Bulletin of Institute of Reproductive Medicine

IRM | Vol. 71 | July 2017

Contents

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PRINTED BY	Phildon 3 Dr Suresh Sarkar Road, Kolkata 700014 Email: phildon10@gmail.com

Institute of Reproductive Medicine

Anti Mullerian Hormone - importance in clinical practice

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Introduction

Anti-mullerian hormone (AMH) has recently been recognized as one of the most dependable biomarkers of ovarian reserve and more importantly of ovarian response to exogenous stimulation with gonadotropin. Even 15 years ago, AMH was considered primarily as a mullerian inhibitory substance (MIS) and its function was mainly concerned with mullerian regression and sexual differentiation in males only. But recently its role in controlling and prediction of ovarian function in women's reproductive period is gaining interest very fast. It is secreted in the female as a protein hormone by *small pre-antral, large pre-antral and small antral follicles in the ovaries*. Apart from predicting ovarian reserve and ovarian responsiveness to stimulation, serum AMH values are being utilized for the diagnosis and pathogenesis of PCOs. Association between AMH and obesity has also been described. The level of serum AMH has also been utilized for recognition and diagnosis of granulosa cell tumour in females. In male, it has a specific indication for recognition of clinical situation of male hypogonadism in the pre-pubertal period.

Objective of writing this chapter will be primarily to discuss in short the basic physiology of AMH in male and female individuals and finally to review the current knowledge about clinical utility of evaluation of AMH with special reference to pathophysiology of female reproduction.

Background of knowledge and study of anti-mullerian hormone:

A. Jost in 1940 first reported that a substance (hormone), other than testosterone, was found in testis which was responsible for regression of mullerian duct in male child.[1] The substance was a protein and was named as mullerian inhibiting substance (MIS). This substance was isolated and characterized 40 years later and the substance was found to be a protein belonging to the family of transforming growth factor-beta (TGF-β) with its gene located on short arm of chromosome 19. In males, AMH is produced by *sertoli cells of testis* since 8th week of intra-uterine life and synthesis and release of AMH continues throughout whole life. In females, AMH is produced by granulosa cell of *small pre-antral, large pre-antral, small antral follicles* from 36th week of intra-uterine life. Throughout reproductive years in females, production and serum levels of AMH will depend on activity and health of the available follicles present in the ovaries. This is not dependent on any feed-back mechanism of hypothalamic pituitary axis. This is the reason why AMH has been considered as a dependable biomarker of ovarian reserve (for details see subsequent part of this chapter).

Source, pattern of production and clinical utility of assessment of AMH in male:

AMH is secreted from sertoli cell of testis from 8th week of gestation and remains at a higher level both in testis as well as in serum until puberty.[2] In males AMH secretion is under control of FSH, and is suppressed by testosterone. In feotal and early neonatal period, androgen receptors are still not expressed in appropriate amount, therefore the suppression effect of testosterone on AMH does not appear. On the other hand, FSH through its receptors on the membrane of sertoli cells stimulates AMH expression.3 Serum levels of AMH are relatively high at this stage and remains high upto 8th year of age (late childhood or early puberty).

With onset of puberty, the effect of LH is much more pronounced due to inhibitory effect of inhibin-B on FSH secretion. Leydig cells become more mature and active which dramatically increases testosterone secretion. Increased testosterone induces maturation of sertoli cells. Through inhibitory effect of testosterone over FSH stimulation, AMH expression from sertoli cell is down regulated and consequently serum levels of AMH decline. The level of serum AMH reaches adult value which is maintained throughout

life.[3,4] The role of AMH in adult men is not very clear. *But estimation of anti-mullerian hormone in childhood as a marker of adult hypogonadism* has a tremendous clinical significance. It is known that testicular AMH production increases in response to FSH stimulation and is totally inhibited by androgen. Therefore low AMH level in late childhood is typical of precocious puberty whereas high AMH level at the age of physiological puberty indicate its delay (delayed puberty). Therefore, assessment of AMH values at this age in male may help in accurate diagnosis of onset and duration of puberty.[4]

Physiology of AMH in females:

It has already been stated that AMH is produced by granulosa cells of foetal ovary from 36th week of intra-uterine gestation. The production and presence of AMH in follicular fluid and serum continue till menopause. In women, serum AMH concentration in pre-pubertal girl is low, but the level is elevated in pre-pubertal daughters of women with polycystic ovary syndrome.[5] Concentration of AMH is maximum in small pre-antral, large pre-antral and small antral follicles. AMH is undetectable in primordial and large antral follicles.

Absence of AMH in larger follicles suggest that this is an important prerequisite for selection of dominant follicle. Also AMH is ***not expressed in the oocytes, the corpus luteum and the atretic follicles or the theca cells. In other words granulosa cells of the smaller follicles are the only sources for production of AMH.*** To understand the function of AMH in the process of folliculogenesis and ovulation it is essential to recollect the basic physiology of the process of folliculogenesis. This is detailed below –

Anti Mullerian Hormone (AMH) – influence on follicular recruitment, follicular preservation and mono-follicular development:

AMH is expressed by granulosa cells of the ovary during the reproductive years. It has a role in folliculogenesis and follicular preservation.[6] Unlike E2, it is not controlled by gonadotropin. AMH has got antagonistic and regulating effect on FSH, E2 and aromatase in follicular micro-environment. AMH performs two functions: (a) controls formation of primary follicles and their preservation by inhibiting unnecessary and excessive recruitment of follicles at two stages of migration (from primordial to small and large pre-antral, and from small to large antral follicle) (b) AMH also indirectly helps in monofollicular development. Monofollicular development is achieved by decreasing responsiveness of gonadotropin sensitive follicles to FSH so that instead of many follicles developing simultaneously, only one may reach the stage of dominant follicle because all pre-antral and small-antral follicles produce AMH (see Fig-1). As they reach large-antral stage, AMH, through FSH inhibition in each follicle, reduces their further growth. But during the last 20 days of 80 day cycle, when some of the follicles become FSH sensitive, the production of AMH is suppressed by rising level of FSH (direct Antagonistic effect). Still AMH does not allow many follicles to grow rapidly and prevents them from becoming dominant.

But one large follicle has more dominant FSH receptors. As it grows it produces less AMH enabling this follicle to grow faster. Rest of the follicles cannot grow because of weak follicle FSH receptors and dominant AMH control, thereby resulting in mono-follicular development. Hence AMH helps indirectly in mono-follicular ovulation.

Estimation of serum AMH (female, 25-40 yrs – 0.90 – 3.0ng/ml – normal range) level on any day of menstrual cycle will indicate ovarian stock of follicles ('reserve') which have not yet been recruited in the gonadotropin sensitive pool. There is a wide variation in reported ranges of normal values of AMH in different publications. This is because the procedure of AMH estimation is yet to be standardized. A small report on future thought of standardization of AMH values has been included in last paragraph of this chapter. Though there is a wide difference between upper and lower limits of normal range of AMH, for all practical purposes, the lower normal limit has been accepted as 1ng/ml. Granulosa cells of polycystic ovaries produce more AMH than granulosa cells of non polycystic ovaries. Higher values of AMH can be used as 'markers' for diagnosis of PCOS.

In summary, the specific functions of AMH during follicular growth and ovulation are –

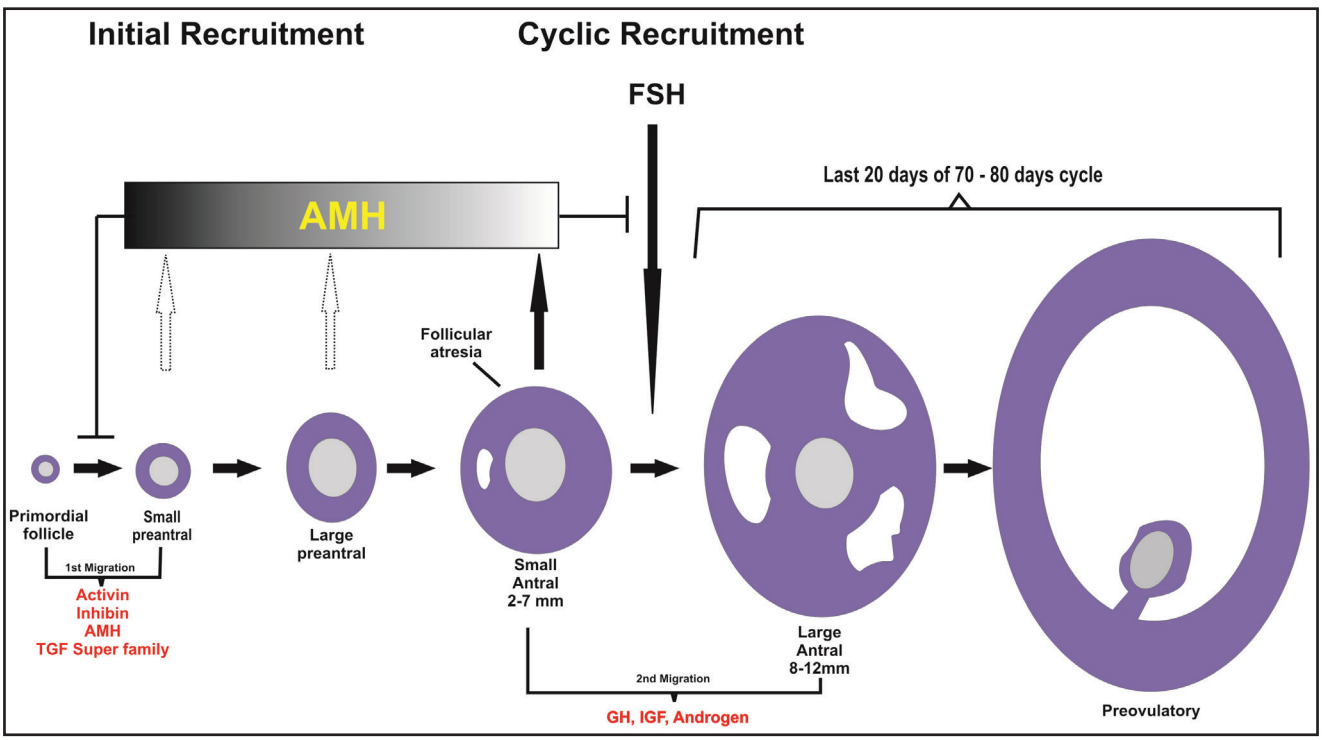
a) Follicular preservation or prevention of unnecessary wastage of follicles
b) Restricting too many follicles to become FSH sensitive
c) Restricting selection of more than one follicle

to become 'dominant' (preventing the risk of multiple pregnancy)

Clinical significance of AMH assessment in females:

A) ***As a marker of ovarian reserve:*** AMH has now been accepted as the most dependable 'marker' of ovarian reserve, - better than the conventional markers like FSH, E2 and Inhibin-B. In conjunction with AFC (antral follicle count) AMH currently has become the most reliable marker of prediction for ovarian reserve. AMH is considered to be superior marker than others because : -

- AMH is exclusively produced by granulosa cells of small antral and pre-antral follicles, and it is the only marker which remains stable throughout the menstrual cycle[7]
- Though some observers[8] noted variation of AMH level in follicular phase in young ovaries, but from practical consideration such fluctuations are of little clinical significance
- Level of AMH precisely reflects the number of pre-antral follicles and therefore oocyte pool in the ovaries, - which pre-determines the germinal reserve of the ovaries, - essential for reproduction
- Plasma level of AMH also correlates tightly with a number of mature follicles (AFC equal to antral follicle count) as assessed by trans-vaginal sonography and also with AMH concentration measured in the follicular fluid. That is, plasma level of AMH and follicular fluid level AMH are similar
- Moreover, during reproductive years of women, plasma level of AMH is a better marker of ovarian reserve, compared to basal FSH, LH and Inhibin-B, because AMH is generated in a 'paracrine' manner and is not dependent on 'feed-back' mechanism of hypothalamic pituitary gonadal axis
- Therefore AMH can be measured on any day of menstrual cycle, and usually a single measurement is sufficient; hence this investigation is more economical
- This is because AMH level is not influenced by endogenous gonadotropin status and reflects only the follicular population
- This is evident from the fact that after



AMH control of folliculogenesis (Fig 1)

The diagram represents stages of folliculogenesis starting from primordial follicle (resting pool) – small pre-antral, large pre-antral, small antral, large antral – preovulatory - finally ending in ovulation. Preantral (small and large) and small antral follicles produce AMH. AMH is produced by granulosa cells and production is not dependent on FSH or does not have any feedback mechanism. The function of AMH is to prevent undesirable follicular migration at two stages of follicular development. The first stage of migration occurs from primordial (resting pool) to preantral (active pool) stage every 70 to 80 days. The process continues from intra uterine life till menopause. During this period of 70 to 80 days, follicles run for maturity and finally undergo apoptosis. The number recruited in the first stage of migration will depend on the number existing in the resting pool. Ordinarily 200 to 300 follicles are recruited every 80 days from 'resting' to 'active' pool.

The second phase of migration occurs when small antral follicles pass on to the large antral follicles. At this stage some of the follicles become FSH sensitive. FSH sensitivity of follicles starts around the age of puberty and continues throughout the reproductive period. This occurs during the last 20 days of 80 days follicular maturation cycle. A cohort of follicles (30-40 in number) amongst the many follicles recruited in 80 day cycle become gonadotropin sensitive in the late luteal phase of a menstrual cycle. When gonadotropin starts rising, one of the gonadotropin sensitive follicles becomes dominant which is destined to ovulate. Others become atretic. The exact controlling factor of migration is not known. But it appears that, the first phase of migration is brought about by inhibin, activin, AMH and TGF super family. The second phase of migration is brought about by growth hormone, IGF-1 androgen etc. This is the reason for using growth hormone and androgen in poorly responding women in IVF stimulation cycles. Growth hormone and androgen may make some of the follicles gonadotropin sensitive and recruit them to become co-dominant follicles in the stimulation cycle.

AMH is produced by pre-antral (small and large)and small-antral follicles which prevent excessive migration at both the stages. AMH also helps in monofollicular development by inhibiting FSH activity in co-dominant follicle (see previous section of this chapter)

Follicles exist in the ovaries in two functional states; a) in resting pool (primordial, primary) b) active pool (pre-antral $\rightarrow \rightarrow \rightarrow$ preovulatory). After puberty, the active pool becomes sub divided into two sub groups; a) gonadotropin insensitive b) gonadotropin sensitive. The value of AMH which is estimated during child bearing period represents amount of AMH being synthesized by the follicles in the gonadotropin insensitive pool,. because follicles of gonadotropin sensitive pool are unable to synthesize adequate AMH.

a single injection of GnRH agonist, when endogenous FSH and LH levels increase, serum AMH value remains unchanged[9]

- Similarly when serum FSH level is suppressed as during pregnancy, AMH level remains unaffected[10]
- During ovarian hyperstimulation as in ART treatment cycles, serum AMH level increases not because of changes in endocrine level but because of changes in follicular dynamics. During ovarian stimulation with gonadotropin, numerous small antral follicles become large antral follicles. Consequently many small antral follicles have to be recruited in fresh batches from the pool of 'resting' or 'primordial' follicles. This additional recruitment of small antral follicles contributes to additional production of AMH which has been suggested as possible cause of elevated AMH levels in hyper-stimulated cycle
- AMH values have a higher sensitivity and specificity as a marker of ovarian reserve than other contemporary markers like FSH, E2 or Inhibin-B[11]
- Lastly, it has been demonstrated that[12] in a group of women with PCOS undergoing IVF treatment, the outcome of the procedure on the basis of AMH determination, as a percentage of successful pregnancy, could be predicted with sensitivity of 75% and specificity of 77.3%

B) ***AMH as marker of ovarian responsiveness to exogenous gonadotropin stimulation:*** Ovarian 'reserve' and ovarian 'responsiveness' are two different terminologies in reproductive medicine. Ovarian reserve denotes the number of 'small antral follicle' (small pre-antral, large pre-antral, small antral) and the 'oocyte pool' present in a woman during the reproductive years, on the day on which the reserve assessment through AMH estimation is being done. Whereas, ovarian responsiveness is defined as a 'correlation' between the number of antral follicles seen by AFC before treatment and the number of oocytes retrieved after ovarian stimulation.[9] Logistic regression analysis for prediction of poor response reveals that serum AMH levels had a better predictive value than serum FSH, E2 and Inhibin-B, - and the predictive value of AMH and AFC were almost equal. If other markers like baseline FSH, Inhibin-B and E2 are included along with AMH and AFC, the reliability of the prediction will be further enhanced.

As already stated, assessment of only serum AMH levels have several advantages. The significant advantages are, - a single assessment is enough and more importantly, in contrast to FSH, Inhibin-B and E2, level of AMH remains relatively constant during the follicular phase and also approximately during the entire menstrual cycle.[13,14]

Role of AMH in predicting ongoing pregnancy is however limited.[15] But it has been reported that D3 AMH value is high in women who are likely to be pregnant following ART treatment. Several reports indicate that AMH levels are correlated significantly with higher number of 6-celled embryos and with better embryo morphology score.[16,17,18]

C) ***AMH as marker of ovarian ageing:*** With ovarian ageing even in young women, as in premature ovarian failure (POF), the number of primordial follicles decline. AMH declines earlier compared to decline of basal FSH, E2 and Inhibin-B. The relatively early decrease of AMH level, compared to FSH, E2 and Inhibin-B (D1/D2) and follicle numbers with chronological age, has been widely accepted.[19,20] From these observations it appears that AMH values have greater sensitivity than FSH, E2 and Inhibin-B values in predicting ovarian follicular reserve

D) ***AMH estimation in polycystic ovarian syndrome (PCOS):*** In general the level of AMH increases in women with PCO or PCOS. The increased level of plasma AMH in PCOS women may be explained by the following facts –

- PCOS women have two to six fold increased number of follicles (primordial, small pre-antral, large pre-antral and small antral) possibly due to the effect of hyperandrogenimia.[21]
- This is because the follicular development is arrested at 6 to 9mm diameter before dominant follicle selection.[22] This is one of main reasons for increased level of AMH in PCOS women as AMH is produced by small antral (2-5mm in diameter) follicles only.[23,24]
- In a recent study, it was found that AMH production per granulosa cell was also increased upto 75% which was proportionately enhanced compared to controls.[25] Hence, a combination of excess follicles and aberrant follicular function was responsible for increased level of AMH in PCOS women
- Other possible causes of increased AMH in these women may be related to increased level of testosterone, androstenedione and free androgen index (FAI).[26,27] Another study attempted to establish a direct correlation between LH excess and elevated level of AMH in PCOS women
- Similarly, hyperinsulinaemia in PCOS, women through androgen excess, has been correlated with excess AMH. The observation of reduction of AMH and androgen level following treatment with Metformin without significant decrease in follicle number corroborates this hypothasis.
- Adult women with PCOS or pre-pubertal girls destined to have PCOS in future will have different levels of AMH in different periods of their lives. Normally in non-PCOS women level of AMH declines with advancing age. A similar decline is observed in women with PCOS also but *at a slower rate*. This is because ovarian ageing is delayed due to negative effect of AMH on primordial follicular recruitment (follicular preservation). High AMH levels are observed in adolescent girls aged between 12 and 18 with polycystic ovarian syndrome compared to controls.28 In addition, increased AMH levels have been detected in girls aged between 4 and 7 years born of mothers with PCOS[29]
- High level of AMH has also been observed in lean women with PCOS. This has been explained through a recent observation that '*stress*' is implicated

in the pathogenesis of anovulatory PCOS.[30]

- Women with stress are likely to have increased oxidative stress31 as well as products of oxidation and advanced glycation end products (AGEs).[32] AGEs is specific finding in lean women with PCOS. Elevated level of AMH and AGEs are commonly observed in normal weight and lean women with PCOS.[33] The concentration of AGEs and AMH is found to be higher in anovulatory PCOS women compared to normal non-PCOS women
- From these observations it has been suggested that elevated level of AGEs and AMH may contribute to the mechanism of anovulation in PCOS women

E) ***Role of AMH in assisted reproduction:*** It was during assessment of ovarian reserve for the procedure of assisted reproductive technology, serum AMH level proved to be the best prognostic marker of ovarian response to controlled ovarian stimulation, - specially when a single marker is determined.[34,35] AMH has a prognostic value for both the number of oocyte likely to be retrieved during follicular aspiration or as poor responder to ovarian stimulation in IVF cycle.[36] AMH levels can also predict women likely to have hyper-response during multi-follicular development through ovarian stimulation with human gonadotropin. High AMH level in the stimulation cycle indicates presence of large number of selectable follicles, - and hence has been suggested as a marker of increased live birth rate.[37] Other workers have indicated that *high follicular fluid AMH* rather than plasma AMH level are better markers of high pregnancy rate in IVF cycle.[38] AMH levels are also not suppressed in women who undergo repeated oocyte donation (3-6 cycles) proving that ovarian ageing is not affected in oocyte donors which was previously suspected.[39]

F) ***AMH and Obesity:*** Information is scanty about relationship between AMH and obesity. In late reproductive years (35-45 years) obese women have lower AMH level (upto 65%) compared to normal weight women of similar age. Though the correlation has not been fully explained, three reasons (hypothesis) have been suggested:[40] – (a) catabolism of AMH may be negatively affected by obesity (b) ovarian potential could be reduced by obesity (c) obesity itself may cause ovarian dysfunction. AMH in general (irrespective of age) is lower in obese and over-weight women with PCOS than normal weight women with the syndrome than the healthy controls.[41] A correlation also exists amongst LH levels, LH & AMH levels in obese and non-obese women with PCOS.

Normal weight women with PCOS have higher LH value than obese women with PCOS. Lower LH value observed in obese PCOS women may be due to aromatization of androgen to oestrogen which takes place in peripheral fat resulting in suppression of LH.[42] Therefore higher AMH values seen in normal weight and lean PCOS could be due to high LH value. This is one of the reasons why clinically lean PCOS women are more susceptible to hyper-stimulation syndrome in COS protocol. This is supported by finding of in-vitro study that LH added to culture of granulosa cells from women with PCOS triples the amount of AMH produced.

Summarizing the correlation among obesity, AMH and LH it has been observed that lean or normal weight women compared to their obese counterpart have high LH and therefore elevated level of AMH.

G) ***Impact of weight loss in obese PCOS women on clinical features and level of AMH:*** Weight loss by bariatric surgery or through hypo-caloric diet and life style changes do not have any positive impact on AMH level or on menstrual irregularities, specially on those women who have elevated level of AMH before treatment. However, some improvement in menstrual abnormalities has been observed in those

women who were relatively younger and had pre-treatment lower AMH level.[43]

Standardization of plasma and follicular AMH level assessment procedure:

Inspite of many valuable information likely to be achieved with regard to male and female reproductive function from AMH assessment, unfortunately till now the assessment procedures currently available have not yet been standardized (the methods have been discussed in a previous chapter and is being summarized again).

Even a few years back, AMH values were not standardized. Recently there has been an evolution of AMH assessment from laboratory versions to the commercially available diagnostic systems lab (DSL) and Immuno-tech Beckman Coulter (IBC) assessment. Recently published studies have used either the DSL or IBC assessment methods. But using these two different assay procedures have also created problems because values reported by different authors have varied substantially. IBC assay provides values of AMH which are higher than those provided by the DSL assay. Currently, the problem has been solved to a large extent as Beckman Coulter has purchased the patents of all previous versions and initiated AMH Generation-II assay. AMH Generation-II assay is highly specific and has been developed to standardize the measurement of AMH-between methods.[44] A similar precision and excellent correlation between-assay agreement should be obtained when laboratory change from the DSL (diagnostic system laboratory) to AMH generation-II Elisa[45] assay. Therefore it has been suggested that performance of AMH generation-II assay is ideal for determination of physiological role of AMH in men and women.

At present, in clinical practice, the normal level of plasma AMH has been accepted as 1-3 ng/ml. Levels between 0.7 to 0.9 ng/ml is recognized as low normal, while levels below 0.3 ng/ml is considered as very low level. Level above 3 ng/ml is considered very high and may be a diagnostic means for PCOS women. But still there is a wide variation in the level for clinical interpretation.[46,47]

Take home message:

- Anti-mullerian hormone, previously known as mullerian inhibitory substance (MIS), was believed to be a protein hormone, and besides testosterone, was found in the testis of a male child. Its function was described to be related to mullerian regression in male child and sexual differentiation in both sexes
- Anti-mullerian hormone is produced by sertoli cells of testis in the male and granulose cells of small follicles (small pre-antral, large pre-natral and small antral) of the ovary in the female. The production of AMH starts from 8th and 36th week of intra-uterine life of male and female offspring respectively. In males, during intra-uterine and pre-pubertal period AMH production is maximum, because of uninhibited action of FSH while from puberty, AMH level declines under the influence of androgen produced by leydig cells stimulated by rising level of LH. Thereafter, the value of AMH remains at a lower level through-out the period of man's life. The function of AMH in adult man's life is not known
- In males low level AMH before the age of 8 years indicates onset of precocious puberty while a high level of AMH at or beyond 8 years denotes delayed puberty
- In females, production of AMH is not under gonadotropin control. AMH is believed to be produced through paracrine action of follicles themselves. The follicular and plasma level of AMH therefore represents primarily the amount and quality of follicular number and dynamics
- Only small pre-antral, large pre-antral and small antral follicles are able to produce AMH. On the other hand, the granulosa cells of primordial and large-antral follicles do not produce AMH. In fact, except granolosa cells of pre-antral and small antral follicles, no other cells within the ovary like oocyte, theca cells, cells of corpus luteum etc cannot produce AMH
- Physiologically, during a normal menstrual cycle, recruitment of a cohort of gonadotropin sensitive follicles occurs from a pool of small pre-natral and antral follicles. These are the follicles where the level of AMH is very high

- Therefore estimation of AMH level as a 'marker' of 'follicular reserve' has gained much importance in reproductive medicine
- Moreover the production of AMH in these follicles are not dependent on endogenous gonadotropin and therefore there is no cyclic variation of AMH level and it remains constant throughout the entire menstrual cycle
- Level of AMH also determines the quality of follicles likely to be recruited during a stimulation cycle. Therefore, AMH level has also been accepted as a good predictive 'marker' of not only ovarian reserve but also of 'ovarian responsiveness' to exogenous stimulation
- In polycystic ovary syndrome, because of inability of follicles to mature in consecutive menstrual cycle, there is crowding of immature (small pre-antral, large pre-antral and small antral) follicles in the ovary which are responsible for over production and accumulation of excess AMH in follicular fluid and plasma. Therefore estimation of AMH (high level) can be accepted as an additional diagnostic marker of PCOS and prediction of hyperstimulation (OHSS)
- Low level of AMH has also been considered as a marker of ovarian ageing, poor response to ovarian stimulation and prediction of outcome in ART cycle (poor responder)
- Obesity, AMH and LH values in PCOS women have some interrelated correlation. Normal weight and lean PCOS rather than obese PCOS women have higher level of plasma LH and AMH values. Therefore it may be an explanation for having a higher risk of developing hyperstimulation which commonly occurs in lean compared to obese PCOS women
- Effect of weight loss in obese PCOS women, resulting in improvement of clinical features has been observed to be more favourable in women who had pre-treatment low AMH level and also are younger in age
- Lastly, it must be admitted that AMH level assessment procedures, either in follicular fluid or in plasma, have not yet been precisely standardized. Recently AMH generation-II Elisa assay method is emerging as a popular method of standardized AMH assessment. It has been agreed that performance of AMH generation-II assay is ideal for assessment of physiological role of AMH in men and women

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Fertility management in Kallmann syndrome: a step towards optimization

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Introduction

Kallmann syndrome (KS) is a genetically heterogeneous disorder, occurring in one per 50,000 women, characterized by hypogonadotropic hypogonadism with hyposmia or anosmia.

Hypogonadism is due to GnRH deficiency which results from failure of embryonic migration of GnRH producing neurons because of gene mutation.[1]

These patients usually present with primary amenorrhoea and delayed puberty. In KS, successfully achieving a pregnancy poses a challenge to the clinicians. We report an interesting case series of three cases of infertility with female partner having KS.

Case Report

Case 1: A 34 year old married female came for infertility evaluation. She was diagnosed with KS at our clinic at the age of 17; since then she was started on cyclical hormone therapy (HT) and counseled about need of future fertility treatment. Karyotype was 46XX and KALL-1 gene was identified. During infertility evaluation, her BMI was 27.4 kg/m^2, her hormonal profile was abnormal (LH 1.05IU/l, FSH 1.77IU/l, E2 12pg/ml) and AMH was 2.3ng/ml. On ultrasonography, uterine size was 6x3.4x2cm with bilateral small ovaries (Volume -1.9cc and 2.1cc). Tubal patency and husband's semen analysis was normal. Ovarian stimulation was undertaken with gonadotropins using 150IU HMG for 8 days starting from cycle day 2. It was increased to 225 IU in view of poor follicular growth. Later, as the patient developed five follicles >14 mm and six follicles of size 10-14mm, they were counseled regarding switchover to IVF. After 22 days of stimulation and a total HMG dose of 4575 IU, hCG 10000 IU was given for final oocyte maturation. Endometrial thickness (ET) was 13mm and serum estradiol (E2) was 2660 pg/ml on the day of hCG. Eight oocytes were retrieved, five embryos were formed and two 8-celled embryos were transferred on day 3. However, she did not conceive in that cycle. After 6 months, frozen embryo transfer (FET) was done. Two good quality embryos were transferred after endometrial preparation with estradiol valerate (6mg daily) and vaginal progesterone (600mg for 3 days prior to embryo transfer). Luteal support with estrogen-progesterone was continued till 12 weeks of gestation. A viable single intrauterine fetus was confirmed on ultrasound at 7 weeks and a healthy baby was born at term.

Case 2: A patient of KS, diagnosed at the age of 20, presented to our clinic at 30 years with primary infertility. She was on HT for the past ten years. Her FSH, LH and E2 were very low and ultrasound showed uterine size of 20x29x58mm and small ovaries (Vol-2.1cc and 2.4cc). She underwent ovulation induction with HMG 225IU from day 2 to day 15 resulting in two follicles ≥ 14mm and was advised timed intercourse after confirmation of ovulation. Luteal support of estrogen and progesterone was continued till 12 weeks after confirmation of pregnancy. She also delivered a healthy baby at term.

Case 3: A 31-year female with BMI 17.2 kg/m2 came with infertility and anosmia. She had withdrawal bleeding only following estrogen progesterone combination. Pelvic examination was normal. Her basal FSH, LH and E2 were 0.91 IU/l, 0.07IU/l and 9.8pg/ml respectively. USG revealed hypoplastic uterus (20x24x48mm) and bilateral small ovaries (Vol 1.3cc and 1.5cc). CT scan of brain, karyotype were normal and KALL-1 gene was present. She was also diagnosed with KS. Other causes of infertility were ruled out. She had poor ET (5mm) even after 12mg of estrogen therapy till day 21. Couple was counseled regarding small sized uterus and poor endometrial response to estrogen. After six months of priming with estrogen-progesterone, ovarian

stimulation was started. A dose of 150IU HMG for five days resulted only in 4 follicles of <10mm. This was followed by 225IU HMG for 19 days leading to five follicles of >14mm and three follicles of <14mm. Six oocytes were retrieved and ET was 7.1mm on day of retrieval. Five embryos were formed and two cleavage-stage embryos were transferred. Her βhCG after 14 days of embryo transfer was 52 IU but eventually led to a miscarriage. Endometrial preparation of the patient was attempted three times with high dose estrogen for FET but endometrium never crossed 5mm when observed till day 30. Since surrogacy was not acceptable to the couple, they were counseled for second ovum pick up considering previous optimum endometrial thickness after ovarian stimulation. She was given HT for one year followed by ovarian stimulation with 225IU daily dose of HMG for 17 days. Total 7 follicles of >14mm developed, 6 oocytes were retrieved and ET was 7.3mm on the day of ovum pickup. Two good quality blastocysts were transferred. She again conceived but ended up in missed abortion

Discussion

In patients with KS, the key aim, after diagnosis, is to induce and maintain secondary sexual characteristics by hormone therapy. Once these patients attempt to conceive, they require induction with gonadotropin in high dosages and for prolonged duration. Various ovulation induction protocols have been attempted for infertile women with this syndrome, such as using pulsatile GnRH, combination of recombinant FSH and LH, or HMG.[2]

In KS patients, approximately 120 pregnancies have been reported in the literature since 1990.[3] Most commonly used protocol for ovulation induction in women with KS is HMG.[4,5] In the above case series, all three patients with KS achieved pregnancy with HMG induction. One patient achieved pregnancy with ovulation induction, second with fresh embryo transfer and the third with frozen embryo transfer. Out of the three, two delivered at term and both children were doing well at one year of follow up. Both these successful outcome patients received cyclical hormone therapy since adolescence. Their ovaries responded satisfactorily to gonadotropins and the uterus was well responsive to estrogen therapy. These two patients were diagnosed with KS during their pubertal years and were on hormone therapy since then. The third patient was diagnosed with Kallmann syndrome at 31 years and HT was given only for six months for priming before starting ovulation induction. Since her uterine size was very small (20x24x48mm), growth of endometrial thickness was monitored with incremental doses of estrogen. Endometrial thickness was only 5 mm in spite of high dose of estrogen and guarded prognosis was explained to the couple. During ovarian stimulation with high dose gonadotropins, her endometrial thickness increased to 7.1 mm. She conceived after embryo transfer but unfortunately had a missed abortion. After managing these three cases, we extrapolated that it is important to treat these women with hormone therapy from puberty onwards, not only to attain secondary sexual characteristics but also for satisfactory fertility outcome. In another case series, the author reported that follicular response to gonadotropins was insufficient in patients not previously primed with hormone therapy in comparison to hormone primed patients.[5] Ovulation induction and conception can be achieved sooner and with less cost if they are previously primed.[5]

A case report of Kallmann syndrome suggested that testosterone supplementation before ovarian stimulation dramatically improved follicular response to gonadotrophins in patient who was previously resistant to gonadotropin stimulation.[6] Similarly, Balasch et al reported that LH priming in hypogonadotrophic hypogonadism women before ovarian stimulation with FSH may reduce the dose required for preovulatory follicular development.[7] Although it may be challenging to attain fertility in Kallmann syndrome but with persistent efforts results are not always disappointing.

Acknowledgments

The authors would like to thank Dr. Saeeda Wasim for helping in editing the manuscript.

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: Not required

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Is Letrozole Better for Ovulation Induction?

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Introduction

Over the past five decades, clomiphene citrate (CC) continues to be the first line treatment primarily for ovulation induction and also for ovulation augmentation in unexplained infertility and in intrauterine insemination (IUI) cycles.[1] However, it is reported that 20-25% of women fail to ovulate due to CC-resistance.[2] In view of this, administration of gonadotropins is considered to be the conventional option in such cases. Though use of gonadotropins is highly effective, it is associated with inevitable risk of multiple pregnancies and ovarian hyperstimulation in a significant proportion of women.[3] As an alternative management of gonadotropins, use of laparoscopic ovarian drilling in CC-resistant women has also been advocated.[4] Addition of CC with Gonadotropins (FSH/hMG) helps in decreasing the dose of total amount of gonadotropins required for optimum stimulation and make it more cost-effective in women who fail to respond to only CC treatment.[5] Acceptable pregnancy rates with CC and sequential hMG ovulation induction protocol in IUI following previous CC and IUI treatment failure have also been reported.[6] However, supra-physiological level of E2 is an undesirable consequence of both CC and gonadotropin stimulation. Apart from risk of hyperstimulation and multiple pregnancies, adverse effects of supra-physiological level of E2 have been observed at several levels. These are - Dyssynchrony between endometrium and embryo maturation during 'implantation window' period, abnormal expression of endometrial pinopodes, defective endometrial oestrogen – progesterone receptors, abnormal endometrial blood flow and abnormal integrin expression. These are some of the reasons for low pregnancy rate in spite of having good ovulatory response following CC induction in anovulatory infertility.

These limitations motivated researchers to find out an alternative drug which will be less expensive than gonadotropin and at the same time will be safe, simple and equally if not more effective than clomiphene. Letrozole was considered to be an alternative acceptable molecule.

How and Why Letrozole?

In women with intact hypothalamic-pituitary-ovarian axis, the commonest cause of anovulation is polycystic ovarian syndrome (PCOS). One of the significant causes of anovulation in PCOS women is 'static' (not pulsatile) elevated or normal level of oestrogen. Static level of oestrogen through 'negative feed-back' mechanism on 'hypothalamic-pituitary (HP) axis' inhibits adequate release of pituitary FSH. Low (not absent) level of FSH results in inadequate growth and development of follicles, - not allowing them to reach maturity and pre-ovulatory follicle leading to non-ovulation. At the same time, tonic elevated level of oestrogen through 'positive feed-back' effect on HP axis results in release of static elevated 'tonic' level of LH. There is no LH surge and therefore anovulation.

Aromatase inhibitors (letrozole) by inhibiting oestrogen synthesis temporarily release hypothalamic-pituitary block by tonic elevated oestrogen thereby normalizing fluctuating (and not tonic) release of pituitary FSH which helps in restoration of normal ovulatory cycle. Therefore, letrozole was considered to be an effective drug for induction of ovulation.

Literature review:

Several research groups have studied the new group of drugs (aromatase inhibitors) for ovulation induction in the past few years.[7–9] Letrozole, a potent and highly specific nonsteroidal aromatase inhibitor, has been observed to be effective in inducing ovulation in anovulatory and ovulatory infertile women with inadequate response to CC. Initially, letrozole was primarily used as a potent reversible oral aromatase inhibitor which acts a chemotherapeutic agent in postmenopausal women with metastatic breast cancer.[10] Being a chemotherapeutic agent, when the drug was used for ovulation induction, concerns have been raised that it may have some teratogenic

effect on oocyte and embryo. Moreover, the resulting hypo-estrogenism may have adverse impact on bone mineral metabolism leading to osteoporosis. The other controversy relating to the use of letrozole as a first-line agent, before CC has been used, is based on the fact that in normo-gonadotropic women, aromatase inhibition is likely to be effective only when baseline estradiol is elevated. The cut-off level of the elevated baseline estrogen is not yet demarcated. Hence use of letrozole as a primary ovulation-inducing drug replacing clomiphene warrants further investigation. An abstract presented at American Society for Reproductive Medicine (ASRM) meeting 2005 regarding increased teratogenic risk of cardiac malformations with letrozole[11] and other safety concerns eventually led to the ban on this drug in India in 2011. Nevertheless, there is an increased concern on the factuality of the observation or a mere co-incidence due to the shortcomings and biases of this study.

In the later years, various studies indicated that letrozole is not associated with increased teratogenic risk.[12,13] Our earlier study showed that the overall rate of congenital malformations among children born to mothers who conceived naturally or after letrozole or CC treatment was observed to be comparable.[12] Our group has conducted one of the largest-ever randomized clinical trials to explore the efficacy of letrozole in ovulation induction on 1387 infertile PCOS women who failed to conceive with CC treatment.[14] This study showed that letrozole appears to be a suitable ovulation inducing agent in polycystic ovary syndrome (PCOS) women with CC failure and is found to be most effective when baseline oestradiol level >60 pg/ml. It is well known that infertility itself is a risk factor and is associated with increased malformation risk as compared to the general population. Several published studies, both controlled and noncontrolled, comparing letrozole with CC alone or in combination with gonadotropins confirm the effectiveness of letrozole as an ovulation inducing agent.[15–18] Based on these various reports, Government of India, Ministry of Health and Family Welfare removed the ban on use of letrozole as ovulation induction agent.

Evolution of aromatase inhibitors for clinical use

Aromatase inhibitors suppress estrogen production by inhibiting the conversion of androgens to estrogens. Letrozole, the drug commonly used in clinical practice, has been developed following extensive trial through three generations of aromatase inhibitors. Third generation aromatase inhibitors like letrozole and anastrozole have been a great leap forward in the treatment of breast cancer. Their clinical efficacy, excellent tolerability and safety profile compare favourably with that of tamoxifen, which has been the cornerstone of endocrine therapy for years.

Concept leading to the use of letrozole for induction of ovulation

The goal of ovulation induction is to induce monofollicular development and subsequent ovulation in anovulatory infertile women. As discussed in previous paragraphs, anovulation in PCOS or any normogonadotropic anovulatory cycle is due to the block of hypothalamic receptors by static elevated supraphysiological level of oestradiol, which is preventing the release of pulsatile luteinizing hormone-releasing hormone (LHRH). Decline in static elevated oestrogen level can help in restoration of synchronized and pulsatile LHRH release. Antiestrogenic effect of letrozole was the concept behind using it for ovulation induction. This was first reported in literature by Mitwally et al.,[19] in anovulatory women resistant to ovulation induction by CC.

Need of an alternative drug for ovulation induction other than clomiphene citrate

Several drawbacks with CC had been the reason for lookout for an alternative ovulation inducing agent in certain cases. CC remains bound with oestrogen receptors for 60 days because of its long half life. In case, CC fails to induce ovulation or establish pregnancy, other ovulation inducing drugs cannot be initiated before 60 days. It is thought that dose of 150 mg or more will confer no benefit. CC induces ovulation in 70-85% of patients while only 20-40% will conceive. The pregnancy rate per cycle is around 10-20%. About 20-25% anovulatory women are clomiphene resistant.

CC has unfavourable effects on endometrial thickness and cervical mucus due to its antiestrogenic effect. The incidence of miscarriage after CC therapy has been reported to be about 23.6%. It has been shown that with prolonged CC use, along with low

endometrial thickness, there is also decreased uterine blood flow during early luteal and peri-implantation phase. There have been evidences suggesting that supra-physiological serum luteinizing hormone (LH) level from day 9 until the LH surge, together with premature luteinisation and higher serum oestrogen levels throughout the cycle can lead to higher chances of either non-conception or miscarriage.

Difference in mechanism of action of CC and letrozole

In CC, hypothalamic receptors are bound to oestrogenic component of CC and therefore these receptors become unaware of presence of supra-physiological levels of circulating estrogens, allowing hypothalamus to release effective synchronized pulsatile LHRH, thereby leading to LH surge and ovulation.

Letrozole causes direct inhibition of oestrogen synthesis thereby allowing follicle-stimulating hormone (FSH) to induce active folliculogenesis. This hypo-estrogenic state is quickly reversible due to the short half-life of letrozole (45 hours). There is no antioestrogenic effect on endometrium. Also there is temporary elevation of testosterone to an optimum level which is beneficial as it increases the follicular sensitivity to gonadotropin. Excess levels of androgen cause detrimental effects whereas a very low level of testosterone impairs follicular development.

Common features in mechanism of action of CC and letrozole

Though the drugs act in different ways, there are some common features in their mechanism of actions. These are: (a) Release of hypothalamus from negative tonic feedback effect of static normal or elevated level of oestrogen (b) Allowing release of pulsatile gonadotropin-releasing hormone (GnRH) (c) FSH & LH ratio is synchronized (d) LH surge is effective for ovulation. These have been illustrated in Fig-1 & Fig-2.

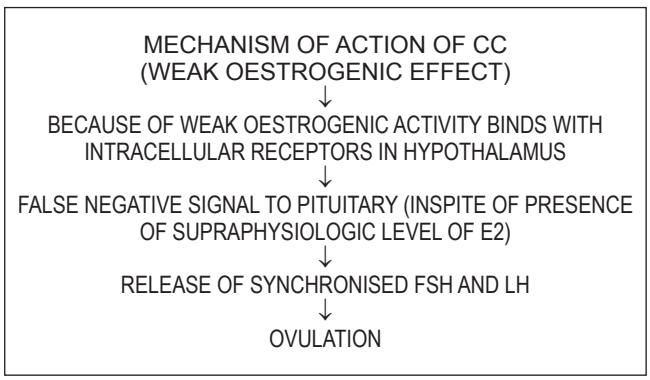

Fig-1: mechanism of action (ovulation induction) with CC

Role of aromatase inhibitors in different types of infertility

Ovulation induction in anovulatory women with PCOS

Letrozole versus CC in PCOS women has been tested in several randomized trials.[14,20–23] However, the efficacy of letrozole in ovulation induction remains unclear. One of the largest randomized controlled trials conducted in our institute comparing efficacy of letrozole with continuous gonadotropins and CC-gonadotropin combination for ovulation induction in 1387 PCOS women after clomiphene citrate failure concluded that the ovulation and pregnancy rate with letrozole was significantly higher with letrozole compared to CC-rFSH combination (79.30% vs 56.95%, p value <0.0001 and 23.39% vs 14.35%, p value <0.0001 respectively).[14] Also there was a significantly lower cycle cancellation rate with letrozole compared to CC-rFSH (20.70% vs 43.05%, p value <0.0001). Another group had reported comparable pregnancy rate with letrozole and CC-hMG therapy in a pilot study.[24] An analysis of four early randomized studies had observed a significantly higher pregnancy and delivery rate in women treated with aromatase inhibitor compared with CC.[25] Nonetheless, the trials involved were heterogeneous with a limited number of patients.

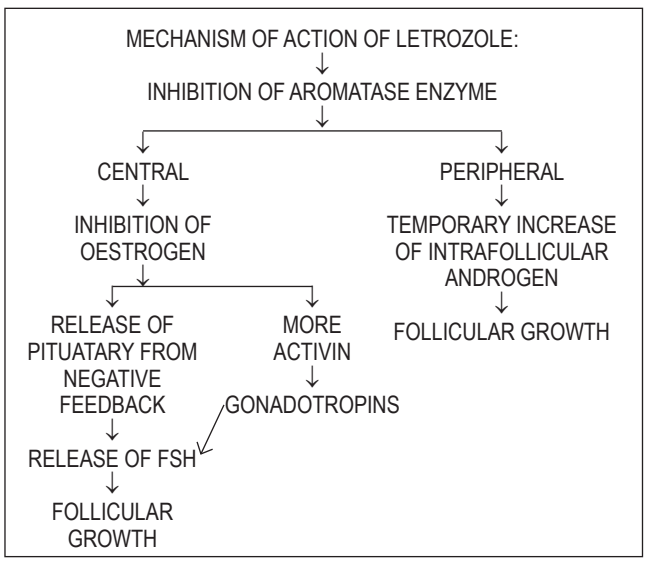

Fig-2: mechanism of action (ovulation induction) with letrozole

A recent well-designed double blind multicentre randomized control trial comparing letrozole versus clomiphene for infertile PCOS women has concluded that letrozole was associated with higher live birth and ovulation rates. Therefore, letrozole is considered to be superior than CC as a treatment for anovulatory infertility in women with PCOS.[26] Similar findings were observed by other studies.[27,28] A meta-analysis published in 2015 analysed 4999 ovulation cycles (2455 with letrozole, 2544 with CC) indicated that live birth and pregnancy rates were higher in patients with PCOS following treatment with letrozole as compared to CC. However, there was no difference in ovulation rate/cycle, miscarriage rate or multiple pregnancy rate between the two drugs.[29] A study by Liu et al. on 141 CC-resistant PCOS women showed comparison between letrozole and laparoscopic ovarian drilling (LOD). They found letrozole had superior reproductive outcomes compared with LOD in women with CC resistant PCOS and that letrozole could be used as 1st line treatment for women with CC-resistant PCOS. The number of cycles with synchronised follicular and endometrial growth was also significantly higher in letrozole group.[30] A study comparing efficacy of letrozole with tamoxifen observed that tamoxifen was inferior to letrozole in terms of ovulation and pregnancy rate.[31]

Ovulation induction/stimulation in unexplained infertility

Aromatase inhibitors are recommended as an alternative drug to CC in women with unexplained infertility, either alone or with gonadotrophins. Nonetheless, it is likely to be less efficacious compared with treatment in PCOS women. Letrozole results in lesser number of mature follicles (mono-ovulation) in comparison to CC because it has less anti -estrogenic effects in the later part of follicular phase. Thus, it may not be the first choice in patients with unexplained infertility. A meta-analysis of seven randomized control trials showed comparable clinical pregnancy rates between aromatase inhibitor and CC in women with unexplained infertility.[32] These findings are in good agreement with another large trial where no statistically significant difference was observed between 100 mg of CC versus 5 mg of letrozole in terms of clinical pregnancy rate in unexplained infertility.[33] A recent large multicentre trial on 900 women with unexplained infertility concluded that letrozole resulted in lower frequency of multiple pregnancies but also lower live birth rates as compared to gonadotropins. However, when letrozole was compared to clomiphene alone, pregnancy rates were similar.[34]

Safety concerns with letrozole

Concerns had been raised regarding the use of letrozole for ovulation induction, as it might interrupt the normal aromatase function in tissues during early fetal development and can be potentially teratogenic.[35] This issue was discussed in the Annual Meeting of the American Society for Reproductive Medicine in 2005. An abstract presentation by the authors discussing the use of letrozole for infertility treatment may be associated with a higher risk of congenital cardiac and bone malformations in the newborns.[11] Following this, Novartis Pharmaceuticals, the company that developed letrozole for breast cancer treatment, issued a warning to infertility clinics asserting that it does not advocate letrozole's use for infertility treatment. In October 2011 the Ministry of Health and Family Welfare, India issued a directive to suspend the use of letrozole in infertile women with immediate effect citing concerns regarding its safety. A study analysing 911 newborns born after infertility treatment with either CC or letrozole found no difference in overall rates of major and minor congenital malformations between the two groups.[36] In a recent retrospective trial from Asian sub-continent analysing 646 women, congenital malformations were found to be comparable following natural conception, letrozole and CC.[12] Most recent trial by Tatsumi et al. (2017) reported that no increase in the risk of major congenital anomalies or adverse pregnancy or neonatal outcomes was observed in letrozole treated women compared with natural cycles in women undergoing ART.[37] Considering these reports, Indian Health Ministry has recently removed the ban on letrozole for use in infertility. Therefore, letrozole may be considered as a safe option for ovulation induction.

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Work Statement of Patients from March to May 2017

Total No of Gynaecological & Obstetric Cases attended		**960**
No of Gynaegological Cases	734	
No of Obstetric Cases	226	
Gynaegological Cases		
Infertility		**622**
Primary	437	
Secondary	185	
Other Gynaecological Cases		38
History of Recurrent Spont Miscarriage(> 3)		35
History of Unexplained Spont Miscarriage (<3)		26
History of Recurrent Pregnancy Loss		13
Categorization of Infertility/ Gynaecologocial Cases		
Female Factor	245	(39.39%)
Male Factor	241	(38.74%)
Unexplained	96	(15.43%)
Combined Factor	40	(6.43%)
Total No of IVF & IUI Cycles		**667**
IVF Fresh Cycle		193
ET Done	144	
ET not done	49	
Cryo Cycle		62
IUI	**412**	
Obstetric Cases		
Pregnancy folowing		
Medical treatment (Induction Ovulation)		46
Surgical Treatment		19
During investigation		47
Intrauterine Insemination		43
IVF-ET including FET Cycle		71

Pregnancy Loss		**3**
Elective Termination		
Spont. Termination		
RSM (> 3)	0	
USM (<3)	2	
RPL	1	
Other type of Pregnancy Loss	**0**	
Viable Deilvery		**49**
CS	49	
Normal	0	
Sucessful Delivery after		
IVF	16	
IUI	6	
OI	12	
Hydrotubation	0	
Spont.	2	
During investigtion	13	
Baby outcome		
Alive		55
Singleton	43	
Male	25	
Female	18	
Twins		12
Male	4	
Female	8	
Neonatal Death		0
Still Born		0
Gynaecological Surgery		73
Laparoscopy + Hysteroscopy		32
Hysteroscopy		21
Laparoscopy		2
Vaginoplasty		1
Ectopic		3
Mcdonald		13
D/C		1