INTRODUCTION

Placing the human ovum with spermatozoa outside the women and transferring the resulting embryo to her uterus is a relatively recent, and rapidly evolving approach to overcome human fertility problems. The first reports of implantation and pregnancy following the use of this technique in human were published during the 1970s. Since then, the techniques known as in vitro fertilization (IVF) and embryo transfer (ET), have resulted in new insights into gamete interactions and early embryonic development, as well as numerous pregnancies. However, IVF tends to be less successful for the treatment of male factor infertility than the treatment of tubal factor infertility ***(Tourney et al., 1992).***

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Intracytoplasmic sperm injection (ICSI) is now one of the most successful and viable techniques in assisted fertilization ***(Nagy et al., 1995).***

In those who have no identifiable or correctable causes, ICSI provides new hope for infertility ***(Chow et al., 2006).***

ICSI can improve previous fertilization limitation on conventional IVF ***(Zhonghua et al., 2005).***

Recent advances in the understanding of ovarian stimulation, the techniques of oocyte retrieval, the handling of gametes, the methods of assisted fertilization and improved conditions of culture media have steadily increased the fertilization rate in cases of assisted reproduction. Fertilization rates of 70-80% can now be expected when conventional insemination or intracytoplasmic sperm injection (ICSI) are carried out. However, there has not been a corresponding increase in implantation rates, which have remained steady at 10-15% for a long time ***(Huang et al., 2000).***

Folliculogenesis, the primary function of the mammalian ovarian follicle is the release of an oocyte capable of being fertilized by a sperm. This involves the growth and maturation of the follicle as well as the enlargement, ovulation and resumption of meiotic division of the egg ***(Richards, 1980).***

Human oocyte maturation is considered as the reinitiation and completion of the first meiotic division from the germinal vesicle stage (prophase I) to metaphase II, and the accompanying cytoplasmic maturation necessary for fertilization and early embryonic development. The cytoplasm of the oocyte is of key interest in oocyte maturation. The best way to improve embryo quality is to improve oocyte quality ***(Kwang et al., 1998).***

Oocyte quality affects early embryonic survival, the establishment and maintenance of pregnancy and fetal development. Quality, or developmental competence, is acquired during Folliculogenesis as the oocyte grows, and during the period of oocyte maturation ***(Krisher, 2004).***

Determination of oocyte and embryo quality are one of the most important goals of embryologists in human IVF. Several methods are employed for determining oocyte and embryo quality ***(Revelli et al., 2009).***

Biochemical characteristics of the follicular fluid play an important role in the prediction of oocyte quality, fertilization and ultimately the embryo quality in noninvasive methods ***(Revelli et al., 2009).***

Anti-mullerian hormone (AMH) is one member of transforming growth factor ***(Das et al., 2008).***

AMH plays a fundamental role in gonadal differentiation during fetal period and inhibits the formation of mullerian ducts in male fetus ***(La Marca et al., 2009).***

Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance is a unique biomarker of follicular status. It has become known as an important regulator of follicle development ***(Seifer et al., 2007).***

It is a glycoprotein exclusively produced by granulosa cells of preantral and small antral follicles. AMH is secreted by the ovarian granulosa cells into blood flow and follicular fluid

in adult female, although its concentration is much higher in the follicular fluid ***(La Marca et al., 2009).***

AMH is produced presumably independently of follicle-stimulating hormone (FSH) ***(Durlinger et al., 2002).***

AMH is involved in the inhibition of the growth from primordial to primary follicle as well as in follicle recruitment and selection ***(Durlinger et al., 2001).***

Moreover, it attenuates the FSH-dependent increase in aromatase activity during early follicle development and reduces the number of LH receptors in FSH-stimulated granulosa cells ***(Jasson et al., 1998).***

Serum AMH has been well studied as an excellent marker of ovarian reserve. Serum AMH levels have shown great sensitivity to ovarian aging and significant correlation with antral follicle count, and this relationship was stronger than that for other hormonal markers ***(Shin et al., 2008).***

In addition, it has been a useful predictor of the ovarian response to controlled ovarian stimulation (COS). All of these evidences show that serum AMH quantitatively reflects the size of the ovarian follicle pool; in other words, AMH is a quantitative marker of ovarian reserve and response ***(Seifer et al., 2002).***

Aim of the Work

This study aims at studying the relation between follicular fluid AMH, oocyte maturation and embryo grading and pregnancy outcome in ICSI patients.

Chapter 1

OVULATION INDUCTION BY GONADOTROPINS

Ovulatory disturbances are present in about 15–25 % of couples presenting for an infertility evaluation. Most infertile anovulatory patients fall into the WHO group II (normogonadotropic anovulation) category. These women are well estrogenized and have normal FSH levels, but LH may be elevated. In contrast, WHO group I anovulation or hypogonadotropic hypogonadism (HH) is a much less frequent condition, characterized by reduced hypothalamic or pituitary activity and resulting in abnormally low serum concentrations of FSH and LH and negligible estrogen activity. The guiding principle for treatment of women with anovulatory infertility should be restoration of the feedback system which selects a single follicle for ovulation. Treatment with gonadotropins should be restricted to women who are resistant to clomiphene ***(ACOG, 2002).***

Pharmaceutical preparations containing biologically active gonadotropins for ovulation induction have been in use for about 75 years. For years, human menopausal gonadotropin (hMG) has been the only urinary gonadotropin available for clinical use. The FSH and LH content of hMG is theoretically equal 75 IU of FSH and 75 IU of LH, albeit with different FSH/LH ratios. In addition, hMG has a low specific activity

and is of <4% purity, as only ~3–4% of the protein content is gonadotropin. Over the past 20 years, urinary FSH-only preparations became new therapeutic options for ovulation induction. In the mid-1980s, urinary 'purified' FSH (pFSH) (with <1% LH contamination but still having 95% protein impurity) was developed, which was followed by the availability of highly purified FSH (FSH-HP) in 1993. FSH-HP contained <0.1% LH contamination, and was the first highly pure biologic extract (~4% impurity), and, as a result of this, could be injected subcutaneously (s.c.), unlike the earlier preparations which had to be administered intramuscularly (i.m.). Specific FSH activity was about 2000 IU/mg protein for the new hMG, compared with 8000 IU/mg of FSH-HP ***(Giudice et al., 2001).***

Recombinant human FSH (rhFSH) which is completely devoid of both LH activity and non specific urinary proteins, represents the final transition to a true drug. Two rhFSH preparations have been registered as follitropin alpha which was marketed first in 1995 (Gonal-F; Merckserono International, Geneva, Switzerland), and follitropin ß (Puregon; NV Organon, Oss, the Netherlands). Both follitropins are structurally identical to native FSH, and each comprises the α and ß subunits which compose this gonadotropin; the nomenclature for these recombinant products does not refer to those subunits, but is merely a means of distinguishing chronologically one from another. Like rhFSH, recombinant

human LH (rhLH) (Luveris; Merck-Serono International) and recombinant hCG (rhCG) (Ovitrelle and Ovidrel; Serono International) are produced under the most stringent manufacturing conditions, and have been assessed successfully for clinical use ***(Fonjallaz and Loumaye, 2001).***

Gonadotropin use for OI may be complicated by multiple pregnancies, particularly triplets and higher order, and by ovarian hyperstimulation syndrome (OHSS).Their use as first-line treatment should be limited to those women who have hypopituitary or hypothalamic amenorrhea. Gonadotropins should not be used in the treatment of "unexplained infertility" for patients who ovulate, but fail to conceive, until a minimum of three cycles of CC or TMX (tamxoifen) ***(Dickey et al., 2005).***

In the natural cycle, FSH levels are highest during the early proliferative phase, and decrease in response to the rising level of estrogen produced in developing follicles. Beginning on approximately the seventh cycle day, the largest follicle, usually 8–9 mm, because it has the greatest number of FSH receptors, begins to monopolize the decreasing FSH production and becomes the "dominant" follicle destined to ovulate. The dominant follicle increases in size by approximately 2 mm a day and acquires LH receptors (when it is 8–10 mm), enabling the oocyte to mature and ovulation to occur, while non-dominant follicles, deprived of sufficient FSH to stimulate further development, become atretic. After 5–7 days, when the dominant follicle is 20–24 mm, a surge in LH occurs that

results in ovulation approximately 36 hours later. During this time estradiol levels double approximately every two days, follicle volume exactly parallels estradiol levels ***(Porchet et al., 1994).***

FSH threshold hypothesis

In gonadotropin treatment cycles, a constant dose of FSH is administered, which, if sufficiently high, will enable non-dominant follicles to continue to develop and to acquire LH receptors and ovulate when the LH surge occurs ***(Porchet et al., 1994).***

The threshold hypothesis for FSH proposes that there is a minimum FSH level needed to initiate an ovarian response, and that an increase of 50% above the FSH threshold level induces multiple follicular development. The threshold serum FSH level necessary to induce follicular growth has been found to be 7.8 mIU/mL in WHO Group 1 women and normally cycling women suppressed with GnRH analogues. In WHO Group II women the threshold ranges from 6.8 to 9.8 mIU/mL. The duration and extent to which FSH level is above the threshold level determines the number of follicles that are capable of ovulation. According to the threshold theory, constant FSH levels $\geq$ 11.7 mIU/Ml are necessary for multiple follicular development. In a retrospective study of the relationship between serum FSH levels during gonadotropin stimulation and in-vitro fertilization (IVF) outcome, the highest ongoing pregnancy rate per retrieval occurred when steady-state FSH levels were 10.0–15.0mIU/mL. Lower FSH

levels were associated with fewer follicles and lower pregnancy rates (figure 1) ***(Shoham, 2002).***

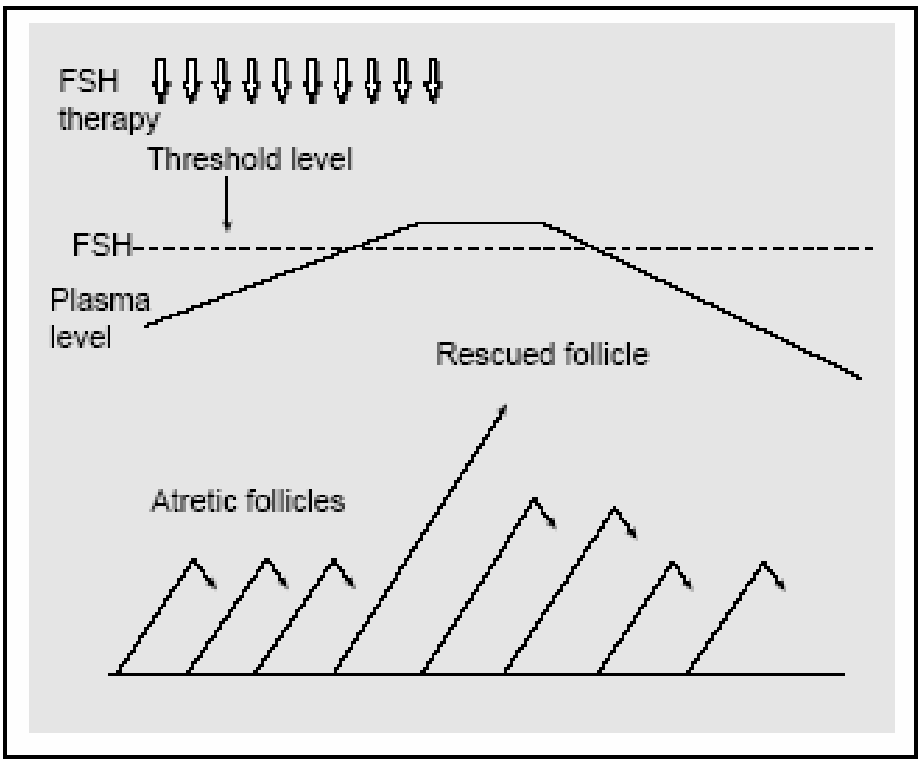

Fig. (1): The threshold theory. When the follicle-stimulating hormone (FSH) level is above threshold, a follicle will be 'rescued' (continue to growth) ***(Shoham, 2002)***.

AMH as a predictor of follicular recruitment

A key feature of the use of GnRH agonists prior to ovarian stimulation is maximal follicular recruitment. The number of follicles recruited per day during GnRH agonist ovarian stimulation differs by 1.9 between younger (<30 years old) and older (>35 years old) women ***(Fleming et al., 2006).*** In comparison, when categorized by different AMH concentrations, this differential is 2.9 follicles per day, indicating that the AMH concentration is a better predictor of follicular recruitment than age ***(Fleming et al., 2006).*** Furthermore, after 10 days of FSH stimulation, higher circulating concentrations of AMH are correlated with increased numbers of follicles recruited and, thus, an increased risk of excessive response and OHSS ***(Fleming et al., 2006).*** However, at extremely low concentrations of AMH, this test may lose some of its sensitivity. For example, AMH concentrations in women nearing menopause, although universally low, have been observed to vary considerably during longitudinal observations ***(Robertson et al., 2011).*** The authors of this study proposed that these distinct AMH patterns that emerge as ovarian follicle reserve decreases with age may be reflective of the intermittent pattern of the emergence of follicles close to menopause. Therefore, it may be more accurate to say that AMH is a marker of potentially functional follicles rather than an indicator of total follicle number ***(Fleming et al., 2013).***

The window for LH: the 'threshold' dose and 'ceiling' value concepts:

Although LH is essential for estrogen synthesis and maintenance of follicular dominance, there is clinical evidence that excessive stimulation of the ovaries by LH adversely affects normal preovulatory development. Depending on the stage of development, follicles exposed to inappropriately high concentrations of LH enter atresia or become prematurely luteinized, and oocyte development may be compromised. Thus, developing follicles appear to have finite requirements for stimulation by LH, beyond which normal development ceases. Whereas each follicle has a threshold beyond which it must be stimulated by FSH to initiate preovulatory development, it may also have a 'ceiling' within which it should be stimulated by LH, unless further normal development is terminated. The amount of LH activity actually necessary for normal follicle and oocyte development, however, is not known, but is likely to be very low, since less than 1% of follicular LH receptors need to be occupied in order to elicit a maximal steroidogenic response, and, accordingly, resting levels of LH (1–10 IU/l) should be sufficient to provide maximal stimulation to theca cells ***(Balasch, 2009)***.

Current concepts of gonadotropic control of ovarian function and clinical evidence have established that both a 'threshold' and a 'ceiling' for LH levels (framing the so-called LH 'window') exist during the follicular phase of menstrual and induced cycles. Therefore, levels of LH should be neither too high nor too low during ovulation induction. During the

second half of the follicular phase, as plasma FSH concentrations decline, the LH-dependent phase of preovulatory follicular development proceeds normally only if LH is present at concentrations over the threshold level and beneath the ceiling value (Fig. 2) ***(Balasch, 2009)***.

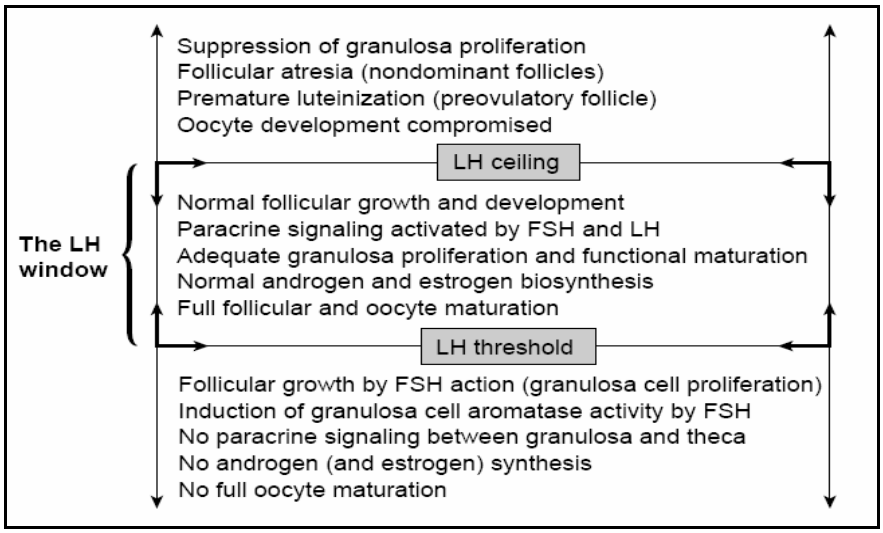

Fig. (2): Diagram illustrating the 'luteinizing hormone (LH) window' ***(Balasch, 2009)***.

The threshold hypothesis for LH maintains that serum levels of 1.8–2.0 mIU/mL are required for continued steroido-genesis and oocyte maturation, and that if LH exceeds a ceiling level, non-dominant follicles become atretic and development of oocytes may impaired ***(Shoham, 2002).***

A- GnRH Agonist/FSH—Long Down regulation Protocol figure (3):

This protocol is currently the protocol of choice for first cycle patients and previously normal or high responders in many

IVF programs. A GnRH-a, is given for 10–14 days before starting gonadotropin treatment (''long-protocol''), and may be commenced in the mid-luteal phase of the previous cycle or on day 2 of the cycle and it was found that pituitary suppression was more effective when the therapy commenced in the mid-luteal phase of the previous cycle rather than in the early follicular phase of the treatment. Another advantage in commencing the analog in the mid-luteal phase was the decreased occurrence of ovarian cysts compared with commencing the GnRH agonist at the beginning of the follicular phase ***(Urbancsek and Witthaus, 1996).***

Suggested criteria for pituitary–ovarian downregulation are E2 levels <180 pmol/L, LH levels <2 IU/L, and progesterone levels <2 nmol/L. FSH commences after achievement of adequate down regulation and continues by daily injections according to individual endocrine and ovarian ultrasonic response until the day before the human chorionic gonadotropin (HCG) injection, 5000 IU IM injection is given when the follicular cohort consists of at least three follicles of 17–20 mm in diameter and serum E2 level is appropriately rising. Egg pick-up follows 36 hr later ***(Urbancsek and Witthaus, 1996).***

The mean desensitization phase with an agonist in the long protocols is about 3 weeks. Several investigators have tried to shorten this long duration of administration, leading to the so-called 'early cessation' protocol. The paradoxical drop of serum LH following early cessation, which leads to significantly lower estradiol levels on the day of hCG, may

have a deleterious effect on IVF outcome, the early discontinuation protocol may improve ovarian response based on a hypothetical effect on the ovary, and was therefore additionally tested in poor responders. Although the number of retrieved oocytes was significantly higher and the amount of required gonadotropins was reduced after early cessation in comparison to the long protocol, this new approach reported no further advantages in these patients in terms of pregnancy and implantation rates. In conclusion, the currently available data do not favor an 'early cessation' protocol, but this approach might have some beneficial effects in poor responders ***(Garcia-Velasco et al., 2000).***

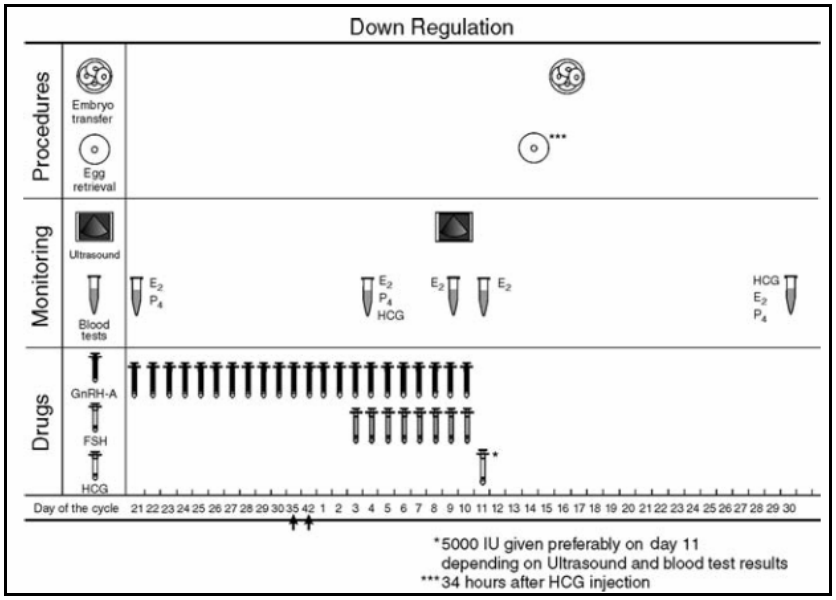

Fig. (3): A representation of the luteal-phase commencement long downregulation protocol using FSH for ovarian stimulation for assisted reproductive technology ***(Healy et al., 2007)***