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Serum bioactive and immunoreactive follicle-stimulating hormone in oligozoospermic and azoospermic men: application of a modified granulosa cell bioassay

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Serum follicle-stimulating hormone (FSH) levels measured by radioimmunoassay (RIA) usually correlate well with the rate of spermatogenesis. However, in certain cases this correlation does not exist. The purpose of this study was to establish a reliable bioassay of FSH for the andrological clinic. Follicle-stimulating hormone was measured by both standard RIA and bioassay in 98 men subgrouped into normospermic, oligospermic, and azoospermic. Bioactivity of FSH was determined using in vitro cultures of granulosa cells utilizing progesterone measurements for assessing FSH activity. Results of FSH levels obtained by both methods correlated well ($r = 0.55$, $P < 0.01$) within themselves, and both correlated negatively and significantly with sperm concentration. The ratio between bioactivity and immunoreactivity of FSH did not correlate with sperm density. Thus, the decrease in sperm concentration and other sperm variables resulting from a germinal epithelial dysfunction was not mediated or associated with low biological activity of FSH. The application of this method can be of clinical value in cases where a discrepancy is found between serum RIA-FSH levels and sperm quality.
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Follicle-stimulating hormone (FSH) has a major role in regulation of normal spermatogenesis.[1] The observation that increased FSH serum levels are associated with severe germinal cell damage[2,3] is only partially explained by a decrease in inhibin produced by the damaged Sertoli cells.[4,5]

Clinicians in andrological units have shown that patients with elevated FSH might have quite normal spermatogenesis and vice versa.[2,3] This hints at the possibility that the FSH measured radioimmunologically does not necessarily always reflect the bioactivity of that molecule.

Biochemical changes in the FSH molecule have been demonstrated to be a result of changes in the hormonal milieu around the hypophysis.[6] Therefore, it was postulated that among men with different degrees of pathology in spermatogenesis, an altered, less active form of FSH is secreted by the hypophysis. This supposition could not be tested until recently. With the development of the in vitro granulosa cell aromatase bioassay for FSH,[7,8] it became possible to measure serum bioactivity of FSH in both physiological and pathological conditions.

In this study, using a modification of the above-mentioned bioassay, we measured the bioassay-FSH activity in a group of men with normal and abnormal numbers of spermatozoa in the ejaculate. Serum bioassay-FSH levels were compared with

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radioimmunoassay (RIA)-FSH levels and to other hormonal and sperm quality variables.

MATERIALS AND METHODS

Patients

Ninety-eight white young men (20- to 35-years-old) were seen in the outpatient infertility clinic. All were physically examined and found to be in general good health and had normal external genitalia. None were on any hormonal treatment in the 6 months preceding the study, or under any drug treatment that might interfere with the hypophyseal-gonadal axis, such as cimetidine or spironolactone. Each patient delivered at least two semen specimens for analysis after 3 days of sexual abstinence.

Blood Sampling and Hormone Determinations

Blood samples were drawn between 7:30 A.M. and 8:30 A.M. to avoid any diurnal variation. Testosterone (T) and luteinizing hormone (LH) were measured by RIA using commercially available kits.[9] Serum FSH was measured in duplicate by RIA (Amerlax FSH; Amersham International, Amersham, United Kingdom). The results were expressed in terms of the Second International Reference Preparation 78/549.

Bioassay-FSH Determination

Bioassay Reagents

The reference standard was the human pituitary preparation LER-907 (FSH biopotency 20 IU/mg, LH biopotency 60 IU/mL, which uses the Second International Preparation of human menopausal gonadotropin standard) from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, Maryland. Human chorionic gonadotropin (hCG, 300 IU/mg Chorigon; Ikapharm, Ramat Gan, Israel), porcine insulin (26.8 U/mg Actrapid; Novo Company, Rotterdam, Holland), L-Glutamine, fetal bovine serum (FBS), McCoy 5-A (serum-free medium), and a penicillin-streptomycin solution (Biolab, Jerusalem, Israel) were also used. Androstenedione, 1-methyl-3-isobutylxanthine, diethylstilbestrol (DES), and polyethyleneglycol (PEG) (molecular weight 8,000) were purchased from Sigma Chemical Co., St. Louis, Missouri; ^{125}I Progesterone (P) from Dupont Company, Boston, Massachusetts. Monoclonal antiprogesterone antibodies were produced by Dr. F. Cohen of the Department of Hormone Research, The Weizmann Institute, Rehovot. Microwells used were of the Nunclon-type 16 mm. (Nunc, Vyborg, Denmark).

Granulosa Cell Assay

Serum for bioactivity estimation was kept in −20°C and thawed no more than twice to avoid a decrease in biopotency known to occur after repeated thawing. Serum bioactive FSH was measured in quadruplicate according to the method described by Jia et al.[8] and Wide et al.[9] with the indicated modifications described: granulosa cells were obtained from immature, 25-day-old, DES-treated female rats. One hundred thousand cells were cultured in each well containing 0.5-mL supplemented McCoy 5-A medium. Supplementation included 2 mM L-glutamine, 100 μg/mL penicillin + streptomycin, 10^{-6} M androstenedione, 10^{-7} M DES, 0.125 mM 1-methyl-3-isobutylxanthine, 1 μg/mL insulin, and 30 ng/mL hCG.

Serum samples were pretreated with 12% PEG (dissolved in McCoy 5-A medium) and a constant serum volume of 20 μL per culture was added. The standard curve was prepared by the addition of 20 μL per culture of increasing concentrations of LER-907 (from 0 to 4 mIU/well). A constant volume of 20 μL pretreated FBS was added to all standard cultures (FBS was proven to be free of FSH activity with no inhibitory effect on the FSH-stimulated granulosa cells). Culture was carried out for 48 hours in 95% humidified air + 5% CO_2 at 37°C. The P produced by the granulosa cells was measured by RIA. The sensitivity of the assay was 0.03 mIU of LER-907 per culture and the intra-assay and interassay variation was 10% and 16%, respectively. Figure 1 illustrates the calibration curve for the bioassay-FSH.

Four changes were introduced in the granulosa cell bioassay, as described by Jia's[7,8] and Hsueh's[10] laboratories: (1) FBS added to maintain constant volume of the standard plates replaced the serum from women taking oral contraceptives. Fetal bovine serum was found to contain almost no FSH activity and thus the test sensitivity was improved. (2) Incubation period was reduced to 48 instead of 72 hours. This shorter incubation period was used to avoid the effect of LH on P or estrogen (E) synthesis, provided receptors for LH are induced on the granulosa cells by FSH only after 48 hours in culture.[11] This possible interference should be seri-

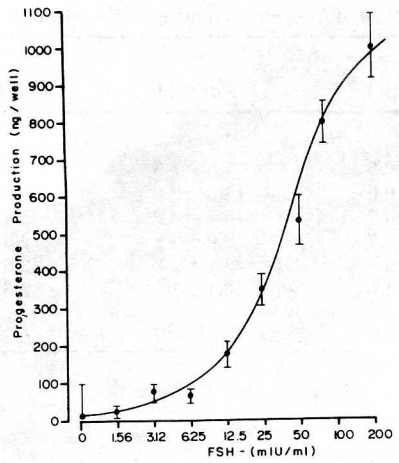

Figure 1 Standard curve used for the bioactivity-FSH determination. The curve was obtained using increasing concentrations of FSH LER-907 standard added to 100,000 granulosa cells incubated for 48 hours. Constant volumes of fetal bovine serum were added to all wells. Progesterone in medium was analyzed by RIA. Numbers are mean ± standard error (SE) of 8 runs in each point of the curve.

ously taken into consideration, because both LER-907 standard and human serum contain LH. (3) In addition to the shorter incubation period, the number of cells per culture was doubled from 50,000 to 100,000. (4) Progesterone was measured instead of E as a marker for bioactivity of FSH in this setting because maximum P production is reached at 48 hours of incubation, whereas E reaches its peak production only on the 3rd incubation day.[11]

Statistical Analysis

Immunoreactive FSH data and the RIA values for P were analyzed using a program that utilizes a weighted logit-log regression analysis. Calculation of FSH biopotency in samples was performed using a standard curve fitted with a second-degree polynomial. Comparisons of mean immunoassay and bioassay FSH, bioactivity to immunoreactivity ratio, and T and LH levels before and during treatment were done by Student's *t*-test or by one-way analysis of variance (ANOVA), where appropriate. Pierson's coefficient of correlation was used to demonstrate correlations between variables. Statistics were performed at the Tel-Aviv University, Center for Applied Mathematics, Unit for Statistical Services.

RESULTS

Table 1 summarizes the basic data of the five subgroups participating in the study. The subgrouping was done according to sperm characterization of the ejaculate.[12,13] Although the men included in the normal control group were found to have a normal number of spermatozoa in their ejaculate, they should not be considered fertile because none were proven fertile by fatherhood. Testosterone levels were similar in all five groups. Mean serum LH levels were significantly higher in severe oligozoospermic and azoospermic males as compared with the other groups ($P < 0.05$). All four parameters of semen characterization (concentration, motility, vitality, morphology) differed significantly ($P < 0.0001$) between the groups. Severe oligozoospermic and azoospermic men were significantly older than infertile normozoospermic men.

Table 1 Basic Data on the Clinical, Sperm Quality, T and LH Levels in Serum of the Five Groups of Men Participating in the Study[a]

Group	No. of men	Age	Body mass index	Sperm quality: Concentration (average)	Sperm quality: Motility	Sperm quality: Vitality	Sperm quality: Morphology	Serum hormones: T	Serum hormones: LH
×10⁶/mL		y		×10⁶/mL		%		μg/mL	mIU/mL
Infertile normozoospermia (>60)	29	26 ± 1	23.9 ± 0.4	120 ± 7	49 ± 2	67 ± 2	40 ± 2	5.4 ± 0.4	4.9 ± 0.7
Mild oligozoospermia (11 to 60)	32	31 ± 2	24.2 ± 0.8	38 ± 2	36 ± 2	68 ± 2	29 ± 2	5.4 ± 0.4	5.8 ± 0.8
Moderate oligozoospermia (1 to 10)	16	31 ± 2	25.4 ± 0.8	5 ± 1	14 ± 3	54 ± 6	20 ± 2	5.5 ± 0.5	6.6 ± 0.7
Severe oligozoospermia (<1)	7	33 ± 2	26.0 ± 1.0	0.5 ± 0.5	5 ± 5	20 ± 5	5 ± 2	5.5 ± 0.6	9.8 ± 0.6
Azoospermia	14	33 ± 1	25.9 ± 0.9	—	—	—	—	5.5 ± 0.6	11.8 ± 2.4
Total	98	31 ± 1	24.7 ± 0.4	49 ± 5	28 ± 2	51 ± 3	25 ± 2	5.4 ± 0.2	7.0 ± 0.7
Analysis of variance in each column		$P = 0.04$	$P = 0.47$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.99$	$P = 0.03$

[a] Values are means ± SE.

Table 2 Description of Immunoreactive and Bioactive Levels in the Five Groups of Men Participating in the Study[a]

Group according to sperm concentration	No. of men	RIA-FSH	Bio-FSH	FSH-Ratio
$\times 10^6$/mL		mIU/mL		
Infertile normozoospermia (>60)	29	4.7 ± 0.5	11.5 ± 1.6	2.8 ± 0.4
Mild oligozoospermia (11 to 60)	32	6.6 ± 0.6	14.5 ± 2.2	2.8 ± 0.3
Moderate oligozoospermia (1 to 10)	16	6.6 ± 0.7	15.2 ± 3.2	2.4 ± 0.4
Severe oligozoospermia (>1)	7	11.6 ± 3.7	19.6 ± 3.8	2.8 ± 0.4
Azoospermia	14	13.0 ± 2.8	23.7 ± 2.8	2.6 ± 0.2
Total	98	8.1 ± 1.0	16.1 ± 1.3	2.6 ± 0.2
Analysis of variance in each column		$P = 0.00001$	$P = 0.015$	$P = 0.75$

[a] Values are means ± SE.

Serum bioassay-FSH (Table 2, Fig. 2) paralleled the RIA-FSH levels over the whole range of FSH levels in all groups ($r = 0.55$, $P < 0.001$). Follicle-stimulating hormone levels were significantly higher, both immunologically and biologically, in severe oligozoospermic and azoospermic men as compared with men with >10 million spermatozoa per milliliter. No difference was found between FSH levels as determined by either method between both groups of mild and moderate oligospermic males, compared with normozoospermic men. The bioactivity to immunoreactivity ratio of FSH in men with all five groups of sperm quality did not differ, and a rather constant ratio of 2.4 ± 0.2 was calculated for the five groups (Fig. 3).

The negative correlation between RIA-FSH and sperm density was found to be statistically significant ($P < 0.01$). Bioassay-FSH levels demonstrated a similar negative link with identical statistical significance. The bioactivity to immunoreactivity ratio bears no correlation to sperm density ($r = 0.41$, $P > 0.5$).

Among the 98 men, 81 were normogonadotropic by the RIA method (1 to 8 mIU/mL), whereas 17 were hypergonadotropic. Testing their bioactivity to immunoreactivity ratios did not yield any difference. That is to say, among hypergonadotropics, the secreted FSH does not differ biologically from that in normogonadotropics.

DISCUSSION

The role of FSH in the regulation of male fertility was a subject of research over many decades. In 1972, it was demonstrated that the hormone binds to specific membranal receptors on the Sertoli cells producing cyclic-adenosine monophosphate (c-AMP) by activating adenylate cyclase.[13] Cyclic-AMP controls some of the events taking place in the Sertoli cells, such as the DNA-dependent RNA production of an androgen-binding protein. This protein pumps T from the interstitial tissue into the cells. Testosterone is essential in the control

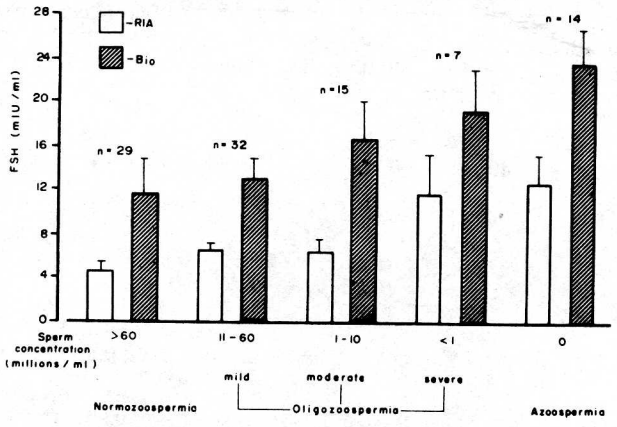

Figure 2 Serum FSH values (mean ± SE) measured by RIA and bioactivity FSH in the five groups of men (n, group size).

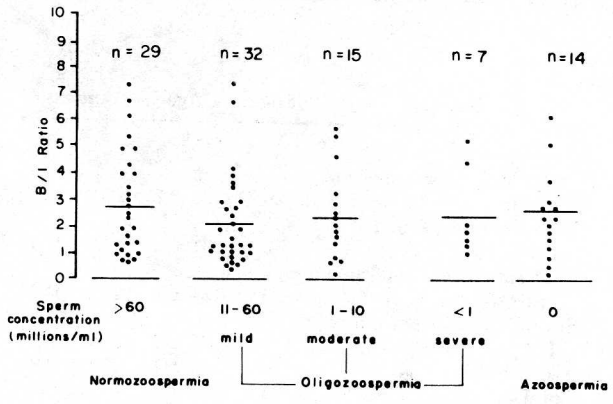

Figure 3 Calculated individual bioactivity to immunoreactivity ratios for all members of the study population, grouped by sperm concentration (n, group size). Horizontal bar indicates the calculated mean in each subgroup.

of the seminiferous tubules and is responsible for maturing spermatids.[14]

Studies have suggested that the endocrine milieu, especially the one surrounding the hypophysis, influences the biosynthesis of FSH molecules and determines the production of different bioactive FSH molecules.[6,15,16] It is known that the anterior hypophysis secretes a "family of isohormones" with different biological activities.[6,16] Until recently, however, there was no test available to measure and define the biological activities of these FSH isohormones.

The rationale for the present study was to use the bioassay for FSH to identify cases with oligozoospermia, but with normal levels of immunoassay-FSH, and possibly partially biologically inactive cases. This might have led us to treat such men with external FSH to compensate for the low activity of stimulation. However, this was not found to be the case in our study group.

Using an already published sensitive in vitro FSH bioassay with some modifications, we were able to measure bioactivity of FSH levels in serum samples from normozoospermic, and different severity oligospermic and azoospermic men. Our data confirmed previous findings by Jia and Hsueh[7] and Jia et al.[8] of a good correlation between serum bioactive FSH and immunoreactive FSH determined by RIA. However, in a single report in the literature, Wang et al.[17] claimed to have found lower bioactivity to immunoreactivity ratios among men with azoospermia and severe oligospermia. Their bioactivity to immunoreactivity ratio among normal fertile men was 2.7, similar to our findings (bioactivity to immunoreactivity ratio, 2.8). Contrary to the constant bioactivity to immunoreactivity ratio among all semen quality groups in our study, they found decreased bioactivity to immunoreactivity ratios among men with severe pathology. This discrepancy can only be partly explained by the different grouping methods used and by the fact that although the total number of cases was similar, they had a group of 15 men with severe oligospermia ($<5 \times 10^6$/mL), whereas in our study group only 7 such men were identified.

The serum LH and T pattern in our group are similar to those already published.[2] Although serum LH levels were significantly elevated among severe oligozoospermic and azoospermic men, their T levels were within the normal range and not different from the other men. This suggests either a less biopotent LH among men with azoospermia or a concomitant partial decreased activity of Leydig cells due to feedback mechanism. In men with azoospermia, RIA-FSH was significantly elevated. This might be a result of decreased inhibin secretion by the dysfunctional Sertoli cells.[4,5] Bioactive FSH levels followed the same pattern as RIA, and as a result, the mean bioactivity to immunoreactivity ratio was similar in this group as in the others. In contrast to the reports by Wang et al.[18] and Diebel et al.,[16] in our study group both serum immunoreactive FSH and bioactive FSH levels correlated negatively and in a statistically significant manner with sperm concentration. As a result of the above, FSH bioactivity to immunoreactivity ratios did not correlate with sperm density.

Our data suggest that the decrease in sperm quality is a result of germinal epithelium insufficiency and is not mediated nor associated with low biological activity of FSH. There might still be another mediating factor (inhibin ?) that might better correlate with the biological quality of the FSH among men with sperm pathology. Even more sound would be the supposition that among oligozoosperic men there are some with intrinsic end-organ failure not mediated via FSH, such as men with varicocele, where the reasons for Sertoli cell dysfunction are related to other mechanical factors. Only in some patients with oligozoospermia will the low number of sperm produced be related to low biological activity of FSH.

The modified granulosa cell P production FSH bioassay provided the possibility to measure bioactive FSH levels in men. Still needed is an accurate definition of the underlying pathology among larger numbers of men with oligozoospermia, and perhaps the isolation of cases with a discrepancy between RIA-FSH levels and sperm quality using bioactive FSH tests. This would enable drawing sound conclusions on the role of bioactive FSH among these men, and perhaps to compensate the relatively low activity of FSH with externally added FSH to solve their fertility problems.

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