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ORIGINAL ARTICLE

Serum Inhibin B: A Direct And Precise Marker Of Ovarian Function

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ABSTRACT

Background Inhibin B is a glycoprotein hormone produced mainly by granulosa cells of the ovary in early folliculogenesis. It selectively suppresses the secretion of pituitary FSH and has local paracrine actions in the gonads. Its measurement is useful for investigating female reproductive dysfunction.
Objective The objective of this study was to examine serum levels of inhibin B in the assessment of ovarian function in patient with premature ovarian failure.
Material & Method Serum from premature ovarian failure (n=34; group A), menopause (n=8; group B) and normally cycling fertile women (n=5; group C) was prospectively collected and stored at –80°C. Serum concentration of inhibin B was measured using specific solid phase sandwich ELISA. FSH level was measured using microparticle enzyme immuno assay (MEIA) for comparison. Independent sample t test was used to see the mean significance differences between groups.
Results Inhibin B level was undetectable (i.e., <15pg/ml) in group A & B women. The mean value in group C women was 51.8pg/ml (range 26-75). Respective values of FSH were 78.8miu/ml (range 25-150), 100.7miu/ml (range 62-150) & 5.96miu/ml (range 4.2-7.9). Inhibin B level was significantly lower in group A & B than group C women ($p<0.0001$) whereas differences were insignificant between group A and B women. Similarly FSH level was significantly higher in group A & B than group C women ($p<0.0001$). We found wide variation in FSH level in group A women. In 5 women FSH level was below 40miu/ml and was related to exogenous estrogen intake more than 3 months of blood sampling.
Conclusion This study demonstrated that inhibin B is a better predictor for ovarian failure than FSH and uninfluenced by exogenous estrogen intake (if taken >3 months before).
Key Words Premature Ovarian Failure; Inhibin B & FSH
Key Messages Inhibin B is a better predictor for ovarian function than FSH

Introduction

Inhibin B is a heterodimeric glycoprotein consisting one α-subunit and one βB subunit [1]. Inhibin B expression and secretion in women is positively correlated with granulosa cell function, oocyte number & oogenesis [2],[3] and negatively correlated with FSH [4],[5],[6]. It is regarded as a serum marker of oogenesis and may offer an improved diagnosis of ovarian function [7],[8].[9]. Human FSH is a glycoprotein and consist of α and β subunits. In the workup of female infertility, FSH is the classical endocrine parameter to discriminate

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between ovarian impairment and other disorders. Several studies confirm that FSH level is a valuable marker of oogenesis [10],[11],[12],[13],[14]. The current study is designed to examine possible role of inhibin B as a predictor of ovarian reserve in comparison with FSH.

Material & Methods

Subjects

The cases were selected from Department of Reproductive Biology, AIIMS between 2002 & 2003 while they were referred by various hospitals including our own for reproductive hormone analysis for infertility, menstrual irregularity or secondary amenorrhoea. Thirty four premature ovarian failure (POF) women (vide [Table/Fig 1] for diagnostic criteria) were selected for the study from 214 women referred. Suspected POF cases were interviewed in detail for medical, surgical & treatment (particularly exogenous estrogen & cytotoxic chemotherapy) history and examined clinically (general & systemic) to exclude any gross underlying disease as per proforma. Most of the cases were investigated extensively outside (including ultrasonography, reproductive hormones, haemogram, biochemistry, TSH, ANA, etc) before attending our department. All related information was obtained in detail. Cases were selected as per criteria given in [Table/Fig 1]

No one had history of autoimmune disease, surgical removal of gonads, radiotherapy or chemotherapy. All the cases were followed up for at least one year. Five known normally cycling fertile women (group C) and eight menopausal women (group B) were included for comparison. Blood sampling from normally cycling fertile women was collected on day 2 or 3 of menstrual cycle. Postmenopausal women were selected from another ongoing project of the department. None of the cases had exogenous estrogen intake for 3 months before blood sampling although most were treated by estrogen in past (more than 3 months ago). Informed consent was obtained before blood sampling. All blood sampling were in fasting state. Serum was isolated after centrifugation and stored at -80^{0}C for 1 to 10 months before inhibin B & FSH assay. Hemolyzed and bilirubin containing samples were discarded from the study.

Table/Fig 1

Parameters	*Selection Criteria*
Premature Ovarian Failure (Group A)	Age <40 years; Amenorrhoea ≥6 months; No H/o any medical problem. No H/o any exogenous estrogen intake in preceding 3 months. No H/o any chemotherapy. Ultrasonography (no follicular activity), TSH (normal), Blood Sugar (normal), ANA (normal), etc. One serum FSH (previous) ≥40 miu/ml
Menopausal (Group B)	Age ≥45 years; Amenorrhoea ≥6 months; No H/o any medical problem. No H/o Hormone Replacement Therapy. Two serum FSH (one/more months apart) ≥40 miu/ml
Normal Women (Group A)	Proven Fertility with normal menstrual cycle and ≤35 years of age. No H/o any medical problem. No H/o any exogenous estrogen intake including hormonal contraceptives.

Showing selection criteria of three groups of women included in the study

Assays

Serum concentration of FSH was measured using highly specific microparticle enzyme immuno assay (MEIA) using AxSYM automated immunoassay system (Abbott Laboratories, USA). Inter and intra-

assay coefficients of variation was <3%, cross reactivity with TSH, LH & hCG was <1% and detection limit was <0.4 mIU/ml.

Serum concentration of Inhibin B was measured using a commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) specific for the dimeric inhibin-B (Oxford Bio-Innovation Ltd. Oxford, UK via Serotec) [15],[16]. The first antibody is directed to the βB-subunit and the second antibody to the α-subunit and conjugated to alkaline phosphatase. The assay had a cross-reactivity of 0.1% with activin and ~1% with inhibin A. Assay sensitivity/detection limit was 15 pg/ml and the inter/intra-plate coefficients of variation was <7%. Before ELISA samples were pretreated with detergent (SDS), heated to 100ºC and exposed to hydrogen peroxide to enhance sensitivity as well as specificity. Control and known standard (1000, 500, 250, 125, 62.5, 31.25 & 15.6) were used for the study. Patients with inhibin B concentrations below detection limit i.e., 15 pg were assigned as undetectable. All the samples were tested in duplicate.

Microtitre plates were pre-coated with a monoclonal antibody to the beta-B subunit of inhibin. Samples (including control & standard) were incubated in the wells so that the antigen binds to the immobilized antibody via its βB subunit. Following washing a detection antibody was added. This was a monoclonal antibody specific for α subunit of inhibin coupled to alkaline phosphatase. Any unreacted material is then removed by washing before detection of alkaline phosphatase using a sensitive amplified substrate. This resulted in a red reaction product with color intensity proportionate to the concentration of dimeric inhibin B present in sample. At the end of procedure absorbance was read at 490nm wave length after subtracting mean zero standard absorbance using an ELISA reader (Molecular Devices). The absorbance values were plotted in graph paper and results interpreted.

FISH Analysis

Interphase FISH with X centromeric probe was carried out from 0.5 ml EDTA blood in all POF cases as per protocol [17].

Statistics

Data were analyzed by using SPSS statistical software. Descriptive statistics are given as the mean ± SD. Independent sample *t* test was used to see the mean significance differences between groups.

Results

The mean (±SD) age and plasma levels of FSH & inhibin B of group A, B & C are shown in [Table/Fig 2]. The age difference was not significant statistically (p = 0.95) between group A & C. Plasma FSH level was elevated in group A & B in contrast to group C women (P < 0.0001). Women in group A & B had undetectable (≤15 pg/ml) level of serum inhibin B whereas the mean level in group C was 51.8pg/ml. The difference was statistically significant (p <0.0001). Level of inhibin B in group A was no different than in group B.

Plasma FSH of group A/B and group C was inversely correlated with plasma inhibin B. There was not a single case with normal FSH/inhibin B in group A & B however there were five case in group A with non-menopausal (<40miu/ml) FSH. All had history of exogenous estrogen intake preceding 3 months of blood sampling [Table/Fig 3].

Interphase FISH with chromosome X centromeric probe was carried out in all cases of POF (group A). All excepting 2 were disomic for chromosome X (normal). Two cases of chromosome X mosaicism (XX/XXX) were detected however, frequency of trisomic (XXX) cell line was below 10%.

Discussion

In current society, the desire of women to reproduce in later years leads to an increase incidence of infertility. Infertility workup and treatment is frequently time consuming, expensive and unsuccessful leading to economical & psychosocial difficulties. Hence, evaluation of ovarian status to identify women who has a chance of becoming pregnant before initiating expensive treatment becomes more important for proper prediction & counseling. This may decrease anxiety as well as marital disharmony.

Ovarian status can be screened using various tests. FSH, estradiol, ovarian volume, antral follicle count, ovarian biopsy, clomiphene citrate challenge test or gonadotropin analogue stimulation test are commonly used. The clomiphene citrate challenge test is widely accepted method of testing ovarian reserve [18],[19],[20],[21], however, it requires few days to perform and multiple blood sampling. The gonadotropins analogue stimulation test is based on concentrations of FSH, estradiol and LH before and after GnRH analogue administration [22]. Its limitations are expense, need for injections and repeated blood samplings in addition to limited ability to differentiate normal from reduced ovarian

reserve [23]. Hence there is a need for alternative simple test. Simple tests for ovarian reserve are ultrasonography [10],[24],[25] and FSH [11]. However, they have their diagnostic limitations as ovulation may be seen in premature ovarian failure despite high FSH [4] and subjectiveness of ovarian ultrasonography. Ovarian biopsy [26],[27] although reliable is invasive procedure.

Table/fig 2

Parameters	*Age (years) Mean ±SD (SEM & range)*	*FSH(miu/ml) Mean ±SD (SEM & range)*	*Inhibin B (pg/ml) Mean ±SD (SEM & range)*
POF	31.65 ± 5.1	78.8 ± 37.2	1.4 ± 3.7
(Group A)	(0.87 & 22-39)	(6.4 & 25-150)	(0.6 & 0-15)
Menopause (Group B)	47.0 ± 1.3	100.7 ± 33.5	0.5 ± 0.9
	(0.46 & 45-49)	(11.8 & 62-150)	0.3 & 0-2
Normally Cycling Fertile (Group	31.8 ± 4.08	5.96 ± 1.4	51.8 ± 20.5
C)	(1.8 & 26-35)	(0.6 & 4.2-7.9)	(9.2 & 26-75)
Significance	p<0.0001 (A/B)	P=0.13 (NS; A/B)	P= 0.48 (NS; A/B)
	p=0.95 (NS; A/C)	P<0.0001 (A/C)	P<0.0001 (A/C)
	p<0.0001 (B/C)	P<0.0001 (B/C)	P<0.0001 (B/C)

Normal Value of FSH in women in our laboratory on day 2/3 menstrual cycle is <12; NS = not significant

Serum inhibin B & FSH levels in premature ovarian failure, menopausal and normally cycling fertile women

Table/fig 3

Parameters	*FSH Menopausal (≥40)*	*FSH High (13-39)*	*FSH Normal (≤12)*	*Total*
Inhibin B Menopausal (≤15)	29	5	0	34
Inhibin B Low (16-25)	0	0	0	0
Inhibin B Normal (>25)	0	0	0	0*
Total	29	5†	0*	34

*No case of POF with normal FSH & inhibin B

†5 cases of POF with non menopausal FSH

Note most cases (29/34) including 5 with ≤40miu/ml FSH had h/o irregular exogenous estrogen intake 3 months before blood sampling

Inhibin B and FSH values in premature ovarian failure (n=34) cases

Hence there is a clear need for noninvasive, direct and precise marker of ovarian reserve. The identification of a parameter that can discriminate between complete absence of germ cells in the ovary and less severe disturbances of ova production would be of considerable prognostic value for assessment of female infertility. Inhibin B & AMH, which are direct product of the granulosa cells, may be more accurate in this regard. Both AMH and inhibin B are produced by the granulosa cells of preantral and small antral follicles of ovary during early folliculogenesis. Since the number of ovarian follicles declines with increasing age, AMH & inhibin B levels may be used as a direct & precise marker for ovarian ageing. Reports claim that inhibin B [2],[7],[8],[12],[28],[29],[30] and antimullerian hormone [10],[31],[32] are better markers of ovarian function.

This study was aimed to find out role of Inhibin B in assessment of gonadal function in premature ovarian failure women and compared with FSH. This cohort of patient was after exclusion of those suffering from overt/diagnosed medical disease or on medication. None of POF cases resumed spontaneous menstruation or conceived in one year (some up to 2 years) follow-up.

The FSH rise in older women has been well documented [33],[34] and commonly utilized test for ovarian reserve. FSH level of ≥40miu/ml in two occasions at interval of 1 or more month/s is indicative of ovarian failure [35]. Serum FSH levels was high in all POF & menopausal women in our study however there were 5 cases of POF with below menopausal (40miu/ml) FSH. Previous studies demonstrated that POF women with amenorrhea for less than 3 months and lower FSH level (although they remain elevated compared with regularly cycling women) are more likely to ovulate than women with a longer period of amenorrhea. Nevertheless, these did not translate into a better prognosis for pregnancy [4],[36]. A significant limitation of the FSH assay derives from exogenous estrogen intake. The fluctuation in FSH level is commonly seen with exogenous estrogen intake which is frequent. Ovarian failure following chemotherapy or radiotherapy may be reversible [37] and may cause fluctuations in FSH level as well as resumption of ovulation. To overcome this effect we have selected group of patient who had amenorrhoea for more than 6 months, who did not take exogenous estrogen at least for last three months before blood sampling and who did not have any history of chemotherapy/radiotherapy. In-spite of all we found 5 cases of non menopausal FSH and seems apparently due to previous exogenous estrogen intake. Furthermore as FSH is an indirect marker, rise takes long time and mildly raised FSH may be seen in women with reduced ovarian reserve Recent years have witnessed inhibin B as a predictor of ovarian reserve. It is produced mainly by granulosa cells. It is undetectable in menopausal women [38],[39]. Approximately 40% of perimenopausal women starting 2 years before final menstruation [40] have undetectable inhibin B. We found undetectable (≤15 pg/ml) inhibin B in all POF cases as well as in all menopausal women. Its level did not influenced by exogenous estrogen if taken 3 months prior to test. Thus inhibin B might offer an enhanced tool for distinguishing women with premature ovarian failure. Although we did not find any case of POF with normal FSH & inhibin B and both are good markers for oogenesis however we believe that inhibin B is superior as it was not influenced by previous exogenous estrogen intake which is very often with POF cases.

We investigated all POF cases for chromosome X by interphase FISH and found 2 cases of mosaic trisomy X. Significance of low level (<10%) trisomy X mosaicism as a cause relationship warrants for more study with larger number of patients.

We conclude that undetectable inhibin B and high FSH constitute markers of ovarian failure and measurements can be a useful non-invasive one step tool for management as well as counseling of POF women who are seeking infertility treatment.

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Conflict of Interest: None declared

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