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Research paper

Serum inhibin-B and follicle stimulating hormone as predictors of the presence of sperm in testicular fine needle aspirate in men with azoospermia

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ABSTRACT

OBJECTIVE: Inhibin-B (Inh-B) is produced by Sertoli cells and controls Follicle Stimulating Hormone (FSH) secretion through a negative feedback mechanism. The primary aim of this study was to compare Inh-B with FSH as predictors of the recovery of sperm in testicular fine needle aspirate in men with azoospermia. DESIGN: In 51 men with azoospermia basal values of Luteinizing Hormone (LH), FSH, prolactin and testosterone as well as Inh-B values before and 24 h and 48 h after the administration of 300 IU recombinant human FSH were determined. Testicular Fine Needle Aspiration (FNA) was also carried out. Thirty-one young healthy men were also enrolled in the study as controls. RESULTS: There was significant difference between men with azoospermia and controls with regard to the basal Inh-B levels [median (interquartile range) 37.2 (36) vs. 103.0 (90) pg/mL, respectively, $p=0.003$] but not to the stimulated Inh-B levels [40.5 (41) vs. 73.0 (44) pg/mL, $p=0.113$ at 24 h and 34.3 (34) vs. 82.0 (50) pg/mL, $p=0.098$ at 48 h)]. The Area Under Curve in Receiver Operating Characteristic curves were similar for Inh-B and FSH (0.610 vs. 0.716, respectively, $p=0.151$) as far as prediction of sperm retrieval is concerned. CONCLUSIONS: Basal serum Inh-B values are significantly lower in men with azoospermia compared to controls. However, Inh-B is not superior to FSH in predicting the presence of sperm in testicular fine needle aspirate .

Key words: FNA, FSH, Inhibin-B, Male subfertility, Sperm retrieval

INTRODUCTION

Inhibin-B (Inh-B), a dimeric glycoprotein, member of the Transforming Growth factor-β (TGF-β) superfamily, is produced and secreted in males, almost

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exclusively, by Sertoli cells.[1] Inh-B main action seems to be inhibition of Follicle Stimulating Hormone (FSH) synthesis and secretion through a negative feedback mechanism.[2] On the other hand, FSH induces Inh-B secretion.[3] Inh-B is considered as a marker of spermatogenesis, as there is evidence that there is a significant positive correlation between basal serum Inh-B levels and sperm concentration.[4]

The introduction of Intra-Cytoplasmic Sperm Injection (ICSI) has made the recovery of even a single sperm during a testicular biopsy an event of utmost importance for azoospermic men. This recovery is usually attempted through a Testicular Sperm Extraction (TESE) procedure. Cytological analysis obtained by testicular Fine Needle Aspiration biopsy (FNA) has been proposed as a more practical alternative to TESE,[5,6] as it allows evaluation of the tubular status and permits identification of both germ (i.e. spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa) and Sertoli cells. Nevertheless, there are no reliable predictors of the recovery of sperm from testicular tissue in men with non-obstructive azoospermia; serum levels of FSH provide only a rough estimation of a successful outcome during a TESE procedure.[7]

In August 2007 we performed a Medline search with Medical Subject Headings (MeSH) terms "Inhibin-B" and "Biopsy"/"Biopsy, Fine-Needle" as keywords; "humans" and "male" were used as limits. After manual searching of the results, we located 12 studies on Inh-B[2,8-18] that directly examined the role of this hormone as predictor of sperm retrieval during a testicular biopsy in men with azoospermia. Nevertheless, in none of these studies was there any correlation of results obtained with specific cause of azoospermia. Moreover, there were very few reports on FNA, as the majority of the studies had applied TESE.

The primary aim of the present study was to compare Inh-B and FSH as predictors of the recovery of sperm in FNA performed in men with azoospermia (Sub-study "FNA"). A secondary aim was to investigate the clinical value of basal and stimulated serum Inh-B levels as a marker of Sertoli cell function (Sub-study "EFSERT").

SUBJECTS AND METHODS

Subjects

We prospectively studied 51 subfertile Caucasian men of Greek nationality [median age 32, interquartile range (IQR) 5 years] attending the outpatient clinic of the Unit of Reproductive Endocrinology. Inclusion criteria were: i) infertility defined as not achievement of a pregnancy after 12 months of unprotected intercourse and ii) two spermiograms showing azoospermia with a time interval of at least 70 days. Exclusion criteria were: i) fever and ii) use of hormonal preparations during the last three months. Thirty-one healthy Caucasian men of Greek origin from the general population [age 31, (8) years] were used as controls.

Methods

All men with azoospermia as well as controls underwent complete andrologic evaluation, including history, physical examination, hormonal profile [serum FSH, Luteinizing Hormone (LH), total testosterone (T), prolactin, Inh-B, Exogenous FSH Sertoli Reserve Test (EFSERT)] and spermiogram. All men with azoospermia underwent testicular FNA. Imaging (scrotum ultrasonography, colour Doppler, pelvic computed tomography) and genetic (karyotype, Yq microdeletions) studies were performed, as clinically indicated. The study was approved by the Bioethics Committee, Aristotle University of Thessaloniki, Greece.

Blood samples were obtained at 09:00 and centrifuged for 20 min. The serum was separated and stored at -20°C until analysis was performed. The serum levels of FSH, LH, T and prolactin were measured by immunochemiluminescent method (Immulite 2000, DPC, USA). Serum levels of Inh-B were measured by an enzymatically amplified two-site two-step sandwich-type immunoassay (DSL-10-84100 ACTIVE Inhibin-B ELISA kit, Diagnostic Systems Laboratories Inc., Texas, USA). Inter-assay and intra-assay coefficients of variation were 6.2% and 3.5%, respectively. Assay sensitivity was 7 pg/mL.

All men with azoospermia as well as controls underwent EFSERT [estimation of serum Inh-B levels before, 24 h and 48 h after the administration of 300

IU recombinant human FSH, intramuscularly (Gonal-F®, Serono)]. The blood specimens for the EFSERT were collected at 09:00 from the median antebrachial vein for three consecutive days. On day one, two blood samples were collected with a time difference of 30 minutes to measure basal Inh-B levels.

Sperm was obtained by masturbation after 3–5 days of abstinence. The samples were centrifuged at 600 g for 10 minutes and if no sperm was detected in the pellet, a diagnosis of azoospermia was confirmed. Sperm concentration, motility and morphology were evaluated according to the World Health Organization (WHO) criteria.[19]

FNA was performed under local anaesthesia using a 23-G butterfly needle attached to an empty 20-mL disposable syringe in each pole of each testis for a total of four specimens per patient. Each retrieved specimen was vacated into a thin-Prep vial and stained with May-Grünwald-Giemsa. FNA interpretation took place in two steps. Initially, in low magnification, a group of two cytologists and one pathologist, who were blinded to the clinical data, assessed the adequacy of the material. Then, in high magnification, the cells were recognized and the spermatogenesis status was classified according to the Meng system, based on the predominant type of cells:

- Normal spermatogenesis: Cells from all stages of spermatogenesis were detected in adequate number.
- Hypospermatogenesis: Although cells from all stages of spermatogenesis (including sperm) were detected, their number was significantly reduced.
- Maturation arrest: Sperm maturation stopped in the early stages of spermatogenesis. Neither sperm nor spermatids were detected. The arrest was usually at the stage of first class spermatocytes ("complete maturation arrest"). If an occasional sperm was found in any of the four FNA sites, the case was classified as "incomplete maturation arrest".
- Sertoli Cell-Only Syndrome (SCOS): Spermatogenesis cells were completely absent, Sertoli cells being the only cells detected ("complete SCOS"). If an occasional sperm was found in any of the four FNA sites, the case was classified as "incomplete SCOS".

Thus, in this classification system six possible FNA diagnoses exist: in four (normal spermatogenesis, hypospermatogenesis, incomplete spermatogenesis arrest and incomplete SCOS) sperm is present, whereas in the remaining two (complete spermatogenesis arrest and complete SCOS) sperm is absent.

Statistical analysis

Data are expressed as median (IQR). Kruskal-Wallis and Friedman tests were used for the comparison of numerical parameters and the Chi-square test for the comparison of nominal parameters. Mann-Whitney U was used as the *post hoc* test. Receiver Operative Characteristics (ROC) curves were determined for FSH and Inh-B using FNA as the reference method. Logistic Regression Analysis was performed with sperm retrieval as the dependent parameter and FSH, Inh-B and volume of the larger testis as independent parameters. A p value of less than 0.05 was considered statistically significant for all tests. Statistical analysis was performed with SPSS® 15 for Windows (SPSS Inc., USA). Comparison of ROC curves was performed with MedCalc® 9.2 (MedCalc Software, Belgium).

RESULTS

Diagnostic evaluation

According to the clinical, hormonal, sperm, imaging, testicular cytology and genetic results, an etiological classification of the 51 men with azoospermia was accomplished. The majority of the subfertile men were diagnosed as having Idiopathic Non-Obstructive Azoospermia (INOA - n=34, 67%), a clinical entity characterized by absence of any known cause of subfertility and azoospermia. Four men (8%) were diagnosed as having cryptorchidism, three (6%) varicocele [bilateral (n=2), left-sided (n=1)/grade II (n=2), grade III (n=1)] and 10 (19%) other diagnoses: post-infection obstructive azoospermia (n=3), Klinefelter syndrome (n=3), Kallmann syndrome (n=1), 46,XX male syndrome (n=1), Guillain-Barré syndrome (n=1) and sickle cell anemia (n=1).

Sub-study "FNA"

FNA diagnoses in the 51 men with azoospermia

were designated as normal spermatogenesis (n=1), hypospermatogenesis (n=16), incomplete spermatogenesis arrest (n=3), complete spermatogenesis arrest (n=11), incomplete SCOS (n=1), complete SCOS (n=13) and insufficient material (non-diagnostic result, n=6). Thus, sperm extraction was successful in 21 and unsuccessful in 24 cases (non-diagnostic results were excluded from the analysis). FNA diagnoses of men with INOA, cryptorchidism and varicocele are illustrated in Table 1.

There was marginal statistical difference in FSH levels among different FNA diagnoses (Kruskal-Wallis, p=0.055, Figure 1a). On the other hand, there was no significant difference in Inh-B levels among different FNA diagnoses (p=0.108, Figure 1b).

ROC curves were constructed using FNA as the reference method (presence or absence of sperm). The reverse FSH (1/FSH) had an Area Under Curve (AUC) of 0.716 [95% Confidence Interval (CI) 0.556 – 0.844], with a threshold of 16 mU/mL having 74% sensitivity and 74% specificity for sperm retrieval (Figure 2). Similar results were obtained for Inh-B (AUC: 0.610, 95% CI 0.445 – 0.758, threshold: 14.5 pg/mL, sensitivity: 100%, specificity: 29%). In pairwise comparison of ROC curves, no hormone was proved to be superior to the other. No better sperm retrieval predictions were obtained with their combination (Inh-B/FSH - AUC: 0.644, 95% CI 0.473 – 0.792, threshold: 2.1, sensitivity: 61%, specificity: 70%).

Table 1. Fine Needle Aspiration (FNA) diagnosis in men with azoospermia of different etiology.

FNA diagnosis	INOA	Cryptorchidism	Varicocele
n	34	4	3
Normal spermatogenesis	0	0	0
Hypospermatogenesis	11	1	2
Maturation arrest, incomplete	2	1	0
Maturation arrest, complete	7	0	0
SCOS, incomplete	0	0	0
SCOS, complete	10	1	0
Non-diagnostic	4	1	1

SCOS: Sertoli Cell-Only Syndrome, INOA: Idiopathic-Non-Obstructive Azoospermia.

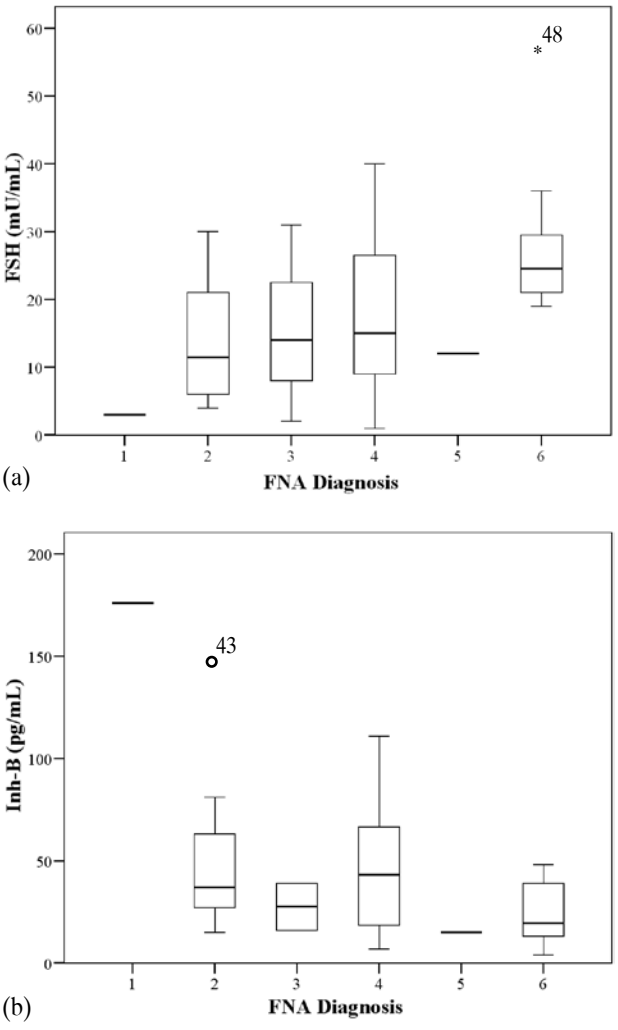

Figure 1. Box-plot graphs of FSH (a) and basal Inh-B (b) versus FNA diagnosis 1: normal spermatogenesis, 2: hypospermatogenesis, 3: spermatogenesis arrest, incomplete, 4: spermatogenesis arrest, complete, 5: SCOS, incomplete, 6: SCOS, complete. Case numbers (48, 43) represent outliers.

In a logistic regression analysis (enter method), neither FSH nor Inh-B could predict presence of sperm in FNA (overall model fit p = 0.07). Specifically, FSH had an odds ratio (OR) for presence of sperm of 0.930 (95% CI 0.861 – 1.004) and Inh-B 0.998 (0.976 – 1.021).

Sub-study "EFSERT"

There was significant difference between men with azoospermia (n=51) and controls (n=31) with regard to the basal Inh-B levels [median (IQR) 37.2 (36) vs. 103.0 (90) pg/mL, respectively, p = 0.003]. Main clinical and laboratory parameters of the control men

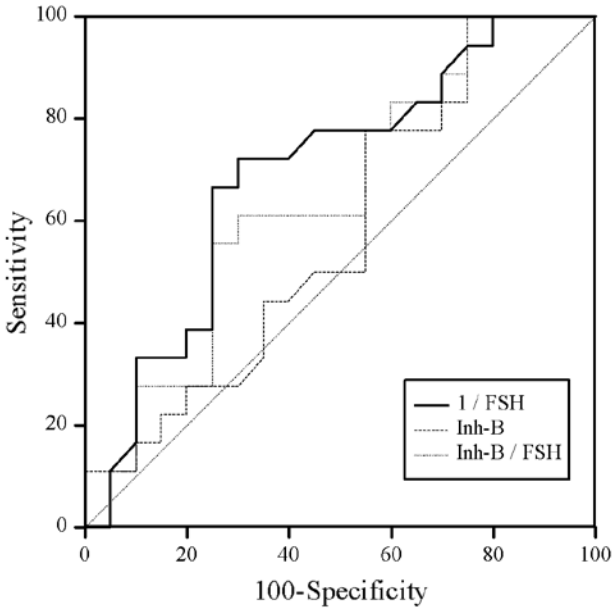

Figure 2. Receiver Operating Characteristic curves of basal inhibin-B, 1/FSH and basal inhibin-B/FSH ratio for sperm retrieval in testicular FNA.

as well as subfertile men with INOA, cryptorchidism and varicocele are illustrated in Table 2.

On the other hand, there were no significant changes between men with azoospermia (n=51) and controls (n=31) with regard to the stimulated Inh-B levels [40.5 (41) vs. 73.0 (44) pg/mL, $p = 0.113$ at 24 h and 34.3 (34) vs. 82.0 (50) pg/mL, $p = 0.098$ at 48 h)]. Stimulated Inh-B levels of the control men as well as of subfertile men with INOA, cryptorchidism and varicocele are illustrated in Table 3.

DISCUSSION

Sertoli cells support spermatogenic activity through multiple paracrine mechanisms, including Inh-B secretion.[20] There is evidence that Inh-B is undetectable in men with SCOS, despite normal testosterone levels,[21] suggesting Sertoli cell damage. Thus, Inh-B is considered as a direct marker of Sertoli cell function and an indirect marker of the spermatogenic status.

Table 2. Main clinical and laboratory parameters in men with azoospermia of different etiology and in controls.

	Controls	INOA	Cryptorchidism	Varicocele	*p* value
n	31	34	4	3	
Left testis volume (mL)	25 (4)	13 (11) [a]	12 (3) [a]	19 (N/A)	<0.001
Right testis volume (mL)	25 (4)	12 (11) [a]	11 (9) [a]	19 (N/A)	<0.001
LH (mU/mL)	3.0 (2)	7.2 (4) [a]	9.5 (15) [a]	4.9 (N/A)	<0.001
FSH (mU/mL)	2.8 (3)	18.7 (15) [a]	25.4 (29) [a]	7.9 (N/A)	<0.001
Prolactin (ng/mL)	7.6 (3)	9.0 (6)	11.4 (5)	20.5 (N/A) [a, b]	0.020
Total testosterone (ng/dL)	592 (440)	371 (197)	418 (258)	388 (N/A)	0.258
Basal inhibin-B (pg/mL)	103.0 (90)	32.9 (37) [a]	24.4 (14) [a]	36.2 (N/A) [a]	<0.001
Sperm volume (mL)	4.1 (1.7)	3.5 (2.5)	3.3 (2.3)	1.5 (N/A)	0.260

INOA: Idiopathic-Non-Obstructive Azoospermia, N/A: Non-Applicable. Data are given as median (IQR). *p* value refers to comparison among all groups (Kruskal-Wallis test), [a] $p < 0.05$ vs. controls, [b] $p < 0.05$ vs. INOA (Mann-Whitney U test). In order to convert total testosterone from ng/dL to nmol/L multiply by 0.0347. In order to convert prolactin from ng/mL to pmol/L multiply by 43.478.

Table 3. Basal and stimulated inhibin-B in men with azoospermia of different etiology and in controls.

	Controls	INOA	Cryptorchidism	Varicocele	*p* value
n	31	34	4	3	
Inhibin-B (pg/mL) at 0 h	103.0 (90)	32.9 (37) [a]	24.4 (14) [a]	36.2 (-) [a]	0.003
Inhibin-B (pg/mL) at 24 h	73.0 (44)	40.4 (42)	25.1 (22)	96.2 (-)	0.154
Inhibin-B (pg/mL) at 48 h	82.0 (50)	31.2 (22)	31.5 (33)	88.0 (-)	0.127
p value	0.102	0.545	0.417	0.307	

INOA: Idiopathic-Non-Obstructive Azoospermia. Data are given as median (IQR). p value refers to comparison among all groups (Kruskal-Wallis test) or within the same group (Friedman test). [a] $p < 0.05$ vs. controls (Mann-Whitney U test).

We conducted a prospective study with the primary aim of comparing Inh-B with FSH as predictors of the recovery of sperm in FNA performed in men with azoospermia. A secondary aim was to investigate the clinical value of basal and stimulated serum Inh-B levels as a marker of Sertoli cell function.

In comparing ROC curves, neither FSH nor Inh-B was proved to be superior to the other as predictor of the recovery of sperm in testicular fine needle aspirate. These results were further supported by the logistic regression analysis, which also failed to provide a model for the prediction of sperm recovery.

In recent studies, attempts have been made to compare serum FSH and Inh-B levels as predictors of the recovery of sperm from testicular biopsies (Table 4): Inh-B proved to be superior[2,8,9,13,14] or equal[10,12,15,16,18] to FSH, while in a couple of studies[11,17] combination proved to be better than any hormone alone. The apparent discrepancy among these studies could be attributed to methodological causes, such as selection criteria for the infertile men, small group sizes,[9,12] lack of a control group[10,11,14-18] and use of different reference methods.[10,12] Compared to the published evidence, the present study has two novel characteristics: i) it includes not only subfertile men with non-obstructive azoospermia but also men with specific clinical diagnoses (i.e. idiopathic, cryptorchidism, varicocele) and ii) the study uses exclusively FNA and not TESE as the reference method.

Many investigators prefer to perform TESE instead of FNA in the evaluation of men with non-obstructive azoospermia (Table 4). One of the main reasons for this preference is the ability of TESE to identify cases of Intra-Tubular Germ Cell Neoplasia (ITGCN – former nomenclature: Carcinoma In-Situ), a condition that is diagnosed with increased frequency in subfertile men.[22] Nevertheless, we consider that testicular FNA biopsy constitutes an important part of the diagnostic and therapeutic approach for subfertile men. Compared to the traditional open testicular biopsy, FNA has significant advantages as it is a fast, low-cost, less traumatic and minimally invasive technique.[23] Furthermore, apart from the cytological diagnosis, FNA makes it possible to collect sperm that can be subsequently used in ICSI procedures, mainly in men with obstructive azoospermia. In general, there are data suggesting that testicular FNA, although inferior to TESE as far as sperm retrieval is concerned,[7,24]

Table 4. Studies in the literature evaluating serum inhibin-B and FSH plasma levels as predictors of the presence of testicular spermatozoa in men with non-obstructive azoospermia.

Study	Reference no.	NOA group (n)	Control group	Reference method	Conclusion
1. Von Eckardstein et al, 1999	17	52	none	TESE	Combination is better than any one hormone alone
2. Ballesca et al, 2000	9	17	51 (22 OA)	TESE	Inh-B is superior to FSH
3. Brugo-Olmedo et al, 2001	2	78	25 (15 OA)	TESE	Inh-B is superior to FSH
4. Vernaeve et al, 2002	18	185	none	TESE	No difference
5. Bohring et al, 2002	11	52	none	TESE	Combination is better than any one hormone alone
6. Bailly et al, 2003	8	75	81 OA	TESE	Inh-B is superior to FSH
7. Tsujimura et al, 2004	15	100	none	TESE	No difference
8. Nagata et al, 2005	14	62	none	TESE	Inh-B is superior to FSH
9. Halder et al, 2005	12	13	5	TESE or FNA	No difference
10. Bettella et al, 2005	10	125	none	TESE and FNA	No difference
11. Liu et al, 2006	13	40	30 (20 OA)	TESE	Inh-B is superior to FSH
12. Tunc et al, 2006	16	66	none	TESE	No difference
13. Present study	-	51	31	FNA	No difference

NOA: Non-Obstructive Azoospermia, OA: Obstructive Azoospermia, TESE: Testicular Sperm Extraction, FNA: Fine Needle Aspiration.

has excellent correlation with it[10,25-28] as well as very good reproducibility: 70% positive and 89% negative predictive value of the first FNA for sperm recovery at a subsequent attempt.[29]

In the present study, we demonstrated significantly higher FSH and lower Inh-B levels in men with azoospermia as compared to controls; in any case, the wide overlapping of values prevents Inh-B from being a useful marker of spermatogenesis. The stimulated Inh-B levels, as estimated by EFSERT, failed to provide additional information for the diagnostic approach of the subfertile men, as the responses were flat in all study groups. These results differ from those of previous studies,[30] in which a higher response of serum Inh-B has been demonstrated in controls as well as in men with mild testicular damage as compared to men with severe testicular damage. A possible explanation for this discrepancy could be the use of a higher dose of exogenous FSH in the other studies.[31]

In conclusion, the present study provides evidence that serum Inh-B is not superior to FSH as predictor of the presence of sperm in testicular fine needle aspirate in men with azoospermia. Thus, given its cost and methodological limitation, Inh-B is not recommended for this purpose. Although basal serum Inh-B levels reflect Sertoli cell function, stimulated levels, as estimated by EFSERT, do not provide additional information. More studies are needed to establish the exact role of Inh-B determination, especially in conjunction with other important molecules of the testicular micro-environment, such as anti-Müllerian hormone, in the evaluation of infertile men.

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