Aus dem Almana Krankenhaus
Abteilung Für Frauenheilkunde und Geburtshilfe,
und
Charité-Universitätsmedizin Berlin
Campus Benjamin Franklin
Abteilung für Frauenheilkunde

DISSERTATION

Outcomes of oocytes in vitro maturation (IVM) in polycystic ovaries and poor responder infertile women

Zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

Vorgelegt der Medizinischen Fakultät der Charité –
Universitätsmedizin Berlin

von
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2008

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Outcomes of oocytes in vitro maturation (IVM) in polycystic ovaries and poor responder infertile women

Submitted for achievement of M.D. degree
Charité-Universitätsmedizin Berlin

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Datum der Promotion: 27/3/2009

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Dissertation

"Ergebnisse der In-vitro-Maturation (IVM)
Von Eizellen bei infertilen Frauen mit
Polyzystischen Ovarien und schlechten Respondern"

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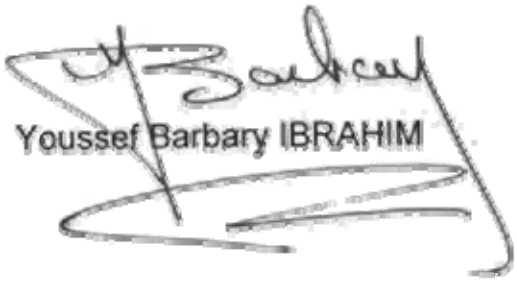

Youssef Barbary IBRAHIM

To

The memory of my mother

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Lists of Abbreviation

BMI:	Body mass index
CC:	Clomiphene Citrate
CCCT:	Clomiphene citrate challenge test
CO_2:	Carbon dioxide
COH:	Controlled ovarian hyperstimulation
E_2:	Estradiol
EFFORT:	Exogenous follicle stimulating hormone ovarian reserve test
EGF:	Epidermal growth factor
ET:	Embryo transfer
FBS:	Fetal bovine serum
FCS:	Fetal cord serum
FSH:	Follicle stimulating hormone
G2:	Grade 2
GAST:	Gonadotrophin releasing hormone agonist stimulation test
GH:	Growth hormone
GIFT:	Gamete intra-fallopian transfer
GnRH:	Gonadotrophin releasing hormone
GnRHa:	Gonadotrophin – releasing hormone analog
GV:	Germinal vesicle
GVBD:	Germinal vesicle breakdown
hCG:	Human chorionic gonadotrophin
HEPES:	4 – (2 –hydroxyethyl) – 1- piperazine – ethanesulfonic acid
hFSH:	Human follicle stimulation hormone
hLH:	Human luteinizing hormone
hMG:	Human menopausal gonadotrophin
ICSI:	Intracytoplasmic sperm injection
IGF-1:	Insulin like growth factor – 1
IGFs:	Insulin like growth factors
IVF:	In-vitro fertilization
IVM:	In vitro maturation

LH:	Luteinizing hormone
MI:	Metaphase I
MII:	Metaphase II
MOD:	Mean ovarian diameter
NO:	Nitric oxide
OHSS:	Ovarian hyperstimulation syndrome
P1:	Primordial oocytes
PCO:	Polycystic ovaries
PCOS:	Polycystic ovary syndrome
r-FSH:	Recombinant follicle stimulating hormone
RNA:	Ribonucleic acid
SHBG:	Sex hormone binding globulin
US:	Ultrasonography
ZIFT:	Zygote intra-fallopian transfer

Acknowledgment

Throughout the development of this thesis, I had the privilege and good fortune of working with many individuals of considerable talent. I would like to express my sincere gratitude to **Dr. M. Samy Ismail**, assistant Professor of Obst. & Gyn., faculty of medicine, Campus Benjamin Franklin, Charitè-universitàts medizin Berlin, for his continuous support that made this achievement a possibility.

I would like to thank **Mr. Ebrahim Almana**, chairman of the board of Almana Group of Hospitals, Eastern Province, Kingdom of Saudi Arabia for his continuous support.

My deepest gratitude and appreciation goes to the IVF unit in Almana General Hospital in Dammam, Eastern Province, Kingdom of Saudi Arabia and its director **Dr. M.D. Kini** for allowing me to conduct this research in their unit. Thanks to **Mr. P.S.R. Murthy** and **Mr. M.M. Adam** for their assistance. Special thanks to **Dr. Hythum Y.O. Ibrahim** consultant OB/GYN for his guidance and support.

I feel a deep sense of gratitude to **Dr. Ahmed A. Bahnassy**, associate Professor of family and community medicine, college of medicine, King Faisal university, K.S.A. for giving much effort to accomplish the statistical work of this study.

Thanks also are due to **Mr. Malte Petersen**, Head of international medical department, Bremen, Germany, for his effort and continuous help.

I am very grateful to **Mrs. Aida Lazo** who helped to assemble and publish this work.

RESUME

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- Team Training Workshop
- Symposium on Coronary Heart Disease

- Essence of Caring-Customer Satisfaction Workshop
- Series of Educational Workshops (12) on Health Care Quality Management topics held quarterly in Saudi Aramco
- Advanced Cardiac Life Support (ACLS)
- Quality Improvement Workshop
- Health Quality Management Symposium
- Symposium, Medical Records
- Leadership in Diagnosis & Management of Bronchial Asthma
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1. Introduction

Since the first successful human pregnancy from in vitro fertilization (IVF) was achieved (Steptoe P. and Edwards R., 1978), assisted reproductive technology has become the frontier of birth infertility treatment and research. There have been continuous improvements in the pregnancy and birth rates with IVF. These improvements have been directly attributed to advances in the hormonal stimulation of patients with various controlled ovarian hyperstimulation (COH) protocols and improved culture media and culture systems for oocytes, sperms, and embryos (Lazendorf S., 2006). However, through all these improvements with stimulated cycles, there has been continued development with natural, unstimulated, or limited-stimulation cycles followed by in vitro maturation (IVM) of oocytes. **Any protocol that would decrease the amount and duration of hormonal stimulation before oocyte retrieval would have an advantage over the more common COH/IVF protocols if the resulting pregnancy rates were the same or improved** (Cha K. et al, 1991). Research in IVM of the human oocyte has shown significant progress and provided hope for certain groups of patients who have infertility problems. Human oocytes recovered from immature follicles, following retrieval, can resume and complete meiosis in-vitro when cultured in media supplemented with recombinant follicle stimulating hormone (r-FSH) and human chorionic gonadotrophin (hCG) (Trounson A., et al, 2001; Hreinsson J. et al, 2003; Lin Y. et al, 2003). Recently published reports show that in vitro matured oocytes could be fertilized, and result in pregnancy (Jaroudi K., et al, 1999), and birth of healthy babies (Chian R., et al, 2001, Suikkari A. et al, 2005). Despite the clinical utilization of IVM in the field of human reproduction, its pregnancy and birth rates remain low compared to in vivo matured oocytes (Lui J., et al, 2003, Lin Y., et al, 2003).

Nevertheless, IVM remaines a low cost procedure and might become an optimum solution to some causes of infertility in certain groups of patients, such as polycystic ovary patients and poor responders.

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, and a common cause of infertility. Approximately 6%-8% of unselected women of

reproductive age suffer from PCOS (Aziz R., et al, 2004). Although its aetiology remains unknown, PCOS is a heterogeneous disorder that may present at one end of the spectrum with the single finding of polycystic ovarian morphology as detected by pelvic ultrasound. At the other end of the spectrum, symptoms such as obesity, hyperandrogenism, menstrual cycle disturbance and infertility may occur either singly or in combination. Metabolic disturbances such as elevated serum concentrations of leutinizing hormone (LH), testosterone, insulin and prolactin are common and may have profound implications on the long-term health of women with PCOS (Balen A. et al, 1995). PCOS may also be a familial condition, possibly autosomal dominant, with premature bolding being the male phenotype (Meyer M. et al, 2000, Wood J. et al, 2003). Women with PCOS have typical symptoms of anovulation, numerous antral follicles in the ovary on ultrasound scan and infertility (Chain R., et al, 1999). In PCOS patients, the dominance of a particular follicle fails to occur and the cohort of the numerous growing follicles accumulates in the cortex (Trounson A., et al, 1994). Clomiphene citrate (CC) continues to be the first line treatment for anovulatory infertility associated with PCOS, although some patients are found to be CC resistant. In these patients, several options might be considered, including oral metformin, ovarian drilling, and gonadotropin therapy (Palomba S., et al, 2006). Induction of ovulation with human menopausal gonadotrophin (hMG) can be successfully achieved in 75% of patients resistant to CC. Some disadvantages exist, such as the requirements of more intensive monitoring, the risk of ovarian hyperstimulation syndrome (OHSS), and higher cost. In the absence of obesity, metformin therapy is capable of reversing most indices of PCOS, but the benefits of metformin are essentially lost as soon as the therapy is discontinued (Zegher F., et al, 2006). Also, some of those patients are extremely sensitive to exogenous gonadotophin when used for assisted reproduction protocols and may develop deep vein thrombosis (Steward J., et al, 1997), and ovarian cancer resulting from prolonged use of fertility drugs. Nowadays, there is an increased interest to avoid these risks, by retrieving oocytes using minimal or no gonadotrophins stimulation and then maturing them in vitro in culture medium.

An adequate ovarian response to ovarian stimulation is a prerequisite for successful in-vitro fertilization. Poor ovarian response and the consequent low number and quality of

oocytes retrieved are frequently associated with a sub-optimal IVF outcome (Ben Rafael Z. et al, 1993). **Poor responders** are patients who fail to achieve an adequate number of mature follicles and/or an adequate serum oestradiol levels after gonadotrophin stimulation. Poor response usually leads to cycle cancellation, or, in cases in which oocytes retrieval is possible, a very low pregnancy rate (Keay S. et al, 1997). It was found that between 5% and 18% of the IVF cycles show poor response (Ben-Rafael Z. et al, 1991; Jenkins J. et al, 1991). Many strategies for the treatment of such patients have been proposed (Sandow J.et al, 1978; Check J. et al, 1990; Ibrahim Z. et al, 1991; Manzi D. et al, 1994; Schoolcraft W. et al, 1997). However, despite multiple different stimulation protocols for IVF, the ideal stimulation for poor responders still remains unknown (Mahutte N. et al, 2002). IVM could be a possible alternative modality for poor responders. Recent reports show that immature oocytes retrieved from these patients were successfully matured in vitro, fertilized, and resulted in live birth (Lin Y., et al, 2003).

In-vitro maturation can be considered for treating infertile PCOS patients. Since the procedure uses low dosage or no exogenous gonadotrophin (Jaroudi K., et al, 1999), it can be attractive for its low cost. The procedure can benefit women suffering from autoimmune disorder like disseminated lupus erythrematosus or cancer by cryopreserving their germinal vesicle (GV) oocytes prior to chemotherapy treatment. In addition, IVM can provide information about the final stages of oocyte maturation (Hreinsson J., et al, 2003).

On the other hand immature oocytes retrieved from patients with PCOS and poor responders were found to have lower maturation rate than mature oocytes retrieved from normal and regular cyclic patients. There is evidence suggesting that in-vitro matured oocytes retrieved from follicles that are in the early stages of atresia are more competent to support embryonic development than those retrieved from actively growing follicles. The presence of granulose cells is vital in providing nutrients and regulatory signals for ovarian follicles. When human chorionic gonadotrophin (hCG) is given before the retrieval of immature oocytes, it was found to assist oocyte maturation, increase the number of procured oocytes and improve the pregnancy rate (Chian R. et al, 1999; Son W. et al, 2002). A rise in the level of serum luteinizing hormone (LH) was reported to

induce the final stage of maturation. When oocytes are matured in-vitro, recombinant follicle stimulating hormone (r-FSH) or urinary gonadotrophins are added to the culture media to improve its maturation (Mikkelsen A., et al, 2001). Recently, both recombinant FSH and LH in addition to hCG have been used to achieve better oocyte maturation (Hreisson J., et al, 2003), but without standardizations of the optimum concentrations which may lead to the best results.

In vitro maturation of oocytes is limited by the culture systems currently used, including the doses and duration of hormones and other factors that are added to the culture media and are needed to initiate and coordinate the events of oocyte maturation. Determining the optimal composition of the culture media, including the proper doses of hormones and an energy source such as pyruvate, could be critical to the rate of oocyte maturation (Roberts R., et al, 2002). While the oocytes are still in the ovary, the basic fundamental physiological system, the ovarian follicle, is still intact, along with the elaborate paracrine and endocrine interactions required for efficient oocyte maturation. Removing oocytes before exposure to hCG or an LH surge removes the elements of the granulosa and thecal cells and any influence they might have on oocyte maturation. To mimic the ovarian follicle system in vitro has been the challenge for making IVM successful. Culture systems, including the media composition, will need to be improved and optimized before IVM can become the method of choice for IVF. Until the culture systems for IVM are improved, it is expected that there might be differences between in vivo- and in vitro-matured oocytes. Although the IVM technique has been applied in the treatment of couples with polycystic ovaries, with the male factor and with unexplained infertility, there are questions that have been raised about its safety. Thus, no reports on the health of IVM children have been published, it was reported that perinatal outcome of the children born was good and the preliminary neurological and neuropsychological follow-up data of was reassuring. The development was found to be within normal range up to two years of age (Suikkari A., et al, 2005).

2. Problem presentation and aim of the work

Problem presentation

Infertile women with polycystic ovaries (PCO) and poor responders have a decreased cycle fecundity and a higher rate of miscarriage (Cha K. et al, 2000). As the expense, complexity and the risks of conventional in vitro fertilization (IVF) treatment continues to limit the availability and success of assisted conception, attention is turning to the prospects of oocytes in-vitro maturation (IVM). In contrast to the conventional assisted reproduction techniques, in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), where mature oocytes are retrieved for fertilization, immature oocytes in the IVM process are retrieved and matured in the laboratory before fertilization and embryo transfer (Chian, R. et al, 2004) b.

The in vitro maturation (IVM) protocol is relatively simple with a short period of treatment and reduced cost compared to conventional IVF. In addition, the side effects of stimulation, in particular ovarian hyper-stimulation syndrome, are eliminated (Mikkelsen A., 2001). The goals of IVM protocols/techniques are targeted to increase the rate of immature oocyte retrieval, optimize the media and culture conditions, and improve oocyte maturation potential, fertilization and pregnancy rates.

The optimum concentration of the hormones used to achieve oocyte in vitro maturation that would lead to the best results is not yet standardized. It is crucial to assess the optimum range of r-FSH concentrations in the culture media that produces best maturation rate (Hreisson J., et al, 2003). The follicle size is another important parameter affecting oocyte maturation and developmental competence. Its effect is still under thorough investigation. Despite the successes and the continuing research effort, the overall efficiency of IVM remains low, and neither clinical nor laboratory procedures can be considered as efficient and routinely feasible as conventional IVF techniques.

2.2 Aim of the work

- To study the effect of recombinant follicle stimulating hormone (r-FSH) concentration in the culture media on oocyte in vitro maturation, fertilization and cleavage.
- To study the effect of the follicles size on oocyte in vitro maturation, fertilization and cleavage.
- To compare the outcomes of in vitro maturation of immature oocytes recovered in situ from infertile women with polycystic ovaries versus poor responders.

3. Materials and Methods

3.1 Objectives

- To determine the effect of recombinant follicle stimulating hormone (r-FSH) concentration in culture media on in vitro maturation outcomes.
- To determine the effect of the follicle size on in vitro maturation outcomes.
- To determine the in vitro maturation outcomes of immature oocytes recovered in-situ from infertile patients with polycystic ovaries (PCO) and poor responders

This study was conducted during the period from April 2000 to December 2004. The results of this study were collected and evaluated from the Assisted Reproduction Unit at Almana General Hospital, Dammam, Eastern`s Province of the Kingdom of Saudi Arabia and in coorporation with the department of Obstetrics and Gynecology, Campus Benjamin Franklin, Charité, Homboldt University. The study was approved by the research ethics board of the hospital. A written informed consent was obtained from all patients.

3.1.1 The effect of r-FSH concentration in the culture media on in vitro Maturation of oocytes

In this experiment, oocytes were collected from 50 women with polycystic ovaries (PCO), (Group 1) who was all scheduled for ICSI. Patients were stimulated with a daily dose of 300 IU r-FSH (Purgeon, Organon, Holland) for 5 days, starting on day 2 of the menstrual cycle until day 6 when a transvaginal ultrasound scan was performed. The aim was to study the influence of follicle stimulating hormone concentration on the in vitro maturation, fertilization, cleavage and pregnancy rates. Recombinant FSH was used in two concentrations of 0.075 IU/ml, 7.5 IU/ml and none as a control. This experiment was performed on patients producing more than 15 germinal vesicles (GV) oocytes. Immediately after collection, the oocytes were equally distributed in three groups of 5 oocytes each in three center-well dishes containing 3 ml Ham's F10 media. Two r-FSH concentrations of 0.075 IU/ml and 7.5 IU/ml were added in two dishes and none as control, respectively. Oocytes in vitro maturation was evaluated 30 hours after

incubation. Oocytes maturation, fertilization, cleavage and pregnancy rates were assessed.

3.1.2 The effect of follicular size on the rate of oocytes in vitro maturation

In this experiment, oocytes were collected from 50 PCO patients (Group 2) who were stimulated with a daily dose of 300 IU (Purgeon, Organon, Holland) for 5 days, starting on day 2 of the menstrual cycle until day 6 when a transvaginal ultrasound scan was performed. The scan showed follicular size ranging between 8-13 mm. The PCO patients were divided into two subgroups according to follicular size on the day of hCG injection. Subgroup 2I had a follicular size of 8-10 mm and subgroup 2II had a size of 11-13 mm. The oocytes retrieved from these patients were incubated for 30 hours in Ham's F10 supplemented with 0.075 IU/ml r-FSH. Oocyte maturation, fertilization, cleavage, and pregnancy rates were then assessed.

3.1.3 In vitro maturation outcomes of immature oocytes recovered in-situ from infertile patients with polycystic ovaries (PCO) and poor responders

This part of the study was conducted on 40 infertile women (Group 3). This group was further divided into two subgroups, 20 infertile women with polycystic ovaries (subgroup 3I) and 20 poor responder infertile women (subgroup 3II). PCO patients (subgroup 3I) were further subdivided into two subgroups, 12 irregular cycling and anovulatory women (subgroup 3Ia), and 8 regular cycling women (subgroup 3Ib). The timing of the start of treatment was random, as most of the patients had irregular menstrual cycles. Table 3.1.1 shows the classification, definition, and the objective for each group and subgroup in the present study.

Table 3.1.4: The classification, definition and objective of the study groups.

Group	Definition	Objective
Group 1	Oocytes collected from 50 infertile women with PCO	To study the effect of different r-FSH concentrations (0.0, 0.075 IU/ml and 7.5 IU/ml) on in vitro maturation of oocytes
Group 2	Oocytes collected from 50 infertile women with PCO	To study the effect of follicular size on the rate of oocyte in vitro maturation
Subgroup 2I	Oocytes from follicles sized between 8 – 10 mm on the day of hCG injection	
Subgroup 2II	Oocytes from follicles sized between 11 – 13 mm on the day of hCG injection	
Group 3	40 infertile women (PCO or poor responders)	To study the in vitro maturation outcomes of immature oocytes recovered in-situ from infertile patients with polycystic ovaries (PCO) and poor responders.
Subgroup 3I	20 infertile women with PCO	
Subgroup 3Ia	12 irregular cycles and anovulatory PCO infertile women	
Subgroup 3Ib	8 regular cycling PCO infertile women	
Subgroup 3II	20 poor responders infertile women	

3.2. Patients' criteria

Infertile women with polycystic ovaries and poor responders were included in this study during their schedule for ICSI in the IVF program. The patients were recruited at random. It was fully explained to each patient that the procedures related to the study were not part of the routine diagnostic procedures required for their infertility assessment.

3.2.1. Patients were recruited after PCO was diagnosed by:

- Pelvic ultrasound (the ultrasonic criteria of PCO were essential for the diagnosis. It included the presence of more than 8 small follicles of 2-8 mm in diameter around a dense core of stroma and a dense ovarian capsule),
- Endocrine and clinical features that varied between regular and irregular cycles,
- Elevated androgen level, LH: FSH ratio >2, and frequently, the clinical features of hirsutism and increased body weight (Adams J. et al., 1985; Hershlag A. et al, 1996).

3.2.2. `Poor responders are patients who fulfilled one or more of the following criteria:

- Failed to achieve estradiol concentration above the level of 200 pg /ml on the day of hCG (Garcia J. et al, 1983).
- Produced less than three mature follicles during the previous stimulation attempts (Serafini P. et al, 1988).
- Failure and/or cancellation of previous IVF cycles due to low quality of oocytes retrieved in previous stimulations (Rienzi L. et al, 2002).

Independent counseling was provided to all patients for IVF /IVM-ET. The experimental procedures involved in the recovery and maturation of primary oocytes were explained. Women with amenorrhea received oral contraceptive Marvelon, (Organon) once daily for 21-45 days to induce withdrawal bleeding at a specified time to the IVF program. A baseline vaginal ultrasound scan was performed for all women between day 1 and 2 of the menstrual bleeding to ensure that no ovarian cysts were present. The baseline hormonal profile was also performed including estrogen, FSH, LH, progesterone, and testosterone on the same day of the baseline ultrasonography and on the day of oocyte retrieval. Ovarian stimulation with r-FSH 300 IU was given daily for five days.

Transvaginal ultrasound scans were repeated on either cycle day 8 and/or the day of hCG administration to exclude the development of a dominant follicle. The size of all follicles on ultrasound scan had to be <10mm in diameter at every scan to proceed to oocytes retrieval, which was performed between days 8 and 10 of the cycle. All patients received a single dose of 10,000 IU of hCG 36 hours before oocytes retrieval.

3.3 Oocytes retrieval and IVM Procedure

Transvaginal ultrasound guided oocytes collection was performed using a specially designed 17-G single-lumen aspiration needle (Casmed, UK) with a reduced aspiration pressure of 7.5 kpa. Aspiration of the follicles was performed under general anesthesia for all patients. All patients received an antibiotic cover of a single dose of 500 mg of metronidazole intravenously during the procedure.

Oocytes were collected in culture tubes containing warm Earl's balanced salt solution with 5000 IU/ml heparin. Immature oocytes were incubated in a culture dish containing 1ml of 3M (Medicult) medium supplemented with r-FSH (Puregon, Organon) (according to the stage of the study) and 5.00 IU/ml hCG (Pregnyl, Organon) at a temperature of 37°C in an atmosphere of 5% CO_2 and 95% air with high humidity. After incubation, the maturity of the oocytes was determined under the stereomicroscope at 30 hours post collection. Oocytes were denuded of cumulus and maturity was determined by the presence of the first polar body. Suitable oocytes were injected with single spermatozoa by micromanipulation (Research Instrument, UK). Following ICSI, each oocyte was transferred into 1ml of Medi-cult IVF medium in a tissue culture dish. Fertilization was assessed 18 hours after ICSI for the appearance of two distinct pronuclei and two polar bodies. Oocytes with two pronuclei were further cultured in Medi-cult IVF medium. Embryos were transferred on day 2 or 3 after ICSI.

3.4 Endometrial priming

For endometrial preparation, patients received estradiol valerate (Progyluton, Schering, Berlin, Germany) orally from the day of oocyte retrieval. The dose was calculated depending on the endometrial thickness on the day of retrieval. If the endometrial thickness measured less than 6 mm, a 10 mg dose was given and if the thickness was more than 6 mm, a dose of 6 mg was given. If it measured less than 6 mm, the couples

were offered embryo cryo-preservation and transfer in a subsequent cycle. Luteal support was provided by 100 mg of progesterone (Gestone, Shire Pharmaceuticals, UK) once daily starting on the day of ICSI and continued, along with estradiol valerate until day 14 from the day of embryo transfer when a blood test for Beta hCG was performed to ascertain pregnancy. If the pregnancy test was positive the luteal support was continued until 12 weeks gestation.

3.5 Media: Components of culture media

Table 3.5.1 Nutrients Mixture Ham's F-10

Old Cat. No. New Cat. No.	041-02390 22390 IX Liquid
Component	*Mg/L*
INORGANIC SALTS:	
CaCI2 (anhyd.)	-
CaCI2 *2 H_2O	44.00
CuSO4 *5 H_2O	0.0025
FeSO4 *7H_2O	0.834
KCI	285.00
KH2PO4	83.00
MgSO4 (anhyd.)	-
MgSO4 *7 H_2O	153.00
NaCI	6900.00
NaHCO3	1200.00
Na2HPO4 (anhyd.)	154.50
ZnSO4 *7 H_2O	0.0288
OTHER COMPONENTS:	
D-Glucose	1100.00
HEPES	5958.00
Hypoxanthine	4.08
Hypoxanthine (sodium salt)	-
DL-68-Thioctic Acid	0.20
Phenol Red	1.20
Sodium Pyruvate	110.00
Thymidine	0.73
AMINO ACIDS:	
L-Alanine	8.92
L-Arginine *HCI	211.00
L-Asparagine	12.98
L-Aspartic Acid	13.30
L-Cysteine	25.00
L-Glutamic Acid	14.70
L-Glutamine	146.00

L-Alanyl – L-Glutamine	-
L-Glycine	7.52
L-Histidine HCl * H_2O[b]	23.00
L-Isoleucine	2.60
L-Leucine	13.10
L-Lysine * HCl	29.30
L-Methionine	4.48
L-Phenylalanine	4.96
L-Proline	11.50
L-Serine	10.50
L-Threonine	3.58
L-Tryptophan	0.60
L-Tyrosine	1.81
L-Tyrosine (disodium salt)	-
L-Valine	3.50
VITAMINS:	
Biotin	0.024
D-Ca Pantothenate	0.72
Choline Chloride	0.70
Folic Acid	1.31
i-Inositol	0.54
Niacinamide	0.62
Pyridoxal HCl	0.21
Riboflavin	0.38
Thiamine HCl	1.01
Vitamin B_{12}	1.36

3.6 Statistical Analysis:

Statistical analysis was done by the student's *t- test*. Frequency data was analyzed by χ^2 contingency tests. Embryo development ratio data was analyzed by analysis of variance. Values were considered significant when $P < 0.05$. Since the oocytes were not matured and inseminated at the same time following maturation in culture, the development stages of embryos were variable both within and between patients.

4. Results

The results of the present study were based on data generated from the three experiments. The mean duration of infertility was 12.3 ± 4.6 years for all patients of the study groups. All patients were under 45 years of age with a range of 21 – 44 years (mean 35.1 ± 5.3 years).

4.1. Results of the first experiment

The first experiment was designed to define the optimum r-FSH concentration. This study's data showed that 0.075 IU/ml was the optimum concentration that provided higher in vitro maturation, fertilization and pregnancy rates compared to 7.5 IU/ml and the control. Details regarding the number of oocytes collected, maturation, fertilization and pregnancy rates after in vitro maturation in media containing different concentrations of r-FSH are shown in tables 4.1.1-4. Recombinant FSH concentration had significantly ($p<0.05$) increased the rate of oocyte maturation from 47% at 0 IU/ml to 81% and 83% at 0.075 IU/ml and 7.5 IU/ml, respectively. Fertilization, cleavage, and clinical pregnancy rates showed a similar trend and significantly increased from 45% to 83% and 80%, from 32% to 80% and 77%, and from 0% to 17% and 14% at the three concentrations, respectively. The 6 and 5 pregnancies resulting from oocyte cultured in media containing 0.075 and 7.5 IU/ml all ended in delivery of healthy children. The results, however, showed that increasing r-FSH concentration to levels more than 0.075 IU/ml did not further improve maturation, fertilization, cleavage and pregnancy rates even when the concentration was increased up to 100 folds.

Table 4.1.1: The effect of different r-FSH concentrations on oocytes maturation, fertilization, embryo`s cleavage and pregnancy rate.

Parameters	**r-FSH Conc. (0.00 IU/ml)**	**r-FSH Conc. (0.075 IU/ml)**	**r-FSH Conc. (7.5 IU/ml)**
GV collected	225	219	230
Matured oocyte	105 (47%)	178 (81%)	186 (83%)
Fertilized oocyte	47 (45%)	147 (83%)	149 (80%)
Cleaved embryos	15 (3%)	119 (80%)	116 (77%)
Transferred embryos	12 (2/ET)	55 (1.4ET)	60 (1.7/ET)
Clinical Pregnancy	0.00	6 (17%)	5 (14%)
No. of patients who had ET	6	39	35

Table 4.1.2: The effect of two different recombinant FSH concentrations (0.00 – 0.075 IU/ml) on oocytes maturation, fertilization, embryo`s cleavage and pregnancy rate.

Parameters	**r-FSH conc. (0.00 IU/ml)**	**r-FSH conc. (0.075 IU/ml)**	***P* - *value***	**Significance**
GV collected	225	219	> 0.05	Non significant
Matured oocyte	105 (47%)	178 (81%)	< 0.05	Significant
Fertilized oocyte	47 (45%)	147 (83%)	< 0.05	Significant
Cleaved embryos	15 (3%)	119 (80%)	< 0.05	Significant
Transferred embryos	12 (2/ET)	55 (1.4ET)	< 0.05	Significant
Clinical Pregnancy	0.00	6 (17%)	< 0.05	Significant
No. of patients who had ET	6	39	< 0.05	Significant

Table 4.1.3: The effect of two different recombinant FSH concentrations (0.00 – 7.5 IU/ml) on oocytes maturation, fertilization, embryo`s cleavage and pregnancy rate.

Parameters	**r-FSH conc. (0.00 IU/ml)**	**r-FSH conc. (7. 5 IU/ml)**	***P* - *value***	**Significance**
GV collected	225	230	> 0.05	Non significant
Matured oocyte	105 (47%)	186 (83%)	< 0.05	Significant
Fertilized oocyte	47 (45%)	149 (80%)	< 0.05	Significant
Cleaved embryos	15 (3%)	116 (77%)	< 0.05	Significant
Transferred embryos	12 (2/ET)	60 (1.7/ET)	< 0.05	Significant
Clinical Pregnancy	0.00	5 (14%)	< 0.05	Significant
No. of patients who had ET	6	35	< 0.05	Significant

Table 4.1.4: The effect of two different recombinant FSH concentrations (0.075 IU/ml – 7.5 IU/ml) on oocytes maturation, fertilization, embryo`s cleavage and pregnancy rate.

Parameters	**r-FSH conc. (0.075 IU/ml)**	**r-FSH conc. (7. 5 IU/ml)**	***P* - *value***	**Significance**
GV collected	219	230	> 0.05	N.S
Matured oocyte	178 (81%)	186 (83%)	> 0.05	N.S
Fertilized oocyte	147 (83%)	149 (80%)	> 0.05	N.S
Cleaved embryos	119 (80%)	116 (77%)	> 0.05	N.S
Transferred embryos	55 (1.4ET)	60 (1.7/ET)	> 0.05	N.S
Clinical Pregnancy	6 (17%)	5 (14%)	> 0.05	N.S
No. of patients who had ET	39	35	> 0.05	N.S

3.3. Results of the second experiment

Based on the optimum concentration resulting from the first experiment, the second experiment was designed to study the effect of the follicle size on oocytes maturation, fertilization and developmental competence.

The results of two different follicle sizes (group 2) are shown in table 4.2.1. Oocytes retrieved from 11-13 mm follicles (subgroup 2II) showed higher rates of maturation, fertilization and pregnancy, than those retrieved from 8-10 mm follicles (subgroup 2I). The above parameters increased from 48 to 70%, from 54 to 76%, and from 11 to 22.5% in the two follicle sizes, respectively. The two pregnancies resulting from 8-10 mm follicles size completed full term, whereas two of the nine pregnancies in the 11-13 mm follicle size subgroup ended in miscarriage and the remaining seven pregnancies ended in the delivery of healthy babies. Follicular size showed significant ($P<0.05$) effect on the assessment parameters.

Table 4.2.1: The effect of follicular size on oocytes-maturation, fertilization, embryo cleavage, and pregnancy rate.

Parameters	**Oocytes from follicles of size 8-10 mm (subgroup 2I)**	**Oocytes from follicles of size 11-13 mm (subgroup 2II)**	***P - value***	**Significance**
GV collected	250	250		
Matured oocyte	120 (48%)	177 (70%)	< 0.05	Significant
Fertilized oocyte	65 (54%)	138 (76%)	< 0.05	Significant
Cleaved embryos	42 (64%)	94 (68%)	< 0.05	Significant
Transferred embryos	33 (1.9/ET)	60 (1.7 ET)	< 0.05	Significant
Clinical pregnancy	2 (11%)	9 (22.5%)	< 0.05	Significant
No. of patients who had ET	17	40	< 0.05	Significant

3.4. Results of the third experiment:

The mean age of group 3 patients was 32.3 ± 5.8 years for subgroup 3I (PCO patients) and 36.4 ± 7.1 years for subgroup 3II (poor responders patients). There was no significant difference regarding the age. The means of parity, abortion, and Hb% were also comparable between the two subgroups. Both the body mass index (29.7 ± 2.3 kg/m^2 vs. 27.1 ± 1.6 kg/m^2) and the duration of the cycles (53.2 ± 21.3 days vs. 30.3 ± 8.6 days) were significantly higher in subgroup 3I (see table 4.3.1).

In the study group (3) patients, there was no difference shown between the two subgroups 3I and 3II in the concentrations of estradiol, progesterone, FSH, and LH on day 2 of the menstrual cycle and on the day of oocytes retrieval (see tables 4.3.2, 4.3.3). From 20 PCO women (subgroup 3I), the mean number of oocytes recovered, matured in vitro, fertilized after insemination, and cleaved in culture were 23.5, 16.1, 7.3, and 4.7 respectively. From 12 irregular PCO women (subgroup 3Ia), the mean numbers of oocytes recovered, matured in vitro, and fertilized after insemination, and cleaved in culture were 18.1, 11.7, 4.2, and 3.1, respectively. The mean numbers of oocytes recovered, matured, fertilized and cleaved from 8 regular cycling PCO women (subgroup 3Ib) were 5.4, 4.4, 3.1 and 1.6, respectively. Oocytes recovered from regular cycling patients (subgroup 3Ib) had a higher developmental potential when compared with irregular and anovulatory patients (subgroup 3Ia) with significantly ($P < 0.05$), higher maturation and fertilization rates (table 4.3.4). Cleavage was not significantly different between the two subgroups, although there was a trend to increased cleavage of embryos in the regular cycling subgroup. Moreover, embryos produced from regular cycling patients had a significantly higher embryo development ratio ($P< 0.05$), indicating the faster cleavage rate of embryos produced from this group of patients. Embryo development ratio is defined as the observed cleavage stage / the expected cleavage stage × 100. Three pregnancies were obtained; one delivered a preterm at 36 weeks and two miscarried at 8 and 10 weeks. From 20 poor responder women (subgroup 3II), the mean numbers of oocytes recovered, matured in vitro, fertilized after insemination, and cleaved in culture were 18.1, 14.5, 5.1 and 3.4 respectively (table 4.3.5). The embryo development ratio was 63.4 ± 2.6.

One pregnancy resulted in the delivery of a full term female baby. Table 4.3.6 shows the mean of oocytes recovered, matured, fertilized, and cleaved in the study group 3.

Table 4.3.1: Patient's criteria in Group 3 (PCO, [subgroup 3I], and poor responders infertile women, [subgroup 3II])

Variable	Subgroup 3I	Subgroup 3II	P-value	Significance
Age (years)	32.3 ± 5.8	36.4 ± 7.1	0.496	Non significant
Parity	0.63 ± 1.2	0.94 ± 1.8	0.251	Non significant
Duration of cycle (days)	53.2 ± 21.3	30.3 ± 8.6	< 0.05	Significant
BMI (kg/m^2)	29.7 ±2.3	27.1 ± 1.6	< 0.05	Significant
Hb g%	13.0 ± 0.7	12.5 ± 1.0	0.126	Non significant

Table 4.3.2: Hormonal profile on day 2 of the cycle in Group 3 (PCO, [subgroup 3I], and poor responders infertile women, [subgroup 3II])

Hormone	Subgroup 3I	Subgroup 3II	P-value	Significance
FSH (IU/l)	7.9 ± 9.8	8.9 ± 6.0	0.844	Non significant
LH (IU/l)	5.5 ± 6. 4	4.8 ± 3.3	0.295	Non significant
Estrogen (pmol/l)	37.3 ± 20.9	32.6 ± 15.4	0.117	Non significant
Progesterone (nmol/l)	1.31 ± 0.48	2.0 ± 2.5	0.132	Non significant

Table 4.3.3: Hormonal profile on the day of oocytes retrieval in Group 3 (PCO, [subgroup 3I], and poor responders infertile women, [subgroup 3II])

Hormone	Subgroup 3I	Subgroup 3II	P-value	Significance
LH (IU/l)	27.7 ± 3.9	81.8 ± 3.24	0.054	Non significant
Estrogen (pmol/l)	1177.4 ± 996.9	1030.0 ± 917.4	0.631	Non significant
Progesterone (nmol/l)	2. 06 ± 0.77	3.06 ± 3.44	0.088	Non significant

Table 4.3.4: Oocytes maturation, fertilization and cleavage in vitro in group 3 (PCO, [subgroup 3I], and poor responders infertile women, [subgroup 3II])

Patients of	No. of	No. of	No. of	No. of	Embryo

group 3	oocytes cultured	oocytes matured	oocytes fertilized	oocytes cleaved	development ratio
(subgroup 3I) PCO patients	250	170 (68%)	56 (22.4%)	45 (18%)	74.2 ± 2.6
(subgroup 3II) Poor responders	200	140 (70%)	60 (30%)	35 (17.5%)	63.4 ± 2.6
P-value	0.251	0.125	0.046	0.223	0.049

Table 4.3.5: Oocytes in vitro maturation, fertilization and cleavage in subgroup 3I (PCO infertile patients)

Patient subgroup 3I	No. of oocytes cultured	No. of oocytes matured	No. of oocytes fertilized	No. of oocytes cleaved	Embryo development ratio
Irregular anovulatory (subgroup 3Ia) (12 patients)	175 (14.6/ patient)	112 (64%)	31 (27.9%)	27 (14.1%)	66.7 ± 3.1
Regular cycle (subgroup 3Ib) (8 patients)	75 (9.4/ patient)	58 (74%)	25 (33%)	18 (18%)	81.5 ± 3.4
Total	250	170 (68%)	56 (22.4%)	45 (18%)	74.2 ± 2.6
P-value	0.046	0.016	0.038	0.049	0.028

5. Discussion

5.1. Polycystic ovary syndrome (PCOS) and the dilemma of its diagnosis

The prevalence of polycystic ovary syndrome (PCOS) in the community was found to be 21% (Farquhar C. et al, 1994; Williamson K. et al., 2001), based on the clinical and endocrinological data. The ultrasound appearance of PCOS was reported in 90% of women with hirsutism and regular cycles, 87% with oligomenorrhea and 32% with amenorrhea (Adams J. et al., 1985; Zawadzki J. et al, 1992). Anovulation, the key feature of PCOS represents as amenorrhea in approximately 55% of cases, and with irregular heavy bleeding in 28% (Michelmore K. et al, 1999). In a recent study, 2004, Aziz R. and his co-workers reported that 6% to 8% of unselected women of reproductive age suffer from PCOS.

The clinical definition of PCOS is characterized by four symptoms: oligomenorrhea to amenorrhea, infertility, hirsutism, and obesity. Some studies demonstrate the absence of one or more of these symptoms (Hershlag A. et al, 1996). Ovarian morphology using the criteria described by Adams J. et al, 1985, (10 or more cysts, 2-8 mm in diameter, arranged around an echo-dense stroma) appears to be the most sensitive diagnostic marker for polycystic ovaries. The present study was conducted during the period from April 2000 to December 2004.

The selection criteria for PCO patients were based on the criteria described by Hershlag A. et al, 1996 and Adams J. et al., 1985. **New diagnostic criteria for polycystic ovary syndrome (PCOS)** were proposed in Rotterdam in 2003, which expanded the previous definition that arose in 1990 during the conference sponsored by the National Institute of Health (NIH) in the USA (Aziz R., 2005). During the U.S. conference, Drs. Zawadski and Dunaif concluded that the major criteria for PCOS should include, in order of importance:

i) Hyperandrogenism and/or hyperandrogenemia
ii) Oligo-ovulation
iii) Exclusion of other known disorders

It is clear that their conclusion identifies PCOS as an androgen excess disorder of exclusion, with ovarian consequences (Zawadski J., Dunaif A., 1992). Clinical

hyperandrogenism has generally been interpreted as hirsutism, since more than 70% of hirsute women are hyperandrogenemic (Aziz R., et al. 2004). Consequently, the diagnostic criteria agreed upon were hirsutism, hyperandrogenemia and oligo-ovulation. The presence of polycystic ovaries by ultrasound was suggestive, but not diagnostic of PCOS. However, it is now clear that many patients with PCOS do demonstrate ultrasound evidence of polycystic ovaries (Carmina E., et al, 1992; Swanson M. et al, 1981; Jonard S., et al, 2003). The Rotterdam conference in 2003 expanded the diagnostic criteria of PCOS. It recommended that PCOS be defined when at least two of the following three features were present:

i) Oligo and/or anovulation

ii) Clinical and/or biochemical signs of hyperandrogenism

iii) Polycystic ovaries. Polycystic ovaries as defined by the 2003 Rotterdam criteria referred to the presence of at least one ovary exhibiting 12 or more follicles measuring 2-9 mm in diameter, regardless of location, and/or a total volume > $10mL^3$, as determined by transvaginal ultrasound (Rotterdam ESHRE/ASRM 2004).

Polycystic ovaries may be associated with several endocrinopathies (Carmina E., et al. 2004). Studies comparing women with polycystic ovaries to normal controls have shown elevated concentrations of LH, LH to FSH ratio, fasting insulin, testosterone and androstendione and reduced concentration of sex hormone binding globulin (SHBG). However, the classical hormone changes are not seen in all patients (Fox et al, 1991: Hamilton-Fairley D. et al, 1993). **In the present study, the hormonal profiles among the patients in group 3 (PCO infertile patients and poor responder infertile patients) were comparable. Both LH and estrogen levels were mildly elevated in PCOS patients (subgroup 3I). This elevation was of no statistical significance.**

It is also concluded that obesity leads to hyperinsulinism (Eden J., et al, 1989; Norman R., et al, 1995, Kousta E., et al, 1999; Cibula D. et al, 2002; De Ugarte C. et al, 2004; Dunaif A., 2006), which causes both hyperandrogenaemia, and raised IGF-1 levels which augments the ovarian response to gonadotrophins (Wu X., et al, 2003). This implies that obesity may be important in the pathogenesis of polycystic ovaries, but further studies are required to evaluate this. Indeed, in a study by Balen et al, (1995*)*

only 38.4% of patients were overweight (BMI >25kg/m^2). **The results of our study showed that the basal body mass index was significantly higher in the PCO patients in comparison to the poor responder women (29.7 kg/m^2 vs 27.1 kg/m^2). Also the duration of the cycles was significantly longer in PCO patients than in poor responder women (53.2 ± 21.3 kg/m^2 vs 30.3 ± 8.6 kg/m^2). Obesity and oligomenorrhea are both two main characteristic features in our PCO patients.**

5.2. Poor Responders

The ovarian response to gonadotrophins in controlled ovarian hyperstimulation (COH) is sometimes difficult. Prediction of poor responder outcome represents a challenge to those carrying out assisted reproduction techniques. Low ovarian response to COH occurs in 9-18% of the cases (Scott R., 1996). The definition of “poor response” varies from one author to another. The original definition of low response was based on a low peak estradiol level, and a small number of follicles and oocytes retrieved (Garcia J. et al, 1983). Serafini P. et al, 1988, defined poor responders as those producing less than three follicles. Other authors (Karande V. et al, 1999) have referred to four follicles as their cut-off point, while Land J. et al, (1996) abandoned the cycle when less than five follicles are produced. Some authors have considered less than six follicles as a reason for cancellation in COH (Rienzi L. et al, 2002). In spite of these differences in definition, poor response cases often lead to cycle cancellation and to another try for better response in a subsequent cycle (Lui J. et al, 2003). **In the present study, poor responder`s patients were patients who failed to achieve estradiol concentration above the level of 200 pg /ml on the day of hCG and failed to develop or developed a maximum of one matured follicle during the previous attempts, with a history of failure and cancellation of previous IVF cycles due to low number and quality of oocytes retrieved in previous stimulations.**

The availability of screening tests to identify patients with poor response to ovarian stimulation would provide physicians with a valuable means of selecting a proper treatment protocol. Tests of functional ovarian reserves can often be used to predict low response to standard protocols (Sharara F. et al, 1998). These tests include basal levels of follicle stimulation hormone (FSH), luteinizating hormone (LH), FSH: LH ratio, estradiol (E2), inhibin B, ovarian biopsies, and follicular density assay (Toner J.P. et al, 1991; Seifer D.B. et al, 1997). Other tests include dynamic ovarian reserve assessment as clomiphene citrate challenge test [CCCT] (Navot D. et al, 1987), gonadotrophin releasing hormone agonist stimulation test [GAST] (Winslow K.L. et al, 1991), and exogenous follicle stimulating hormone ovarian reserve test [EFFORT] (Fanchin R. et al, 1994).

Ultrasonography (US) may help in predicting poor responder`s cases. Measurement of ovarian volume (Lass A. et al, 1997), antral follicle count (Ruess M. et al, 1996), and ovarian stromal blood flow with color Doppler (Engmann L. et al, 1999) are promising techniques for ovarian reserve prediction. All tests considered have provided prognostic information, but sometimes this information is relative. The mean ovarian diameter (MOD) measured in the largest sagittal plane of the ovary correlates with the ovarian volume and could be used for fast assessment of the ovarian status before COH (Farrattareli G. et al, 2002). The regulation and significance of the ovarian and uterine haemodynamics in human reproductive pathophysiology is becoming an important tool. Increased vascularization of ovarian follicles in the course of their development occurs in experimental animals (Koning H. et al., 1989). In women, enhanced vascularization seems to be responsible for the selection and maturation of follicles both in spontaneous and stimulated IVF cycles (Weiner Z. et al, 1993; Balakier H. et al, 1994; Bassil S. et al, 1997). Gonadotrophins, steroids, prostaglandins, and other vasoactive molecules are involved in the regulation of ovarian blood flow (Taymor M., 1996). The importance of nitric oxide (NO) as an intra-and intercellular modulator has been recognized in many biological processes, including ovarian physiology (Anteby E. et al, 1996).

Among the variables correlated with a poor ovarian response to exogenous gonadotrophins are maternal age and serum concentrations of follicle stimulating hormone (FSH) in the follicular phase (Padilla et al., 1996). **In this study, the mean age of poor responder women was higher than the PCO women (36.4 years vs 32.3 years).** Although the difference was not significant, it is well known that the age affects the outcomes of both IVF and IVM, especially the oocyte characters and the final outcome. **The results showed a higher mean level of basal serum FSH in poor responder women than the mean serum FSH level in PCOS women. This difference was not significant.** In particular, within the population of poor responders, women with elevated baseline FSH concentrations on menstrual cycle day three tend to respond poorly in subsequent cycles (Hershlag A. et al, 1990).

Not withstanding the various stimulation protocols that have been devised in the attempt to improve IVF outcome in poor responders to ovarian stimulation (Ben-Rafael Z. et al,

1991; Jenkins J. et al, 1991; Dor J. et al, 1995; Awonuga A. et al, 1997; Schoolcraft W. et al, 1997; Lashen H. et al, 1998), the latter technique remains one of the most controversial and disappointing issues in reproductive medicine.

In vitro maturation is an alternative strategy for the treatment of poor responders. As this group of patients are resistant to gonadotropin stimulation for various reasons and as they require prolonged and higher doses of gonadotrophin stimulation protocols, IVM provides a different approach to a safer and cheaper treatment modality. In addition, natural-cycle IVF combined with IVM might provide more efficient treatment for poor responder infertile women (Chian R. et al, 2004a).

5.3. Oocyte maturation in vivo and in vitro

The feasibility of obtaining full-term pregnancies from in vitro–matured immature oocytes obtained from stimulated and non-stimulated ovaries is well established (Veek L. et al, 1983; Cha K. et al, 1991). The scarcity of subsequent reports points to the fact that the procedure is not even close to being transferred into daily clinical work. The impossibility of judging ooplasmic maturation forces the use of nuclear maturation as the basis for classification of female gametes. There is no way, at the present time, to know whether all oocytes classified as prophase I (PI), for instance, have the same degree of ooplasmic maturation. It is theoretically possible that the oocytes of PI that are able to establish a term pregnancy belong to a subgroup exhibiting more advanced or more favorable cytoplasmic maturation. Furthermore, P I oocytes from primordial follicles (Primordial oocytes) have not experienced the growth phase found in preantral developing follicles (preantral oocytes). This growth phase is synchronized with that of the surrounding follicular somatic cells and implies a substantial increase in oocyte mass (Baker T. et al, 1973; Coticchio G. et al, 2004). It is known that, during the growth phase, oocytes experience an overwhelming amount of protein and RNA synthesis, and accumulates a protein pool allowing the gamete to acquire competence for maturation, fertilization, activation, pre-implantation development, pregnancy establishment and zona pellucida formation, which for the most part occurs during the growth phase of preantral oocytes (Philpott C. et al, 1987). The synthesis of proteins during nuclear maturation may or may not be related to the disappearance of the nucleolemma during transition from PI to metaphase I (MI) (Sun F. et al, 1991).

As a corollary to all investigation performed, it can be safely stated that nuclear, cytoplasmic and somatic cell follicular maturation, although frequently well synchronized in natural and stimulated cycles, is not necessarily so under abnormal in vivo or under in vitro conditions. In the absence of gonadotropins in the medium, the presence of granulose cells is mandatory for oocyte growth and nuclear maturation; the maintenance of gap junctions is necessary for growth but not for nuclear maturation (Herlands R. et al, 1984). The gap junction system seems to be useful in compensating for deficiencies of the mammalian oolemma and may provide the means for exchange of chemicals (amino acids, growth factors, macromolecules), some of which may have a paracrine or

juxtacrine effect when gap junctions are not present (co-cultures). Packer and co-workers (Packer Al. et al, 1994) have described a ligand (kit ligand, stem cell factor or steel factor) that can accelerate oocyte growth when added to an oocyte-cumulus complex system with reduced gap junction /oolemma interaction surfaces. This factor is produced by granulose cells and interacts with the proto-oncogene c-kit produced by the kit locus, present in the oocyte. Furthermore, through the experience obtained in IVF, it is also possible that the oocyte may send messengers (diacylglycerol, for instance) that may provide paracrine or juxtacrine control over granulose cell function. The presence of gonadotropins in the culture medium seems to be beneficial, although not mandatory, for the oocyte to resume meiosis. Even when the oocytes resume meiosis, they do so much later in vitro than in vivo, and it is unknown whether gonadotropins can accelerate the process (Eppig J. et al, 1996).

In the mouse, in vitro fertilization capacity and embryo development of oocytes from primordial follicles require a two-step culture system: organ culture and oocyte-cumulus culture. Epidermal growth factor (EGF) and follicle-stimulating hormone (FSH) enhance the survival of the complex and the completion of meiosis, respectively. Despite this, the development to the blastocyst stage is very low. Alternatively, the success of fertilization and embryo development of oocytes derived from preantral follicles matured in vitro depends on the age of the animal donating the gametes; it is always lower than with oocytes grown in vivo and it is related to the quality of the culture medium (serum-free or with added fetuin). These gametes show less ability to undergo nuclear maturation, to fertilize, to develop to the blastocyst stage and to produce term pregnancies than oocytes grown in vivo.

It has been demonstrated that culture media supplementation with nutritional factors, ions, physical elements, gonadotropins, steroids, growth factors and other substances improve culture conditions and results. Another requirement is the change in culture conditions as embryo development progresses (modifications in oxygen tension, prevention of apoptosis by supplementation with gonadotropins, steroids and growth factors, and the preservation of the granulose cell phenotype) (Morgan P. et al, 1991; Alak, B. et al, 1994; Eppig J. et al, 1996).

Barnes and colleagues, (Barnes F. et al, 1996), examined the maturation and development of primary oocytes recovered from untreated women with regular cycles, or with polycystic ovary syndrome (PCOS) with anovulation or irregular cycles. Oocyte-cumulus masses were placed in a medium supplemented with rFSH and hCG. Insemination of matured oocytes was performed using 4 x 10 of the husband's spermatozoa. The embryo development ratio of these fertilized oocytes, classified as slow, average or rapid, was compared with that of in vitro matured oocytes from regularly cycling women. Oocytes recovered from regular cycling patients have a higher development potential than those from irregular or anovulatory PCOS patients. There was a trend to increased cleavage of embryos in the regular cycling group, which had a significantly higher embryo development ratio (faster cleavage). The highest mean embryo development ratio was obtained in oocytes with a complete cumulus and corona cover from regularly cycling women. Oocytes matured in vitro had a significantly higher rate of arrest at the two-cell stage than oocytes matured in vivo, and the embryo development rate was also much lower. Interestingly, the development ratio of in vitro-matured oocytes from regularly cycling women was no different from that of the in vivo-matured oocytes.

Veeck and colleaguesq produced one of the first clinical papers on the subject, studying a total of 74 oocytes at the P I stage obtained in 44 cycles after conventional ovarian stimulation with human menopausal gonadotropin (hMG)/FSH/hCG without pituitary suppression by a gonadotropin-releasing hormone analog (GnRHa) (Veeck, L. et al, 1983*).* Thirteen percent failed to mature; 7% matured but failed to fertilize; 8% showed abnormal fertilization; 9% failed to cleave and only 59.5% were available for transfer (80% with cleavage and 20% at the pronuclear stage). A total of 44 conceptuses were transferred in 30 patients, but only 15 of them were derived solely from immature oocytes (five were multiple embryo transfers and ten were single embryo transfers). Two gestations were obtained, but only one developed into term (6%) after the transfer of three embryos at the four and five-cell stage, respectively. The other gestation ended in a preclinical abortion. This paper (Veeck, L. et al, 1983) was written in such a way that the most positive aspects are not quite clear to the reader.

To summarize those results in a positive fashion: 87% of the oocytes matured; 93% of the inseminated oocytes fertilized and 92% fertilized normally (two pronuclei); 90.5% of the normally fertilized oocytes cleaved, but the pregnancy rate, when only embryos derived from immature ooctyes were transferred, was 13% and the delivery rate was 6.6%. This work concludes that the procedure is very efficient in obtaining nuclear maturation, fertilization and cleavage of the immature oocytes matured in vitro, but it is inefficient in producing term pregnancies.

Cha and colleagues in 1991 investigated the effect of mature follicular fluid on immature follicular oocytes to evaluate the developmental capacity of embryos after in vitro maturation, fertilization and transfer in a donor ooctye program. Other ooctyes were obtained from biopsy specimens or excised ovaries from 23 women aged 20-50 years, undergoing surgery for benign gynecological pathology. Oocytes were also obtained from patients in a regular IVF program. The culture medium for maturing of the immature oocytes was Ham's F-10 with the addition of 20% human fetal cord serum (FCS) or 50% follicular fluid from mature stimulated follicles. A total of 270 immature oocytes were collected from 23 ovaries. The maturation rate was 35.9% when FCS was used and 55.8% when follicular fluid was utilized. The fertilization rate was 31.6% and 81%, respectively. Oocytes obtained from non-stimulated ovaries produced more normal embryos (78.7%) than those retrieved from stimulated ovaries (61.4%), although the rate of fertilization was significantly lower in the non-stimulated ovaries (73.0% vs. 45.2%).

Very few papers were subsequently published until the work of Trouson and co-workers appeared in 1994 (Trounson A. et al, 1994). They used immature oocytes from women with non-treated PCOS or with polycystic ovaries. Unfortunately, it is well known in programs of IVF that such patients may produce oocytes of low quality, mainly with regard to implantation and term pregnancy rates. On average, more than five times the oocytes were retrieved from women with PCOS than from ovulatory women without polycystic ovaries. Eighty-one percent of cultured oocytes from women with PCOS were mature after 48-54 h. Thirty-four percent of the oocytes inseminated had pronuclei, 23% had two pronuclei, and 56% cleaved to eight cells or more in culture. Only 11% of the total number of oocytes inseminated reached the cleavage stage. No significant

differences in the results were observed between the PCOS and the polycystic ovary groups. Thirteen patients went on to embryo transfer and one (8%) from a PCOS patient established an ongoing pregnancy that was in the second trimester at the time of publication. These results are similar to those obtained by Veeck and co-workers (Veeck, L. et al, 1983).

In 1995, Trounson's group (Barnes F. et al, 1995*)* reported the sequential use of a series of recently developed assisted reproduction and assisted fertilization procedures on immature oocytes from non-stimulated polycystic ovaries (in vitro maturation, intracytoplasmic sperm injection (ICSI), in vitro culture, assisted hatching and improved embryo and uterine synchrony) to obtain blastocyst development and a live birth in a patient with polycystic ovaries. Reviewing the paper in detail, one comes to the conclusion that the procedure had a very low efficiency level and that some circumstances, to which the authors assigned importance, were occurrences over which the physician had no control, e.g. the presence of a preovulatory follicle at the time of retrieval.

In 1996, the Brussels group (Nagy Z. et al, 1996*)* published the retrieval, in a 29-year-old woman, of 14 ooctyes in the germinal vesicle stage (reason unknown) after conventional gonadotropin ovarian stimulation (hMG) under GnRHa (buserilin acetate) suppression. They were co-cultured with their own cumulus cells. After 30 h, nine oocytes reached the M II stage. ICSI was performed in all of them, owing to very poor sperm characteristics; seven showed normal fertilization and five cleaved to the 4-6 cell stage at 42 h after ICSI. All embryos were of very good quality and the best four were transferred. A normal female baby was delivered, with a normal 46XX karyotype on prenatal screening. This procedure was triggered by an undiagnosed problem in the stimulation, ending in the retrieval of all immature (P I) oocytes, and therefore was not part of a planned research program on the clinical use of immature oocytes, although the authors stated that maturation attempts with M I and P I ooctyes were part of their daily practice.

Barnes F. et al, (1996), utilized immature oocytes obtained in ICSI cycles in an attempt to mature them in vitro and performed ICSI after maturation. If they fertilized normally, they were cryo-preserved at the zygote or an early cleavage stage. The pregnancy potential of these cryo-preserved zygotes and embryos has yet to be determined. Cha and associates (1998) repeated a study on PCOS oocytes, trying to determine the maturation, fertilization and subsequent developmental competence of immature follicular oocytes obtained from non stimulated PCOS patients. After in vitro maturation, ICSI was performed; co-culture with a confluent Vero cell monolayer was used. Assisted hatching was performed on every embryo transferred. Out of 369 oocytes collected and 365 cultured, 59.5% matured. Of these, 75.7% fertilized and 82.6% cleaved. The pregnancy rate was 13.3% per transfer and 11% per case.

Racowsky and associates, (1996), evaluated the effect of FSH in the culture medium and related it to the phase of the menstrual cycle in which the oocytes were retrieved and to the size of the cumulus mass in oophorectomy specimens. In oocytes obtained in the follicular phase, the size of the cumulus mass does not seem to have an influence on the response to FSH. The presence of FSH significantly enhanced the maturation of oocytes to the M II stage. Oocytes that are collected during the luteal phase required a significantly larger FSH concentration. It's maturation was influenced by cumulus mass size.

In 1991, Cha et al reported a pregnancy from IVF with oocytes obtained from ovariectomy specimens and matured in culture (Cha K., et al, 1991). The idea that immature oocytes extracted from the ovary could be coaxed into maturing from the germinal vesicle stage to the metaphase II (MII) stage in vitro, and then fertilized and resulted in a pregnancy, has led to further efforts to develop IVM techniques. The goal was then to acquire oocytes from the ovary before an LH surge or an hCG injection and to continue the development of the oocytes in vitro to produce oocytes ready for IVF.

In 1994, Trounson et al, reported the birth of a normal baby with IVM of immature oocytes from a polycystic ovary syndrome (PCOS) patient undergoing IVF who had not

been triggered to ovulate (Trounson A. et al, 1994). Although there have been more than 300 births of babies with IVM procedures, including patients with PCOS (Chian R. et al, 2004b), IVM has not become mainstream in IVF, with ovulation induction cycles with oocyte retrieval of mature (MII) oocytes still the highly favored protocol. Some clinics are reporting no differences in pregnancy rated between IVM and in vivo-matured oocytes (Cha K. et al, 2005). However, in most clinics, the pregnancy and live birth rates with IVM do not match those reported for IVF cases using full hormonal protocols with triggered maturation in vivo. Therefore, only specific patients are currently considered for IVM, most notably PCOS patients who might be more sensitive to the elevated levels of gonadotropins and steroids experienced during an IVF protocol.

Patients at risk for ovarian hyperstimulation syndrome (OHSS) might also benefit from IVM to avoid elevated levels of gonadotropins and estrogen that might trigger or worsen OHSS (Schroder A. et al, 2003). IVM might become the method of choice for patients diagnosed with cancer who want to undergo oocyte retrieval with the purpose of cryopreserving their oocytes. Delaying treatment for cancer in deference to preparing the ovaries for aspirating mature oocytes becomes an excruciating decision for the patient, and offering Ivm could alleviate some of those concerns. Emerging technologies in oocyte banking and oocyte donation might also benefit from abbreviated COH protocols and IVM.

One hurdle that must be overcome before IVM becomes a mainstream procedure is the technical aspect of aspirating and handling immature oocytes. Compared with in vivo-matured oocytes, aspiration of immature oocytes from the ovaries is more technically demanding, requiring adjustments in the aspiration needles, the pressures used, and the skills and patience required to navigate an ovary with small follicles. Furthermore, there is a possibility that patients undergoing IVF with IVM might have a lower pregnancy rate that could be attributed to alterations in the uterus, specifically a diminished endometrial thickness that might impair implantation (Requena A. et al, 2001). Such technical and endocrine concerns can be addressed. More daunting is how the oocytes will be handled and prepared until they are ready to be fertilized.

5.4. Oocyte Retrieval

5.4.1. Timing

The development of the ovarian follicles is monitored by vaginal ultrasound scanning of the ovaries and measurement of serum estradiol. The clinical criteria for human chorionic gonadotrophin (hCG) administration will vary with the stimulation protocol. When the criteria are met, the final maturation of the oocytes is initiated by an intramuscular injection of hCG (5,000 to 10,000 IU) to mimic the endogenous LH surge. After the administration of hCG, the oocytes are expected to ovulate approximately 37 hours later. The oocyte retrieval is scheduled to precede ovulation, approximately 34 to 36h after the hCG injection. During this period cytoplasmic changes take place in the oocyte and meiosis is resumed (Trounson A. et al, 1999).

5.4.2. Anesthesia

The degree of anesthesia may vary depending on the procedure, transvaginal or laparoscopic. For transvaginal procedures, pain relief may be obtained with a paracervical block (e.g. xylocaine) or mild sedation (diazepam) in combination with opiod analgesics (pethidine hydrochloride). Alternatively, spinal or general anesthesia may be used. On average, the oocyte retrieval takes no longer than 10 minutes, minimizing the exposure of the oocytes to the anesthetic agents. These pharmaceutical agents accumulate rapidly in the follicular fluid during the procedure (Soussis I. et al, 1995). There is little evidence that sedative and anesthetic agents have an adverse effect on the post conceptional development of the exposed human oocyte (Coetsier T. et al, 1992). Even though the concentration of these drugs in the follicular fluid is much lower than their serum concentrations (Soussis I., et al, 1995), it is advisable to reduce the procedure time to a strict minimum. **Anesthetic agents such as propofol were found to be detrimental to in-vitro maturation, fertilization and cleavage in mouse oocyte. In this study, the same anesthetic agent was used. Although it may have similar effect, it has not yet been proved on human oocytes and more research is required to study this matter.**

5.4.3. Retrieval technique

The success of oocyte retrieval is dependent on good visualization and accessibility of both ovaries and on the materials and methods used for the oocyte collection. The aspirating needle can be either a single or double lumen needle. Prior to use, the aspiration needle and its Teflon tubing are flushed thoroughly with heparinized handling medium (Trounson A. et al, 1999). The use of syringes to create vacuum for aspiration should be strongly discouraged. Clear evidence shows that the pressure variations and the uncontrolled nature of this method create shearing forces, which result in a high degree of fractured zonae and ploypermy (Lowe B. et al, 1988). Instead, a pedal-operated suction pump with vacuum regulator should be used. The maximum aspiration pressure is set at approximately 7.5 K.p.a.

Wickland et al, 1985, reported a new technique using the transvaginal approach both for the follicular aspiration and the scanning. The transvaginal approach has become the method of choice. It can easily be performed in an outpatient setting under local anesthesia or light intravenous sedation. The transvaginal puncture route has eliminated iatrogenic trauma to the bladder. The vagina is not prepared with antiseptic aqueous solutions as these are embryo toxic. Prophylactic antibiotic cover to prevent pelvic infections is advised by some clinicians, (Meldrum D., 1989), but it is not a routine policy. Prophylaxis is indicated when patients have additional risk factors such as hydrosalpinges. In the present study, all patients received an antibiotic cover of a single dose of 500 mg of metronidazole intravenously during the procedure.

When the follicle is aspirated, the follicular fluid is handed over to the embryologist. To reduce adverse effects of temperature fluctuations on the oocytes, the distance between the patient and the embryology lab should be minimal. Alternatively, transportation by the oocytes should be done with the collecting tubes in warm blocks. Whenever possible, trauma to the ovary should be minimized. In many cases, only one puncture through the ovarian capsule is needed to aspirate all or most of the follicles. This greatly reduces the risk of post operative haemoperitoneum formations.

5.4.4. Follicular flushing

The value of follicular flushing is debatable (Kingsland C., 1991). Its value is evident where low number of follicles is present, as in patients on a natural or a minimal stimulation IVF cycle or in poor responders to controlled ovarian hyperstimulation. Flushing all follicles prolongs the procedure considerably, there by increasing the patient discomfort and raising the overall cost of the procedure. Flushing all follicles up to six times may increase the yield by 20%. Up to 70% of the oocytes are found in the first flush (El-Hussein E. et al, 1992).

5.4.5. Identification and assessment of oocytes

The aspirates of each follicle are assessed by the embryologist (table 5.4.5.1) who determines whether an oocyte is present and grades its maturity. The stage of oocyte maturation is important and can be used to determine the timing of insemination. It is widely accepted that fertilization rates of even mature oocytes are improved with short delays of insemination of between 4 to 6 hours after oocyte retrieval, (Veeck L. et al, 1988). During the handling of the retrieved oocytes, three key factors are important; temperature control, pH control and aseptic techniques. Every effort should be made to maintain the temperature as close as possible to 37°C. It has been shown that a 10-minute exposure to room temperature results in microtubule disassembly and spindle disruption (Pickering S. et al, 1990). Changes of pH should be avoided. The addition of (HEPES) buffer in the handling medium will help to maintain the correct pH while handling the oocytes. Aseptic handling of all human fluid is necessary to prevent infection in the culture media. Gametes and embryos should be handled under a laminar flow. There are several culture methods which are in common use, such as, micro-drop, test tube, organ culture dish, and 4-well dish. After culture, the cumulus-oocyte complex is assessed for maturation by direct and indirect techniques. In the direct technique, the oocyte is examined for the presence of the germinal vesicle or the first polar body. The indirect technique is used to score oocyte maturity while the oocyte remains concealed in the cumulus mass.

Table 5.4.5.1: Morphological parameters used for assessment of oocyte maturity

Oocyte status	Grade	Description	Preinsemination incubation time (h)
Immature	1	Poorly expanded, dense compact cumulus; compact and adherent radiating corona; aggregated granulose cells; oocytes obscured; GV observed; cytoplasm may be dark with clumped organelles.	24-30
Intermediate	2	Expanded cumulus and slightly compact corona (partially radiating); well dispersed granulose; oocyte may be visible.	6-24
Mature	3	Very expanded cumulus and well-dispersed radiating corona; evenly distributed around oocyte; loosely aggregated granulose; clear zona and ooplasm; polar body visible.	4-6
Postmature	4	Expanded cumulus with clumps of cells; radiant corona but often clumped, irregular, and incomplete; visible zona; ooplasm may be granular or dark.	4-6
Atretic	5	Absent cumulus or present in small amounts; corona, if present, is clumped and irregular; dark misshapen ooplasm; visible zona.	4-6

5.4.6. Cryopreservation

Chamayou et al, 2006, studied the consequences of cryopreservation on fertilization, cleavage rates and embryo quality obtained from frozen – thawed oocytes and compared them to the results obtained from sibling fresh oocytes. They concluded that oocyte cryopreservation decreased cleavage rates but did not influence fertilization rate (Chamayou S. et al, 2006). **In the present study, cryo- preservation was used in a total of three cases, two in the PCO group, and one case in the poor responders group. The protocol of oocyte cryopreservation used was a slow-freeze/rapid-thaw procedure, using PROH and sucrose as cryoprotectants. All frozen oocytes were at metaphase II stage. There was no difference noticed on the cleavage or**

fertilization rate of the cyropreserved oocytes compared to the sibling fresh oocytes.

5.4.7. Complications

The ultrasound guided transvaginal technique is a very efficient and simple procedure for oocyte aspiration. However, some dangerous complications may be encountered. Postoperative acute abdomen has been reported in up to 1/250 cases, (Bennett S. et al, 1993). Other complications include hemorrhage, trauma to pelvic structures and infection. Minimal vaginal hemorrhage is the most commonly encountered complication occurring in 9 to 24% of all cases. It is usually self limited and only 1% of these cases require tamponade to stop the blood loss. Minor pelvic infection occurs in approximately 0.3% of all cases. Inoculation with vaginal microbial organisms is the most frequent cause of pelvic infection after transvaginal oocyte retrieval.

In this experiment, no complications were encountered during or following the procedure of oocyte retrieval.

5.5. Embryo culture media

The outcome of clinical in vitro fertilization (IVF) is very much affected by culture condition. Poor culture conditions result in impaired embryo development and a subsequent loss of viability (Staessen C., et al 1994). In general there are three types of culture media: The first type is a simple salt solution with added energy substrate. Examples of this are type of media used in clinical IVF M16, (Whittingham D.G. et al 1971) T6, Earle's (Edwards R.G. 1981) CZB, (Chatot C.L. et al 1989) and KSOM (Lawitts J.A. et al 1992). Derived from such types of media were human tubal fluid medium (HTF), (Quinn P. 1995), and PI (Carrilo A.J. et al 1998).

Such simple media are usually supplemented with either whole serum or serum albumin. The second type is complex tissue culture media. These media are commercially available and are designed to support the growth of somatic cells in culture, e.g. Ham's F-10. Such media are far more complex, containing amino acids, vitamins, nucleic acid precursors, transitional metals, and are usually supplemented with 5 to 20% serum. The third type is the sequential media. These media have been developed to take into account the changes in embryo physiology and requirements that occurs as the embryo develops from the zygote to the blastocyst. Examples of such media include G1/G2 (Gardner D.K., 1994; Barnes F.L., et al 1995).

5.5.1. Composition of embryo culture media

5.5.1.1. Ions

Little is known about the role of ions during pre-implantation embryo development. The mammalian oviduct fluid is characterized by high potassium and chloride concentration, and a high osmolarity (Van Winkle L.J. et al, 1990; Biggers J.D. et al, 1993). It is difficult to interpret the effects of individual ions on embryo development and viability, as some interactions, exist between ions, carbohydrates and amino acids. High potassium levels in culture media have been reported to have a beneficial effect on sperm capacitation (Roblero L.S. et al 1990) and embryo development in vitro (Wales R.G. 1970; Roblero L.S. et al, 1994; Erbach G.T. et al, 1994). The ionic composition of the culture medium is an important consideration as external ion concentration can have a profound effect on

intracellular ion levels and therefore, the regulation of normal cellular processes. The concept is very much demonstrated in the magnesium: calcium ratio in the medium (Hardy K. et al, 1989; Lane M. et al, 1998). Low ratios reduced the ability of the embryos to regulate ionic hemostasis, which directly affects, in turn, the viability of the embryo.

As for phosphate, its role is still controversial. Recent studies showed that in simple culture media containing glucose, the presence of phosphate hinders the development of the human embryo (Quinn P., 1995). However, when phosphate is present in a kind of physiological medium, it does not have such inhibitory effect. The ions in any medium are the strongest contributing factor affecting the osmotic pressure. The optimal osmolarity for the development of the human embryos in culture has a wide range. It varies between 275 and 295 mOsmols.

5.5.1. 2. Antibiotics

Antibiotics such as Penicillin, Streptomycin or Gentamycine have been routinely included in embryo culture medium. However, one study reported improved cleavage rates of human embryo in medium free of antibiotic (Magli M.C. et al, 1996). Washing of embryos in medium supplemented with antibiotics can remove any bacterial contamination.

5.5.1. 3. Hormones and growth factors

The role of the growth factors in the development of the pre-implantation mammalian embryo is not yet clearly identified. Growth factors have been shown to stimulate cleavage, amino acid transport, protein supplies, and blastocoed formation or inner cell mass development. In humans, there is evidence that growth factors are present within the female tract and embryo (Smotrich D.B. et al, 1996). As much as growth factors are potential regulators of human embryo development (Krussel J.S. et al, 1998), it is possible that synergies may exist between two or more growth factors and that simply adding an individual growth factor may not results in a response by the embryo. Furthermore, many growth factors exhibit pleiotropic properties such that cells can be directed down certain pathways of differentiation, even improper pathways, by the exposure to inappropriate growth factor.

Several studies have described an effect of growth hormone (GH) on IVM. These studies concluded that growth hormone added to IVM medium seems to improve the nuclear maturation rate of immature oocytes in stimulated cycles (Lighten A. et al, 1998). The cytoplasmic maturity, evaluated by the distributional pattern of cortical granules, is not affected by the presence of GH in the IVF medium (Ravet S. et al, 2005). Supplementation with gonadotropins – FSH or FSH plus human chorionic gonadotropin (hCG) – increased fertilization (20.7%) and cleavage (15.7%) when compared with culture medium alone (6.7% and 2.2%, respectively).

Alak B. and Wolf D. (1994) found that the highest oocyte yield and quality were obtained when ovaries from the early follicular phase were used. Exogenous human FSH (hFSH) and human luteinizing hormone (hLH) improved GVBD and conversion to the metaphase II (M II) stage of oocytes recovered from the later follicular phase ovaries, but impaired these parameters in those from ovaries in the early follicular or luteal phases. The fertilization rate was not modified.

Schramm and Bavister, 1994, studied the kinetics of meiotic maturation in non-stimulated rhesus monkeys and found that the addition of hFSH, hLH, or a mixture of both to the medium significantly accelerated the time of GVBD (10.8 ± 1.7h; 10.1 ± 1.08h; 8.8 ± 1.1h, respectively) when compared to controls (17.4 ± 2.0 h) but did not modify the extrusion of the first polar body. The same group (Schramm R. et al, 1994) found that rhesus monkeys stimulated with porcine FSH resulted in a greater percentage of cumulus-enclosed oocytes completing meiotic maturation (74% vs. 41%) and showed more activation / fertilization (81% vs. 61%) and cleavage to the 2-4 cell stage (79% vs. 38%) and to morula stages (29% vs. 1%). They also found that only 4% of blastocysts which were obtained in FSH-primed monkeys performed better than similar oocytes from non-stimulated monkeys and were more competent to cleave to the eight-cell stage than were cumulus-enclosed oocytes from non-stimulated animals.

Alak and co-workers (1996), studied the behavior of oocyte-cumulus complex with an intact germinal vesicle and a diameter of >100 μm without degenerative changes and enclosed by two or more layers of cumulus cells. The oocyte-cumulus complex was studied in terms of GVBD, M II maturation and fertilization when increasing

concentrations of recombinant activin A, inhibin A plus activin A or follistatin, were added to the serum-free culture medium.

A significant increase in GVBD was observed when 100 ng/ml of activin A or activin A plus inhibin A was added; the maturation of M II was not modified by activin A alone, but a significant stimulation was observed when the combination of activin A and inhibin A was used. Increasing concentration did not improve either of the end-points. The combination showed a tendency towards accelerated GVBD and enhanced maturation rate. The presence of follistatin negated these effects with a less dramatic influence on M II maturation. The fertilization rate of oocytes exposed to activin A or to the combination was significantly higher than that of controls. The author's conclusion was that activin A and inhibin A are potent stimulators of primate oocyte maturation and fertilization efficiency; the latter indicates an effect of these proteins on cytoplasmatic maturation also. In preliminary trials these oocytes exhibited an increased ability to undergo cleavage after fertilization (Alak B. et al, 1996).

As for the species, the key question of cytoplasmic maturation and its molecular and biochemical nature remains unanswered in the human. Nothing has been done in humans that have not been done previously in other mammals and even in subhuman primates. However, information obtained from other species may not be useful to answer the same question in the human (Das K. et al, 1991).

5.6 Assessment of embryo metabolism and viability

“Viability” is defined as the ability of an embryo to give rise to a live offspring after transfer. When embryos are transferred on day 3, implantation rates of between 5% and 30% have been reported (Plachot M., 1989). In contrast, implantation rates of up to 50% have been obtained by the transfer of human blastocyts (Gardner D., 1998 a). Even in this latter scenario, half of the embryos selected for transfer do not give rise to a baby. Therefore, there is an obvious clinical need to be able to select those embryos most likely to implant after replacement in the uterus (Gardner D., 1998 b). Several procedures for determining the viability of pre implantation mammalian embryos have been used. These include assessment of morphology, development in culture, dye exclusion, fluorescence of degenerate cells, production of fluorescent metabolites, production of platelet activating factor, production of human chorionic gonadotrophin, enzyme leakage, oxygen uptake measurements and nutrient uptake measurements (Shea B., 1981; Cummins J., 1986).

5.7 Discussion of the results

The present study showed that in-vitro matured oocytes retrived from PCOS patients had the potential to undergo successful maturation, fertilization and the resultant embryos showed good developmental competence. Following the IVM procedure, embryo transfer culminated in clinical pregnancies and birth of healthy children. All the oocytes retrieved in this study were at the germinal vesicle (GV) stage. The latter is defined as the stage that represents oocytes arrested at prophase of meiosis–1 with prominent discernable germinal vesicle nucleus.

There are various factors that affect oocyte in-vitro maturation. The most important among these factors are the exposure of the immature oocyte to gonadotrophins in the culture media, and the follicle size at which the oocyte was retrieved. r-FSH, LH, and hCG (Hreinsson J. et al, 2003), and purified gonadotropin (Mikkelsen A. et al, 2001) were used to induce oocyte maturation in vitro. In the present study, the effect of both factors on oocyte maturation, fertilization, cleavage, and pregnancy rates were investigated.

Cha et al reported a pregnancy rate of 27.1% after IVM and IVF-ET in patients with PCOS. They also reported that the combined ET (ZIFT + uterine ET) yielded a significantly higher pregnancy rate than either ZIFT alone or uterine ET alone (Cha K., et al, 2000). Previously, other studies have shown that the pregnancy rate of conventional IVF in PCOS patients was similar to that of conventional IVF in non-PCOS (Mac Dougall M., et al, 1993). A possible mechanism suggested for the lower pregnancy rate of IVM is that some of the oocytes undergoing nuclear maturation after IVM are incapable of undergoing cytoplasmic maturation, thus resulting in poor embryo quality and a higher incidence of pregnancy failure. A number of other factors might lead to a lower success rate of IVM, including suboptimal culture conditions, advancing maternal age, an endocrine disturbance, previous IVF failures, and suboptimal timing of insemination (Picton H., 2002).

There have been reports regarding the increased risk of pregnancy loss in PCOS. A rate of spontaneous abortion of approximately 42%-44% occurs in patients with PCOS (Glueck C., 1999). Hypofibrinolysis due to increased plasminogen activator inhibitor might be an independent risk factor for miscarriage in PCOS. Insulin resistance, the

major patho-physiology of PCOS, has been proposed to play an essential role in the development of spontaneous abortion. The first-trimester spontaneous abortion rate of 62% was decreased to 26% after metformin administration in PCOS (Glueck C., et al, 2002). In the present study in-vitro maturation rates of oocytes cultured in media containing 0.075 IU/ml was 81%, which is higher when compared with similar studies.

Table 5.7.1: Comparison of the oocyte in vitro maturation rates obtained from various studies.

Study	Rate
Barnes F., et al, 1996	62%
Hwu Y., et al, 1998	67%
Jaroudi K. et al, 1999	71%
Child T., et al, 2001	61%
Torousen et al, 2001	71%
Combelles C., et al, 2002	66%
Gaspard O., et al, 2003	58%
Hreinsson J., et al, 2003	55.9%
The present study	81%

The variations in maturation rates between the present study and those mentioned above may be due to the composition of the culture medium used and protein supplement. In the present study, the optimum culture time (30h) was comparable with other studies (Cha K.Y. et al, 1998). Inadequacies of the culture media cannot be ruled out as a possible cause for low IVM success (Combelles C.M. et al, 2002). There is evidence suggesting that culture media used for IVM adequately support nuclear maturation, but failed to produce oocyte with cytoplasmic maturation. While in this study synthetic serum supplement was used as a source of proteins, in other studies fetal bovine serum (FBS) was used. FBS was considered more crucial for bovine oocyte maturation than human. In addition, the base medium used in the study was Hams F10 which is designed to meet the nutritional and maturational needs for human oocyte.

Other reasons for the low maturation, fertilization and the developmental competence may be attributed to polycystic ovary syndrome as the main cause of infertility.

It has been reported that immature oocytes recovered from regulary cyclic non-PCOS patients attained better maturation, fertilization, and developmental competence than those oocytes that were recovered from PCOS patients (Barnes F. et al, 1996). This may be due to the prolonged exposure of immature oocytes to high level of androgens in the case of PCOS patients (Anderiesz C.et al, 1995). The high androgen level results from increased theca cell secretion of androgen and blockage of aromatization in the granulose cell compartment (Almahbobi A.et al, 1996).

Recent reports show that the low maturation rates in IVM could be improved by priming patients with gonadotrophins. Patient priming with r-FSH and hCG before retrieval may have contributed to the increase of the maturation rate in this study. Trounson A. and colleagues (2001) reported a higher maturation rate (71%) in FSH treated women when compared with untreated women (44%). Follicle priming with r-FSH and hCG before oocyte retrieval had significantly increased the rate and speed of oocyte maturation (Trounson A. et al, 2001). It was found that 75% of the oocytes recovered from super ovulated and primed patient reached metaphase II after 30h of culture, while the same percentage was reached after 42-45h of culture.

Despite the relatively high maturation, fertilization and cleavage rates shown in the present study, the pregnancy rate remained relatively low (22.5%) compared to 27% reported by Cha K. et al, 1998 and 40% reported by Chian R. et al 1999. The low pregnancy rates shown in IVM cases in general are partly attributed to abnormality during cytoplasmic maturation (Lin Y. et al, 2003). However the pregnancy rate in other studies reached even higher percentages (40%) following administration of 10000 IU hCG before immature oocyte retrieval (Chian R. et al, 1999; 2002). In the present study, all patients were primed with 5000 IU hCG rather than 10000 IU to reduce the risk of OHSS, and this may have contributed to the low pregnancy rate. According to Chian R. et al (1999), Son W. et al, (2002) and Hreinsson J. et al, (2003), hCG priming improves the percentage of oocytes achieving maturation and hastens the maturation process. The overall oocyte quality might be reduced by the oocytes retrieved from PCOS due to high androgen level. Although hCG priming improved the maturation rate of immature oocyte (Chian R. et al, 2002), there is no evidence to suggest that FSH priming has the similar effect on pregnancy rates except for a few exceptional cases

(Mikkelsen A. et al, 2001). In other studies, FSH priming made no difference to oocyte recovery, maturational and developmental potential, fertilization rate and pregnancy rate (Trounson A. et al, 1998; Lin Y. et al. 2003). The low implantation and pregnancy rate in IVM may be due to asynchrony in the cytoplasmic and nuclear maturation of the oocyte (Jaroudi K. et al, 1999). According to Trounson A. et al, (2001), the cytoplasmic protein necessary for the development of embryos can only be produced upon the oocyte completing cytoplasmic maturation. It was also reported that oocytes from PCO patients show compromised developmental potential compared to regular cyclic patients.

The thickness of the endometrium directly affects the pregnancy rate. According to Gonsen Y. et al, (1990), endometrial thickness of 8.6 mm was found capable of achieving pregnancy. Ultrasonographic studies in cycles of in vitro fertilization have revealed that successful implantation is correlated with endometrial thickness on the day of hCG administration. In a program utilizing clomiphene and timed administration of hCG for the purpose of intrauterine inseminations, no pregnancies occurred when the endometrial thickness measured less than 6 mm. The chance of pregnancy is greatest, no matter which program of ovarian stimulation is being used, if the endometrial thickness is 6- 9 mm or more (Shapiro H. et al, 1993).

There are different priming protocols used in PCOS patients undergoing IVM. Recently proposed pretreatment with human chorionic gonadotrophin (hCG) before immature oocyte recovery showed an improved pregnancy rate with respect to other protocols (Cizek M. et al, 2005). Kovac et al evaluated some factors that could affect the IVM outcome in PCOS patients. They compared the dynamics of estradiol, endometrial thickness on the day of oocyte puncture and the percentage of good quality embryos in two differently primed PCOS groups of patients with FSH or hCG undergoing IVM treatment. They concluded that in the hCG primed group, there was a slight increase in the estradiol level on the day of puncture and a significantly lower mean endometrial thickness. The number of embryos with optimal morphology derived from in vitro matured oocytes following the hCG priming protocol was larger than the number of embryos derived from oocyte in vitro maturation with FSH priming protocol (Kovac V. et al, 2005). In the present study, FSH priming, however, had increased the level of E2, but it did not show any increase in the endometrial thickness or in any of the parameters

studied previously. A similar conclusion was also reported in several other studies (Mikkelsen A. et al, 2001; Lin Y. et al, 2003). **This study showed a mean endometrial thickness of 8.2 mm and all patients showed triple line ring pattern on the day of embryo transfer. The endometrial thickness on the day of hCG was better than other studies which achieved higher pregnancy rate.** Lin et al, (2003), reported 31% pregnancy rate in the FSH primed patients with mean endometrial thickness of 8.2 mm on the day of hCG injection. Poor endometrial thickness was partly responsible for the reduction of pregnancy rate in IVM cycles. It was reported that some endocrinological disturbances occur in IVM patients following oocyte retrieval that may result in FSH and LH rise, and E2 drop (Requena A. et al, 2001). An inadequate E2 level may be harmful to endometrial development and may reduce the pregnancy rate.

Chromosomal anomalies may be another reason for reduced pregnancy rates in IVM cycles. Oocytes from PCOS patients that are matured in vitro have a propensity for problems with chromosomes or microtubules compared with oocytes allowed to mature in vivo before aspiration (Li Y., et al, 2006). The spindles and chromosome arrangements are critical in oocyte maturation in vitro and in vivo. Alterations in the oocyte internal structure, in particular spindles and chromosomes, have critical importance in the ability of the oocyte to be fertilized, develop into a normal embryo, and ultimately produce a healthy live birth. The movement of the chromosomes within the oocyte is controlled by spindles, specialized components of the oocyte infrastructure that are composed of microtubules. This internal infrastructure is a type of scaffolding or super-highway, moving materials and structures within the oocyte. This constantly changing structure is influenced by the oocyte environment, including the hormonal milieu, osmolarity, temperature, pH, and salt concentrations (Mullen S. et al, 2004; Wang W.H. et al, 2002). If the spindle is disrupted, then the movement of chromosomes during meiosis could also be affected (Wang W. et at, 2002).

Li et al, used confocal microscopy and fluorescent immunocytologic staining to analyze the appearance of spindles and chromosomes in IVM oocytes acquired from PCOS patients and compared their results with in-vivo matured oocytes from another group of PCOS patients. They reported that IVM oocytes were more likely to have abnormal chromosome configuration and disorganized meiotic spindle microtubules (Li Y., et al,

2006). While some studies had shown 78.5% chromosomal anomalies in in-vitro matured oocytes from stimulated cycles (Nogueria D. et al, 2000), others reported 20% (Gras L. et al, 1992) and none (Cha K. et al, 2000; Lin Y. et al, 2003). Further research is required to assess the chromosomal anomalies in in-vitro matured oocyte.

Reduced pregnancy rates in the IVM cycles of this study may be related to the low number of embryos transferred. In the present study, an average of 2.3 embryos per patient were transferred. Studies had shown that implantation rates of IVM were relatively low compared to IVF cycles. To overcome such a problem more embryos have to be transferred in IVM cycles (Lin Y. et al, 2003). In this study, in 80% of the cases, only 1.7 embryos were transferred. This may have contributed to the low pregnancy rate achieved in the present study. On the other hand the effect of follicle size on the above parameters showed some interesting results. The data showed that oocyte maturation rate and developmental competence has significantly increased with increase in follicle size. Eppig J.J. et al, (1992), concluded that developmental competence of oocytes depends on the follicle and oocyte size. The growth in size is due to the fact that oocyte synthesizes and stores mRNA and proteins that are essential for the completion of maturation and for the subsequent acquisition of embryo developmental competence (Gosden R. et al, 1995). This probably explains the relatively higher maturation, fertilization, cleavage, and pregnancy rate in oocyte obtained from follicles with 11-13 mm size rather than 8-10 mm. It has been found that the capacity of meiotic competence in animals such as the rhesus monkey (Schramm R. et al, 1993) increases with follicle size and is not strictly correlated with maximum oocyte diameter or formation of antrum. Conversely, human oocyte resumes maturation at a lower rate compared with other species (Edwards R., 1965). This may be due to the fact that meiotic competence occurs late during the growth phase or maturation needs both stimulation and removal of inhibition (Lefevre B. et al, 1987). This also indicates that human oocyte has a size-dependant ability to resume meiosis and complete maturation in-vitro when oocyte diameter increases from 9 to 12 mm. Moreover, meiotic competence was found to be dependant on the size of the follicle and the stage of the menstrual cycle. Human oocytes retrieved from follicles of 9 -15 mm in diameter complete meiotic maturation to metaphase II at a higher rate than oocyte from follicles of

3 - 4 mm in diamter (Whitacre K. et al, 1998). However the minimum follicle diameter from which human oocyte would mature was reported to be 5 mm (Wynne P. et al, 1998).

The effect of follicle size on oocyte maturation, fertilization and subsequent developmental competence has been reported in various other studies (Trounson A. et al, 2001). These reports reached the same conclusion as in the present study. Simonetti S. et al, (1985) found that human oocyte maturation rate significantly increases with follicular size after superovulation with human menopausal gonadotrophin (hMG) and human chornionic gonadotrophin. Scott et al., 1989, found that the proportion of oocyte maturation increased from 9% to 30% when the oocytes were obtained from two follicular sizes of <11 and 12-14 mm, respectively. Follicle size was also reported to affect the fertilization rate. Recent studies showed that immature oocytes retrieved from smaller follicles showed rather lower maturation rate (58%) compared to those retrieved from larger follicles (89%) (Gaspard O. et al, 2003). The higher maturation rate may be due to the fact that larger follicle may produce larger oocytes at metaphase one stage which may have better maturation capacity/potential than germinal vesicle oocyte.

According to Trounson A. et al, (2001), oocyte size is directly related to maturation and subsequent embryo development. This may be due to the molecular cascade of both cytoplasmic and nuclear maturation as initiated when the oocyte reaches a certain size. Following gonadotrophin stimulation, it was found that fertilization rate was lower in oocyte obtained from follicles < 10 mm than those obtained from larger follicle (Wittmaack F. et al, 1994; Dubey A. et al, 1995; Salha O. et al, 1998). The same report found that cleavage rate was also reduced with follicular size. Combelles C. et al, (2002), attributed the low efficacy of IVM to intrinsic differences in oocytes which results in developmental capacity variation due to incomplete or abnormal oocyte growth. It has also been suggested that failure in developmental competence may be due to abberation in cytoplasmic maturation (Moor R. et al, 1998). At the molecular level, meiotic competence in oocytes has been related to germinal vesicle chromatin organization and meiotic cell cycle status.

While in the present study, the pregnancy rate increased with follicle size, other reports found that implantation, pregnancy, and birth rates were independent of follicular size

(Wittmaack F. et al, 1994; Salha O. et al, 1998). **The present data suggested that when oocytes were cultured in IVM media containing 0.075 IU r-FSH or retrieved from follicle size exceeding 10 mm, a comparable or even better maturation rates and developmental competence are achieved. Moreover it has been shown in the present study that the two concentrations of r-FSH 0.075IU/ml and 7.5 IU/ml were equally effective in promoting oocyte maturation. On the other hand, the results also showed that the above parameters were lower when the oocytes were incubated in media containing less than 0.075 IU/ml r-FSH or retrieved from follicles smaller than 10 mm.** In group 3 (PCO infertile patints and poor responder infertile patients), all patients' criteria were comparable. In subgroup 3II (the poor responder infertile patients) the mean age was higher than the PCO infertile patients (subgroup 3I). Although the difference was not significant, but it is well known that the age affects the outcomes of both IVF and IVM, especially the oocyte characteristics and the final outcome. The basal body mass index was significantly higher in the PCO patients (subgroup 3I). Also the duration of the cycles was significantly longer in PCO patients (subgroup 3I). Obesity and oligomenorrhea are both two main characteristic features in PCO patients. The hormonal profiles among the patients in group 3 (PCO infertile patints and poor responder infertile patients) were comparable.

The outcome of IVM was promising in group 3 (PCO infertile patints and poor responder infertile patients). Four hundred and fifty oocytes were retrieved from 40 patients (250 oocytes from subgroup 3I, and 200 oocytes from subgroup 3II). This difference was not significant. The percentage of oocyte maturation was significantly higher in PCO subgroup (3I) than the poor responder subgroup (3II) (68% vs. 57.5%); oocyte fertilization and cleavage in both subgroups were comparable (22.4% vs. 21%, and 18% vs. 16%).

In the irregular cycling PCO patients (subgroup 3Ia), there was a higher number of oocytes recovered and cultured in comparison with the regular cycling subgroup PCO patients (3Ib) (175 vs. 75). This was consistent with the observation of Barnes et al, (1996). In the regular cycling subgroup, the percentage of maturation and fertilization cleavage and embryo development ratio was significantly higher than in irregular cycling subgroup. In fact, the non-vitro oocytes from regular cycling patients appeared to

perform almost as well as those recovered after super ovulation and maturation in vivo. Of those oocytes recovered, 74% matured after 30 hours, 33% fertilized, and 18% cleaved with a mean embryo development of 81.5%. This data suggests that, if more oocytes can be recovered from regular cycling patients, pregnancy expectations may not be very different from those of superovulated patients. The present results indicate that immature oocytes recovered from regular cycling PCO patients exhibit developmental competence when matured, fertilized, and cultured in vitro than oocytes recovered from patients with polycystic ovaries who exhibit menstrual irregularity.

Also, the results indicate that in vitro maturation of oocytes recovered from PCO patients exhibit developmental competence more than the oocytes recovered from poor responder patient. The explanation of this finding may be related to the age of the patients. Most of the poor responders are older than PCO patients with the contribution of other factors like the male factor. PCO patients are younger and their main problem is the competency of in vivo oocytes maturation. In studying the effect of the male factor, all males in the PCO patients had normal sperm count and viability. In poor responders, only 1 case showed subnormal sperm count.

The effect of the male factor could be omitted in this study due to the low number of cases and the inability of any statistical evaluation.

In the present study, factors affecting immature oocyte maturation and developmental competence are not fully explored. There are many gaps that need to be bridged and other factors need to be closely investigated. The effect of growth hormone in culture media during oocyte maturation, chromosomal anomalies as well as the effect of anesthesia is worthy of a thorough investigation.

6. Conclusion

a. In vitro maturation of oocytes represents an optimum solution for PCO and poor responders infertile women. The procedure ensures a shorter period of treatment, and a reduced cost with no side effects of hyperstimulation. Unfortunately pregnancy rates remain low.

b. Recombinant follicle stimulating hormone (r-FSH) supplementation in the culture media with a concentration of 0.075 IU is optimum for improving oocyte maturation, fertilization, cleavage and pregnancy rate.

c. Oocytes recovered from follicles with 11-13 mm in diameter provided higher maturation rates and a better subsequent developmental competence than those retrieved from smaller follicles of 8-10 mm in diameter.

d. Oocytes recovered from regular cycling PCO patients have a significantly higher developmental potential when compared with irregular and anovulatory patient.

e. In vitro maturation of oocytes retrieved from PCO patient exhibit developmental competence over that of the oocytes retrieved from poor responders.

7. Summary

7.1 English Summary

In-vitro maturation (IVM) of the human oocytes is becoming increasingly important in treating some aspects of infertility. In conventional assisted reproduction techniques, (in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)) mature oocytes are retrieved for fertilization. In the IVM process, immature oocytes are retrieved and matured in the laboratory before fertilization and embryo transfer. The in vitro maturation (IVM) protocol is relatively simple with a short period of treatment and reduced cost compared to conventional IVF. In addition, the side effects of stimulation and in particular ovarian hyper-stimulation syndrome (OHSS) are reduced.

This study was designed to evaluate the effect of both the concentration of recombinant follicle stimulating hormone (r-FSH) in culture media, and the follicle size on oocyte maturation, fertilization and cleavage and to compare the outcomes of in-vitro maturation of immature oocytes recovered in situ from infertile women with polycystic ovaries and poor responders.

During the first and second parts of our study, one hundred (100) women in an IVF program were randomly studied. Fifty (50) patients produced 674 germinal vesicle (GV) oocytes that were allocated to study the effect of three concentrations of r-FSH (0.0, 0.075IU/ml, and 7.5 IU/ml) supplemented in culture media on oocyte maturation, fertilization, cleavage, and pregnancy rate. The remaining 50 patients produced 500 GV and were allocated to study the effect of two follicle sizes (8-10 and 11-13 mm) on the same parameters as above.

The results showed that oocytes maturation rate had significantly increased from 47% at 0.0 (control) to 81% and 83% at 0.075 and 7.5 IU/ml of r-FSH concentration respectively ($P<0.05$). Fertilization, cleavage, and clinical pregnancy rates showed a similar trend and significantly increased from 45% to 83% and 80%; from 32% to 80% and 77% and from 0% to 17% and 14% at the three r-FSH concentrations, respectively. The results however showed that increasing r-FSH concentration to more than 0.075 IU did not further improve the rates of the above parameters even when the concentration was increased up to 100 folds. Follicular size, on the other hand, showed significant ($P<0.05$)

increase on the above parameters. Oocytes retrieved from 11-13mm follicles showed higher rates of maturation, fertilization, cleavage, and pregnancy than those retrieved from 8-10 mm follicles. The above parameters increased from 48% to 70%; from 54% to 76%, from 64% to 68%; and from 11% to 22.5%, respectively.

In the present study, the results suggest that r-FSH supplementation in the culture media with a concentration of 0.075 IU is optimum and improved GV maturation, fertilization, cleavage and pregnancy rate. The results also show that oocytes recovered from follicles with 11-13 mm in diameter provide higher maturation rates and a better subsequent developmental competence than those retrieved from smaller follicles of 8-10 mm diameter.

The third part of the study was conducted on forty (40) infertile women, (20) with polycystic ovaries (subgroup 3I) and (20) poor responders (subgroup 3II). They were recruited at random during their treatment with ICSI in the IVF program.

Patients with PCO were identified by pelvic ultrasound, and those who had unsuccessful IVF procedures in stimulated cycles. Their endocrine and clinical features varied between regular and irregular cycles. Anovulatory polycystic ovarian syndrome patients had a characteristically elevated androgen level, LH: FSH ratio >2, and frequently, the clinical features of hirsutism, increased body weight, as well as more than eight of 2-10m follicles in each ovary.

Poor responders are patients who failed to achieve estradiol concentration >200 pg/ml on the day of hCG, or failed to develop or developed a maximum of one follicle during the previous attempts, and who had a history of failure and cancellation of previous IVF cycles due to low number and quality of oocytes retrieved with previous stimulations.

In the third experiment, PCO patients were further divided into two subgroups, subgroup 3Ia included (12) patients who presented mainly with irregular anovulatory menstrual cycles while subgroup 3Ib included (8) regular cycling PCO infertile women.

Results of this part of the study showed that the mean age was higher in the poor responder group than the PCO subgroup, although the difference was not significant. The basal body mass index was significantly higher in the PCO subgroup. The duration of the cycles was significantly longer in PCO patients. The number of oocytes recovered

from the PCO irregular cycling patients was significantly higher than the oocyte retrieved from the PCO regular cycling patients.

Oocytes recovered from regular cycling patients had a significantly higher developmental potential when compared with irregular and anovulatory patients, as well as higher maturation, fertilization and cleavage rates. Moreover, embryos produced from regular cycling patients had a larger embryo development ratio, indicating the faster cleavage rate of embryos produced from this subgroup of patients.

The results indicated that in vitro maturation of oocytes retrieved from PCO patients exhibit developmental competence over the oocytes retrieved from poor responders. The explanation of this finding is that most of the poor responders are older than the PCO patients with the combination of other factors such as the male factor.

In the present study, factors affecting immature oocyte maturation and developmental competence are not fully explored. There are many gaps that need to be bridged and other factors need to be closely investigated. The effect of growth hormone in culture media during oocyte maturation, chromosomal anomalies as well as the effect of anesthesia is worthy of a thorough investigation.

7.2 Deutsche Zusammenfassung

Die In-vitro-Maturation von menschlichen Eizellen gewinnt bei der Behandlung bestimmter Formen der Infertilität zunehmend an Bedeutung. Im Gegensatz zu herkömmlichen unterstützenden Reproduktionsverfahren, der In-vitro-Fertilisation (IVF) und der intrazytoplasmatischen Spermieninjektion (ICSI), bei denen reife Eizellen zur Befruchtung entnommen werden, werden bei der IVM unreife Eizellen entnommen, die vor der Befruchtung und dem Embryotransfer im Labor reifen. Der Ablauf der In-vitro-Maturation (IVM) ist relativ einfach. Die Behandlungsdauer ist relativ kurz, und die Kosten sind niedriger als bei der konventionellen IVF. Zudem werden die Nebenwirkungen der Stimulation (insbesondere das ovarielle Überstimulationssyndrom – OHSS) vermieden. Diese Studie diente der Untersuchung der Auswirkung der Konzentration des rekombinanten Follikel-stimulierenden Hormons (r-FSH) im Kulturmedium und der Follikelgröβe auf die Eizellenreifung, Fertilisation und Zellteilung sowie dem Vergleich der Ergebnisse der In-vitro-Maturation unreifer Eizellen, die in situ von infertilen Frauen mit polyzystischen Ovarien und schlechten Respondern gewonnen wurden.

Im ersten Teil der Studie wurden einhundert (100) Frauen in einem IVF- Programm nach dem Zufallsprinzip untersucht. Fünfzig (50) Patientinnen wurden in das erste Experiment mit 674 Eizellen in Keimbläschen (GV) aufgenommen, um die Auswirkung von drei r-FSH- Konzentrationen (0,0, 0,075 und 7,5 I.E.) im Kulturmedium auf die Eizellenreinfung, Fertilisation, Zellteilung und Schwangerschaftsquote zu untersuchen. Die anderen 50 Patientinnen produzierten 500 GV und wurden in das zweite Experiment aufgenommen, um die Auswirkung von zwei Follikelgröβen (8-10 und 11-13 mm Durchmesser) auf die gleichen Parameter zu untersuchen.

Die Ergebnisse haben gezeigt, dass die Eizellenreifung signifikant ($P<0,05$) von 47% bei einer Konzentration von 0,0 (Kontrollgruppe) auf 81% (bei einer Konzentration von 0,075 I.E. r-FSH) bzw. 83% (bei einer Konzentration von 7,5 I.E. r-FSH) angestiegen ist. Für die genannten r-FSH-Konzentrationen weisen die Fertilisationsrate (Anstieg von 45 auf 83 bzw. 80%), die Zeillteilungsrate (Anstieg von 32 auf 80 bzw. 77%) und die klinische Schwangerschaftsrate (Anstieg von 0 auf 17 bzw. 14%) einen ähnlichen Trend auf. Die Ergebnisse haben jedoch gezeigt, dass eine Steigerung der r-FSH-

Konzentration auf über 0,075 I.E. die Quoten der oben genannten Parameter auch dann nicht weiter verbessert, wenn die Konzentration um das 100-fache erhöht wurde.
Andererseits bewirkte die Follikelgröße ebenfalls einen signifikanten (P<0,05) Anstieg der oben genannten Parameter, Eizellen, die aus Follikeln der Größe 11-13 mm gewonnen wurden, erzielten gegenüber Eizellen, die aus 8-10 mm große Follikeln gewonnen wurden, höhere Raten von Maturation (Anstieg von 48 auf 70%), Fertilisation (Anstieg von 54 auf 76%), Zellteilung (Anstieg von 64 auf 68%) und Schwangerschaften (Anstieg von 11 auf 22,5%).
Die Ergebnisse weisen darauf hin, dass die Zugabe von 0,075 I.E. r-FSH zum Kulturmedium in dieser Studie optimal war und eine Verbesserung von Maturations-, Fertilisations-, Zellteilungs- und Schwangerschaftsrate der GV bewirkte. Die Ergebnisse haben außerdem gezeigt, dass Follikel mit einem Durchmesser von 11-13 mm höhere Maturationsquoten erzielten und sich anschließend besser entwickelten als Eizellen aus kleineren Follikeln von 8-10 mm Durchmesser.
Der dritte Teil der Studie wurde mit vierzig (40) infertilen Frauen mit (20) polyzystischen Ovarien und (20) schlechten Respondern durchgeführt. Sie wurden nach dem Zufallsprinzip während ihrer Behandlung mit ICSI im Rahmen des IVF-Programms rekrutiert.
Patientinnen mit polyzystischen Ovarien (PCO) wurden per Ultraschall und durch erfolglose IFV-Verfahren in stimulierten Zyklen diagnostiziert. Schlechte Responder sind Patientinnen, die auf eine Standard-Ovulationsstimulation schlecht oder überhaupt nicht reagierten. Im dritten Experiment wurden PCO-Patientinnen in zwei Untergruppen unterteilt, von denen 12 Patientinnen vorweigend unregelmäßige, anovulatorische Menstruationszyklen und 8 regelmäßige Zyklen hatten.
Die Ergebnisse dieses Studienteils haben gezeigt, dass die Gruppe der schlechten Responder ein höheres Durchschnittsalter hatte als die PCO-Gruppe. Obgleich der Unterschied nicht signifikant war, ist es allgemein bekannt, dass das Alter einen Einfluss auf die IVF und IVM und insbesondere auf die Eigenschaften der Eizellen und das Endergebnis hat. Der basale Body-Mass-Index war in der PCO-Gruppe signifikant höher. Adipositas und Oligomenorrhoe sind zwei wesentliche Merkmale von PCO-Patientinnen. Die Zyklusdauer war bei PCO-Patientinnen erheblich länger. Bei PCO-

Patientinnen mit unregelmäßigem Zyklus konnten signifikant mehr Eizellen gewonnen werden als bei PCO-Patientinnen mit regelmäßigem Zyklus. Eizellen, die bei Patientinnen mit regelmäßigem Zyklus gewonnen wurden, hatten ein wesentlich größeres Entwicklungspotential als die von Patientinnen mit unregelmäßigem, anovulattorischem Zyklus.

Die Maturations-, Fertilisations-und Teilungsraten waren höher. Zudem wiesen Embryonen von Patientinnen mit regelmäßigem Zyklus eine größere Embryoentwicklungsrate auf, was auf eine schnellere Zellteilung bei den Embryonen dieser Patientengruppe deutet.

Die Ergebnisse habe gezeigt, dass Eizellen nach der In-vitro-Maturation, die bei PCO-Patientinnen gewonnen wurden, besser entrwicklungsfähig sind als Eizellen, die von schlechten Respondern gewonnen wurden. Dies ist neben anderen Faktoren dadurch zu erklären, dass die meisten schlechten Responder alter als PCO-Patientinnen sind.

Diese Studie hat gezeigt, dass die Einflussfaktoren auf die Reifung von unreifen Eizellen und auf die Entwicklungsfähigkeit noch nicht vollständig geklärt sind. Es gilt noch viele Wissenslücken zu schließen und weitere Faktoren näher zu untersuchen.

7.3

4 الملخص العربي

نواتج الإنضاج الخارجي للخلايا البيضية البشرية عند المريضات العقيمات اللاتي لديهن نكيس المبيضين وذوات الاستجابة الضعيفة

أصبح طفل الأنابيب للخلية البيضية البشرية سبيلاً هاماً جداً لمعالجة العقم على خلاف أساليب المعالجة التقليدية للتخصيب ، التلقيح أو التخصيب بالأنابيب وحقن النطاف السيتوبلازمي الداخلي الذي تسترد فيه كل الخلايا البيضية الناضجة كي تخصب ، والخلايا البيضية التي لم تلقح في عملية طفل الأنابيب ستسترد وتجدد وتلقح من جديد في المختبر قبل التخصيب والنقل الجنيني.برتوكول طفل الأنابيب بسيط نسبياً، يتميز بفترة قصيرة من العلاج وكلفة أقل مقارنةً بأساليب المعالجة التقليدية لعلاج العقم وطفل الأنابيب . بالإضافة إلى ذلك فإن الآثار الجانبية للتحريض خصوصاً في حالة متلازمة فرط الإثارة المبيضية تعتبر مقصاة. هذه الدراسة صممت لتقدير آثار كلاً من تركيز نتاج عودة الاتحاد الجيني لهرمون حث الجريبات في وسط مستنبت وحجم الجريب في الخلية البيضية الناضجة المخصبة والمنقسمة مع مقارنة نتائج التلقيح بالأنابيب الخاص بالخلايا البيضية الغير ملقحة والمستردة من المرأة العقيم التي لديها تكيس في المبيضين والاستجابة الضعيفة. خلال المرحلة الأولى من الدراسة تم إخضاع 100 امرأة للدراسة في برنامج التخصيب بالأنابيب، 50 منهن تم إدراجهم بالتجربة الأولى 674 خلية مبيضية ذات الجريب المنتشة تم تحديدها وذلك بغرض دراسة آثار 3 تركيزات لهرمون حث الجريبات (0 ، 0 و 075 ، 0 و 5 ، 7 وحدة) مضافة إلى وسط مستنبت في تلقيح الخلية البيضية ، والتخصيب ، والانقسام ومعدل الحمل ، والخمسين حالة المتخلفين يتم إدراجهم في التجربة الثانية المنتجة لـ 500 جريب منتشة ويتم تحديدها لدراسة أثر حجم اثنين من الجريبات (10 - 8 مم و 13 – 11) بالمشعرات ذاتها. النتائج بينت أن معدل الخلايا البيضية الناضجة ارتفع بشكل ملحوظ < 0, 50 من 47 % بـ (تحكم) 0، 0 إلى 83.81% عند 570.0 و 5.7 وحدة/مل بتركيز متعاقب من هرمون حث الجريبات . معدلات التخصيب والانقسام والحمل السريري أوضحت ميل مماثل وارتفاع ملحوظ من 45% إلى80.83% ، من 32% إلى 80 و 77% ومن 0 إلى 17 و 14 % عند التركيز الهرموني للحث الجريباتي الثالث على التوالي . ولكن النتائج بينت أن ارتفاع تركيز هرمون حث الجريبات عن 570.0 وحدة لم يؤثر إيجابيا على معدلات المشعرات المذكورة آنفاً حتى في حال ارتفع معدل أو ارتفعت نسبة التركيز 100 ثنية . أما الحجم الجريب فقد أوضح بوضوح ارتفاع > 50.0

ما فوق المشعرات. الخلية البيضية البدائية التي تم استردادها من جريبات قياسها 11 – 13 مم أظهرت معدلات أعلى من الإلقاح ، الخصوبة ، الانقسام والحمل بالمقارنة مع تلك التي تم استردادها من 8 – 10 مم جريبي . المشعرات المذكورة أعلاه ارتفعت من 48 – إلى 70 % . ومن 45 إلى 76 % . ومن 64 إلى 68 % . ومن 11 إلى 22.5 % على التوالي . النتائج أظهرت أن في حال كان هرمون حث الجريبات مضاف إلى تركيزات وسط مستنبت بوحدة 0,075 فإنها بذلك تمثل الوحدة المثلى بالنسبة للدراسة الحاضرة مع تحسين معدلات الجريبات المنتشة للإلقاح ، الخصوبة ، الانقسام ومعدلات الحمل . النتائج أوضحت أيضا أن الخلايا البيضية المتسردة من الجريبات والتي تقدر حجمها بـ 11 – 13 مم . بمعدلات خصوبة أعلى وأفضل كفاءة متطورة مقارنة مع تلك المستردة من جريبات صغيرة والتي تقدر حجمها بـ 8 – 10 مم . هذه المرحلة الثالثة من الدراسة تم تطبيقها على 40 امرأة عقيم 20 منهن ذوي مبيض متعددة الكيسات والـ 20 الأخريات ذو استجابة ضعيفة . يتم انتقاء النساء بعشوائية خلال مرحلة العلاج بحقن النطاف السيتوبلازمي الداخلي ضمن برنامج طفل الأنابيب . الأشخاص الذين يعانون من مبيض متعددة الكيسات تم كشفهم من خلال الأشعة الصوتية للحوض ، واللواتي خضعن لعملية التخصيب بالأنابيب ولكن لم تنجح في دورات محرضة .

آما السيدات ذوات الاستجابات الضعيفة واللاتي لم تظهرن أي استجابة أو استجابتهن كانت ضعيفة للبروتوكولات المعتادة والمتعارف عليها لإحداث الإباضة . بالتجربة الثالثة مرضى مبيض متعددة الكيسات يقسمن إلى شعبتين ، 12 سيدة يشخصون بالمعاناة من دورات شهرية لا إباضية غير منتظمة و 8 نساء من مرضى مبيض متعددة الكيسات بدورات منتظمة . نتائج هذه الجزئية من الدراسة بينت أن النسبة الأعلى تكمن في أولئك الأفراد ذوو الاستجابة الضعيفة مقارنة بمجموعة مبيض متعددة الكيسات آخذين بالاعتبار أن نسبة الاختلاف ليست ملحوظة ولكن كما هو معروف أن العمر له تأثير كبير على نتائج كل من طفل الأنابيب و التلقيح بالأنابيب وخاصة على خصال الخلية البيضية والنتائج النهائية . منسب حجم الجسم الأساسي كان مرتفعَ بشكل ملحوظ عند مجموعة مبيض متعددة الكيسات . البدانة وندرة الطمث صفتين رئيسيتين مميزتي لمريضات مبيض متعددة الكيسات. مدة الدورات كان طويلاً بشكل ملحوظ عند مرضى مبيض متعددة الكيسات .

عدد الخلايا البيضية المستردة من مرضى مبيض متعددة الكيسات ذوو الدورات الغير منتظمة كان أعلى بشكل ملحوظ من أولئك المرضى ذوو الدورات المنتظمة .

الخلايا البيضية المستردة من مرضى الدورات المنتظمة لوحظ لديهن كمون تطوراً ملحوظاً بالمقارنة مع مرضى الإباضة الغير منتظمة مع نسبة تلقيح مرتفعة . ومعدلات تخصيب وانقسام عالية. بالإضافة إلى أن

الأشخاص ذوو الدورات المنتظمة كان لديهم نسبة إنتاجية كبيرة للأجنة المتطورة مع الإشارة إلى نسبة انقسام أسرع لإنتاج الأجنة من هذه المجموعة من الأشخاص . النتائج أظهرت أن التلقيح بالأنابيب بالنسبة للخلايا البيضية المستردة من مرضى مبيض متعددة الكيسات تظهر كفاءة متطورة تفوق تلك الخلايا البيضية المستردة من عند الأشخاص ضعيفي الاستجابة . التفسيرات المتوصلة لهذه النتائج و البحوث بينت أن معظم الأشخاص ذوو الاستجابات الضعيفة أكبر عمرا من مرضى مبيض متعددة الكيسات مع اتحاد العوامل . هذه الدراسة أوضحت أن العوامل التي تؤثر على تلقيح الخلايا البيضية الغير ناضجة والكفاءة المتطورة ليست مكتشفة تماماً وأن العديد من الفجوات متواجدة وبحاجة إلى بحث أعمق لربط العوامل ببعضها والتحقق من تلك العوامل التي بحاجة إلى التدقيق .

8. References

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