CLINICAL AND ENDOCRINE RESPONSES TO OVARIAN HYPERSTIMULATION IN FLARE AND LUTEAL GONADOTROPIN-RELEASING HORMONE AGONIST (GnRHa) PROTOCOLS

by

TUAN-ANH THI NGUYEN

M.D, University of Training Center for Health Care Professionals, Vietnam, 2001

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

AUGUST, 2008

© Tuan-Anh Thi Nguyen, 2008
ABSTRACT

**Background:** Due to the “flare effect” associated with the flare protocol, variation in the degree of follicular maturation during stimulation may result in differences in follicle response as compared to the luteal protocol which is based on maximal pituitary suppression and synchronization of follicular maturation. In this study, besides other methods, Anti-Mullerian Hormone (AMH), a novel marker for ovarian reserve, was used as a tool to evaluate the ovarian responsiveness to stimulation.

**Methods:** Women undergoing IVF/ICSI treatment in the UBC IVF Program from January to December 2006 using luteal and flare protocols were retrospectively selected for a total of 40 treatment cycles, 20 cycles in each protocol matched by age, weight, and indication for IVF/ICSI. Serial serum Estradiol (E$_2$) levels and follicle data were obtained from the clinic chart. Follicle stimulating hormone (FSH), Luteinizing Hormone (LH), progesterone (P), androstenedione (D$_4$) and AMH levels were measured from aliquots of frozen serum samples. Hormone responses were evaluated by Area Under the Curve (AUC). Data were analyzed using the t-test and statistical significance was considered present at $P<0.05$. Results are reported as the mean ± SEM.

**Results:** For flare versus luteal protocol, there was a significant difference in the number of total follicles (14.5 ± 1.8 vs 21.3 ± 2.3), medium follicles (3.7 ± 0.6 vs 8.4 ± 1.3), eggs retrieved (8 ± 0.8 vs 14 ± 1.4) and oocytes fertilized (4.4 ± 0.5 vs 8.4 ± 0.7), AMH AUC (62 ± 12 vs 111 ± 13), LH AUC (67 ± 21 vs 20 ± 9), FSH AUC (171 ± 59 vs 112 ± 29), respectively. Mean number of embryos transferred in both groups was similar. Number of pregnancies conceived (5 for flare and 10 for luteal protocol) was not significantly different. Although E$_2$ AUC in luteal protocol was higher than that in flare protocol, the difference was not statistically significant (28,339 ± 2,669 vs 26,905 ± 2,790). Differences in P and D$_4$ AUC between the two protocols were not statistically significant. Correlations with ovarian follicles and eggs retrieved were better for AMH than E$_2$.

**Conclusions:** The luteal protocol exhibited a better ovarian response to stimulation as compared to the flare protocol. As compared to E$_2$, AMH had a better correlation with the number of follicles and eggs retrieved.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................ ii  
TABLE OF CONTENTS ................................................................................................... iii  
LIST OF TABLES ................................................................................................................. v  
LIST OF FIGURES ................................................................................................................ vi  
LIST OF ABBREVIATIONS ............................................................................................. vii  
ACKNOWLEDGEMENTS ................................................................................................. ix  
RATIONALE AND OBJECTIVES .................................................................................. 1

CHAPTER I: GENERAL INTRODUCTION .................................................................... 3  
1.1 Assisted reproduction .......................................................................................... 3  
1.2 Ovarian responsiveness ....................................................................................... 3  
1.3 Normal ovarian physiology (ovarian function) ................................................... 4  
  1.3.1 Folliculogenesis .............................................................................................. 4  
    1.3.1.1 Stages of folliculogenesis ....................................................................... 4  
  1.3.2 Two-cell two-gonadotropin theory ................................................................. 6  
  1.3.3 Luteal function ................................................................................................ 6  
  1.3.4 Intraovarian regulators: ................................................................................... 7  
    1.3.4.1 Prostaglandin ........................................................................................... 7  
    1.3.4.2 Insulin and Insulin Growth Factor .......................................................... 7  
    1.3.4.3 Interleukin ............................................................................................... 8  
    1.3.4.4 Transforming Growth Factor β superfamily ........................................... 8  
    1.3.4.4.1 Activin, inhibin ................................................................................... 9  
    1.3.4.4.2 Bone morphogenic proteins ................................................................ 9  
    1.3.4.4.3 Growth differentiation factor 9 ......................................................... 10  
    1.3.4.4.4 Anti-mullerian hormone .................................................................... 10  
    1.3.4.4.5 Bone morphogenic proteins ................................................................ 9  
    1.3.4.4.6 Growth differentiation factor 9 ......................................................... 10  
    1.3.4.4.7 Anti-mullerian hormone .................................................................... 10  
  1.3.4.4.8 Gonadotropin-Releasing Hormone ....................................................... 11  
    1.3.4.4.8.1 Forms of GnRH .......................................................................... 11  
    1.3.4.4.8.2 Ovarian modulator: ..................................................................... 12  
  1.4 Female infertility and ovarian reserve ............................................................. 12  
  1.4.1 Epidemiology ................................................................................................ 12  
  1.4.2 Oogenesis and the establishment of ovarian reserve .................................... 13  
  1.4.3 Tests for ovarian reserve ............................................................................... 14  
1.5 IVF protocols .................................................................................................... 16  
  1.5.1 Controlled ovarian stimulation and ovarian induction .................................. 16  
  1.5.2 Drugs used for stimulation of ovulation: ...................................................... 16  
    1.5.2.1 FSH, LH: ............................................................................................... 16  
    1.5.2.2 hCG: ...................................................................................................... 17  
    1.5.2.3 GnRH and GnRH agonist ..................................................................... 17  
  1.5.3 Controlled hyperstimulation protocols .......................................................... 18  
  1.5.4 Evaluation of ovarian function: .................................................................... 19  
    1.5.4.1 Antral Follicle Count ............................................................................ 19  
    1.5.4.2 Endocrine characteristics of ART cycles .............................................. 20
CHAPTER II: CLINICAL AND ENDOCRINE RESPONSES TO OVARIAN HYPERSTIMULATION IN FLARE AND LUTEAL GnRHα PROTOCOLS ....... 22

2.1 Introduction........................................................................................................ 22
2.2 Materials and methods.................................................................................. 24
  2.2.1 Subjects....................................................................................................... 24
  2.2.2 Methods....................................................................................................... 25
    2.2.2.1 Hormone assays: ................................................................................... 25
    2.2.2.2 Statistical analysis................................................................................. 26
2.3 Results............................................................................................................... 27
  2.3.1 Characterization of the study population: .................................................. 27
  2.3.2 Dynamic patterns of several hormonal concentrations during IVF cycles... 27
  2.3.3 Variation in follicular response between two protocols ......................... 36
  2.3.4 AMH and E2 as a marker of ovarian responsiveness............................... 39
  2.3.5 Factors influencing the hormonal value and the outcome in flare and luteal
      protocols......................................................................................................... 40
2.4 Discussion......................................................................................................... 42
  2.4.1 The follicular responses of the two protocols on ultrasound.................. 43
  2.4.2 The follicular responses of the two protocols evaluated by endocrine patterns
      ....................................................................................................................... 45
  2.4.3 The correlation between basal clinical, endocrine and ovarian responses... 51
  2.4.4 Summary....................................................................................................... 52

Chapter III SUMMARY AND CONCLUSIONS......................................................... 54
3.1 Summary........................................................................................................... 54
3.2 Conclusion........................................................................................................ 56

BIBLIOGRAPHY ....................................................................................................... 57
LIST OF TABLES

Table 2.1 Characterization of the study population .......................................................... 27
Table 2.2 Pearson correlation value between basal clinical, endocrine and ovarian
responses ......................................................................................................................... 41
LIST OF FIGURES

Figure 2.1. Hormonal dynamic patterns of (A) E₂, (B) FSH, (C) LH, (D) P, (E) D₄, (F) AMH throughout the treatment cycles.................................................................................................................. 29
Figure 2.2. Comparison of the outcome between the flare and luteal protocols ............ 37
Figure 2.3. The area under the curve of the hormonal data (A) AUC AMH, AUC D₄, AUC FSH, AUC LH, AUC P; (B) AUC E₂.................................................................................................................. 39
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>Anti-Mullerian Hormone</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted Reproductive Technologies</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3’5’-monophosphate</td>
</tr>
<tr>
<td>CCCT</td>
<td>Clomiphene Citrate Challenge test</td>
</tr>
<tr>
<td>COH</td>
<td>Controlled ovarian hyperstimulation</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>D4</td>
<td>Androstenedione</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E2</td>
<td>Estradiol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immuno Assay</td>
</tr>
<tr>
<td>ET</td>
<td>Embryo transfer</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth differentiation factor</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GnRHa</td>
<td>GnRH agonist</td>
</tr>
<tr>
<td>HCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HMG</td>
<td>Human menopausal gonadotropin</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin growth factor binding protein</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MIS</td>
<td>Mullerian Inhibiting Substance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OHSS</td>
<td>Ovarian hyperstimulation syndrome</td>
</tr>
<tr>
<td>P</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PCO</td>
<td>Polycystic ovaries</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2α</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>rFSH</td>
<td>Recombinant human FSH</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to express my deep appreciation to my supervisor, Dr. Basil Ho Yuen, for providing me the opportunity to pursue this study, for his patient guidance, support and encouragement over the last two years.

I would like to thank the supervisory committee members, Dr. Dan Rurak (Chair), Dr. Anthony Cheung and Dr. Peter Leung for their advice and encouragement. I would also like to thank Dr. Keith Choi for being an external examiner.

I would like to thank the staff in Dr. Ho Yuen’s lab; Mrs. Lydia Sy and Mrs. Theresa Yang for their support and for helping me in the technical aspects. I would also like to thank Songling Poon from Dr. Leung’s lab for her assistance in the AMH assay.

And finally I would like to thank my family for providing me continual support and always encouraging me, especially to my late father.
RATIONALE AND OBJECTIVES

Rationale

Two kinds of controlled ovarian hyperstimulation (COH) protocols, which remain the standard of care for in vitro fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI) patients, are the long and short protocol, also known as luteal and flare protocols, respectively. In both protocols, Gonadotropin-Releasing Hormone agonist (GnRHa) is administered before exogenous Follicle-Stimulating Hormone (FSH) administration to suppress the pituitary. In the flare protocol, GnRHa is given in the early follicular phase followed by FSH. On the other hand, in the luteal protocol, GnRHa is given from the mid-luteal phase of the preceding cycle. Because GnRHa is given continuously, different from the pulsatile pattern in normal cycles, first there is a flare-up effect with the increase in FSH and Luteinizing Hormone (LH) concentration but after that the continuous exposure to GnRHa inhibits pituitary hormone secretion. Normally pituitary desensitization is generally achieved within 5 days of initiating GnRHa treatment (Bider et al., 1989). In the luteal protocol, because of the long time exposure to GnRHa, the pituitary desensitization is more profound than in the flare protocol. Luteal protocols based on complete pituitary suppression, maintaining plasma FSH levels low so that all recruitable follicles remain at a resting state, prior to inducing ovarian stimulation while with flare protocols due to incomplete pituitary down regulation there is a greater variation in the degree of follicular maturation as compared to luteal protocol at the time of FSH administration give rise to the difference in the follicular response between the two protocols.

We hypothesized that there is a difference in the follicular response in flare versus luteal protocols for controlled ovarian hyperstimulation during IVF/ICSI. The ovarian response in luteal protocol will be superior to flare protocol due to the differences in the degree of pituitary suppression and thus the follicular synchronization between the two protocols at the beginning of the treatment.

Objectives

This hypothesis was tested by:

1) characterizing the serum estradiol (E₂), FSH, LH, progesterone (P), androstenedione (D₄), Anti-Mullerian Hormone (AMH) level, follicle data (from
ultrasound observations) in flare versus luteal protocol to evaluate ovarian follicle response between flare and luteal protocols,

2) determining the correlation of the baseline clinical data, total FSH doses, hormonal values and the outcome in flare versus luteal protocols.

Our results provide data on dynamic patterns of a set of reproductive hormones, especially AMH - a novel hormone for prediction of ovarian reserve - in the luteal and flare protocols. This may be useful for further studies to assess AMH profile in IVF/ICSI cycle monitoring and validate the usefulness of AMH in clinical practice.
CHAPTER I: GENERAL INTRODUCTION

1.1 Assisted reproduction

From its infancy until now, more than 1.5 million IVF babies were born worldwide. This approach helps millions of infertile couples to have their own biologic children. The need for IVF and its derivatives never stops growing, since up to 10-15% of couples may suffer from some form of infertility (Speroff and Fritz, 2005). However, assisted reproductive technologies (ART) therapy is still an expensive therapy whether the source of funding is public insurance, private insurance or family income. In most developed countries it costs about 15% of the average family’s total annual income for a single ART cycle (Collins, 2004).

In addition, there are the emotional and psychological stresses that both patients and health care providers have to undergo during the treatment cycles. This is particularly important when the cycles fail.

1.2 Ovarian responsiveness

Ovarian stimulation is an integral part of assisted reproduction treatments. Ovarian response to gonadotropin treatment, besides other factors, determines the outcome of treatment, as the number and quality of oocytes retrieved are related to the chance of achieving a pregnancy. There are a number of patient-related factors which might predict ovarian response, factors such as age of the patient and antral follicle count. In addition, it has been shown that genetic factors such as the patient's FSH-receptor genotype also determine individual response to FSH treatment (Sudo et al., 2002; Greb et al., 2005; de Koning et al., 2006; Jun et al., 2006; Loutradis et al., 2006; Wunsch et al., 2007). Besides patient-related factors, the choice of drugs for ovarian stimulation plays a significant role. However, accurate prediction of ovarian response is still a topic of clinical concern. There still exists unpredictable “poor responders” and so far no consensus has been found concerning the optimal stimulating protocol for those women.
1.3 Normal ovarian physiology (ovarian function)

1.3.1 Folliculogenesis

1.3.1.1 Stages of folliculogenesis

In the human, folliculogenesis starts when nongrowing follicles leave the ovarian reserve. This culminates with the production of a single dominant follicle during each menstrual cycle. This process can be divided into three main steps; (1) initiation, (2) early follicle growth, and (3) selection and maturation of the preovulatory follicle.

At birth, the ovaries contain about 2 million follicles. This endowment is progressively depleted as the follicles leave the primordial follicle pool to enter the growing phase. However, during an average woman’s reproductive lifetime only around 400 follicles sequentially mature and ovulate. The majority of these growing follicles will be lost as a result of atresia. The initiation of growth of primordial follicles occurs continuously and randomly. The mechanism for determining which follicles and how many will start growing during any one cycle is unknown. This initial recruitment is a process that starts just after follicle formation, long before pubertal onset. When follicles enter the growth phase, the oocyte enlarges, and the granulosa cells proliferate markedly from flattened granulosa cells to single and multiple layers of cuboidal granulosa cells. It takes several months to develop from the primordial to the preovulatory stage (Gougeon, 1996; McGee and Hsueh, 2000). In terms of morphology, the follicles are categorized into primordial, primary, preantral and antral follicle stages. Primordial follicles are 50 µm in diameter. When they become preantral follicles, their size increase up to 2-5 mm in diameter, and they continue growing, reaching 20mm in diameter at the preovulatory stage. However, in terms of the responsiveness of the follicles to FSH stimulation, there are two main stages: the first stage is FSH-independent and the second stage is FSH-dependent. Follicular growth to the stage of antrum formation is independent of gonadotrophic stimulation. This notion is confirmed in studies of hypophysectomized women in whom preovulatory Graafian follicles develop within two weeks after ovarian stimulation with gonadotropins (Schoot et al., 1992). Antrum formation and further growth to the stage at which follicles acquire the capacity to reach full maturation require tonic stimulation by FSH. Prior to the onset of puberty, blood concentration of FSH do
not rise sufficiently to sustain development beyond this stage. Therefore many antral follicles become atretic. After puberty, as each menstrual cycle begins, FSH concentration rises beyond a critical “threshold”, and multiple follicles are recruited to begin a more advanced stage of maturation. Since preantral folliculogenesis is quite asynchronous, because of the continuous exit of follicles from the primordial pool, there will always be a maturationally distinct distribution of early antral follicles within the ovaries. Therefore, there are always some follicles available for continued maturation under the influence of FSH.

Responsiveness to FSH is only acquired once granulosa cells are formed. And as FSH concentration rises during the early follicular phase, follicles will be stimulated. When estrogen concentration increases, FSH secretion will be inhibited, thus depriving other follicles of their gonadotropin support. Due to the increase in its responsiveness to FSH and LH, one of these follicles becomes selected to ovulate while the remainder becomes atretic. Through indirect observational study, it is believed that the cohort size of healthy early antral follicles recruited during the luteo-follicular transition is around 10 per ovary (Hodgen, 1982; Pache et al., 1990). While granulosa cells from early antral follicles respond only to FSH, those from maturing dominant follicles show receptors to both FSH and LH, and are therefore responsive to both gonadotropins (Sullivan et al., 1999; Filicori et al., 2002b). At the mid-follicular phase, the dominant follicle reaches 10mm in diameter, and increasingly synthesizes estradiol. According to the two-cell theory, estradiol is synthesized from the substrate androstenedione derived from the theca cells by the induction of the aromatase enzyme. Tonic stimulation by FSH and LH maintains estrogen secretion by the dominant follicle, which grows to 20mm in diameter before it ovulates in response to the mid-cycle LH surge.

The mechanism in which only one follicle is recruited, dominant and finally ovulated is related to the concepts of FSH threshold and LH ceiling. Each growing follicle possesses a threshold requirement for stimulation by circulating FSH that must be surpassed to ensure ongoing preovulatory development (Macklon and Fauser, 1998). The follicle which is destined to be ovulated is more sensitive to the FSH level than the others, in other words, its FSH threshold is lower than the remaining follicles. This helps it to continue growing while the FSH level keeps decreasing as the result of estrogen negative
feedback. The maturing follicular development also needs a certain LH level, but not sufficient to exceed the LH ceiling, at which the ovulation might be triggered (Hillier, 1994; Shoham, 2002).

1.3.2 Two-cell two-gonadotropin theory

As the follicles develop to the preantral and antral stage, the granulosa cell layer works in concert with the theca cell layers to produce estradiol, a phenomenon well-known as the two-cell two-gonadotropin theory (Hillier et al., 1980). LH receptors are found mostly on the theca cells and FSH receptors only on the granulosa cells (Kobayashi et al., 1990). Tonic LH concentration is required to stimulate theca cell androgen production. Since granulosa cells do not express enzyme P450c17, which is necessary for the conversion of 21-carbon substrate to androgens, they are dependent on the source of androgens from the theca cell, in order to make estrogen. However, the theca cells themselves lack the enzyme P450arom to convert androgens to estrogen, and this process is stimulated by the rising of FSH levels (Sasano, 1994). Studies on primates (Karnitis et al., 1994; Zelinski-Wooten et al., 1995) have shown that follicles still develop after administration of FSH to monkeys made LH deficient by using GnRH antagonist. Estradiol was also produced but in very limited amounts, mostly from the androgens supplied from the adrenal glands. These results confirmed the essential role of FSH in follicular recruitment. With further growth of the follicle, FSH induces the development of aromatase-linked receptors on granulosa cells.

1.3.3 Luteal function

The estradiol levels increase sharply as the follicles go further to the preovulatory stage. Peak levels of estradiol are achieved and the onset of the LH surge occurs. This surge promotes the luteinization of the granulosa cells in the dominant follicle. This increases progesterone receptor expression in granulosa cells, and results in the expression of vascular endothelial growth factor (VEGF) (Doldi et al., 1997; Anasti et al., 1998). Progesterone is the main hormone during the luteal phase, reaching peak level 7 days post ovulation. If implantation takes place, human chorionic gonadotropin (hCG), produced by the developing trophoblast, ensures progesterone production from the corpus luteum until the placenta is fully formed, and can take over this hormonal function. If
implantation does not take place, the corpus luteum regresses and this results in a fall in progesterone and estrogen levels, which lead to the menses, and another cycle starts.

1.3.4 Intraovarian regulators:

Whereas gonadotropin and steroid hormones play important roles throughout folliculogenesis, it is the contribution of locally produced ovarian factors that allows individual follicles to grow and develop within this hormonal milieu. The most exciting progress that has been made during the past few years is the unraveling of the complex intraovarian control mechanisms, especially those produced by the oocyte. These mechanisms coordinate folliculogenesis, including activation of resting follicles, early growth, and terminal maturation.

1.3.4.1 Prostaglandin

Prostaglandins (PGs) are produced in the follicle during folliculogenesis, and are important intrafollicular regulators that control proliferation and differentiation (Li and Tsang, 1995). However, PGs are more well-known as the key mediators in the ovulatory cascade.

1.3.4.2 Insulin and Insulin Growth Factor

The insulin growth factor (IGF) system is composed of two ligands, IGF-I and IGF-II, two receptors, type I and type II, and six IGF-binding proteins (IGFBPs). These IGFBPs bind IGF-I and -II with high affinity and decrease IGF bioavailability (Monget and Monniaux, 1995). In the human ovarian follicles, the predominant IGF is IGF-II and its actions are modulated by insulin-like growth factor-binding protein-4 (IGFBP-4), the IGFBP-4 protease, and the pregnancy-associated plasma protein-A (PAPP-A). These peptide components are synthesized by the granulosa cells of the developing follicle (Alexiadis M et al., 2006).

IGF-II is synthesized in theca cells in small antral follicles and in granulosa cells in dominant follicles. In small antral follicles, IGF-II acts in an autocrine fashion in theca cells and in a paracrine fashion in granulosa cells. When the follicles become dominant, granulosa-derived IGF-II acts in an autocrine manner in granulosa cells. IGFs amplify the effects of FSH when follicles enter into the gonadotropin-dependent stages of follicular
development. It potentiates FSH-stimulated cAMP production, aromatase activity and LH receptor expression by granulosa cells. In the late stages of folliculogenesis, the decreases in IGFBPs lead to the increase in IGF bioavailability, and further intensify FSH action (el-Roeiy et al., 1993; Monget et al., 1996; Monget and Bondy, 2000).

1.3.4.3 Interleukin

In the human ovary interleukin-1 (IL-1) is expressed in the granulosa cells, and its actions in the human follicle during folliculogenesis are presumably both on the granulosa and theca cells because IL-1 receptors are found in both these cells (Hurwitz et al., 1992). It was demonstrated that IL-1 promotes granulosa cell proliferation, suppressing apoptosis, enhancing glucose uptake, DNA synthesis, and it facilitates angiogenesis. It inhibits FSH-induced estradiol synthesis and progesterone production from granulosa cells. It also inhibits gonadotropin-stimulated androstenedione production in the theca cells, and also FSH-induced LH receptor formation in immature granulosa cells. IL-1 increases PG production, PGE more than PGF2α. PGF2α is also involved in granulosa cell proliferation and differentiation. Besides this, IL-1 plays a role in inducing ovulation.

1.3.4.4 Transforming Growth Factor β superfamily

Transforming Growth Factor β (TGF-β) superfamily is a family of peptides known to be involved in the regulation of ovarian development and function. It consists of more than 35 structurally related members, and has a key role in multiple aspects of follicle development. These aspects include several different functions: primordial follicle recruitment, granulosa and theca cell proliferation/atroresia, steroidogenesis, gonadotropin receptor expression, oocyte maturation, ovulation, luteinization and corpus luteum formation. The TGF β superfamily is widely distributed throughout the body, its proteins function as extracellular ligands involved in numerous physiological processes during both pre and postnatal life. Based on their structural characteristics, members of this superfamily have been further classified into several subfamilies. There are six subfamilies which include the prototypic TGF-β subfamily (comprising TGF-β1, TGF-β2, TGF-β3), an extensive bone morphogenetic protein (BMP) subfamily (with some 20 members), the growth and differentiation factor (GDF) subfamily (at least nine members),
the activin/inhibin subfamily (including activin A, AB, B, inhibin A, B) the glial cell-
derived neurotrophic factor (GDNF) subfamily (including GDNF, artemin and neurturin),
as well as several additional members such as AMH; also known as MIS. Not all of these
factors are present at every stage of follicular transition.

1.3.4.4.1 Activin, inhibin

In addition to their feedback action on the pituitary FSH, activin and inhibin also
play a role in ovarian follicle growth and maturation (Miro and Hillier, 1996; Zhao et al.,
2001). Activin stimulates FSH-induced granulosa cell growth and early follicle growth,
while inhibin stimulates late follicle growth. Note that there is a shift of activin control at
the early stage, to the inhibin influence at the later stage of follicle development.

Activin and inhibin also regulate steroidogenesis in the ovary by upregulating
FSH receptor expression in granulosa cells, and augments LH-stimulated androgen
production in theca cells.

1.3.4.4.2 Bone morphogenic proteins

Bone morphogenic proteins (BMPs) comprise almost half the members of the
TGFβ superfamily, not all of which have been shown to act in the ovary.

BMP-4 and BMP-7 are expressed by ovarian stromal cells and/or theca cells and
act as positive regulators of the primordial-to-primary follicle transition, and they
enhance follicle survival (Lee et al., 2001).

BMP-15, an oocyte-derived growth factor, has been shown to stimulate
proliferation of undifferentiated granulosa cells in an FSH-independent manner (Otsuka
et al., 2000). However, the role of BMP-15 in early preantral follicle development is
species variable; studies on mice with null mutation in the BMP-15 gene are only mildly
subfertile with a weak ovarian phenotype. In contrast, ewes homozygous for
inactivating BMP-15 mutations are completely infertile, with follicle development
arrested at the primordial stage (Yan et al., 2001; Juengel et al., 2002).

In addition to their well-documented roles in early follicular development, there is
increasing evidence to support critical roles of BMPs in growing antral follicles. BMP-6
suppresses FSH action; its absence at this time would be necessary for continued follicle
development, via the action of FSH (Otsuka et al., 2001a; Otsuka et al., 2001b). BMP-6
and BMP-15 inhibit gonadotropin-stimulated progesterone secretion. BMP-2 promotes estradiol, and inhibin A secretion (Souza et al., 2002).

In general, multiple BMPs with granulosa, oocyte or thecal origin interact in a complex manner to promote follicle survival by maintaining cell proliferation and preventing premature luteinzation and/or atresia.

1.3.4.4.3 Growth differentiation factor 9

Growth differentiation factor -9 (GDF-9), similar to BMP 15 and BMP-6, is derived from the oocyte in early stage follicles. GDF-9 has an obligatory role in promoting primary follicle progression. Studies on mice with a null mutation in the GDF-9 gene are infertile, and show arrested follicle development at the primary stage (Dong et al., 1996). This conclusion is confirmed by the finding that GDF-9 treatment in vivo or in vitro enhances the progression of early to late stage primary follicles in the rat (Vitt et al., 2000; Nilsson and Skinner, 2002). The effect of GDF-9 on steroidogenesis is similar to that of BMP-15.

1.3.4.4.4 Anti-mullerian hormone

The study of Durlinger et al showed that the ovaries of mice null for anti-mullerian hormone (AMH) contain fewer primordial follicles, and three times more growing follicles than their wildtype counterparts. This suggests a release of inhibition on primordial follicle recruitment (Durlinger et al., 1999). In addition, the exposure of neonatal mouse ovaries to AMH will decrease the number of growing follicles (Durlinger et al., 2002a).

AMH is first expressed by the granulosa cells of primary follicles, and it continues to be expressed by granulosa cells until the early antral stage of development (Baarends et al., 1995; Durlinger et al., 2002a), implying a continued functional involvement in follicle development. At the same time, AMH inhibits granulosa cell proliferation in preantral and small antral follicles through attenuating the responsiveness of preantral and small antral follicles to FSH (Durlinger et al., 2001). Thus, AMH may play a role in the selection of the dominant follicle (Visser and Themmen, 2005). The expression of AMH by growing follicles is variable (Baarends et al., 1995). Those that express reduced levels of AMH may be more sensitive to FSH, and thus more likely to be
selected for continued growth up to the preovulatory stage, with one follicle attaining dominance.

1.3.4.4.5 Gonadotropin-Releasing Hormone

There are a number of Gonadotropin Releasing Hormones (GnRH). They are all decapetides. They were first found in the hypothalamus, and play a key role in the process of reproduction. The amino acid sequence of GnRH-1 has been shown to be in the order pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2. It is produced by hypothalamic neurosecretory cells and released in a pulsatile manner into the hypotalamo-hypophyseal portal circulation. This hormone is responsible for increase in synthesis, storage, activation and secretion of leutinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH synthesis is under the control of catecholaminergic neurons, which produce noradrenalin or dopamine as neurotransmitters. Its activity is modulated, partly by auto stimulation of its own receptor and partly by short feedback loops from the pituitary, but mainly by the ovarian steroids i.e. progesterone (P) and estradiol (E2) (Cheng and Leung, 2005)

1.3.4.4.5.1 Forms of GnRH

The most widely recognized and common structural variation among the different forms of GnRH resides in amino acids between 5 and 8 in the sequence.

The hypothalamic GnRH, which is also referred to as GnRH-I or type one mammalian GnRH, was first isolated and sequenced during the early seventies.

The second type, a midbrain GnRH or GnRH-II was first identified in the chicken brain, and is also referred to as chicken GnRH-II or cGnRH-II (Millar et al., 2004). It is structurally conserved in species ranging from teleost fish to humans. This second GnRH form differs from GnRH-1 by three amino acid residues at position 5, 7, and 8(His5Trp7Tyr8 GnRH-1) (Pfleger et al., 2002). One of the specific biological functions of GnRH-II is to serve as a potent inhibitor of K+ channels in the amphibian sympathetic ganglion. Inhibition of these ion channels facilitates rapid excitatory transmission of conventional neurotransmitters, and may provide a general neuromodulatory mechanism for GnRH-II in the nervous system (Millar, 2003).
The third type, a telencephalic GnRH, also called type III GnRH or GnRH-III, and exists preferentially in the terminal part of the olfactory neuronal cell in the brain (Troskie et al., 1998; Millar et al., 2004).

1.3.4.5.2 Ovarian modulator:

GnRH is also thought to be an important paracrine/autocrine gonadal regulator and its receptor mRNA has recently been characterized in human granulosa cells (Cheng and Leung, 2005). In the ovary, GnRH is considered to act differently during follicular maturation, and has been shown to be both steroidogenic and antisteroidogenic. It has been shown to stimulate basal steroidogenesis, but also to attenuate Gonadotropin-induced cAMP and progesterone production. There are different reports demonstrating the effect of this peptide on ovarian steroidogenesis. Acute stimulatory effects on progesterone production are seen in both cultured rat and human granulosa and luteal cells. However, an inhibitory effect of LH-stimulated progesterone production has also been shown to be leutolytic under in vivo and in vitro conditions.

In addition to its classic function in stimulating pituitary gonadotropin secretion, GnRH I also acts as a local autocrine/paracrine factor in the human ovary; where it regulates steroidogenesis, cell proliferation and apoptosis (Cheng and Leung, 2005).

1.4 Female infertility and ovarian reserve

1.4.1 Epidemiology

The infertility rate worldwide is around 10-15% (Speroff and Fritz, 2005). Besides male-factor and unexplained infertility, female infertility accounts for about one third or more of the cases of infertility.

Nowadays, there is an increase in the proportion of women over 35 seeking professional help because of infertility. In 1995, statistics showed that one of every five women in the United States is having a first child after 35, a marked increase over earlier years (Speroff and Fritz, 2005). It has not included yet the majority of women with impaired fecundity who had not obtained any professional services for any reason. This reflects both the deferment of marriage and postponement of pregnancy in marriage as women in modern society have a tendency to commit to the work place.
Thus a major problem we are facing now concerning female infertility is advanced maternal age. It is obvious that aging impacts on fertility by the depletion of ovarian reserve; problems can involve either quantity or quality, or both. Furthermore, even when pregnancy is achieved in these women, the greatest obstacle is still the risk of higher rate of spontaneous abortion.

1.4.2 Oogenesis and the establishment of ovarian reserve

In the human, oogenesis starts in the second month of pregnancy after the migration of the primordial germ cells to the gonadal ridges, where sexual differentiation occurs. The primordial germ cells continue to proliferate in the embryonic ovary, and become oogonia. By the twentieth week of pregnancy, about 7 million oogonia are present; thereafter some will undergo mitotic and meiotic division and become arrested at the diplotenne stage of prophase I; these are called primary oocytes (Wise et al., 1996; Alberts et al., 2004). The oocytes are surrounded by a layer of epithelial pregranulosa cells to form primordial follicles. Most of these follicles remain in the resting stage until they either degenerate or enter the growth phase. After birth, the pool of follicles at the resting stage constitutes the ovarian reserve. At birth, the size of the ovarian reserve varies among individuals. The number of oocytes present immediately after birth is thought to be finite, and to represent the total number that a female is allocated for her lifetime (Wise et al., 1996). However, the recent evidence has suggested that ovarian germ cells can be renewed in the adult (Johnson et al., 2004; Oktem and Oktay, 2008).

In humans as in other mammals, the size of the ovarian reserve decreases dramatically with age, either by apoptosis or by entry into the growth phase. The rate of follicular depletion might vary among subjects, and accelerates from approximately 38 years of age onward. This leads to a stock at menopause estimated at between less than 100 and 1000 resting follicles per ovary (Faddy et al., 1992; Gougeon et al., 1994). The range of the menopausal age between women may be related to variable depletion rates, differences in the initial number of resting follicles, or both. It is recognized that the age at which menopause occurs is genetically programmed, as the menopausal age of a given woman is correlated with the timing of menopause in her mother and sisters. In addition,
it is also influenced by parity and environmental factors such as nutrition, race, and socioeconomic status (Fauser, 2000).

1.4.3 Tests for ovarian reserve

When the ovarian reserve is reduced, the induction of multifollicular growth remains a challenge. Among several factors which could be associated with reduced ovarian response, reduced ovarian reserve, either in older patients or in young patients, represents the most frequent etiological factor. Whatever is the etiology, one of the main problems is how to identify potential low responders. Therefore, analysis of the ovarian reserve of the patient in ART becomes clinically important. It is mandatory to tailor the best ovarian stimulation regimen. Ovarian reserve can be considered normal where stimulation with the use of exogenous gonadotropins will result in the developing of at least 8-10 follicles, and the retrieval of a corresponding number of healthy oocytes at oocyte pick-up (Fasouliotis et al., 2000).

Advancing female age is an important determinant of decreased ovarian reserve or responsiveness. In addition to the female age, other parameters of ovarian function need to be considered in the assessment of ovarian responsiveness. Antral follicle count can reflect more accurately the actual functional ovarian reserve than the women’s age. Unfortunately this tool depends a lot on the sonographer’s skill. The Clomiphene Citrate Challenge test (CCCT) (Navot et al., 1987) and exogenous FSH and gonadotropin agonist stimulation tests are other methods that have been used to evaluate the ovarian reserve. Nevertheless, as the performance of these tests leaves much to be desired, there is a clear need for better tests to predict ovarian reserve. These following tests are those currently available and applied to evaluate ovarian reserve: ovarian volume, ovarian blood flow, antral follicle count (Syrop et al., 1995; Zaidi et al., 1996; Tomas et al., 1997), basal FSH (Muasher et al., 1988; Scott et al., 1989), estradiol (Licciardi et al., 1995; Smotrich et al., 1995), inhibin B (Seifer et al., 1997) and recently AMH testing (Seifer et al., 2002).

As the women’s age advances, the growing follicle population declines and its associated loss of estrogen and inhibin feedback to the pituitary leads to an augmentation of FSH levels. Therefore elevated FSH levels on cycle day 3 (greater than 12 IU/L) are associated with poor performance in IVF (Toner et al., 1991). However, basal FSH
concentration varies dramatically from cycle to cycle, and it is believed that a significant diminution of the ovarian follicle reserve has already occurred by the time FSH level changes. On the other hand, due to the negative feedback of E₂ on FSH level, the validity of FSH measurement depends on the time in the cycle when the sample is collected, optimal when the E₂ level is lowest. As a result, it is necessary to combine basal day 3 FSH and E₂ measurement to obtain a higher performance test. Furthermore, Frattarelli and colleagues proved that elevated basal E₂ concentration alone cannot predict the outcome (Frattarelli et al., 2000).

Inhibin B is secreted by the granulosa cells of small, FSH-selected antral follicles, and its serum levels are high during the early follicular phase of the menstrual cycle; this reflects the number and health of follicles recruited from the primordial pool. The population of primordial follicles declines after many rounds of follicle selection, atresia, and ovulation; as a result, less inhibin B is produced. Therefore, inhibin B levels are indicative of ovarian reserve. Seifer et al found low day 3 serum inhibin B concentration was associated with a reduction in the number of eggs retrieved, a higher cancellation rate, and a decreased clinical pregnancy rate (Seifer et al., 1997). In contrast, another study by Scott et al evaluating the predictive value of basal inhibin B levels in 292 ART patients showed that inhibin B concentration did not add any significant clinical value beyond that obtained by basal FSH (Scott, 2004). Because of that, we still need to search for another better marker for ovarian reserve; then came AMH.

Basal FSH and AFC are commonly employed in the assessment of ovarian reserve. Recently AMH has emerged as a potentially important test of ovarian reserve. A meta-analysis concluded that AMH may be comparable to AFC for this purpose (Broer et al., 2008). AMH, similar to inhibins and activins, is a member of the TGFβ superfamily. It is also produced by the granulosa cells in preantral and small antral follicles. It inhibits the initial recruitment of primordial follicles into the growth pool and reduces the sensitivity of the growing follicles to FSH. Compared to FSH, estradiol, and inhibin, one of the greatest advantages of AMH is that its level is relatively stable during the menstrual cycle. However, it starts declining slowly far before menopause actually happens. This is prior to the other hormonal changes, or the symptoms of menopause. It is the advantage of AMH that it can be considered a superior marker for prognostic ovarian reserve. It is
found that AMH level is strongly correlated with the number of antral follicles seen on ultrasound scan and the number of oocytes retrieved (van Rooij et al., 2002). There was also a strong negative association between low AMH levels and poor ovarian response to stimulation.

1.5 **IVF protocols**

1.5.1 **Controlled ovarian stimulation and ovarian induction**

Ovarian stimulation first used for the anovulatory cases, nowadays is also used in controlled ovarian hyperstimulation and in vitro fertilization-embryo transfer (IVF-ET). Different from ovulation induction, in which the idea is to induce ovulation of only one single dominant follicle from stimulation, in IVF treatment the purpose of ovarian stimulation is to induce as many mature healthy oocytes as possible, with lesser complications such as ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies.

The strategy of IVF stimulation regimens is to override the natural process of unifollicular recruitment and maturation (characteristic of the human cycle) by manipulating ovarian exposure to FSH and LH during the follicular phase of the ovarian cycle.

1.5.2 **Drugs used for stimulation of ovulation:**

1.5.2.1 **FSH, LH:**

hMG (human menopausal gonadotropin) was first purified from postmenopausal urine by Donini and was named Pergonal®. It contains an equivalent amount of 75 IU FSH and 75 IU LH in vitro bioactivity. However, when extracted from urine, < 5% of the total protein content is the major active agent, FSH. The specific activity of these products does not usually exceed 150 IU/mg protein (Giudice et al., 1994).

Although hMG preparations are effective and relatively safe, they showed local side effects such as pain and allergic reactions, probably due to protein impurities (Li and Hindle, 1993). Moreover, the high content of LH associated with FSH in hMG preparations causes some premature LH surge, and thus uncontrolled ovulation which is the worst failure for IVF treatment.
With the development of new technologies, urinary FSH (Metrodin ®) and highly purified FSH became available by using specific monoclonal antibodies to bind the FSH and LH molecules in the hMG material in such a way that unknown urinary proteins could be removed. Metrodin has a specific activity of 100-200 IU of FSH/mg of protein, whereas Metrodin HP (highly puried) has an activity of approximately 9000IU/mg of protein.

A significant breakthrough in ART medicine was the appearance of recombinant DNA technology. This provided a purified human FSH from cell culture supernatant. This new rhFSH (Puregon®, Gonal F®) helps avoid the side-effects of LH in ART treatment (Jennings et al., 1996; Rose, 1998).

1.5.2.2 hCG:

hCG has been used as a surrogate LH surge to trigger final ovulation based on the degree of homology between the two hormones. FSH, LH and hCG have the identical structure of the α subunits; only the β subunits are unique to each hormone, and once linked to the α chain determine specific hormone function (Pierce et al., 1976; Ryan et al., 1988). Because the plasma metabolic clearance rate of hCG is slower than that of LH, it is more stable and long lasting as a therapeutic agent.

1.5.2.3 GnRH and GnRH agonist

GnRH is secreted from the hypothalamic neuronal cells in a pulsatile fashion and it acts via the coupled G-proteins. However, because native GnRH has a short plasma half-life and is rapidly inactivated by enzymatic cleavage, its analogs were created by a structural change at the position of enzymatic breakdown; this allows the longer half-life and higher receptor activities. Although more than 1000 GnRH analogs have been synthesized and tested, only a few have been introduced into clinical practice. Since all GnRH agonistic analogs are small polypeptide molecules which are very susceptible to gastrointestinal proteolysis, they need to be administered parenterally. It is the methods of administration and potency that makes these analogs different. The biopotency of the oral and rectal administration of analogs is very low, about 0.0 – 1% compared to the parenteral administration. Intranasal spray is extremely effective, however its bioavailability is only 3-5% of subcutaneous administration (Lemay et al., 1983).
Because of the relatively fast elimination of intranasal spray, it requires frequent doses to obtain continuous stimulation and downregulation, for example Nafarelin is given twice a day. In contrast, intramuscular injections could maintain therapeutic levels for 28-35 days. Thus monthly injections are sufficient for maintaining downregulation. Acute administration of GnRH agonistic analogs increases gonadotropin secretion (the “flare up” effect) and usually requires 7-14 days to achieve a state of pituitary suppression. Prolonged administration of GnRH agonistic analogs leads to downregulation of GnRH receptors, which was first described by Knobil and coworkers (Belchetz et al., 1978).

1.5.3 Controlled hyperstimulation protocols

It is well documented that reproductive function declines as the women become older. That age-related diminution in reproductive potential is related to changes at the oocyte level rather than at the uterine level. Ovarian responsiveness is the most important factor to help in deciding cycle cancellation or to increase or decrease the dosage of exogenous gonadotropins. A decline in ovarian responsiveness might be due to a decrease in quantitative ovarian responsiveness, or a decrease in ovarian reserve. The parameters of ovarian response include the number of mature follicles, i.e., potential oocytes.

So far, there is no consensus in the literature on the definition of the “low responder” or “poor ovarian reserve” (Olivennes et al., 1993; Ben-Rafael et al., 1994). Some authors regard less than four mature follicles at the time of human chorionic gonadotropin (hCG) administration, or a peak estradiol (E$_2$) of less than 500pg/ml, as “low responders” or “poor ovarian reserve” (Scott, 2004). However, this is not always the case.

The protocol used in the early days of IVF is the combination between clomiphene citrate and FSH. FSH preparations initially used were hMG (containing both LH and FSH bioactivity), later followed by purified urinary FSH, and more recently, recombinant FSH. One concern of the clinician in IVF treatment is the occurrence of premature LH surge during IVF cycles that could lead to an uncontrolled period of ovulation. The emergence of recombinant FSH (no LH included) partly helps to solve that problem and results in better outcomes in protocols using rFSH instead of urinary
FSH (Daya et al., 1995; Out et al., 1995; Recombinant Human FSH Study Group, 1995; Bergh et al., 1997; Daya and Gunby, 1999; Hoomans et al., 1999; Frydman et al., 2000). Later on, the development of GnRH agonist, and its application into the IVF cycles, avoids the exposure to high LH which resulted in premature luteinization of follicles. Today, the most used protocol for IVF cycles is long pituitary downregulation with the GnRH agonist, also called luteal protocol because GnRH is administered from the mid-luteal phase of the previous cycles (Daya, 2000). Flare protocols, in which GnRH agonist is administered at the beginning of the cycles, are not as successful for the average ART patient as the luteal phase suppression protocols. However, to some extent, they do offer an opportunity to obtain controlled ovarian hyperstimulation in some patients who cannot be stimulated with other protocols (Greenblatt et al., 1995).

1.5.4 Evaluation of ovarian function:

1.5.4.1 Antral Follicle Count

Several studies have attempted to correlate certain morphological features on baseline ultrasound with subsequent response to ovulation stimulation. Syrop and colleagues found a correlation between total ovarian volume and peak E₂ concentration, number of eggs retrieved, number of embryos obtained, and clinical pregnancy rate. However, because this study did not exclude patients with polycystic ovaries (PCO), it is believed that it is the PCO patients who exhibit an exaggerated response that accounted for the correlation (Syrop et al., 1995). Subsequent studies confirmed the same result: a relationship between ovarian volume and the ovarian response even after excluding the PCO patients. They concluded that the women with more antral follicles at the beginning of the cycle and larger ovaries, will develop more follicles, and have more embryos for transfer. There would also be a higher clinical pregnancy rate, but unfortunately a higher risk of developing ovarian hyperstimulation syndrome (Danninger et al., 1996). Tomas and colleagues even reported that the baseline antral follicle count was more representative of the actual functional ovarian reserve than the women’s age (Tomas et al., 1997). Recent introduction of 3-D ultrasound into practice provides a better tool for sonographic ovarian assessment (Kyei-Mensah et al., 1996).
From studies investigating the effect of follicular size on collection, fertilization and pregnancy rates, the authors concluded that optimal oocyte recovery and fertilization rates can be obtained from follicles between 14 and 24 mm in diameter on the day of oocyte collection. When the follicles exceed 24 mm or remained lower than 14 mm in diameter, the oocyte recovery rate starts to decrease (Tan et al., 1992a; Wittmaack et al., 1994).

1.5.4.2 Endocrine characteristics of ART cycles

The frequently used means for monitoring IVF cycles, nowadays, is ultrasound and hormonal evaluation. The early predictive values for pregnancy and cancellation rates currently applied include age, and basal day 3 serum FSH concentration. Also, later predictive values in the cycle are the magnitude of elevated serial serum E\textsubscript{2} measurement, in combination with the responsive growing follicles determined by ultrasound. A large retrospective study has shown that pregnancy rates decreased markedly as basal day 3 FSH concentrations rose (Scott et al., 1989).

Among the methods used clinically to monitor follicular maturation in IVF cycles, combined serum E\textsubscript{3} and ultrasound is the widest method applied. No study has shown that either serum E\textsubscript{2} or ultrasound alone is superior to the other for monitoring follicular maturation in IVF cycles (Wikland and Hillensjö, 2004).

Estradiol, the major estrogen secreted by the human ovary, is converted from both testosterone and androstenedione derived from theca cells in response to LH. Yet, testosterone is also produced from androstenedione. Androstenedione, therefore, is considered as a main precursor for estradiol synthesis, and it can reveal the degree of LH suppression.

More recently, antimullerian hormone (AMH) is considered as the best marker for evaluating the ovarian reserve, and hence the responsiveness of ovarian stimulation. AMH, also known as mullerian inhibiting substance (MIS) belongs to the transforming growth factor- β (TGF-β) superfamily. During fetal development in males, AMH is essential for the regression of the Mullerian ducts, the anlagen of the female genital tract (upper part of the vagina, uterus and fallopian tube). In men, AMH is expressed strongly in the Sertoli cells from the moment of testicular differentiation during fetal development.
until puberty, when it starts to decline. In contrast, in women AMH is produced by ovarian granulosa cells from about week 36 of gestation, peaks during reproductive life, and then steadily decreases approaching menopause (Lee et al., 1996; Teixeira et al., 2001).

Studies in AMH knock-out mice compared to wild-type mice proved that AMH has an inhibitory effect on two periods of follicular development. It inhibits the initiation of primordial follicle recruitment, as well as the responsiveness of growing follicles to FSH (Durlinger et al., 1999; McGee and Hsueh, 2000; Durlinger et al., 2001). On the other hand, there was also evidence that AMH can induce the growth of preantral follicles (McGee et al., 2001).

AMH expression starts in the granulosa cells of primary follicles, is highest in secondary, preantral and small antral follicles, and gradually diminishes in the subsequent stages of follicle development (Weenen et al., 2004). Concentrations of AMH are much higher in small antral follicles than preovulatory follicles, and this accounts for the observed fall in circulating AMH after gonadotropin stimulation.

Whereas concentrations of other ovarian hormones vary considerably through the menstrual cycle, AMH levels do not show substantial fluctuation. Compared to FSH and inhibin B; serum AMH levels on day 3 of the menstrual cycle are best correlated with antral follicle counts (de Vet et al., 2002; La Marca et al., 2006). The absence of serum AMH in ovariectomized women demonstrates that the ovary is the only source of the circulating AMH (La Marca et al., 2005). Studies show that AMH serum levels were significantly and inversely correlated to FSH serum levels. It is appropriate to the preceding studies that AMH inhibits the responsiveness of growing follicles to FSH, and FSH may down-regulate the AMH expression in adult rat ovaries (Baarends et al., 1995).
CHAPTER II: CLINICAL AND ENDOCRINE RESPONSES TO OVARIAN HYPERSTIMULATION IN FLARE AND LUTEAL GnRHa PROTOCOLS

2.1 Introduction

In controlled ovarian hyperstimulation, the purpose is to obtain as many healthy mature oocytes as possible without increasing the risk of ovarian hyperstimulation syndrome. As a result, identification of the prospective ovarian response is one of the crucial concepts for an optimized clinical management of COH for IVF/ICSI (Arslan et al., 2005). The availability of new hormonal preparations and clinical experiences has resulted in significant modifications of ovarian stimulation regimens. From its infancy until now, starting with clomiphene citrate and hCG as stimulation protocol, then gonadotropin and hCG, the introduction of GnRHa into IVF protocols helped to prevent premature luteinization, improve the stimulation of follicular development and the quality of developing oocytes (Meldrum et al., 1989; Muasher, 1992). However, study showed that the ovarian responsiveness is different from protocols in which GnRHa is given at different time points during the cycle treatment. The luteal protocol in which GnRHa is administered in the luteal phase of the previous cycle may enhance oocyte recovery and lower cycle cancellation compared to the flare protocol in which GnRHa is administered in the early follicular phase. On the other hand, some studies have shown that flare and luteal protocols have comparable outcome. Therefore, considering the duration of GnRHa administration, flare protocol might be more cost effective. However, in general luteal protocol seems to be more favored for COH than flare protocol.

Different from the natural cycles in which only one follicle become dominant and ovulated from the cohort of growing follicles, the main goal in COH is to achieve a synchronous cohort of mature oocytes ready for retrieval. Thus, it requires a synchronization of the cohort of follicles at the initiation of the treatment cycle. These follicles are ready for the FSH-dependent developmental stage and grow at the same speed, reaching the same level of maturation on the day of oocyte collection. The more synchronized the cohort, the more synchronous oocytes can be collected. Luteal estradiol and GnRH antagonist administration in the early follicular phase was shown to coordinate follicular growth by improving the homogeneity of early antral follicles.
(Fanchin et al., 2005b). However, there are limited studies that have investigated the degree of synchronization between the long-term and short-term GnRHa administration

Traditionally, the ovarian response is assessed by the serum E$_2$ pattern and the subsequent follicular response as monitored by ultrasound. Later on, measurement of basal cycle day 3-FSH and E$_2$ levels were considered predictors of COH response and IVF outcome. (Muasher et al., 1988) Nevertheless, due to the variation of serum FSH from cycle to cycle and its late substantial rising when the perimenopausal transition is already present, it is not a very reliable screening test. Therefore, many other tests have been introduced as candidates for the examination of the ovarian reserve such as clomiphene citrate challenge test (Navot et al., 1987); GnRH test (Muasher et al., 1988); GnRH agonist test (Padilla et al., 1990); basal cycle ovarian volume, antral follicle count and ovarian stromal blood flow via ultrasound (Syrop et al., 1995; Zaidi et al., 1996; Tomas et al., 1997); inhibin B measurement (Seifer et al., 1997); and AMH (Seifer et al., 2002).

AMH, as a member of TGFβ superfamily, has a function in follicular development. Expressed in the granulosa cells of primary follicles, AMH is highly expressed in small antral follicles and gradually diminish at the later stage of follicle development, AMH levels reflect the number of small follicles (Pigny et al., 2006; Visser et al., 2006). This is confirmed by the high AMH level in PCO patients. The constancy of serum AMH levels throughout the menstrual cycle is still controversial. While in earlier studies, serum AMH levels were found relatively steady during the cycle (Hehenkamp et al., 2006; La Marca et al., 2006; Streuli et al., 2007; Tsepelidis et al., 2007), a recent study did not confirm its stability (Wunder et al., 2008). However, compared to other hormones, such as FSH, E$_2$, or inhibin; serum AMH is still less variable (Fanchin et al., 2005c) With all of those characteristics, AMH is considered a promising and reliable marker for ovarian reserve and thus ovarian response.

As mentioned above, luteal protocol with GnRHa administration from the mid-luteal phase of the preceding cycle suppress the pituitary completely after 14 days of treatment. Whereas, in flare protocol due to the “flare effect” of GnRHa, the pituitary is incompletely suppressed. Therefore, at the time of starting exogenous FSH administration,
the cohort of follicles will be more synchronous in the luteal protocol than that in flare protocol.

In this study we investigated the differences in ovarian response in flare and luteal protocols. We hypothesized that ovarian response in luteal protocol will be superior to that in flare protocol due to the differences in the degree of follicular synchronization between the two protocols at the beginning of the treatment.

2.2 Materials and methods

2.2.1 Subjects

All women undergoing IVF /ICSI treatment in UBC Reproductive Health Department from January 2006 to December 2006 were selected for a total of 40 treatment cycles, 20 cycles in each protocol. Women in flare and luteal protocols were matched by age, body mass index (BMI), and indication for IVF/ICSI. Typically, the luteal protocol was employed as the first line approach to stimulation while the flare protocol was usually selected based on patient characteristics such as female age, previous response to stimulation and patient preference.

2.2.1.1 Ovarian stimulation:

The study involved women using GnRH agonist only; those using GnRH-antagonists were excluded. With the flare protocol, Buserelin acetate (Suprefact, Aventis Pharma, Canada) 500 micrograms daily is administered subcutaneously from day 3 of the cycle for 3 days then maintained at 250 micrograms daily. Recombinant FSH (rFSH) (Gonal-F; Serono, Canada or Puregon; Organon, Canada) is administered subcutaneously in a fixed dose (150 IU to 300IU) for 2 days from day 5 of the cycle. In the luteal protocol, Naferelin acetate (Synarel; Pfizer, Canada) 200 micrograms twice daily is sprayed intranasally from day 21 of the preceding cycle and continuously 14 days afterward. rFSH is administered on the 15th day of Naferelin administration protocols after the determination of a serum estradiol level < 200pmol/l to ensure that the pituitary is completely suppressed. The cycle monitoring procedure and the criteria for hCG administration were similar in the two protocols. Basal FSH on cycle day 3, and estradiol serum levels are routinely measured. During the period of treatment, serial estradiol
serum concentrations were routinely measured to evaluate ovarian response. FSH dose was administered on an individual basis according to the ovarian response, as assessed by sequential transvaginal ultrasonography and serum estradiol measurements. When there are two or more leading follicles (≥ 18 mm in diameter) in association with a consistent rise in serum estradiol concentration, human chorionic gonadotropin (hCG, Profasi; Serono, Canada or Ovidrel; Serono, Canada) was administered. Oocyte aspiration guided by vaginal ultrasonography was performed 32-36h after hCG administration. Depending on the cause of the infertility, oocytes were inseminated by standard IVF procedures or by ICSI. Up to three embryos per patient were transferred 2-3 days after fertilization. Luteal phase is supported by micronized progesterone administration (Prometrium; Schering, Canada, 300mg twice per day). Pregnancy was diagnosed by increasing serum concentration of β-hCG 14 days after embryo transfer and was confirmed by the subsequent presence of an intrauterine gestational sac under ultrasonography.

The age, body weight, days stimulated, dose of FSH administered, number of eggs retrieved, number of eggs fertilized, number of embryos transferred, clinical pregnancies, and number of follicles detected on ultrasound and E$_2$ concentrations were collected from the patient records. FSH, LH, P, D$_4$ and AMH were assayed retrospectively in frozen serum samples from available cycles.

2.2.2 Methods

2.2.2.1 Hormone assays:

E$_2$, FSH, LH and D$_4$ assay were performed using the Immulite chemiluminescent system. The sensitivity for E$_2$, FSH, LH and D$_4$ were 55pmol/L, 0.1 IU/L, 0.1 IU/L, 0.1 nmol/L, respectively. Inter and intraassay coefficients of variation (CV) were 9% and 5% for E$_2$; 7% and 4% for LH and FSH and 16% and 6% for D$_4$, respectively. Progesterone was assayed by radioimmunoassay (RIA) method, using $^{125}$I as tracer. The sensitivity was 0.06nmol/L and the inter and intraassay CV were 9% and 4%, respectively. Materials for E$_2$, FSH, LH, D$_4$ and P assays were purchased from Inter Medico, Toronto, Ontario; Canada.

Serum AMH levels were determined in duplicate using commercially available AMH-ELISA kits (Beckman Coulter Canada Inc, Mississauga, Ontario) according to the
manufacture’s instructions. Results are expressed in pmol/L. The detection limit of this assay is 0.7 pmol/L. Inter and intra-assay CV were 22% and 10%, respectively. Samples with hormone values below the assay detection limit were assigned values equal to the detection limit of that assay.

2.2.2.2 Statistical analysis

For statistical analysis, area under the curve (AUC) was used to summarize the information from a series of measurements on one individual by applying the following formula (Altman, 1991).

With n+1 measurements \(y_i\) at times \(t_i\) (\(t = 0, \ldots, n\)) the AUC is calculated as follow:

\[
\frac{1}{2} \sum_{i=0}^{n-1} (t_{i+1} - t_i) (y_i + y_{i+1})
\]

Then the AUC of each hormonal assay was compared between the two protocols by using the unpaired t-test. T-test was also used to test the differences in the outcome such as the number of total follicles, eggs retrieved, eggs fertilized and the number of small, medium and large follicles between the protocols. Results were expressed as mean ± standard error of mean (SEM). \(\chi^2\)-test was used for testing differences in clinical pregnancy rate between protocols.

To determine the influence of baseline clinical data, such as age, weight, BMI (body mass index), total FSH dose and the hormonal values on the differences in the outcome between the protocols, we also correlated the baseline data as well as the hormone doses and values and the outcome (the number of total follicles, follicles aspirated, eggs retrieved and eggs fertilized) by Pearson correlation analysis.

The SPSS statistical software was used for all statistical analyses. \(P \leq 0.05\) (two tailed) was considered significant.
2.3 Results

2.3.1 Characterization of the study population:

Because in this study we matched the patients according to age, weight and indication, there was no significant difference in age and weight between two protocols. The days of stimulation were also the same in the two protocols, with an average of 11 days. Flare protocol had a longer duration of infertility than luteal protocol but this was not significant. The mean of total FSH dose were significantly higher in flare protocol compared to luteal protocol.

Table 2.1 Characterization of the study population

<table>
<thead>
<tr>
<th></th>
<th>Flare protocol</th>
<th>Luteal protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>37 ± 0.5</td>
<td>36.8 ± 0.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>63.7 ± 2.9</td>
<td>63.5 ± 2.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9 ± 1.1</td>
<td>24.1 ± 1.1</td>
</tr>
<tr>
<td>Period of stimulation (d)</td>
<td>11.6 ± 0.3</td>
<td>11.2 ± 0.3</td>
</tr>
<tr>
<td>Duration of infertility (y)</td>
<td>5.7 ± 0.7</td>
<td>4 ± 0.7</td>
</tr>
<tr>
<td>FSH dose (IU/L)</td>
<td>2425 ± 216</td>
<td>1837 ± 107</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; cycle</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; cycle</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; cycle</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

In this study we included all of the major causes of infertility such as tubal (35%), endometriosis (10%), male factor (25%), unexplained (25%), and male combined with ovulatory dysfunction (5%).

2.3.2 Dynamic patterns of several hormonal concentrations during IVF cycles

Because the duration of treatment varied from patient to patient, if we had chosen the day of FSH treatment as the starting point of the dynamic patterns, it would yield
inaccurate changes of LH level putatively occurring as a sharp peak at ovulation. For example day + 14 from the onset of FSH treatment would have been the day of ovulation for some patients but still far for others. As a result, we used the day of hCG injection as day 0 and the other days were counted backward from the day of hCG injection (i.e. d -1, d-2, d-10, -11, etc...)

The dynamic patterns of E$_2$ concentration during the IVF protocols can be divided into 2 stages (Figure 2.1 A): for the first 7 days E$_2$ level was very low, plateaued, then increased slightly. The second stage was the last 4 days of stimulation in which E$_2$ level increased quickly up to around 9000 pmol/L for the luteal protocol and 6000 pmol/L for the flare protocol. For the first stage E$_2$ level was almost the same in two protocols until the last 4 days. However, the difference in E$_2$ level for the last 4 days was not large enough to make a difference between the two protocols. The E$_2$ level, which always peaked on the day of hCG in both protocols, confirmed the maximum development of follicles on that day.

Overall, FSH level was always higher in flare compared to luteal protocol (Figure 2.1 B). The lower level of FSH in the beginning of the luteal protocol reflected the more profound degree of pituitary suppression by GnRHa in this protocol. In both protocols FSH increased slightly during the cycles combined with exogenous FSH, reaching a peak two days before hCG administration.

The LH level in the luteal protocol was low and plateaued with little variation for the last 3 days of treatment, reached peak value 2 days before hCG but the peak value was not much higher than the LH level on the other days (Figure 2.1 C). LH levels in luteal protocol confirmed the suppression of GnRH on the pituitary. In contrast, LH levels in flare protocol were much higher and fluctuated more than in the luteal protocol for any day. The peak level of LH in the flare protocol occurred at the first stage of treatment reflecting the “flare effect” on the pituitary.

While progesterone in the luteal protocol was very stable and plateaued until the last 4 days and reached the peak level on the day of hCG administration, progesterone levels in the flare protocol fluctuated with a greater variation (Figure 2.1 D). That made the peak day of progesterone level in the luteal protocol in the middle of the treatment
cycle. However, it appeared that progesterone level in flare protocol was consistently higher than that in the luteal protocol.

There was no significant difference between these protocols in terms of androstenedione (Figure 2.1 E). It started at different points and reached the peak level on the last two days of the cycle.

AMH concentrations in the luteal protocol started at a higher level than that in flare protocol (Figure 2.1 F). For the first stage, AMH levels fluctuated in both protocols, with the level in the luteal protocol always higher than that in the flare protocol reflecting an increased number of follicles in the luteal protocol. During the last 6 days of the treatment cycle AMH levels decreased slightly in both protocols until the last day. In the luteal protocol the peak day occurred on the first day of the FSH administration while in the flare protocol the peak day was around the middle of the cycle after FSH was given for a few days.

Figure 2.1. Hormonal dynamic patterns of (A) E2, (B) FSH, (C) LH, (D) P, (E) D4, (F) AMH throughout the treatment cycles

The cycles were counted from the day of hCG backward until the first day of FSH administration. Each point at the given time is the mean value ± SEM
Figure A shows a graph of estradiol levels (pmol/L) over days before hCG administration. The graph compares the Flare protocol (dashed line) and the Luteal protocol (solid line). The x-axis represents the days before hCG administration, ranging from -14 to 0 days. The y-axis denotes estradiol levels ranging from 0 to 10,000 pmol/L.
Flare protocol

Luteal protocol

FSH

-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0

days before hCG administration

IU/L

-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0

days before hCG administration

IU/L
Flare protocol

Luteal protocol

Days before hCG administration

IU/L

LH

- - - - - - Flare protocol

- - - - - - Luteal protocol
![Graph showing Flare and Luteal protocols with Progesterone levels on the y-axis and days before hCG administration on the x-axis. The Flare protocol is indicated by a dashed line, and the Luteal protocol is indicated by a solid line. The graph shows fluctuations in Progesterone levels with peaks and troughs corresponding to the number of days before hCG administration.]
E

Androstenedione

- - - - Flare protocol
- - - - Luteal protocol

days before hCG administration

nmol/L
2.3.3 Variation in follicular response between two protocols

Follicular response is determined by the number of total follicles on the day of hCG given and the number of eggs retrieved. The luteal protocol had more total follicles and yielded more eggs than the flare protocol (21 ± 2 vs 14.5 ± 1.8, p < 0.05; 14 ± 1.4 vs 8 ± 0.8, p < 0.001; respectively). The follicles then were subdivided into large (>15mm), medium (10-14mm) and small (<10mm) follicles (Figure 2.2). When comparing the number of large, medium and small follicles between the protocols, only the difference in the number of medium follicles was statistically significant (3.7 ± 0.6 in flare protocol vs 8.4 ± 1.3 in luteal protocol, p = 0.003). In addition, the stratified t-test for the first cycle between the two protocols confirmed that the number of eggs retrieved in the luteal protocol was significantly higher than that in the flare protocol (17.4 ± 2.5 vs 9.7 ± 2, p < 0.05, respectively). In addition, the number of eggs fertilized in the luteal protocol is also higher than that in flare protocol (8.4 ± 0.7 vs 4.4 ± 0.5, p < 0.001). Among 20 cycles for each protocol, 10 cycles in the luteal protocol had a clinical pregnancy while there were just 5 cycles having a pregnancy in the flare protocol. However, the difference in clinical pregnancy between two protocols was not significantly different.
Figure 2.2 Comparison of the outcome between the flare and luteal protocols
A. The mean value ± SEM of the outcome (the number of total follicles, eggs retrieved, eggs fertilized)
B. The mean value ± SEM of the follicles based on their sizes (< 10mm = small follicles, 10-14mm = medium follicles, > 15mm = large follicles)
C. The mean value ± SEM of the follicular response (number of total follicles, eggs retrieved, eggs fertilized) stratified by the number of cycles

a denotes a significant difference between the flare and luteal protocols.
A. Flare protocol
B. Luteal protocol

B. Graph showing the number of follicles for different follicular sizes:
- Small
- Medium
- Large

C. Graph showing the number of follicles/eggs for different outcomes:
- Total follicles
- Eggs retrieved
- Eggs fertilized

Legend:
- Flare protocol
- Luteal protocol
- 1st cycle
- 2nd & 3rd cycle

Note: The graphs include error bars and asterisks indicating statistical significance.
2.3.4  AMH and E$_2$ as a marker of ovarian responsiveness

AUC AMH, AUC FSH, AUC LH were significantly different between luteal and flare protocols 111 ± 13 vs 62 ± 12, p = 0.01; 113 ± 6 vs 170 ±13, p < 0.001; 20 ± 2 vs 67 ± 4.7, p < 0.001, respectively while the differences in AUC E$_2$, AUC P, AUC D$_4$ were not statistically significant. These data show that the pituitary suppression in the luteal protocol is more profound than that in the flare protocol regarding the difference in LH and FSH concentration.

Figure 2.3. The area under the curve of the hormonal data (A) AUC AMH, AUC D$_4$, AUC FSH, AUC LH, AUC P; (B) AUC E$_2$.

a denotes a significant difference between the flare and luteal protocols
2.3.5 Factors influencing the hormonal value and the outcome in flare and luteal protocols

There were no significant correlations between age and the outcome such as the number of total follicles, the number of follicles aspirated, the number of eggs retrieved and eggs fertilized. FSH dose correlated with body weight but the correlation was not strong. FSH dose correlated with the number of stimulated days. Interestingly, the FSH dose negatively correlated with AHM baseline and AMH on the day of hCG administration. Besides, FSH dose also weakly and negatively correlated with the number of total follicles, but it did not significantly correlate with the number of eggs retrieved.

The E$_2$ level on the day of hCG administration, FSH level at the beginning of the treatment cycles, FSH level on the day of hCG administration, AMH level at the beginning of the treatment cycles and on the day of hCG administration correlated with outcome including follicles and oocyte data (Table 2.2).
Table 2.2

Pearson correlation value between age and outcome; between FSH dose and body weight, the number of stimulated days, FSH dhCG, AMH baseline, AMH dhCG and outcome; between E₂, FSH, AMH level and outcome

<table>
<thead>
<tr>
<th>Factors</th>
<th>Age</th>
<th>FSH dose</th>
<th>E₂ dhCG</th>
<th>FSH baseline</th>
<th>FSH dhCG</th>
<th>AMH baseline</th>
<th>AMH dhCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>r = 0.3</td>
<td>p = 0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days stimulated</td>
<td>r = 0.5</td>
<td>p = 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH dhCG</td>
<td>r = 0.6</td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMH baseline</td>
<td>r = -0.6</td>
<td>p &lt; 0.001</td>
<td>r = 0.4</td>
<td>p = 0.07</td>
<td>r = -0.4</td>
<td>p = 0.004</td>
<td>r = -0.6</td>
</tr>
<tr>
<td>AMH dhCG</td>
<td>r = -0.5</td>
<td>p &lt; 0.001</td>
<td>r = 0.36</td>
<td>p = 0.02</td>
<td>r = -0.3</td>
<td>p = 0.046</td>
<td>r = -0.6</td>
</tr>
<tr>
<td>Total follicles</td>
<td>r = 0.15</td>
<td>p = 0.3</td>
<td>r = -0.3</td>
<td>p = 0.04</td>
<td>r = 0.4</td>
<td>p = 0.009</td>
<td>r = -0.4</td>
</tr>
<tr>
<td>Follicles</td>
<td>r = 0.05</td>
<td>p = 0.2</td>
<td>r = -0.3</td>
<td>p = 0.07</td>
<td>r = 0.5</td>
<td>p = 0.001</td>
<td>r = -0.6</td>
</tr>
<tr>
<td>Eggs retrieved</td>
<td>r = 0.07</td>
<td>p = 0.6</td>
<td>r = -0.2</td>
<td>p = 0.1</td>
<td>r = 0.4</td>
<td>p = 0.003</td>
<td>r = -0.6</td>
</tr>
<tr>
<td>Eggs fertilized</td>
<td>r = 0.9</td>
<td>p = 0.9</td>
<td>r = - 0.1</td>
<td>p = 0.5</td>
<td>r = - 0.7</td>
<td>p &lt; 0.001</td>
<td>r = - 0.5</td>
</tr>
</tbody>
</table>
2.4 Discussion

The study population included women who underwent IVF/ICSI and was matched by age, weight and indication. It is already known that advanced maternal age will lower the success of treatment by compromising the ovarian response or pregnancy rate. It may be the result of ovarian depletion or the increase in aneuploidy associated with the advanced maternal age. As the luteal protocol was the first line choice for stimulation in our study population, it is more likely that those who belonged to the luteal protocol were younger than those who used the flare protocol. To exclude the bias of the outcome between the two protocols due to the aging discrepancies, we matched the women in two protocols by age. The average age in the current study was $37 \pm 0.5$ (range from 33 to 43), which is appropriate for our infertility population.

Weight is another confounding factor. Studies have already shown that the higher BMI of the patient, the more FSH dose is needed for ovarian stimulation. It seems that weight alone is more influential on FSH dose than BMI is. For example, although two patients have the same BMI, those who are overweight have the tendency to be resistant to stimulation, need more FSH dose and hence are poor responders. In this study we matched women between two protocols by weight to control the bias of FSH dose. Although only weight was matched, the mean BMI between the two groups was the same ($23.9 \pm 1.1$ in flare protocol vs $24.1 \pm 1.1$ in luteal protocol).

In addition, the ovarian response might be different in those who had previous surgery on the ovary. Those who have multiple etiology of infertility were probably less successful in treatment than those who have only one etiology. The pregnancy rate is affected by both female and male factor. Furthermore, minimal or mild endometriosis can cause the endocrine discrepancy as studies have already shown low serum AMH levels in mild endometriosis patients compared to the control group (Lemos et al., 2008). Therefore, in our study indication was also matched to exclude the potential bias.

A previous study in our center showed that those with a reduced GnRHa dose had a higher cancellation rate and lower pregnancy rate with a higher chance of premature LH surge (Gamelin and Cheung, 2007). We, therefore, excluded cycles with a GnRHa dose reduction at the onset of gonadotropin treatment from our study.
It is believed that due to the more profound pituitary suppression in luteal protocol, a greater dose of exogenous FSH is required in order to achieve adequate follicular growth. Several studies comparing the two protocols have shown that the number of FSH ampoules in the luteal protocol was equal or greater than that in the flare protocol. In contrast, our results revealed the mean total FSH dose in the luteal protocol is lower than that in the flare protocol, even though the average number of days of stimulation was almost the same in the two protocols. The idea of using the flare protocol is to take advantage of the flare effect to reduce the amount of exogenous FSH administration as the major cost in ovarian stimulation is that of gonadotropin. Our result indicates that flare protocol did not help in financial saving since the FSH dose was the same or even higher in the flare protocol.

2.4.1 The follicular responses of the two protocols on ultrasound

Our results have shown that the number of total follicles, eggs retrieved, and eggs fertilized were higher for the luteal protocol than for the flare protocol. By further analyzing the results of follicular growth by subdividing the follicles into three groups based on the size, we found that the luteal protocol patients revealed a higher number of medium follicles than the flare protocol patients (Figure 2.2 B). Due to the limitation of the retrospective study, we cannot exclude the possibility that the number of small follicles was not accurately recorded. The difference in the number of total follicles may be largely due to the difference in the number of medium follicles between two protocols. It is known that the relative size of follicles is usually positively related to the maturity of the oocyte. However, the number of large, medium and small follicles was recorded only on the day of hCG administration, not on the day of egg retrieval. It would be more informative if we recorded the number of large, medium and small follicles on the day of egg retrieval because the cohort of the medium follicles at the higher margin can become a large follicle during the last 36 hours from giving hCG to egg retrieval. Taken together, this may explain why there was no significant difference in the number of small and large or leading follicles between two protocols, yet the differences in the number of oocytes retrieved and fertilized between two protocols were significantly different. Furthermore, sometimes the medium follicles still can yield a good quality oocyte although it is not
easy to retrieve the oocyte from the smaller follicles. Loumaye et al have also shown that the flare protocol appeared to reduce the oocyte fertilization rate and embryo quality when compared with the luteal protocol (Loumaye et al., 1989). Several studies thereafter also proved the superiority of the luteal protocol compared to the flare protocol. In Tan’s study, even though GnRHa was administered from day 1 of the menstrual cycle in their long protocol group, the concept of pituitary desensitization is still the same as gonadotropin was only started at least 14 days after GnRH administration (Tan et al., 1992b; Greenblatt et al., 1995; Tasdemir et al., 1995; Cramer et al., 1999; Tavmergen et al., 2002; Loutradis et al., 2005). Their results were consistent with our results in term of the outcome between two protocols. On the other hand, there were some lines of evidence supporting the flare protocol in which they concluded that the outcomes between the two protocols were comparable while the flare protocols seem more economical considering the FSH doses and the duration of treatment cycle. However, a meta-analysis from Cochrane Database Review on twenty-six randomized control trials comparing the outcome between long-term and short/ultrashort protocols concluded that so far the long-term (luteal) protocol is still superior to the short (flare) protocol (Daya, 2000).

Even though most cycles included in this study were the first cycle (11 cases of first cycle in the flare protocol and 15 cases of first cycle in the luteal protocol) and did not exceed the third cycle for any particular woman, the increasing number of cycles might be a biasing factor as the flare protocol was more likely applied for those who failed with previous cycles and thus more likely to be the poor responders with poor ovarian reserve. We limited this possibility by including in the flare protocol only those who had a normal value of FSH (< 12 IU/L) before starting of the treatment cycles. When considering only the first treatment cycle, although the number of total follicles was no longer significant between the two protocols, the number of eggs retrieved and eggs fertilized remained significantly higher in the luteal protocol. It confirmed the quality of the follicles and oocytes in those using luteal protocols and the effectiveness of luteal protocols.

Fifty percent of the cycles resulted in a pregnancy in luteal protocol (10 out of 20 cycles) compared to twenty five percent in flare protocol (5 out of 20 cycles). However, the difference was not statistically significant most likely due to the small sample size of
the study. It is said that the flare protocol after an unsuccessful luteal protocol increases the pregnancy rate per cycle slightly. In our study, among 5 cases of successful pregnancy using flare protocol, 2 cases were changed to the flare protocol due to a previously unsuccessful luteal protocol. The remaining 3 cases were stimulated with flare protocols at the first attempt.

2.4.2 The follicular responses of the two protocols evaluated by endocrine patterns

As we already know that although follicle growth is driven by FSH, the increased follicular $E_2$ biosynthesis is associated with the dominant follicle development (Sanyal et al., 1974; Bomsel-Helmreich et al., 1979). Therefore the augmentation of estradiol concentration at the end of the treatment positively reflects the accumulation of the follicular estradiol concentrations. Follicles lower than 10mm in diameter had a low $E_2$ level and there was no correlation between follicle size and $E_2$ level in this size range (Sanyal et al., 1974; Westergaard et al., 1986; van Dessel et al., 1996). $E_2$ dynamic patterns in the current study confirmed this concept. The sudden increases in $E_2$ level indicated the shifting of follicles from 10mm in diameter into the cohort of dominant follicles. Also the higher concentration of $E_2$ on the day of hCG administration in the luteal protocol compared to the flare protocol in part suggested that the number of dominant follicles in the luteal protocol is higher than that in flare protocol.

Although $E_2$ level is probably associated with the number of leading follicles, there is a concern that the high levels of estrogen during ovarian stimulation may have the adverse effects on the oocyte, the uterus and implantation. A recent study analysed 1196 cycles using the long protocol with GnRHa and FSH concluded that there were no differences in the quality of oocytes retrieved and fertilization rate between the group with $E_2$ level of 4401-11185 pmol/L and group with $E_2$ level $> 15153$ pmol/L. They found only the implantation rate of the group with $E_2$ level $> 15153$ pmol/L was significant lower than other low $E_2$ groups (Chen et al., 2007). However, in our study, mean peak $E_2$ concentration on the day of hCG only reach to around 9000 pmol/L with the highest range is 12587 pmol/L. At this level of $E_2$, neither quality of the oocyte nor the implantation rate should be affected.
In addition, a high E\textsubscript{2} level is also associated with increased risk of OHSS, one of the complications of IVF treatment. OHSS often occurs in those with PCOS, a younger age which were not found appropriate with our study population. In our study, among 40 cycles in both protocols there was no case developing OHSS although 9 cycles in the luteal protocol and 4 cycles in the flare protocol had E\textsubscript{2} level on the day of hCG > 10000 pmol/L.

FSH, LH

The lower FSH level in the luteal protocol reflected the more profound degree of pituitary suppression in the luteal protocol than in the flare protocol. On the other hand, the high FSH level in the flare protocol may be partly due to the significant exogenous FSH administration in this protocol. Furthermore, the higher FSH level in the flare protocol could be a consequence of the poor ovarian reserve. Although we just included those who had the normal basal FSH level (<12 IU/L) in the flare protocol, the variability of FSH level between cycles could make it an unreliable criteria for excluding those patients with poor ovarian reserve.

For the half first of the treatment, FSH level after several days of treatment slightly increased to the level at which it is considered as a threshold to allow the selection of the cohort of dominant follicles. Once the FSH level reach that threshold around the day -7 of the cycles, the cohort of follicles enter the gate and continue developing. It is consistent with the onset of E\textsubscript{2} increase from day -7 in both protocols.

The greater degree of pituitary downregulation in the luteal protocol was confirmed again with the dynamic patterns of LH level during the cycle. Different from FSH, LH level was not affected by the FSH administration, especially with the high purity of the new recombinant FSH preparations.

The higher and more fluctuating LH level in the flare protocol raised the concerns of premature LH rise and thus premature luteinization which is associated with a less favourable outcome because of poor oocyte quality and decreased fertilization and implantation rate (Loumaye, 1990; Manzi et al., 1995). This could be one of the possible explanations for the lower number of eggs retrieved, eggs fertilized and pregnancy rate in the flare protocol that we found in the present study.
Progesterone

The cut-off point of P level in defining premature luteinization in previous studies varied from one study to another, ranging from 0.9 to 2.0 ng/ml (equivalent to 3 - 6.3 nmol/L). In our study, the mean P level in the flare protocol from day -4 to the day of hCG injection increased to \( \geq 6 \) nmol/L while the corresponding P level in the luteal protocol did not exceed 6 nmol/L. However, it was still unclear whether this increase in P is the consequence of elevated LH levels from day -8 to day -5 during the treatment cycle or of the increase in the granulosa cell steroidogenetic activity due to the higher rFSH exposure during the second half of the treatment cycle in the flare protocol. Contrary to the common belief that it is the LH activity in exogenous gonadotropins administration that causes the premature luteinization, it was already recorded that there was a strong positive correlation between the administered FSH dose and follicular phase P levels (Filicori et al., 2002a). Although the number of eggs retrieved and eggs fertilized were significantly lower in the flare protocols compared to the luteal protocols in our study, the premature luteinization in the flare protocol was probably the manifestation of diminished ovarian reserve rather than the consequences of LH surge since the elevated LH levels did not exceed 10 IU/L. It was also reported in the literature that premature luteinization is related to poor ovarian reserve (Younis et al., 2001). It might be the case in the present study since it is consistent with the higher dose of FSH administered we found in the flare protocol.

Androstenedione

As the pituitary is more down regulated in the luteal protocol than in the flare protocol, it is expected that the D\(_4\) level in the luteal protocol should be lower than that in the flare protocol. However, we did not find any significant difference in the D\(_4\) level between the two protocols. The higher level of D\(_4\) in the luteal protocol in the beginning of the treatment cycle can be explained by the increase in the D\(_4\) level often observed after 12 to 15 hours of FSH administration as in Fanchin’s study (Fanchin et al., 1995). In addition, variation in the duration of FSH treatment from patient to patient resulted in fewer samples collected as we went further backward from the day of hCG administration, for example on the day -13, there was only one sample in the luteal protocol and no sample in the flare protocol for comparison. It is, therefore, difficult to
compare the D4 level between the two protocols in the beginning of the treatment cycle. However, after the “flare effect” of FSH administration, the D4 level in our study was consistent with the level of its kind in the COH cycles with GnRHa suppression, ranging from 3.5 to 7 nmol/L (1 to 2 ng/ml) (Akaboshi et al., 1998; Fanchin et al., 2003a), lower than COH cycles without GnRHa pretreatment. Then, together with exogenous FSH administration, D4 level increased gradually, reaching peak around the day of hCG injection, consistent with results in the literature (Martin et al., 1997).

Evidence has already established an association between androgens with oocyte quality and fertility in animal models. Administration of androgens can result in follicular atresia and degenerate oocytes in rats (Hillier and Ross, 1979; Azzolin and Saiduddin, 1983). The impact of ovarian androgens on human reproduction was noted from PCOS women. The high level of androgens in the follicular fluid in these women results in the arrested follicles and the reduction of ovarian mass by wedge resection could restore the ovulatory function and fertility. However, the role of androgens in reproductive outcomes is still unclear. A previous study has demonstrated that the level of testosterone in follicular fluid of those patients using the short protocol was significantly higher than those using the long protocol (Filicori et al., 1996). This explained the better outcomes in the long protocol in their study. In our study, we just investigated the dynamic patterns of D4 instead of the full set of androgens as D4 is the main precursor of E2. Although the outcomes were inferior and the LH level was significantly higher in the flare protocol, we failed to confirm a higher level of D4 in the flare protocol. Therefore, from our result, the mechanism of this topic is still ambiguous and requires further investigations in the future.

AMH

In our study AMH level in the luteal protocol was found significantly higher compared to the flare protocol. For the first half of the cycle, mean AMH level in the luteal protocol range from the 10 to 20 pmol/L. In constrast, the corresponding mean AMH level in the flare protocol was lower, ranging from 3.5 to 12 pmol/L. It is already recorded that the mean AMH level in the follicular phase in normoovulatory women ranges from 15 to 20 pmol/L (Tsepelidis et al., 2007). However, in that study the supply of AMH ELISA kits were from a different manufacturer which provided a lower corresponding level of AMH than our assay (Fréour et al., 2007). In the present study, the
higher AMH level in the luteal protocol was consistent with the significantly higher number of total follicles noted in this protocol. It has already known that there is a strong correlation of AMH concentration and the antral follicle count on ultrasound.

Previous immunohistochemistry studies on human ovaries have already shown that AMH expression is first observed in the primary follicles, highly expressed and secreted by secondary, preantral and small antral follicles ≤ 4 mm in diameter, and almost lost in follicles ≥ 8 mm in diameter which means AMH is a main product from the small antral follicles (Weenen et al., 2004). It was confirmed later by AMH mRNA expression in the adult human ovaries which is expressed at low levels in the primordial follicles, highly increase in the primary and preantral follicles, drops with further follicular development to express at the low level in the large antral and ovulatory follicles (Modi et al., 2006). The dynamic patterns of AMH concentration during the treatment cycle in our study showed the decline in the AMH level from the beginning of FSH treatment to the day of hCG injection in both protocols This reflected that AMH level is associated with the shifting of the small follicles through the medium follicles to large follicles. That trend of AMH concentration occurred in both protocols. However, there was a sharper decline in AMH level in the luteal protocol than in the flare protocol, especially apparently for the last 5 days of the cycle, suggesting the greater number of medium-to-large follicle transition in the luteal protocol. Given that AMH is highly expressed in small follicles and since its level was significantly different between two protocols, suggests that there must be a significant difference in the number of small follicles between these protocols. In the current study, we failed to prove that. As mentioned above, probably the number of small follicles which is the main source of AMH secretion was not fully recorded since the small follicles, especially ≤ 2mm in diameter, were hardly measurable by a routine ultrasound examination. However, a difference in the number of small follicles between the two protocols was reflected by the difference in the number of medium follicles as the small follicles were growing up thereafter. The dynamic patterns of AMH in our study are similar to that reported in previous studies (Fanchin et al., 2003a). However, different from our study, only the luteal protocol was included in their analysis. So far our study is the first study comparing AMH concentrations on the daily basis between the luteal and flare protocol in IVF/ICSI treatment.
The function of AMH during folliculogenesis has not been clearly established. Some studies suggested that AMH inhibits the primordial to primary follicle transition and FSH-sensitivity during follicular development (Durlinger et al., 1999; Durlinger et al., 2001; Durlinger et al., 2002a; Durlinger et al., 2002b). On the other hand, other studies have shown that AMH could enhance FSH-induced follicle growth (McGee et al., 2001; Schmidt et al., 2005). Whether AMH is secreted independently or regulated by any other hormones is still a question. A recent study found that even though AMH level declined after endogenous FSH inhibition by oral contraceptive pills, the drop in AMH and FSH concentrations was a coincidental phenomenon indirectly related to the reduction of follicle activity (Arbo et al., 2007). However, our results, which show that AMH levels negatively correlate with FSH dose and FSH concentrations, suggest that AMH could possibly be regulated by FSH.

The traditional evaluation of the ovarian response currently used in clinical practice is E\textsubscript{2} measurement and follicle count on ultrasound. In this study we applied a new tool to evaluate ovarian response, AMH. Interestingly, although there was the difference in the number of eggs retrieved and fertilized between the two protocols exhibiting the difference in the ovarian responsiveness in these protocols, E\textsubscript{2} AUC during the treatment cycles between two protocols was not significant while AMH AUC was found significantly different between two protocols. It has been suggested that AMH is superior to E\textsubscript{2} as a marker of ovarian responsiveness. Nevertheless, whether AMH can replace E\textsubscript{2} measurement in cycle monitoring or not is still the matter of debate and future research. So far, clinicians still have been familiarity with the response of E\textsubscript{2} in which to adjust the FSH dose. Further assessment is needed to validate the clinical usefulness of AMH in practice in the future.

Furthermore, the failed cycle is a cancelled cycle due to either a poor response to COS or a high risk of OHSS. For a long time AMH was considered as a marker for ovarian reserve, however since the strong correlation of AMH with the number of follicles, it can be a promising marker for predicting OHSS as well. A recent study already demonstrated that AMH level can predict the likelihood of OHSS in women undergoing IVF/ICSI treatment and AMH is superior to E\textsubscript{2} in anticipating OHSS (Lee et al., 2008). AMH levels in both protocols in our study did not reach the AMH threshold.
(24 pmol/L or 3.36 ng/ml) believed to be able to predict the potential of developing OHSS as in their study. Using the cut off $E_2$ value on the day of hCG (1431 pg/ml = 5253 pmol/L) for predicting OHSS in their study, most of the patients in our study have a high risk of OHSS. No case of OHSS, however, was found among the women undergoing luteal and flare protocol with that high level of $E_2$. Nevertheless, due to differences in AMH ELISA assays employed in their study and ours, the use of circulating AMH concentrations in predicting OHSS require further study. However, our present results support the finding of AMH as a reliable marker of the ovarian response to COH which is of value not only for poor response but also for OHSS.

2.4.3 The correlation between basal clinical, endocrine and ovarian responses.

It is already known that the number of available follicles lessens with advanced age, successful IVF treatment is, therefore, usually age-related (Szamatowicz and Grochowski, 1998). However, in the current study there were no significant correlation between age and the outcome such as the number of total follicles, the number of follicles aspirated, the number of eggs retrieved and eggs fertilized. This can be explained by the age-matching between protocols in our study. Interestingly, our results showed that the higher AMH baseline level and AMH level on the day of hCG administration, the lower FSH dose need to be administered, reflecting the reliability of AMH in evaluating the ovarian reserve. Among the hormonal values that were expected to predict the outcome (such as $E_2$ level on the day of hCG administration, FSH level at the beginning of the treatment cycles, FSH level on the day of hCG administration, AMH level at the beginning of the cycles and on the day of hCG administration), the correlation between AMH level at baseline and on the day of hCG administration and the outcome (number of follicles, eggs retrieved) was strongest. Over the last several years, numerous studies have determined the relationship of serum AMH and the ovarian status. From the literature, it is already recorded the strong and positive correlation between AMH and the ovarian reserve as well as the number of oocytes retrieved after COH (Fanchin et al., 2003b; Laven et al., 2004; Eldar-Geva et al., 2005; Fanchin et al., 2005a; Penarrubia et al., 2005; Fleming et al., 2006; van Rooij et al., 2002). These series of studies have proved the superiority of AMH in predicting cycle cancellation, compared to current markers such as
FSH, E₂ and inhibin B. (Nakhuda et al., 2007; Seifer and Maclauglin, 2007). It remains efficient in both IVF and ICSI treatments (Lekamge et al., 2007). In the latest meta-analysis, it was shown that so far only antral follicle count is a current marker which has the same level of accuracy and clinical value for the prediction of poor response and failure to conceive as AMH (Broer et al., 2008). We did not find any significant difference between two protocols in terms of pregnancy rate due to the small sample size. However, whether AMH can predict pregnancy outcome or not is still a matter of controversy, as many factors intervene in the determination of the ultimate pregnancy rate (Hazout et al., 2004; Ficicioglu et al., 2006; Smeenk et al., 2007).

2.4.4 Summary

In the current study, 40 cycles using the luteal and flare protocols were included and matched between protocols in terms of age, weight and indication. In addition, only those who had a normal basal FSH value were included. If it is not the first treatment cycle, only those whose treatment cycle did not exceed the third cycle meet the inclusion criteria. Ovarian responses and main outcomes such as number of total follicles, eggs retrieved and eggs fertilized and pregnancy rate were compared between 20 cycles in each protocol. The dynamic patterns of series hormones such as E₂, FSH, LH, P, D₄ and AMH were evaluated and then compared between the two protocols. The number of total follicles, eggs retrieved and eggs fertilized were significantly higher in the luteal protocol than in the flare protocol. When the total follicles were subdivided according to follicle size into small, medium and large follicles, only the difference in the number of medium follicles was found to be significant. The number of pregnancies conceived was 5 (25%) for flare protocol and 10 (50%) for luteal protocol, but was not significantly different. Mean AMH and E₂ level were higher in the luteal protocol while mean FSH, LH, P and D₄ level were lower. Among the hormones measured, only AMH, FSH and LH AUC were significantly different between the two protocols; the remaining hormonal AUCs did not differ significantly. The total FSH dose was significantly higher in the flare protocol compared to the luteal protocol. However, it did not show significant differences when considering only the first treatment cycles. AMH baseline and AMH on the day of hCG injection were positively and quite strongly correlated with the outcomes (total
follicles and eggs retrieved). Their correlation were stronger than the correlation of E$_2$ on the day of hCG, FSH baseline and FSH day hCG with the outcome.
Chapter III SUMMARY AND CONCLUSIONS

3.1 Summary

Over the past several decades ART has continued developing. Ovarian stimulation is an integral part of the IVF process. The ultimate purpose in COH is to obtain as many fertilizable oocytes as possible without increasing the OHSS risk. The development of medical ovarian stimulation started with clomiphene citrate and hCG, through gonadotropin and hCG, then recently GnRHa following exogenous rFSH. So far, the latter stimulation protocol, which helps to prevent premature luteinization and improve the quality of developing oocytes, has been the most popular in IVF treatment (Meldrum et al., 1989; Muasher, 1992). However, the different time points of GnRH administration during the cycle treatment were shown to provide dissimilar ovarian responsiveness. The underlying mechanism of this diversity was attributed to the synchronization of mature oocytes available at the time of oocyte retrieval which derived from the homogeneity of a cohort of selected follicles. Still limited studies investigated the degree of synchronization between the long-term and short-term GnRHa administration.

Serum E₂ pattern and follicular responses evaluated by ultrasound were traditional methods and currently used in IVF cycle monitoring. Basal day 3-FSH was also a predictor of COH response and IVF outcome (Muasher et al., 1988). Thereafter, many other tests were introduced to examine the ovarian reserve and hence the ovarian responsiveness such as CCCT (Navot et al., 1987), GnRH test (Muasher et al., 1988), inhibin B (Seifer et al., 1997). However, the performance of these tests is still unsatisfactory. In addition, E₂, FSH and inhibins belong to a feedback system and their concentrations are dependent on each other. Moreover, serum FSH level varies widely from cycle to cycle. AMH, a member of TGFβ superfamily, has a function in follicular development. Highly expressed in the granulosa cells of small antral follicles, with a gradual reduction in expression in the later stages of follicle development, AMH levels reflect the number of small follicles (Pigny et al., 2006; Visser et al., 2006). A high AMH level was also found in PCO patients. Although the constancy of serum AMH level during the menstrual cycle is still controversial (La Marca et al., 2006; Wunder et al.,
AMH level seems independent of FSH (Arbo et al., 2007). Studies have already confirmed the strong and positive correlation between AMH and the number of small follicles (Fanchin et al., 2003b). As a result, AMH is considered a promising and reliable marker for ovarian reserve and thus ovarian response.

In the present study the differences in ovarian response between the flare and luteal protocols, which are the two most popular protocols currently used in IVF, were investigated. Our hypothesis is that the luteal protocol will provide a better outcome than the flare protocol based on the differences in the degree of follicular homogeneity between the two protocols at the onset of FSH administration.

The selected patients are those using flare and luteal protocols in UBC IVF center during the year 2006. The patients were mainly in their first cycle and no patient exceeded to the third cycle of treatment. Those cycles then were matched by age, weight and indication. In the flare protocols only those who had normal FSH level were included. Follicle data on ultrasound and E_2 profile were retrospectively collected. Hormonal assays of FSH, LH, P, D_4, AMH were performed on available frozen samples of each treatment cycle.

Our results revealed that the mean total FSH dose in luteal protocol is lower than that in flare protocol, even though the average number of days of stimulation was almost the same in these protocols. The number of total follicles, of eggs retrieved, and of eggs fertilized was higher for the luteal protocol than for the flare protocol. By further analyzing the results of follicular growth by subdividing the follicles into three groups based on the size of the follicles, we found that the luteal protocol patients revealed a higher number of medium follicles than the flare protocol patients. Number of pregnancies conceived was 5 (25%) for flare protocol and 10 (50%) for luteal protocol, but was not significantly different. AUC AMH, AUC FSH, AUC LH were significantly different between luteal and flare protocol while the differences in AUC E_2, AUC P, AUC D_4 were not statistically significant. Compared to the correlation between E_2 day of hCG, FSH baseline, FSH day of hCG and the outcome, the correlation between AMH at baseline and on the day of hCG administration and the follicle data was strongest.
3.2 Conclusion

In conclusion, although luteal protocol requires a rather long treatment period in order to achieve pituitary suppression, this protocol can provide more follicles, more eggs retrieved, more eggs fertilized than the flare protocol. As compared to E₂, AMH had a better correlation with the number of follicles and eggs retrieved. In this study for the first time we present the dynamic patterns of AMH level during the treatment cycle, associated with other hormonal patterns in the two standard IVF protocols. However, because of limitations associated with retrospective studies, we could not completely exclude selection bias associated with the choice protocol. A well-designed randomized control trial in the future might be a solution. However, our data can serve as a reference for further studies on the application of AMH in cycle monitoring in IVF practice.
BIBLIOGRAPHY


Fanchin R, Louafi N, Mendez Lozano DH, Frydman N, Frydman R and Taieb J (2005a) Per-follicle measurements indicate that anti-mullerian hormone secretion is modulated by the extent of follicular development and luteinization and may reflect qualitatively the ovarian follicular status. Fertil Steril 84:167-173


Frydman R, Howles CM and Truong F (2000) A double-blind, randomized study to compare recombinant human follicle stimulating hormone (FSH; Gonal-F) with highly purified urinary FSH (Metrodin HP) in women undergoing assisted reproductive techniques including intracytoplasmic sperm injection. The French Multicentre Trialists. Hum Reprod 15:520-525


Karnitis VJ, Townson DH, Friedman CI and Danforth DR (1994) Recombinant human follicle-stimulating hormone stimulates multiple follicular growth, but minimal estrogen production in gonadotropin-releasing hormone antagonist-treated monkeys: examining the role of luteinizing hormone in follicular development and steroidogenesis. J Clin Endocrinol Metab 79:91-97


Li J and Tsang BK (1995) Prostaglandins mediate the stimulation of deoxyribonucleic acid synthesis by transforming growth factor alpha in hen granulosa cells during ovarian follicular development. Biol Reprod 52:1050-1058


Souza CJ, Campbell BK, McNeilly AS and Baird DT (2002) Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. Reproduction 123:363-369


Streuli I, Fraissé T, Pillet C, Ibecheole V, Bischof P and de Ziegler D (2007) Serum antimullerian hormone levels remain stable throughout the menstrual cycle and after oral or vaginal administration of synthetic sex steroids. Fertil Steril in press


68
hormone (LH): a role for LH in the final stages of follicular maturation. J Clin Endocrinol Metab 84:228-232


Wunder DM, Bersinger NA, Yared M, Kretschmer R and Birkhäuser MH (2008) Statistically significant changes of antimüllerian hormone and inhibin levels during the physiologic menstrual cycle in reproductive age women. Fertil Steril 89:933


