receptor coactivator, ARIP3, which interacts with and

stabilizes the AR N- and C-terminal protein interaction, thus amplifying the expression of the firefly luciferase enzyme. A constitutively active Renilla luciferase plasmid is used to control for transfection efficiency. Firefly and Renilla luciferase enzyme activities were measured with the Dual Luciferase kit (Promega, Madison, Wisc) according to the manufacturer's instructions, using a Turner 20/20 luminometer. The results are quantified based on the ratio of firefly:Renilla luciferase activities for known and test samples. The values from samples containing known concentrations of added testosterone (see above) were used to plot a standard curve of relative luciferase activity to testosterone concentration. Utilizing the standard curve, the biologically active androgen in each test sample was determined based upon its relative luciferase activity. Bioactive androgen concentrations were quantified in serum (30 μL) and intratesticular fluid (1 μL). The sensitivity of this assay is equivalent to 0.069 ± 0.03 nmol/L of testosterone; inter- and intra-assay CVs are 23% and 6%, respectively. All samples were assayed simultaneously and run in quadruplicate.

Semen Analysis

Semen samples were obtained by masturbation into a sterile container after a defined period of abstinence of 2 to 3 days. The ejaculate volume was measured and the sperm concentration was determined using a Microcell counting chamber (Conception Technologies Inc, La Jolla, Calif). The coefficient of variation of this assay was 9% (Overstreet et al, 1999). Proficiency testing was conducted quarterly by the laboratory through the American Association of Bioanalysts. The average total sperm count was calculated for each volunteer from 3 separate semen samples obtained over a 1-week period.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to detect differences among groups. For comparisons between 2 group means, the Student's *t* test was used. Means were considered to differ at *P* values less than .05. Pearson's correlation was used to analyze the degree to which variables are related.

Results

The mammalian cell recombinant protein assay was used in this study to quantify androgen bioactivity in human serum and intratesticular fluid. In initial studies, the possibility that interfering substances might affect the results obtained with this assay was assessed by quantifying bioactive androgens in serial dilutions of human serum and human testicular fluid samples. This approach also tests whether dilution alters the free vs bound equilibrium. Assays were run in quadruplicate, with 4 different volumes tested. The results are shown in the Table. ANOVA revealed no significant differences ($P > .05$) in androgen bioactivity with the different volumes of human serum or human testicular fluid.

The specificity of the assay was evaluated by measuring androgen bioactivity after adding various steroids to fetal calf serum stripped of endogenous steroids by absorption to dextran-coated charcoal. For this analysis, 0.5 nM testosterone, 6 nM testosterone, 0.5 nM dihydrotestosterone, 6 nM estradiol, 6 nM progesterone, or 6 nM testosterone plus the anti-androgen hydroxyflutamide (600 nM) were tested (Figure 1). An approximate 10-fold increase in androgen bioactivity was seen with the addition of 6 nM testosterone as compared to 0.5 nM testosterone, and androgen bioactivity was completely suppressed by the presence of hydroxyflutamide. DHT had approximately 10-fold higher bioactivity than testosterone, as would be expected (Raivio et al, 2002). Neither estradiol nor progesterone demonstrated androgenic activity. In addition, testosterone-free human serum (Diagnostic Systems Laboratories, Webster, Tex) did not demonstrate detectable androgenic activity in this assay.

The effect of sample volume on the measurement of androgen bioactivity (mean ± SEM) in human serum and testicular fluid specimens

Volume (μL)	Androgen Bioactivity (nM)
Human serum	
10	9.26 ± 0.08
20	8.37 ± 0.83
30	7.46 ± 0.12
40	9.47 ± 0.53
Human testicular fluid	
1.0	1234 ± 19
1.5	1105 ± 48
2.0	856 ± 142
2.5	968 ± 161

The mean total (immunoreactive) serum testosterone concentration for the 22 patients entered into this study, measured by RIA, was 11.7 ± 0.7 nM, and the androgen bioactivity in the serum of these same subjects, measured by the androgen bioactivity assay, was 8.1 ± 0.5 nM. This represented a significant ($P < .01$), 30% decrease from the total concentration (Figure 2). The total testosterone and bioactive androgen concentrations in intratesticular fluid were, in both cases, approximately 100-fold higher than the respective values in serum; the concentration of total (immunoreactive) testosterone was 1226 ± 86 nM, and that of bioactive androgen was 835 ± 74 nM (Figure 2). The 30% decrease in intratesticular bioactive androgen from immunoreactive intratesticular testosterone was significant ($P < .01$). As depicted in Figure 3, total testosterone and bioactive androgen concentrations in the serum of individual men were highly correlated ($r = 0.624$; $P = .0019$). In contrast, total testosterone and bioactive androgen concentrations in the intratesticular fluid did not correlate ($r = 0.037$; $P = .87$).

We wished to determine whether serum levels of total testosterone and/or bioactive androgen concentrations

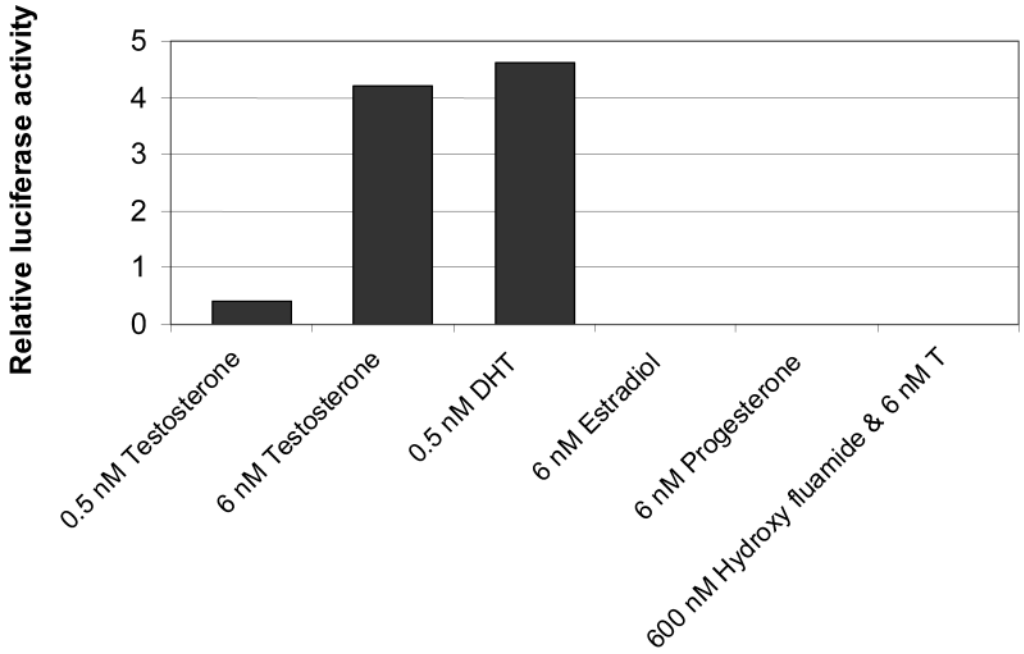

Figure 1. Specificity of androgen bioactivity assay. Testosterone, estradiol, progesterone, or testosterone plus hydroxyflutamide were added to charcoal-stripped fetal calf serum.

were predictive of intratesticular concentrations. Total testosterone concentration in the serum was not correlated with testosterone concentration in intratesticular fluid ($P = .38$), but it was weakly correlated with intratesticular bioactive androgens ($r = 0.46$; $P = .03$). The concentration of bioactive androgens in the serum was not correlated either with total testosterone ($P = .64$) or with bioactive androgen concentration ($P = .35$) in intratesticular fluid.

SHBG in the serum, and SHBG/ABP within the testes, are considered to be the major androgen-binding molecules in the human (Joseph, 1994). We hypothesized, therefore, that the difference between total testosterone and bioactive androgen levels in the serum and within the testes might result from the binding of androgens by SHBG/ABP. Surprisingly, the mean concentration of SHBG in testicular fluid was relatively low, 52.4 ± 9.7 nM. Moreover, although the difference between total testosterone concentration and bioactive androgen activity in the serum is relatively small compared to the difference between these parameters in intratesticular fluid, the concentration of SHBG in the serum, 46.1 ± 6.4 nM, was not significantly different from that in the testis.

The average sperm count of the men entered into this study was 156.4 ± 16.5 × 10^6 per mL, with a range of 36–294 × 10^6 per mL. Although each of the men was

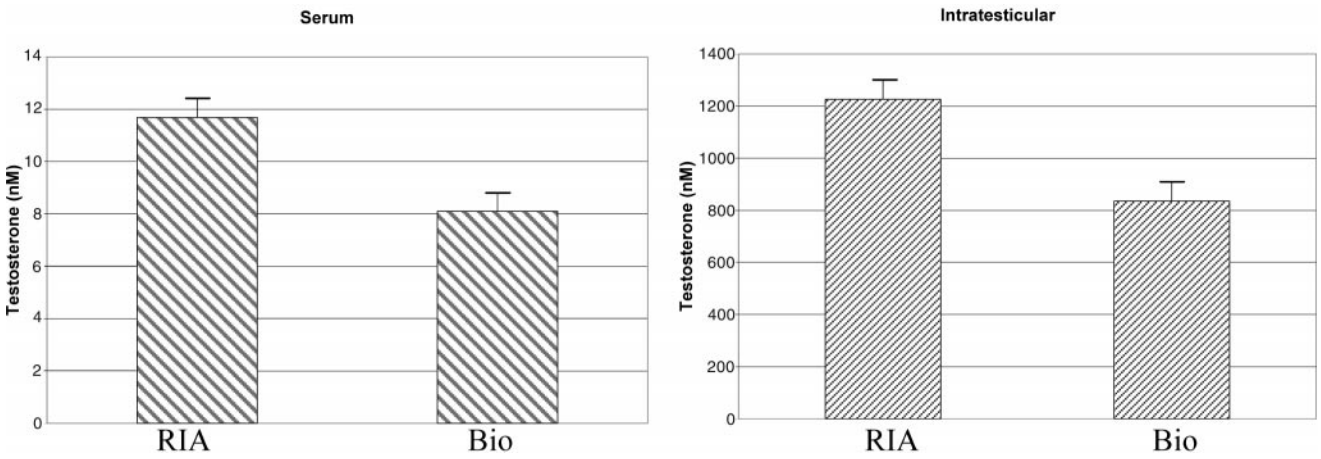

Figure 2. Comparison of the mean (±SEM) total concentration of testosterone, as measured by radioimmunoassay (RIA), and androgen bioactivity (Bio), as measured by the bioactivity assay, in serum and intratesticular fluid.

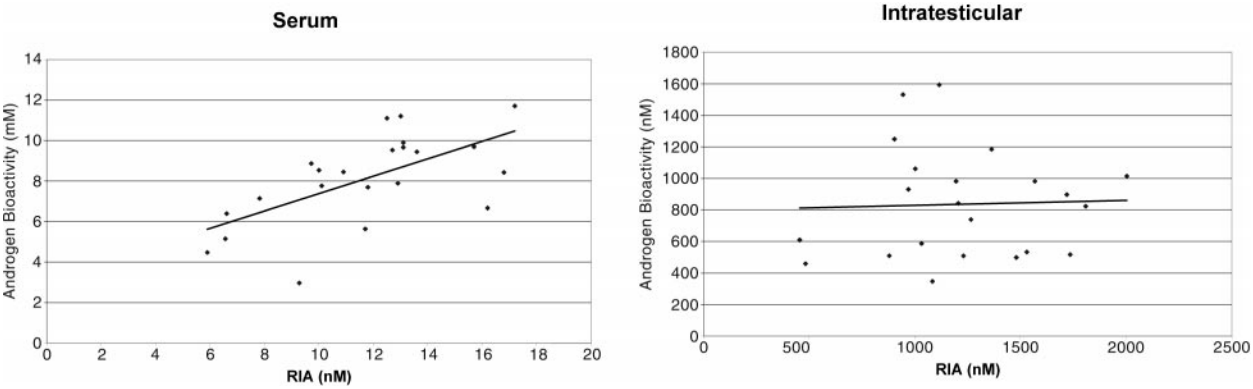

Figure 3. Correlation between total testosterone and androgen bioactivity in serum and intratesticular fluid.

fertile, the range of sperm counts was broad. We wished to determine whether the intratesticular androgen concentration of these men was predictive of their sperm counts. No correlation was found between sperm count and intratesticular total testosterone concentration ($P = .5$) or bioactive androgen concentration ($P = .25$) in individual men.

Discussion

The androgen bioactivity assay used in our study is a functional assay that measures the total bioactivity of androgens within a fluid sample. The data presented herein show that the concentration of bioactive androgens within the normal human testis is roughly two thirds that of total (immunoreactive) testosterone. Although there was a good correlation between total and bioactive androgens within serum, this was not the case in testicular fluid. In fact, the bioactivity of androgens within human testicular fluid could not be predicted either by measuring immunoreactive or bioactive testosterone in the serum.

We hypothesized that the difference between total testosterone and bioactive androgens within the testis would be accounted for by ABP/SHBG, the primary extracellular androgen-binding proteins within the testis. However, the concentration of SHBG/ABP within the testes, approximately 52 nM, was found to be far too low to account for the roughly 400-nM difference between total testosterone and bioactive androgen concentrations measured in human testicular fluid. Equally surprisingly, there was no difference between the concentration of SHBG in serum and SHBG/ABP within the testes. We have no explanation for the differences in total testosterone and bioactive androgen concentration in testicular fluid, except to suggest that testosterone might be made unavailable by binding to other, as yet unknown, molecules in addition to its binding to SHBG/ABP.

Studies of the rat have shown that intratesticular testosterone concentration can decline by over 50% without a significant effect on sperm count (Santulli et al, 1990). If the relationship between intratesticular testosterone concentration and sperm production in man is similar to that of the rat, a wide range of intratesticular testosterone concentrations in normal, fertile men would be anticipated. Therefore, one would hypothesize that intratesticular testosterone concentration would not predict sperm numbers. Indeed, we found this to be the case; no correlation was seen between sperm counts and either testosterone or bioactive androgen activity within the testis of the men entered into this study, all of whom were fertile. As intratesticular androgenic bioactivity falls below a threshold value, a direct relationship would be anticipated between sperm number and bioactive androgen concentration. This prediction is being tested at present.

There are subsets of subfertile men who might benefit from therapies designed to increase intratesticular testosterone concentration. Unfortunately, it is difficult to identify such men. For example, the results discussed above indicate that standard RIA of serum androgens would not reliably identify men whose intratesticular testosterone levels were low. This is also true in other circumstances. For example, in men who receive testosterone-based contraceptive regimens, peripheral testosterone concentrations are maintained at normal levels, while intratesticular testosterone concentrations are low, presumably because local production by Leydig cells is reduced following suppression of LH secretion (Jarow and Lipshultz, 1990). There is an obvious advantage in measuring testosterone within testicular fluids, particularly by the use of an assay that measures biologically active androgens. The disadvantage of such an approach is that the procedure is invasive. Nonetheless, knowledge of the quantitative relationship between intratesticular bioactive androgen concentration and sperm production might make it possible to assess the prognosis for effective androgen replacement therapy in at least some subfertile men.

Acknowledgments

We thank Drs Olli Janne and Jorma Palvimo (University of Helsinki, Finland) for providing the plasmids, pMLBD-hAR, pVP16-(5–538)rAR, pFLAG-ARIP3(1–572), and pG5-luc used in the androgen bioassay.

References

Awoniyi CA, Santulli R, Chandrashekar V, Schanbacher BD, Zirkin BR. Quantitative restoration of advanced spermatogenic cells in adult male rats made azoospermic by active immunization against luteinizing hormone or gonadotropin-releasing hormone. *Endocrinology.* 1989; 125:1303–1309.

Chen H, Chandrashekar V, Zirkin BR. Can spermatogenesis be maintained quantitatively in intact adult rats with exogenously administered dihydrotestosterone? *J Androl.* 1994;15:132–138.

Huhtaniemi I, Nikula H, Parvinen M, Rannikko S. Pituitary-testicular function of prostatic cancer patients during treatment with a gonadotropin-releasing hormone agonist analog. II. Endocrinology and histology of the testis. *J Androl.* 1987;8:363–373.

Jarow JP. Nonsurgical treatment of male infertility: empiric therapy. In: Lipshultz LI, Howards SS, eds. *Infertility in the Male.* St Louis, Mo: CV Mosby Co; 1997:410–423.

Jarow JP, Chen H, Rosner TW, Trentacoste S, Zirkint BR. Assessment of the androgen environment within the human testis: minimally invasive method to obtain intratesticular fluid. *J Androl.* 2001;22:640–645.

Jarow JP, Lipshultz LI. Anabolic steroid-induced hypogonadotropic hypogonadism. *Am J Sports Med.* 1990;18:429–431.

Joseph DR. Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. *Vitam Horm.* 1994;49:197–280.

Khan MS, Ewen E, Rosner W. Radioimmunoassay for human testosterone-estradiol-binding globulin. *J Clin Endocrinol Metab.* 1982;54: 705–710.

McLachlan RI, O'Donnell L, Stanton PG, Balourdos G, Frydenberg M, de Kretser DM, Robertson DM. Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones, and germ cell populations in normal young men. *J Clin Endocrinol Metab.* 2002;87:546–556.

Morse HC, Horike N, Rowley MJ, Heller CG. Testosterone concentrations in testes of normal men: effects of testosterone propionate administration. *J Clin Endocrinol Metab.* 1973;37:882–886.

Overstreet JW, Fuh VL, Gould J, et al. Chronic treatment with finasteride daily does not affect spermatogenesis or semen production in young men. *J Urol.* 1999;162:1295–1300.

Palermo GD, Cohen J, Rosenwaks Z. Intracytoplasmic sperm injection: a powerful tool to overcome fertilization failure. *Fertil Steril.* 1996; 65:899–908.

Raivio T, Palvimo JJ, Dunkel L, Wickman S, Janne OA. Novel assay for determination of androgen bioactivity in human serum. *J Clin Endocrinol Metab.* 2001;86:1539–1544.

Raivio T, Tapanainen JS, Kunelius P, Janne OA. Serum androgen bioactivity during 5alpha-dihydrotestosterone treatment in elderly men. *J Androl.* 2002;23:919–921.

Roberts KP, Zirkin BR. Androgen binding protein inhibition of androgen dependent transcription explains the high minimal testosterone concentration required to maintain spermatogenesis in the rat. *Endocrine J.* 1993;1:41–47.

Santulli R, Sprando RL, Awoniyi CA, Ewing LL, Zirkin BR. To what extent can spermatogenesis be maintained in the hypophysectomized adult rat testis with exogenously administered testosterone? *Endocrinology.* 1990;126:95–101.

Thonneau P, Marchand S, Tallec A, et al. Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988–1989). *Hum Reprod.* 1991;6:811–816.

Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gunsalus GL. On the androgen microenvironment of maturing spermatozoa. *Endocrinology.* 1984;115:1925–1932.

Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. *Endocrinology.* 1989;124: 3043–3049.