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Evaluation of the Correlation of Some Follicular Fluid Cytokines with Intracytoplasmic Sperm Injection Outcome

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In the name of god

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Abstract

Infertility is a problem concerning about 10-15% of the individuals in the fertile period due to both female and male causes. Mammalian reproduction hinges upon the timely ovulation of a fully differentiated oocyte. In human, the developmental competence is embryo specific, and this specificity arises in the preovulatory oocyte. The quality and viability of oocytes will be intimately linked to the intraovarian/perifollicular cytokine milieu. Follicular fluid (FF), an oocyte microenvironment, is a unique biological fluid rich in growth factors and cytokines that exert paracrine and autocrine effects on implantation and the events of oocyte maturation. Furthermore, FF is easily available during oocyte pick up and represents an optimal source of non-invasive biochemical predictors of oocyte quality.

To determine the prognostic value of intrafollicular concentrations of some cytokines from women undergoing ovarian stimulation in the outcome of intracytoplasmic sperm injection/embryo transfer (ICSI/ET) cycles. A total of 80 patients were included in this study following ovarian stimulation and ICSI. Follicular fluids were collected at the day of oocyte retrieval. Ten cytokines including: tumor necrosis factor- alpha (TNF-α), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, CCL8/IL-8, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), and interferon gamma (IFN-γ) were measured using magnetic multiplex immunoassays. ROC curve was used to evaluate the performance of each estimated cytokine on the prediction of pregnancy and severe OHSS. Only the concentration of IL-5, IL-4, and GM-CSF in FF were significantly different ($p < 0.05$) between ICSI cycles that resulted in pregnancy and those that failed. Elevated FF IL-5 levels were associated with poor oocyte quality, which decreases the chance of both biochemical and clinical pregnancy. Higher FF GM-CSF associated to decrease of mature oocytes, while higher FF IL-4 concentrations were linked to good ICSI outcome through increased fertilization rate. In conclusion, the elevated intrafollicular concentrations of IL-5 seem to be a negative predictor to the pregnancy outcome in ICSI cycles. According to ROC curves, IL-5, TNF-α, and IL-10 had a medium predictive ability in prediction of pregnancy and the chance of severe OHSS, respectively.

Key Words: Cytokines, Interleukin-5, ICSI outcome, Female infertility.

Acknowledgments

I would like to dedicate the fruit of these efforts to the spirit of my deceased brother "Ihsan"

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List of Contents

Page

List of Abbreviations

2PN	**Two-pronuclei**
AMH	Anti-mullerian hormone
ART	Assisted Reproductive Technologies
ASA	Acetylsalicylic acid
AUC	Area under curve
BMI	Basal metabolic rate
CC	Cumulus cells
CXCL	Chemokine C-X-C motif ligand
COH	Controlled ovarian hyperstimulation
COX	Cyclooxygenase
CSF	Colony stimulating factor
E2	Estradiol
ECM	Extracellular matrix
EMBIC	European network for research, Embryo Implantation Control
FF	Follicular fluid
FR	Fertilization rate
FSH	Follicle stimulating hormone
GC	Granulosa cell
G-CSF	Granulocyte-Colony stimulating factor
GDF-9	Growth and differentiation factor-9
GH	Growth hormone
GM-CSF	Granulocyte macrophage - Colony stimulating factor
GnRH	Gonadotropin releasing hormone
GV	Germinal vesicle
HB-EGF	Heparin binding-epidermal growth factor
hCG	Human chorionic gonadotropin
HIV/AIDS syndrome	Human immunodeficiency virus /Acquired immune deficiency
ICSI	Intracytoplasmic sperm injection
IFN-γ	Interferon-gamma
IL	Interleukin
IR	Implantation rate
IUI	Intrauterine insemination
IVF	In vitro fertilization
LGL	Large granular lymphocytes
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
MCP-1	Macrophage-chemoattractant protein

M-CSF	Macrophage-Colony stimulating factor
MII	Metaphase-II
MMP	Matrix metalloproteinase
NK	Natural killer cell
NO.	Number
OHSS	Ovarian hyperstimulation syndrome
OPU	Oocyte pickup
OR	Odds ratio
PB	Polar bodies
PBMC	Peripheral blood mitogen- stimulated cells
PCOS	Polycystic ovary syndrome
PG	Prostaglandin
PRL	Prolactin
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
SART	Society for assisted reproductive technology
TC	Theca cell
TGF-β	Transforming growth superfamily beta
Th1	T helper cell type 1
Th2	T helper cell type 2
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

List of Tables

Table **Page**

List of Figures

Figure **Page**

1

INTRODUCTION

1.1 Infertility

Infertility is defined as inability to achieve pregnancy after one year of regular and unprotected intercourse (Jose-Miller et al., 2007). It can take several different forms, including resolved infertility (pregnancies that occur after one year without medical intervention), primary infertility (never pregnant), or secondary infertility (failure to conceive after having previously delivered an infant without the use of infertility treatment). Early environmental, chemical, or occupational exposures (e.g., in utero, in childhood) could permanently change fecundity or biologic capacity by affecting gynecologic, urologic, or pregnancy health (National Public Health Plan, 2014).

Nearly seven million men and women in 2015 in the U.S. experienced infertility and needed help through some sort of assisted fertility treatment, such as In vitro fertilization (IVF). According to the Society for Assisted Reproductive Technology (SART), nearly 175,000 cycles of IVF were conducted in 2013, which shows a sixty-five percent increase since 2003 (RMANJ, 2015). The live birth rates per cycle range between 30-40% in women <37 years of age undergoing their first IVF cycle, with significantly worst outcome in repeated cycles or when age is more than 38 years or other negative prognostic factors (i.e. endometriosis or premature ovarian failure) are present (Papaleo et al., 2011). Declining live birth rates in IVF have been observed over the last decade (2004-2014) in almost regions of the world, though intensity of change varied between regions. Strongest negative associations were observed with mild stimulation protocol of ovulation induction, extended embryo culture to blastocyst stage (BSC), elective single embryo transfer (eSET) in Japan, Australia/Newzeland and Canada (Gleicher et al., 2017).

1.1.1 Infertility Causes

In 2014, the National Public Health Action Plan for the prevention and management of infertility summarized the known and potential, but not include the following causes:

1) Reproductive age; includes establishing biomarkers, determining the predictors and correlates of early depletion of the ovarian reserve, in addition to the effects of age on semen quality and reproductive function.

2) Important developmental periods; identifying factors that affect fertility during certain developmental periods (e.g., preconception, in utero, puberty, transgeneration) to identify the best time for intervention.

3) Infectious diseases; the proportion of cases of tubal factor infertility attributable to infectious diseases and the role of specific infections, such as chlamydia, gonorrhea, mycoplasmas, trichomoniasis, bacterial vaginitis, tuberculosis of the reproductive tract, microbial organisms associated with reproductive tract infections, epididymoorchitis, prostatitis, and mumps.

4) Chronic conditions and diseases; including endocrine and metabolic diseases such as primary ovarian insufficiency, polycystic ovary syndrome (PCOS), hypothalamic amenorrhea, menstrual cycle defects, endometriosis, uterine leiomyomata, thyroid disorders, metabolic syndrome, diabetes, autoimmune disorders, meiotic aneuploidy, cystic fibrosis, varicocele, testicular disorders, multiple sclerosis, general urologic health, and immune-mediated disorders.

5) Behavioral factors; such as diet, exercise, sleep, psychological and physiological stress, caffeine consumption, tobacco and alcohol use, weight gain or loss, nutritional disorders, illicit or prescription drug use, and illicit use of anabolic steroids and growth hormones.

6) Iatrogenic causes; such as chemotherapy or associated medications for testicular or ovarian cancer and antiretroviral therapy for HIV/AIDS.

7) Occupational and environmental hazards; such as radiation, repetitive motion or posture, injury (e.g., reproductive or urinary tract trauma such as that experienced during military duty), or natural or synthetic chemicals and compounds with hormonal activities (e.g., endocrine disruptors).

8) Genetic influence; such as male karyotype abnormalities, Y chromosome microdeletions, or androgen receptor gene abnormalities. (National Public Health Plan, 2014).

For women, **age** remains the greater predictor for convenience. After age, anti-mullerian hormone (AMH) is the most important determinant to the viability of women's oocyte reverse, because fertility is not only about the number of oocytes available, but also ids the quality of those oocytes. After this age, oocytes become more susceptible to chromosomal imbalance and the frequency of chromosomal aberrations increases in human embryos, which limits their reproductive potential (RMANJ, 2015). Furthermore, advanced reproductive age is associated with early and later pregnancy complications in addition to infertility, as age a recognized risk factor for spontaneous abortion (Liu and Case, 2011).

Regards to the **ovulatory disorders,** the World Health Organization (WHO) categorizes ovulatory disorders into three groups: group I is caused by hypothalamic pituitary failure (10%), group II results from dysfunction of hypothalamic-pituitary-ovarian axis (85%), and group III is caused by ovarian failure (5%).Women in group I typically present with amenorrhea and low gonadotropin levels, most commonly from low body weight or excessive exercise. Women in group II include those with PCOS and hyperprolactinemia. Women in group III can conceive only with oocyte donation and IVF (Lindsay and Vitrikas, 2015).

Women with moderate to severe **endometrioses,** the inflammatory microenvironment in endometriosis women, affecting follicular function and thereby possibly contribute to the reproductive dysfunction in endometriosis (Wu et al., 2017).

PCOS patients are typically characterized by production of an increased numbers of oocytes during stimulation in an IVF cycle; however, these women suffer from poor-quality oocytes and embryos, lower fertilization, cleavage and implantation rates, and higher miscarriage rates (Qiao and Feng, 2011). Early pregnancy loss may be related to PCOS per se due to hypereandrogenemia, abnormal synthesis and release of adipokines from excessive visceral tissues in metabolically obese normal weight women reduced insulin resistance/ hyperinsulinemia (Elkholi and Nagy, 2016).

Table 1.1 causes of female factor infertility (jose-miller et al., 2007)

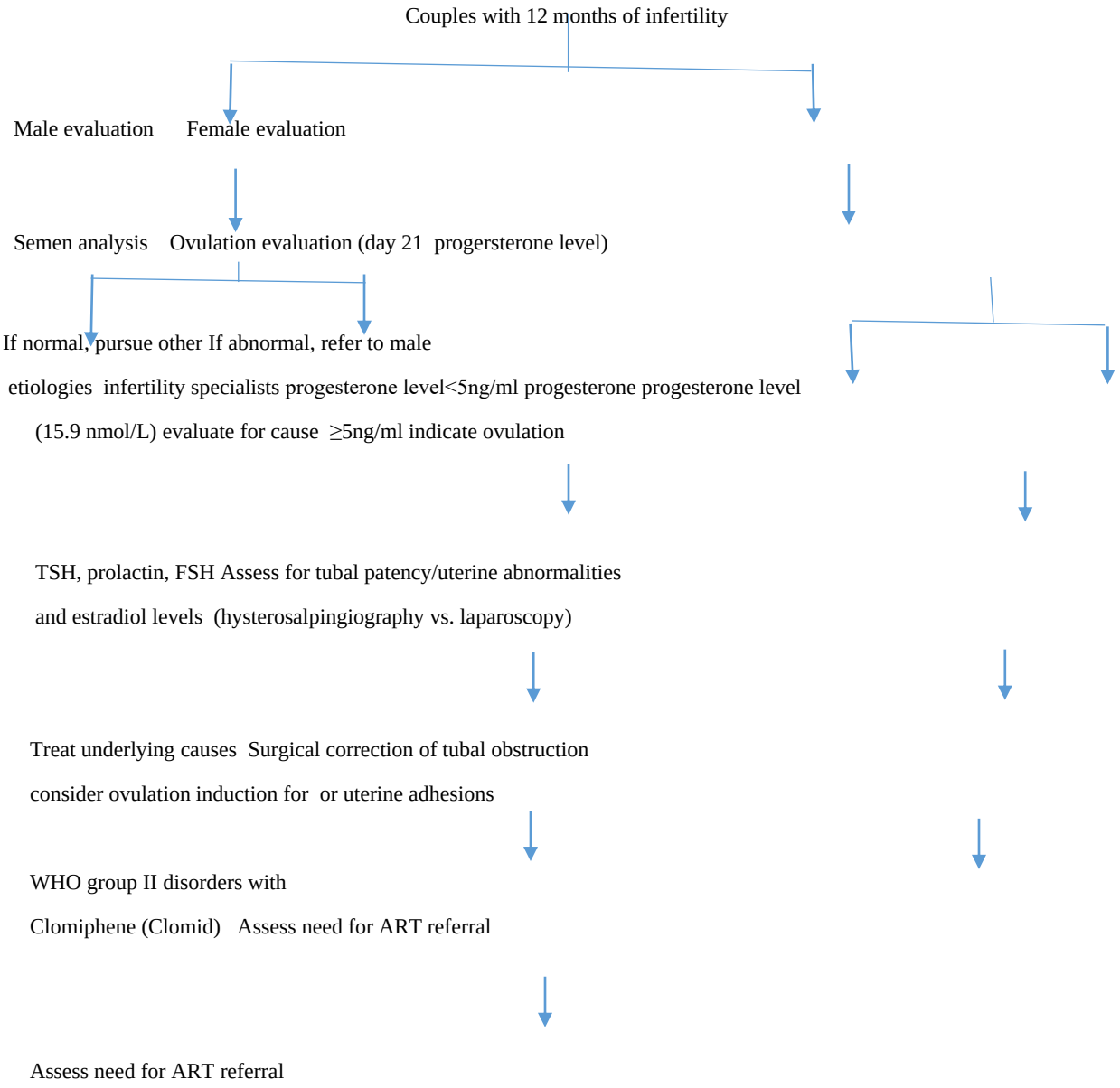
Causes of female factor infertilty
Ovulation disorders (40 percent)
Aging
Diminished ovarian reserve
Endocrine disorders (e.g., hypothalamic amenorrhea, hyperprolactinemia, thyroid disease, adrenal disease)
Polycystic ovary syndrome
Premature ovarian failure
Tobacco use
Tubal factors (30 percent)
Obstruction (e.g., history of pelvic inflammatory disease, tubal surgery)
Endometriosis (15 percent)
Other causes (about 10 percent)
Utrine /cervical factors (more than 3 percent)
Congenital uterine anomaly
Fibroids
Polyps
Poor cervical mucus quantity/quality (caused by smoking/ infection)
Uterine synechiae

1.1.2 Infertility Evaluation

Evaluation may be initiated sooner in patients who have risk factors for infertility or if the female partner is older than 35 years. A history and physical examination can help direct the evaluation. Men should undergo evaluation with a semen analysis. Abnormalities of sperm may be treated with gonadotropin therapy, intrauterine insemination (IUI), or in IVF. Ovulation should be documented by serum progesterone level measurement at cycle day twenty-one. Evaluation of the uterus and fallopian tubes can be performed by hysterosalpingography in women with no risk of obstruction. For patients with a history of endometriosis, pelvic infections, or ectopic pregnancy, evaluation with hysteroscopy or laparoscopy is recommended. Women with anovulation may be treated in the primary care setting with clomiphene to induce ovulation. Treatment of tubal obstruction generally requires referral for subspecialty care. Unexplained

infertility in women or men may be managed with another year of unprotected intercourse, or may proceed to assisted reproductive technologies (ART), such as IUI or IVF (Lindsay and Vitrikas, 2015).

Table 1.2 Evaluation of infertility (Lindsay and Vitrikas, 2015).



1.2 Ovarian follicles

The functional units within the ovary are its follicles, whose development is orchestrated by an extensive network of cytokines and growth factors which operate as paracrine mediators across

the intrafollicular microenvironment in response to the prevailing gonadotropin and steroid milieu. The ovary is sensitive to conceptus-derived human chorionic gonadotropin (β-hCG) which prolongs the life of the corpus luteum and ensures the successful establishment of pregnancy (Orsi et al., 2014). Human folliculogenesis and follicle maturation are complicated developmental processes through which a mature follicle is differentiated from primordial follicles, yielding one mature follicle that is eventually selected to ovulate, and release a mature oocyte. A series of extra- and intra-ovarian factors causing abnormalities during folliculogenesis, follicular growth and oocyte meiotic maturation processes have been identified that alteration of many factors may directly or indirectly impair the competence of maturating oocytes through endocrine and local paracrine/autocrine actions, resulting in a lower pregnancy rate in patients with PCOS. The extra-ovarian factors identified included gonadotropins, hyperandrogenemia and hyperinsulinemia. The intra-ovarian factors included members of the epidermal, fibroblast, insulin-like and neurotrophin families of growth factors, as well as the cytokines (Qiao and Feng, 2011).

The majority of oocytes in fetal ovaries are depleted before birth, and only about 400 will ovulate during the normal fertile life span. Studies on animals have shown that apoptosis is the mechanism behind oocyte depletion and follicular atresia. In human, depletion of ovarian follicles during fetal development occurs through the mechanism of apoptosis, which is particularly extensive in the oocytes during the first three quarters of gestation. During adult life, apoptosis is mainly located in the granulosa cells of growing follicles. Thus, in fetal as well as in adult ovaries, the destiny of early follicles may be determined by intrinsic mechanisms of apoptosis in the oocyte, whereas later, during follicle stimulating hormone FSH-dependent stages of follicular development, granulosa cell apoptosis plays a major role in follicular demise. Several agents, such as the bcl-2 family, are likely to participate in the regulation of apoptosis in the human ovary (Vaskivuo et al., 2001).

Oocytes are sequestered in primordial follicles before birth and remain quiescent in the ovary, often for decades, until recruited into the growing pool throughout the reproductive years. Therefore, activation of follicle growth is a major biological checkpoint that controls female reproductive potential (McLaughlin and Mclver, 2009).

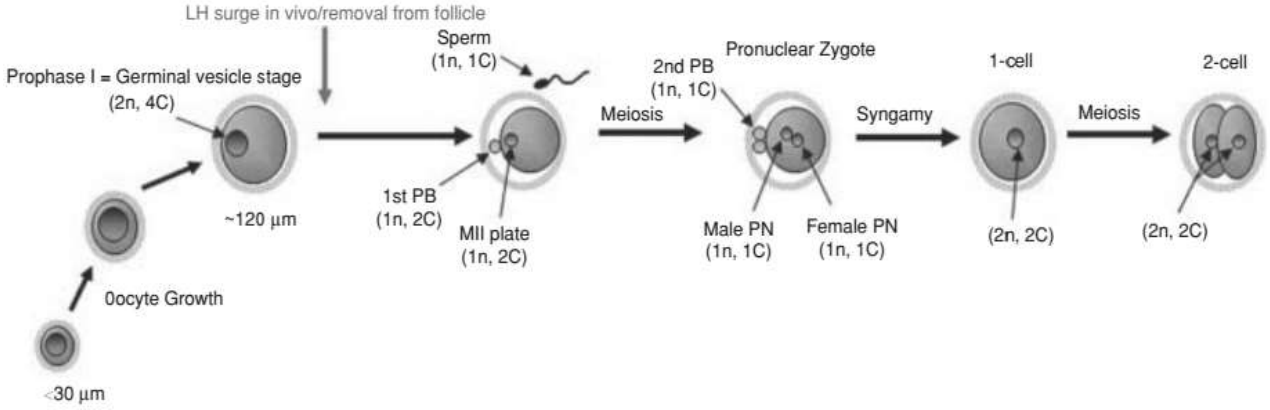


figure 1.1 schematic representations of oocyte growth and development (lonergan, 2013)

1.2.1 Ovarian follicles Structure

The ovarian follicle is a defined developmental unit consisting of a single oocyte surrounded by layers of somatic granulosa and theca cells. During postnatal life, most oocytes exist in dormant primordial follicles within a single layer of a few “flat” squamous, nonproliferating granulosa cells. Upon growth activation, the granulosa cells switch to a cuboidal morphology and begin to proliferate while the oocyte initiates a growth phase. Follicle development then consist of continued proliferation and differentiation of the granulosa layers, acquisition of an additional cell layer outside the basement membrane (termed the theca), and continued oocyte growth. Eventually, a fluid-filled cavity forms within the granulosa layers (termed the antrum), giving the follicle an asymmetrical character. By this time, the granulosa cells in closest association with the oocyte are physically separate from those that make up the follicle wall; the granulosa cells in these locations are definitive lineages: the steroidogenic mural population (in the wall) and the cumulus population (surrounding the oocyte). Should the follicle survive to finish the term of growth and maturation, the egg within its cumulus layers is expelled from the surface of the ovary during ovulation (Uslu and Johnson, 2013).

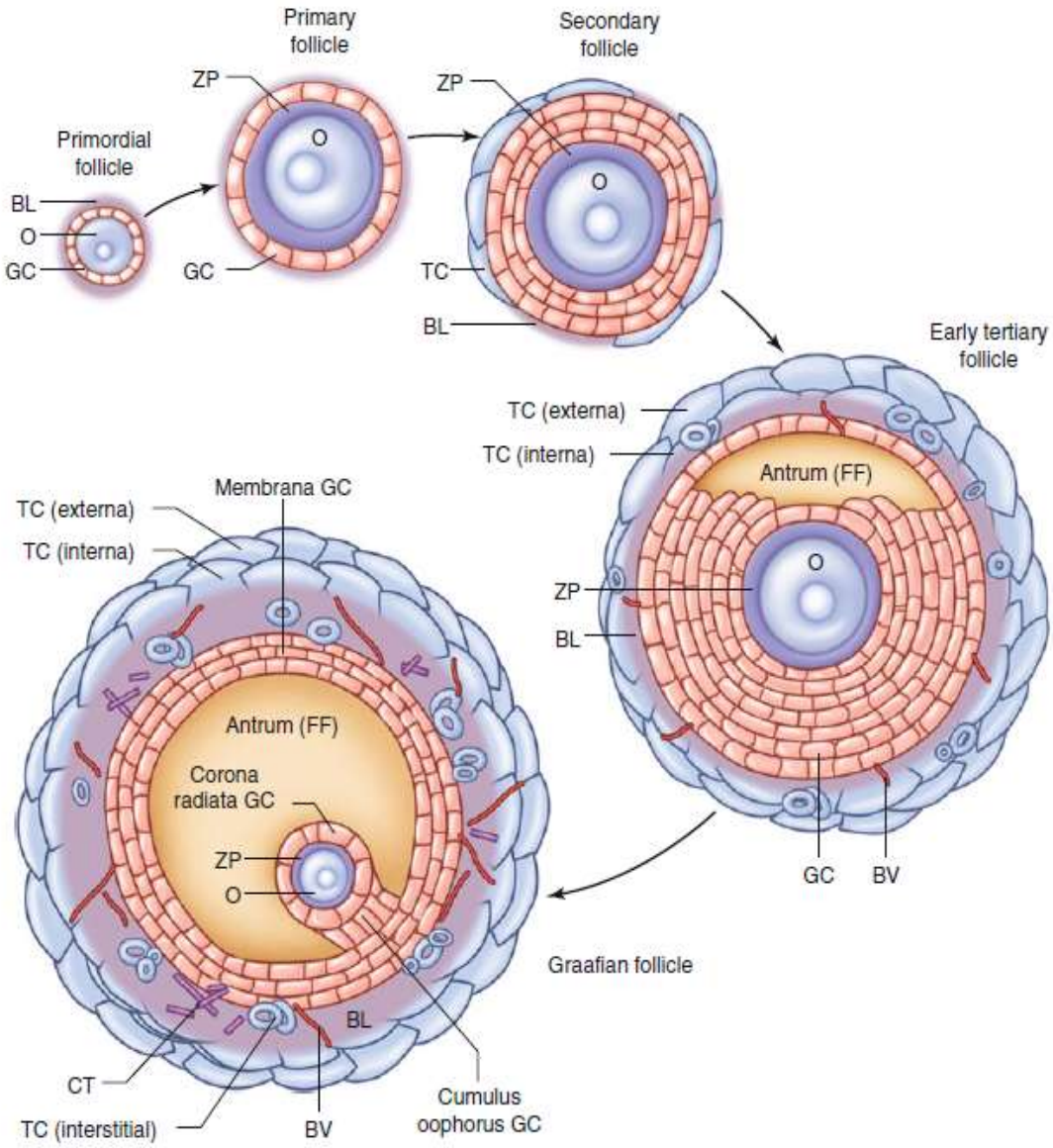


Figure 1.2 Schematic representation of development of the primordial follicle to the pre-ovulatory Graafian follicle (Orsi et al., 2014).

The preantral to early transition is the penultimate stage of follicular development in terms of gonadotropin dependence and follicle density (growth versus atresia). Follicular growth during the preantral-early antral transition is tightly regulated by intra-ovarian oocyte-granulosa-theca-cell interactions. Formation of the theca cell layer is a key event that occurs during this transitional stage. Granulosal factors appears to stimulate the recruitment of the theca cells from cortical stromal cells, while oocyte-derived growth differentiation factor-9 (GDF-9) is involved

in the differentiation of theca cells during this early stage of development, promotes follicular survival and growth during the preantral to early antral transition by suppressing granulosa cell apoptosis and follicular atresia, and enhances preantral follicle growth by up-regulating theca cell androgen production. Thecal factors also promote granulosa cell proliferation and suppress granulosa cell apoptosis. The dysregulation in these interactions may lead to ovarian pathology such as PCOS and gonadotropin poor-responsiveness (Orisaka et al., 2009).

1.2.2 Ovarian follicles function

The ovary is a unique organ which fulfils a plethora of physiological functions ranging from steroid hormone biosynthesis, oocyte production and the support of early pregnancy through involvement in the regulation of growth, behavior and immune function in response to an array of endogenous and environmental cues. While multiple cytokine-mediated interactions between the granulosa/theca cell compartments and the oocyte of developing follicles are central to the coordination of ovarian cyclicity, its systemic effects are instead attributable to the interactions between ovarian follicles, the hypothalamus and pituitary implicating an array of peptide and steroid hormones. During the follicular phase, primordial follicles are called upon to initiate a process of follicular development which culminates in ovulation. Supporting establishment and maintenance of pregnancy through the production of progesterone from postovulatory follicle. Once the ovary has exhausted its reserve of follicles throughout reproductive life, many of its functions are lost as women enter the menopause. The oocyte-bearing follicles, are composed of multiple somatic cell types which communicate through extensive paracrine cytokine/growth factor-mediated networks to orchestrate oocyte development and cyclical steroid hormone production (Orsi et al., 2014).

Follicular phase: In particular, the luteinizing hormone (LH) surge induces terminal growth and maturation of both the dominant follicle, whose follicular fluid volume rapidly increases, and its oocyte resumes meiosis. The combination of estrogen and FSH promotes LH receptor expression on granulosa cells (GCs) which forfeit estrogen production in favor of the LH-stimulated progesterone synthesis which characterizes the luteal phase (Orsi et al., 2014).

Folliculogenesis: It occurs in the ovarian cortex and begins with the recruitment of a **primordial follicle** into the pool of growing follicles and ends with either ovulation or death by atresia; the

entire process can take up to a year in women. Folliculogenesis is divided into two phases. The first phase, termed the pre-antral or gonadotropin independent phase, which is characterized by the growth and differentiation of the oocyte and is principally controlled by locally produced growth factors through autocrine/paracrine mechanisms. This phase covers the transition of the primordial follicle to the primary pre-antral follicle. By contrast, although the second phase also features continued follicular growth, this occurs in response to pituitary gonadotropins. **The secondary follicle**: Theca development is accompanied by neoangiogenesis which promotes the development of a perifollicular vascular network within the layer which rapidly expands as the follicle increases in size (Orsi et al., 2014).

Antral follicle: As oocytes reach the end of their growth phase, they acquire competence to undergo two aspects of maturation: cytoplasmic and nuclear. The cumulus oophorus regresses before ovulation, leaving the oocyte surrounded by a layer several cells thick, termed the corona radiata. Prior to ovulation, the remaining attachment of the cumulus-oocyte complex connection to the now markedly thinned zona granulosa breaks down altogether. By this stage, the follicle bulges under the ovarian surface. The overlying surface epithelium cells become flattened and atrophic and the thin intervening stroma becomes degenerate and avascular. Components of the antral follicle, including three distinct somatic cell types (theca, granulosa and cumulus), the basal lamina, and follicular fluid, each have active and regulatory roles in oocyte differentiation. Mammalian reproduction hinges upon the timely ovulation of a fully differentiated oocyte. This event is the culmination of a complex and dynamic developmental relationship between the oocyte and the antral follicle housing it; the antral follicle constitutes a specialized microenvironment or niche, uniquely suited to the needs of the oocyte as it approaches ovulation. During this time, the oocyte must complete its final growth, capacitation, and nuclear and cytoplasmic maturation. Its microenvironment, the antral follicle, is in turn responsible for the integrity of these processes and the production of a high quality oocyte (Hennet and Combelles, 2012; Orsi et al., 2014).

Ovulation: Whether or not a dominant follicle will in fact reach ovulation depends on the frequency and amplitude of LH pulses continually induced in the pituitary. These LH pulses will increase in intensity as more time elapses from the previous cycle's ovulation event (due to luteolysis of the corpus luteum and diminishing progesterone levels), and eventually culminate in

a surge sufficient to induce ovulation. They are likely to be assisted in this role via the LH-induced immune effector cell infiltrate and alterations in the local cytokine microenvironment (Hennet and Combelles, 2012; Orsi et al., 2014).

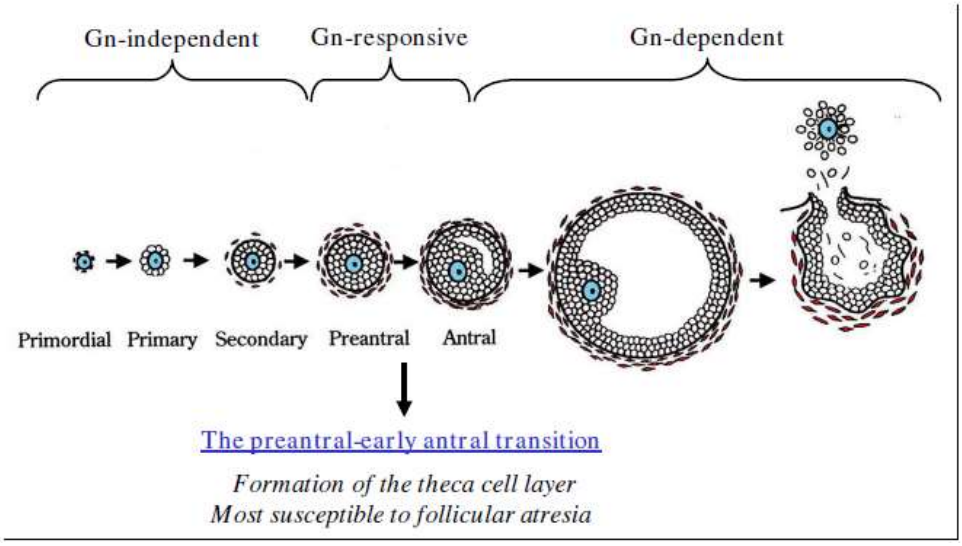


Figure 1.3 schematic representations showing the transition of the follicle from preantral to the early antral stage (Orisaka et al., 2009)

Steroidogenesis: Hormones govern the antral phase of folliculogenesis, and as such they are a pervasive influence in almost every aspect of somatic cell support to the oocyte. Although some hormones, such as FSH and LH, are produced externally, others such as estradiol and androgens are produced within the follicle by granulosa and theca cells. The regulation of intrafollicular hormone levels is, in and of itself, a critical aspect of the antral follicle microenvironment. For instance, growth hormone (GH) in follicular fluid enhances the FSH-dependent estradiol production by granulosa cells. Estradiol, in turn, upregulates androgen secretion by theca cells, a product that provides granulosa cells with the substrate necessary for further estradiol production. Androgen production by theca cells is also stimulated by GH and dependent on LH levels. Granulosa cells can synthesize inhibins that sensitize theca cells to LH, thus facilitating further estradiol synthesis through the supply of androgens until the LH surge (Knight and Glister, 2001; Kwintkiewicz and Giudice, 2009). A dominantly estrogenic environment is correlated with oocyte competence. The hormones produced locally within the follicle, such as

progesterone, anti-Mullerian hormone, and estradiol, will be involved in a variety of follicular modifications, signaling cascades, and metabolite production (Hennet and Combelles, 2012).

It appears that ovarian function and steroidogenesis is a highly-integrated process that regulated by endocrine, paracrine and autocrine factors that act through both cytoplasmic and nuclear mechanisms. Of the several cytokines, TNF-α is considered the main cytokine involved in luteal function (Dor et al., 1996).

1.3 Follicular fluid

Follicular fluid (FF), an oocyte microenvironment, is a unique biological fluid in which the events of oocyte, follicular maturation and somatic cell-germ cell communication occur. Because of the intimate proximity of FF to the maturing oocyte, this fluid provides a unique window into the processes occurring during follicular maturation (Hashish et al., 2014; Zamah et al., 2015).

FF is easily available during oocyte pick-up and represents an optimal source on non-invasive biochemical predictors of oocyte quality (Revelli et al., 2009), so the identification of the specific components within FF may provide a better understanding of intrafollicular signaling, as well as reveal potential biomarkers of oocyte health for women undergoing ART has been a goal of embryologists and researchers in clinical IVF for decades (Revelli et al., 2009; Van Blerkom, 2012; Zamah et al., 2015).

FF is a pool of considerable biological significance since its composition indicates the environment in which the oocyte and granulosa cells are growing and maturing. It is well established that oocyte quality determines the embryo's developmental potential after fertilization, in common with all other cells, the oocyte and granulosa cells are bathed in extracellular fluid. It has, however, become conventional to reserve the term 'follicular fluid' for that fraction of the extracellular fluid that accumulates in the antrum of larger follicles. Furthermore, it buffers the internal environment of the follicle against the influence of external conditions presented by the blood stream (Gosden, 1988).

FF represent the intermediate stage between the information encoded in the genome and the functional cell's phenotype. Their analysis in biological fluids may provide more information than the direct study of gene expression, mRNAs or proteins. It may provide the actual functional status of a biological system and its cell and therefore may better explain the

contradictory actions and functions of cytokines as given by gene expression studies. Follicular fluid provides an ideal microenvironment, which is abundant and easily available during IVF cycles and might be an optimal non-invasive predictor of implantation in IVF-ET (Chimote et al., 2010).

Human FF has extracellular vesicles, especially CD9-positive extracellular vesicles, which play important role for sperm-oocyte interaction and embryo implantation. In contrast to other body fluids such as saliva, lacrima, urine and ejaculate, FF do not contain highly coagulant tissue factor-exposing vesicles with no procoagulant activity (Franz et al., 2016).

Addition of high concentration of FF to the maturation medium reduced embryonic development rates, but in lower concentrations, FF slowed the meiotic progression and migration of CG and contributed to increase inner cell mass in bovine embryos produced *in vitro* (Navakanitworakul et al., 2016). CoelhoCruz et al. (2014) showed that the addition of higher concentrations of FF to the bovine oocytes maturation medium reduced the embryonic developmental rate, while in lower concentration; FF enhanced the number of cells in bovine embryos. However, the flushing of the endometrial cavity with FF after oocyte retrieval neither improved nor adversely affected clinical pregnancy and implantation rates in subfertile women undergoing intracytoplasmic sperm injection (ICSI) (Sarhan et al., 2016).

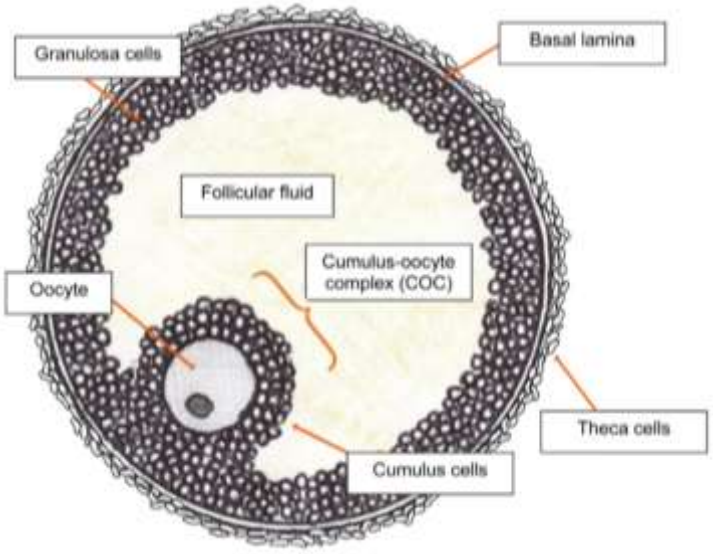


Figure 1.4 Schematic representation of an antral follicle (Hennet and Combelles, 2012).

1.3.1 Physiochemical features of FF

1. FF **absorbency**: more than 415-455 nM wave length is correlated with fertilized oocytes (Revilli et al., 2009).

2. FF **pH**: between 7.2-7.4, if it increases to 7.6 it will combined with negative IVF/ICSI. Ultrasound guided oocytes retrieval preserved the normal slightly alkaline follicular fluid pH, while using laparoscopy in oocytes retrieval has been associated with more alkaline pH and poor IVF outcomes. Oocytes in preovulatory follicles are surrounded by fluid that is more alkaline than plasma. Hence, the acidic environment treated by CO_2 may be deleterious to subsequent reproductive function of the oocyte (Daya et al., 1988).

3. FF **viscosity** and FF **refractive index**: These characteristics have no effect on oocyte quality, maturation grade, fertilization capacity, and IVF outcome. Furthermore, these two parameters have no difference between thawed frozen fluids and fresh fluids (Fisch et al., 1996).

1.3.2 Follicular fluid constituents

The composition of follicular fluid in Graafian follicles is similar but not identical to ovarian venous plasma. Differences between the two fluids are attributed to a blood-follicle barrier, which restricts the passage of large molecules. The follicle epithelium has been characterized as 'leaky' on the basis of both chemical and electrical criteria. The presence of an intervening acellular zona pellucida is both a physical barrier between the oocyte and the follicular milieu and a biological filter that limits the diffusion of molecules to those up to ~60,000–70,000 Daltons (Van Blerkom, 2012).

The rate of FF accumulation is much greater during preovulatory activation of the follicle (secondary fluid) than at preceding stages (primary fluid), suggesting that gonadotrophic hormones have a major influence on the rate of swelling. The evidence for water transport following an osmotic gradient set up by active transport of Na' has been inconclusive. The conventional view that fluid forms from transudation of plasma rests on circumstantial evidence and is less likely to account for primary than for secondary fluid (Gosden, 1988).

According to Revilli et al. (2009), the most important chemical constituents of FF which have used as biomarkers and could be related to IVF/ICSI outcome are:

a) Hormones; Gonadotropins, growth hormone, prolactin, estrogens, progesterone, androgens and corticoids.

b) Growth factors and Cytokines; Insulin-like growth factors, Anti-mullerian hormone, Bone morphogenetic protein-15, inhibin, and activin. Interleukins (IL): IL-1β, IL-2, IL-10, IL-12, IL-1, IL-6, leukemia inhibitory factor (LIF), tumor necrosis factor-alpha (TNF-α), and granulocyte-colony stimulating factor (G-CSF).

c) Sugars; Hyaluronan and Myo-inositol

d) Prostanoids; prostaglandins F2α (PGF2α) and PGE2

e) Proteins, peptides and amino-acids; Antigen CD44, Leptin, endothelin-2, Alpha1-antitrypsin, Oocyte maturation inhibitor, homocysteine, beta-endorphin, lactoferrin, angiotensin II, prorenin, Vascular Endothelial Growth Factor (VEGF) and amino-acids.

f) Reactive Oxygen Species (ROS); antioxidant factors, and nitric oxide.

1.4 Oocyte developmental competence / quality

Oocyte developmental competence is the ability of the oocyte to complete meiosis and undergo fertilization, embryogenesis, and term oocyte development (Lonergan, 2013). Bidirectional somatic cell–oocyte signaling is essential to create a changing intrafollicular microenvironment that controls primordial follicle growth into a cohort of growing follicles, from which one antral follicle is selected to ovulate a healthy oocyte. Such intercellular communications allow the oocyte to determine its own fate by influencing the intrafollicular microenvironment, which in turn provides the necessary cellular functions for oocyte developmental competence. These coordinated somatic cell–oocyte interactions attempt to balance cellular metabolism with energy requirements during folliculogenesis, including changing energy utilization during meiotic resumption (Dumesic et al., 2014).

In human, the developmental competence is embryo specific, and this specificity arises in the preovulatory oocyte. This gives reason for optimism that investigations capable of displaying a comprehensive picture of the biochemistry of each follicle's fluid will be informative and clinically beneficial in infertility treatment (Van Blerkom, 2012). Different ovarian stimulation protocols for IVF are being developed to obtain elevated number of oocytes at retrieval with the aim to increase success rates. Variety of factors play a role in oocyte competence including environmental conditions, oocyte interactions with cumulus cells (CC) and FF and various

chemical components (Papaleo et al., 2011). Follicular diameter positively associated with acquisition of oocyte competence in several species. Circulating pulses of LH during the preovulatory period may be beneficial for the final maturation of the oocyte. Ovulatory follicle size remains an easily measurable attribute of the follicle that is highly associated with its ability to produce estradiol. Perhaps the critical maturational component is the production of sufficient estradiol by the ovulatory follicle to prepare follicular cells for lutenization and progesterone synthesis needed to prepare the uterus for pregnancy (Geary et al., 2013).

Although small differences exist between the human and bovine models, the bovine is currently a more completely characterized model for antral folliculogenesis (Hennet and Combelles, 2012).

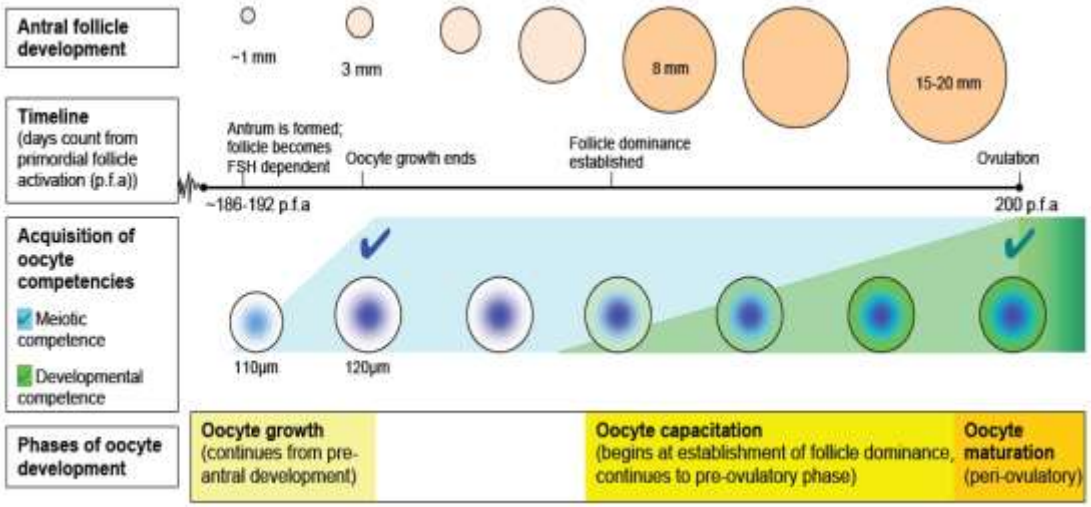


Figure 1.5 The acquisition of oocyte competencies, and phases of oocyte development, in relation to antral folliculogenesis in the bovine (Hennet and Combelles, 2012).

Increasing evidence is emerging that controlled ovarian hyperstimulaton (COH) with exogenous gonadotropins may be detrimental to oogenesis, embryo quality, and endometrial receptivity (McLaughlin and Mclver, 2009). COH perturbs intrafollicular cytokine networks, in terms of both cytokine levels and their interrelationships. This may impact oocyte maturation/fertilization and embryo developmental competence (Baskind et al., 2014).

1.4.1 Morphological assessment of oocyte Quality/competence

Because it may be a governing factor in influencing IVF outcomes, oocyte selection and the identification of the best oocytes would help to limit embryo overproduction and to improve the results of oocyte cryostorage programs (Revelli et al., 2009). However, morphological evaluation of oocyte quality is difficult in conventional IVF cycles. According to the Global Fertility Academy, the functional viability of oocyte comprising of the following morphological assessments of oocyte quality; fertilization rate; and implantation rate (Guelman and Patrizio, 2009):

Oocyte evaluation after retrieval (Day 0)

Morphologically, the ideal oocyte is generally receptive to fertilization, which is characterized by the presence of the cumulus cells still around the oocyte, and well expanded producing the typical “sunburst-like effect”.

Day One: Fertilization Check

During this granulation phase, the sperm head decondensed. Subsequently, the second polar body was extruded, which was followed by the central formation of the male pronucleus. At about the same time, the female pronucleus formed and was drawn towards the male pronucleus until the two abutted. Both pronuclei then increased in size, and their nucleoli moved around and arranged themselves near the common junction. A sign of occurred fertilization is the presence of two pronuclei (PN) and two polar bodies (PB) at a precise time from insemination that is 16 to 18 hours later.

Day Two/Three: Multicell Grading

On day two the single cell zygote should divide into an embryo (approximately two to four cells). On day three the embryo should continue to divide (four to eight cells). Good embryo has no fragmentation and the size of the blastomeres is even and regular. Zygotes showing pronuclei with approximately the same number and alignment of nucleolar precursor bodies had a good prognosis in terms of subsequent implantation.

Day Four

On day four, embryos begin their transition from a multicell embryo to a more advanced developmental stage. Embryos should begin compacting and forming morula. Cells of a morula - stage embryo are not as distinct as in previous days; therefore, these embryos do not receive quality grades.

Day Five: Blastocyst Stage

A blastocyst is a highly developed embryo that is composed of two different cell types: one group of cells, called the inner cell mass, leads to fetal tissue and another group of cells, called the trophoectoderm, forms the placenta. It is important to note that this approach will not necessarily yield higher numbers of blastocysts but, more importantly, blastocysts that are more viable as assessed by increased implantation rates (Ebner et al., 2003; Guelman and Patrizio, 2009; Mehta et al., 2013).

1.4.2 FF biomarkers for assessment of oocyte quality/ competence

A relationship between oocyte quality and FF hormones is expected, since the formation of FF coincides with the oocyte maturation phase. High concentrations of LH and E2 in individual FF samples were related to oocyte nuclear maturation, fertilization and embryo grading during ART. Oocytes with higher levels of estradiol resulted in good embryo grading suggesting the effect of estradiol on oocyte cytoplasmic maturation which has a crucial role in the oocyte developmental competence (Sarhan et al., 2016). In successful ICSI cycles, the levels of LH and GH were higher in follicles from which oocytes gives rise to embryos with best morphology and fast cleavage rate as compared with other follicles from which a mature oocyte were cryopreserved for later use (Mendoza et al., 2002). Follicular environment rich in estradiol, progesterone, and testosterone is key to good oocyte development. High levels of progesterone and to a lesser extent, testosterone would be crucial for determining good oocyte quality and key for normal fertilization, as well as an essential step for success in assisted reproduction (Carpintero et al., 2014). de los Santos et al. (2012) quantified the FF concentrations of estradiol (E2), progesterone (P), FSH, LH, testosterone (T) and androstendione (D4) in unstimulated (control) and stimulated (COH) groups. T (testosterone) was higher in the COS group, while D4, E2 and LH were significantly higher in unstimulated cycles. Mehta et.al (2013) was the first that successfully

explored AMH in the pooled FF microenvironmental milieu of mature pre-ovulatory follicles and established it as a potent biochemical indicator of oocyte viability and correlated to increase fertilization rate, clinical pregnancy and embryo implantation rates in convential IVF cycles. Lower FF AMH might allow for the aromatization of substrate androgens to be converted to estrogens, also necessary for preparing the endometrium for the ensuing embryo implantation process.

The analysis of follicular metabolic composition could be considered as an additional tool in oocyte selection. Alterations in protein constituent of FF from fertile patients with varying etiologies such as advanced female age, endometriosis or PCOS. The circulatory and FF proteins were changed in response to hCG and could be used as markers of premature or appropriate lutenization to allow for improved ovarian stimulation outcomes (Zamah et al., 2015). The metabolic composition of the FF of women undergoing ART is associated with oocyte and embryo quality. FF apolipoprotein A1 (ApoA1) and total protein concentrations were negatively linked with oocyte quality may be due to the involvement of metabolic aberrations in the FF rather than basal metabolic rate (BMI)-related changes as having potential hazardous effects on oocyte quality (Valckx et al., 2012).

Female obesity aggravates FF oxidative stress, which in turn negatively affects the success of ART. Obese women with higher BMI have malonaldehyde (OMDA), NO2/NO3 (Nitrite/Nitrate) ratio, markers of oxidative stress. Moreover, the clinical pregnancy rate was lower in obese women undergoing ICSI (Shaeer et al., 2014). Saturated fatty acids and the ratio of SFA to polyunsaturated fatty acids were correlated negatively with a number of mature oocytes. Linoleic acid was positively correlated, while the level of arachidonic acid was negatively correlated with fertility percentage in IVF/ICSI cycles (Shaaker et al., 2012). In poor ovarian response patients, the alterations in gene expression of some FF lipids including phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, and diacylglycerols causes an alteration in the glycerolipid balance (Cataldi et al., 2013).

Overall these aforementioned, alterations in different metabolic constituents can be used as a diagnostic tool for the success of IVF/ICSI cycles after ovarian hormonal stimulation. However, the quality and viability of oocytes will be intimately linked to the intraovarian/perifollicular cytokine milieu. The complexity of cytokines roles and interactions is undeniable, and existing

data handling approaches still fall short of providing a holistic representation of their biological properties. With the advent of high-throughput, multiplex analytical platforms calls for the use of similarly sophisticated modelling approaches in order to reveal the true nature of cytokines in their physiological context (Field et al., 2014).

1.5 Cytokines and Fertility/Infertility

Cytokines are key regulators of ovarian physiology, particularly in relation to folliculogenesis and ovulation, where they contribute to creating an environment supporting follicle selection and growth. Their manifold functions include regulating cellular proliferation/differentiation, follicular survival/atresia, and oocyte maturation. (Piccinni, 2007; Guzeloglu-Kayisli et al., 2009; Field et al., 2014). Some of the redundancy and pleiotrophy between certain cytokines that share an accessory signal transducing subunit resulting in their pro- as well as anti-inflammatory properties (Heinrich et al., 2003). Several cytokines, such as transforming growth superfamily beta (TGF-β members), are involved at all stages of folliculogenesis while the production of others including members of the glycoprotein gp130 cytokine family (IL-11 and LIF), colony-stimulating factors (CSFs), IL-1, IL-15, and IL-33 is stage-dependent (Field et al., 2014). Some cytokines such as IL- 1β influence oocyte fertilization and embryo quality while others correlated to successful pregnancy such as IL-8, IL-18, and MIP-1β (Sarapik et al., 2012).

Cytokines have traditionally been divided into families, depending on the origin of immune cell and the immunological effects that they bring about. $CD4^+$ T helper (Th) cells are the major immune cells that involved in cytokine production. Those can be divided into three functional subsets based on their cytokine production: Th1 cells produce IFNγ, IL-2 and TNFβ and that considered the main effectors of cell-mediated immune responses. Th2 cells produce IL-4, IL-5, IL-6 and IL-10, the anti-inflammatory cytokines, the main effectors of antibody-mediated humoral immunity. The third T helper cell populations are Th0 cells that can be converted to either Th1 or Th2 cell type and can produce Th1, Th2 cytokines, TNFα and granulocyte macrophage- colony stimulating factor (GM-CSF). A distant family of cytokines is the pro-inflammatory cytokines that includes IL-1, TNF-α, IL-6 and leukemia inhibitory factor (LIF). This family is primarily produced by macrophages, non-immune cells such as epithelial and stromal cells of the endometrium, the decidual, and cytotrophoblast cells of the placenta, is

involved in the inflammatory events associated with tissue damage and repair (Dimitriadis et al., 2005; Laird et al., 2006; Chatterajee et al., 2014).

In normal pregnancy, local modulation of pro-inflammatory and anti-inflammatory cytokines resulting in a Th2-type biased high levels during placental formation and most of the gestation period. Towards the end of the third trimester and at term, an opposite bias is initiated as a consequence of downregulation of Th2-type cytokines and upregulation of Th1-type pro-inflammatory cytokines (Dealtry, 2000). Therefore, a predisposition to a premature shift from anti- to pro-inflammatory balance may indicate a pathologic pregnancy such as abortion or preterm birth in human (Marzi et al., 1996; Velez et al., 2008) and animal models as well (Yang et al., 2016).

Granulocyte-colony stimulating factor (G-CSF) is the only known cytokine in FF that entirely associated with assisted reproductive techniques (ART) success which ws confirmed by three distinct experiments conducted by the European network for research, Embryo Implantation Control (EMBIC) (Lèdèe et al., 2010). G-CSF is a non-invasive biomarker for implantation, as it correlates with the potential of the corresponding embryo to result in a live birth in IVF (Lèdèe et al., 2008). It also predicts the oocyte competence and subsequent birth, combined with FF IL-15 (Lèdèe et al., 2011). Moreover, G-CSF provides a better choice of embryos, limits multiple pregnancies and reduces embryo cryostorage according to the oocyte morphology alone (Lèdèe et.al, 2012). Other cytokines have a contradicted and conflicted results respective to their correlation to IVF/ICSI outcome such as IL-1β (Rehman et al., 2014; Wu et al., 2017), IL-6 (Amato et.al, 2003; Bedaiwy et.al, 2007), and TNF-α (Mendoza et al., 2002; Yildizfer et al., 2012). In addition, some other cytokines were rarely studied or have no effect in ART treatments such as IL-3 and IL-5.

Here, we will summarize the most important **cytokines** in serum and FF which had been used as biomarkers and could be correlated to fertilization, oocyte quality, embryo quality, and IVF/ICSI outcome and their role in pregnancy with or without disease conditions:

1.5.1 IL-1β

IL-1β is produced from FF, GC, and macrophages during the proliferative phase of the cycle. It reduces the expression of LH receptors in rat granulosa cells; participates in determining whether follicles undergo atresia or progress to ovulation; upregulates granulosa cell and intraovarian

macrophage nitric oxide production, and acts as an autocrine regulator of ovarian function responsible for orchestrating cumulus-cell expansion in mice (Field et al., 2014). IL-1β is associated with successful implantation and endometrial changes. It induces COX-2, in addition to the induction of cytokine production including LIF, macrophage-colony stimulating factor M-CSF (CSF-1), and GM-CSF (CSF-2) (Castro-Rendón et al., 2006). Lower IL-1β in FF is correlated to tubal factor infertility in PCOS patients (Sarapik et al., 2012), while in endometrial secretions no statistically significant differences were seen between the groups with and without successful pregnancy (Rahiminejad et al., 2015). It is possible that IL-1 stimulates those events in oocyte cytoplasmic maturation that are necessary for normal fertilization, but without improving post-fertilization embryo viability (Mendoza et al., 2002). In severe endometriosis patients, follicular fluid IL-1β significantly was higher than those in the serum, and inversely was correlated to the follicular estradiol concentration (Wu et al., 2017). Low plasma levels and high FF levels of IL-Iβ and IL-IRα were associated with successful pregnancy outcomes after IVF (Laird et al., 2006). The circulatory and pooled FF intrafollicular levels of Il-1β was correlated to pregnancy outcome in ICSI cycles (Asimakopoulos et al., 2010).

1.5.2 IL-2

Placental IL-2 is associated with increase abortion rates in mice, whereas low placental production of IL-2 observed in mice with normal pregnancy outcomes (Laird et al., 2006). Marzi et al. (1996) found that the low IL-2 in peripheral blood mitogen- stimulated cells (PBMC) were linked to normal pregnancy, and its higher levels were associated to pathologic pregnancy especially in the third trimester of pregnancy. In women undergoing unexplained recurrent spontaneous abortion, the significantly increased in IL-2 serum concentration as compared to normal pregnant women at similar stages of gestation was obsereved (Raghupathy et al., 2000). Yang et al. (2016) showed that IL-2 in PBMC was upregulated during early pregnancy.

1.5.3 GM-CSF (CSF-2)

GM-CSF is produced in large amounts under inflammatory conditions by activated cells of immune system. It is critical to the activation of immune response in monocytes, macrophages and dendritic cells. GM-CSF not only promotes the survival of macrophages, but also supports their differentiation toward a proinflammatory phenotype (Utshach and Zlotnik, 2016). In ovary,

GM-CSF expressed from GC and theca cell (TC) and participates in ovulation and lutenization by enhancing macrophages recruitment (Field et al., 2014). In mice, GM-CSF is produced by murine decidual cells in response to invasive trophoblast (Castro-Rendón et al., 2006). GM-CSF was higher in the FF from the antagonist superovulation protocol and negatively correlated with Eotaxin-1 (CCL11) (Malhotra et al., 2013). However, the intrafollicular GM-CSF is not related to corresponding ongoing pregnancy rates (Lèdèe et al., 2012).

1.5.4 TNF-α

TNF-α is a multifunctional hormone-like polypeptide, which has a wide spectrum of biological activities, including stimulation of cell proliferation, differentiation and induction of apoptotic death. Macrophages are a main source of TNF-α in ovary, and TNF-α is expressed in oocytes, corpora lutea, theca, and granulosa cells. TNF-α exerts its effects by binding to two distinct receptors: TNF-α-R1, mainly responsible for transduction of the death signals, and TNF-α-R2, chiefly implicated in cell proliferation. It appears that in ovary, TNF-α can trigger either proliferation or apoptosis depending on the cell type and the stage of follicular development (Artini et al., 2007).

TNF-α facilitates ovulation by inducing localized apoptosis at the ovarian surface-follicular interface, extracellular matrix (ECM) remodeling; upregulation of collagenases in preparation for ovulation, a process involving local macrophage protease activity; induces macrophage-chemoattractant protein (MCP-1) and M-CSF. It is also an important cytokine in antral follicle growth and selection (Field et al., 2014). TNF-α is an important mediator of ovulation in terms of decreasing the number of released oocytes and inducing granulosa cell death of un-ruptured follicles which could induce follicle atresia via apoptosis and autophagy for remodeling ovarian tissues. TNF-α is considered to be one of the important factors in the preovulatory stage; however, the actual role of TNF-α and its effects in the ovary are not completely understood (Yamamato et al., 2015). In infertile women with PCOS in the course of IVF, TNF-α levels was higher in FF than serum, and the intrafollicular TNF-α concentrations were inversely correlated to the E2 levels (Amato et al., 2003). TNF-α concentration was positively associated with total glycerides. The percentage of top-quality embryo decreased in the PCOS and metabolic syndrome, with elevated lipolysis condition within the FF of these patients (Niu et al., 2017). A relationships between leptin and resistin as well as TNF-α is expected, because double-sided

effects are being observed among them. Resistin increases TNF-α, which in turn induces resistin. Leptin stimulates both resistin and TNF-α, that increases serum leptin concentrations (Yildizfer et al., 2015). Low concentrations of TNF-α in endometrial secretion from ICSI patients might be associated with improved endometrial receptivity and IVF outcome (Rahiminejad et al., 2015). TNF-α mechanistically contributes to cerebral edema by increasing blood brain barrier permeability and is underlying factor in the development of cerebrovascular abnormalities associated with preeclampsia complicated by placental ischemia (Warrington et al., 2015).

1.5.5 IL-4

Cumulus oophorus T cells produce higher levels of IL-4 and LIF than the T cells of peripheral blood or the ovary. The factors present in the microenvironment of the T cells are that could be responsible for the cytokine profile of decidual and cumulus oophorus T cells (Piccinni et al., 2001; Foster et al., 2010). During pregnancy, the uterine mucosa is characterized by a large number of maternal immune cells (natural killer NK cells, macrophages, dendritic cells and CD3+ T cells) which are found in close contact with trophoblast cells. In the cumulus oophorus that surrounds the oocyte during ovulation and the embryo during the first 72 h before the implantation of the blastocyst, immune cells (CD3+ T cells and macrophages) are also present. These T-cells produce cytokines that may provide a microenvironment suitable for preimplantation development of the mammalian embryo. It appears as an immunological mechanism may play a role in pregnancy and embryo development. T-cell LIF, M-CSF, IL-4 and IL-10 production at the fetomaternal interface could contribute to the maintenance of pregnancy (Piccinni, 2007). The resolution of inflammation pregnancy plays an important role throughout pregnancy and is largely mediated by immune cells that produce IL-4 and IL-10 (Chatterjee et al., 2014). Increased plasma levels of IL-10, IL-4, and TNF-α was associated with failure of implantation, while increased plasma levels of IL-2, IFN-γ, and TNF-α was associated to increase abortion rates (Laird et al., 2006).

1.5.6 IL-5

IL-5 is a dimer of 15 kDa, JAK/STAT dependent cytokine signals through IL-5R. It is produced from haemopoeitic and non- haemopoeitic cells with pleiotropic activities on various target organs (Takatsu et al., 2011). It functions on myeloid cells, increment of chemotaxic activity and

adhesion capacity, tissue remodeling, and wound healing (Akdis et al., 2011). IL-5 is produced by PBMC from women in their first trimester in normal pregnancy, and women with recurrent spontaneous abortion after 24 and 96 hours of culture (Raghupathy et al., 2000). It was suggested that Th1-type cytokines (IFN-γ and TNF-α), which promote allograft rejection, may compromise pregnancy, whereas the Th2-type cytokines (IL-4, IL-5, IL-10) inhibiting the Th1 responses, promote allograft tolerance and therefore may improve fetal survival (Piccinni, 2007). Higher IL-5 levels were observed in the endometrial secretions obtained from women with stimulated cycles than in others with natural cycles (Boomsma et al., 2009).

1.5.7 IL-6

IL-6 is a multifunctional cytokine that regulates various aspects of the immune response, acute phase reaction and hematopoiesis and has some functional redundancy with IL-11 and LIF (Dimitriatis et al., 2005). It is expressed from FF, TC, and GC, and is involved in the survival/apoptosis decision in antral follicles and cumulus cell expansion. IL-6 activates the JAK/STAT pathway and the MAPK cascade. Its receptors can promote granulosa cell survival, which is involving IL-6 and its soluble receptor, IL-6 (sR), a key regulator of follicle growth and atresia in humans (Field et al., 2014). It belongs to the same family of LIF and IL-11 and share the similar receptors using gp130 to signal within the cell and thus might be expected to have similar effects on cell functions. Infertile women due to primary infertility have low peripheral blood IL-6 compared to fertile ones (Laird et al., 2006). Previous investigations showed contradicted results. Low FF IL-6 levels in IVF patients were associated with increased likelihood of clinical pregnancy and IL-6 was correlated significantly and directly proportioned with estradiol at the day of hCG injection (Altun et al., 2011). Opposite results were reported when lower FF IL-6 were associated with a negative IVF outcome through their effect on oocyte quality and/or implantation either alone (Southcombe et al., 2013) or accompanied by elevated peri-ovolatory FF IL-12 (Bedaiwy et al., 2007). Whereas other studies failed to found any significant correlation between intrafollicular or circulatory IL-6 with successful and unsuccessful ICSI cycles (Asimakopoulos et al., 2010; Mahdi, 2011). Increased intrafollicular IL-6 levels were correlated to PCOS patients (Amato et al., 2003). Elkholi and Nagy (2016) showed that IL-6 levels were elevated in metabolically obese normal weight and PCOS patients.

1.5.8 IFN-γ

IFN- γ, a proinflammatory cytokine, is well known for its important role in innate and adaptive immunity against intracellular infections and for tumor control. It has a strong impact on bone marrow output during inflammation, as it affect the differentiation of most hematopoietic progenitor cells. It acts through upregulation of suppressor or cytokine signaling molecules, which impairs signaling of several cytokine receptors (de Bruin et al., 2014). IFN-γ is essential for remodeling of spiral arteries (Piccinni, 2007). It has minimal antiviral activity and is the major immunoregulatory product and co-inducer of the Th1 cytokine pathway (Akdis et al., 2011). Levels of IFN-γ and IL-10 was increased in serum of women with reproductive failure (Mahdi, 2011) and were correlated to endometriosis, while low FF IL-1β and IFN-γ were correlated to tubal factor infertility (Sarapik et al., 2012).

1.5.9 CXCL8/IL-8

IL-8 is expressed from GC and TC and it participates in antral follicle growth and selection by recruitment of neutrophils, granulocyte chemotaxis, neoangiogenesis and perifollicular blood flow. In ovulation and lutenization, it is produced from GC, TC, mast cell and macrophages to stimulate progesterone, inhibit estradiol and leukocytes particularly neutrophils recruitment (Field et al., 2014). IL-8 is found to be associated with successful pregnancy following IVF treatment (Sarapik et al., 2012). Recently, a study of Wu et al. (2017) showed no significant differences in serum and FF concentrations of IL-8 in endometriosis patients.

1.5.10 IL-10

IL-10, a Th-2 cytokine, is co-produced from the previously mentioned cells together with IL-4 (Piccinni et al., 2001; Foster et al., 2010). It is a potent anti-inflammatory cytokine as regulator of TNF-α in the amniotic fluid of pregnant women (Velez et al., 2008). Decreased levels of IL-4 and IL-10 promote persistent inflammation and depending on the level, stage, and systemic versus local effects can lead to a spectrum of gestational complications such as failure of implantation, miscarriage and increased abortion rate (Laird et al., 2006; Chatterjee et.al, 2014). In OHSS patients, there is a significant increase of CD11c+HLA-DR+ dentritic cells in FF (Shi

et al., 2015). In metabolically obese normal weight and PCOS patients, IL-10 levels was low significantly than control (Elkholi and Nagy, 2016).

1.6 Implantation

Embryo implantation is a progressive process that requires communication between two different organisms, the embryo and endometrium. It consists of three consecutive phases: apposition, adhesion and invasion (Huang, 2006). Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage, and a synchronized dialogue between maternal and embryonic tissues. In addition to the well-characterized role of sex steroids, the complexity of embryo implantation and placentation is exemplified by the number of cytokines and growth factors with demonstrated roles in these processes (Guzeloglu-kayisli et al., 2009).

A plethora of cytokines and growth factors are involved in the human embryo implantation process. There is a lot of redundancy in their functions and they seem to regulate each other (Chimote et al., 2010). Members of the gp130 cytokine family, IL-6, IL-11, LIF, the TGF-β superfamily, the colony-stimulating factors, and the IL-1 and IL-15 systems are crucial molecules for a successful implantation. These cytokines act through preparation of the endometrium for implantation and during implantation and placental formation. Chemokines are also important, both in recruiting specific cohorts of leukocytes to the implantation site and in trophoblast trafficking and differentiation (Dimitriasis et al., 2005; Guzeloglu-kayisli et al., 2009). In human, the immune-modulatory effect on lymphocytes and other cells such as natural killer cells could be stimulated by PGE2. This effect is mediated by colony stimulating factors like GM-CSF and other cytokines produced by cells localized in the decidua (Castro-Rendón et al., 2006).

At implantation, the cytokine balance is largely neutral. There is some evidence for a Th1-type bias, although this largely reflects the presence of TNF-α which is likely to be acting in a non-immune fashion. During placental formation, there is a clear bias towards Th2-type cytokines. These may serve to regulate maternal inflammatory and cytotoxic activity by acting upon local macrophages, NK and maternal large granular lymphocytes (LGL) cells (Dealtry et al., 2000). Disturbances in the normal expression and action of these cytokines result in an absolute or partial failure of implantation and abnormal placental formation in mice and human (Guzeloglu-kayisli et al., 2009).

Several theories hypothesize that the cross-talk between trophoblast and endometrium at preimplantation and immediately post-implantation is mediated, in part, by cytokines. However, no theory explains the distant role of cytokines in the process of implantation. Here, we will present a three selected theories:

One theory proposed by Dimitriasis et al. (2005) is insisted that during preimplantation phase, cytokines are secreted by uterine glands, stromal fibroblasts, decidual cells and trophoblast. These can act separately or in concert on trophoblast, on endometrial epithelium and during decidualization. Once trophoblast invasion is in progress, an array of cytokines produced by glands, decidual cells and leukocytes [predominantly uterine-specific natural killer (uNK) cells and macrophages] are likely to promote cellular differentiation, trafficking of leukocytes and trophoblast and continuing decidualization (Dimitriasis et al., 2005).

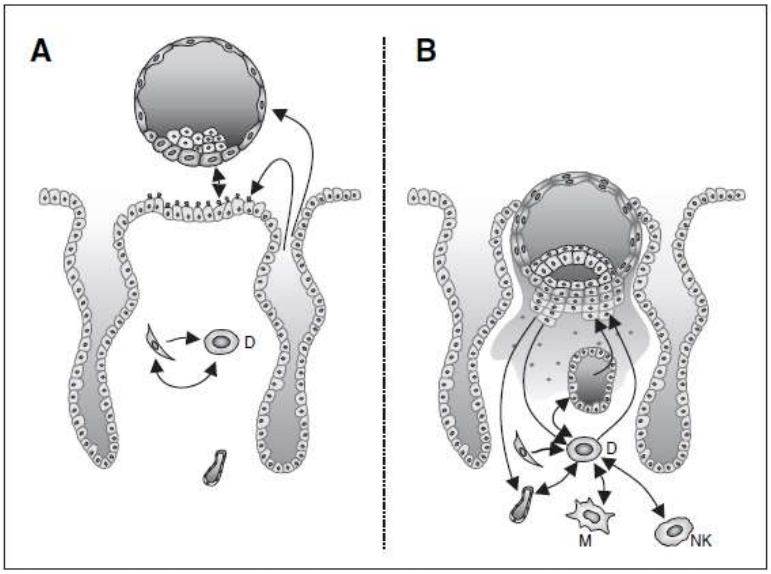


Figure 1.6 Cross-talk between trophoblast and endometrium, preimplantation (A) and immediately post-implantation (B) is mediated, in part, by cytokines in the directions shown by the arrows. (Dimitriasis et al., 2005).

Another theory proposed a model for the interaction between cytokines and mediators of inflammation associated with embryo implantation. The effect of E2 is mediated by the nidatory action of LIF in the endometrium, which is regulated by IL-1 and heparin binding-epidermal growth factor (HB-EGF). Both factors induce trophoblastic differentiation and angiogenesis through VEGF. Prostaglandin modulates IL-1 and possibly CSF-1 production. Both contribute to

syncytio-trophoblast and anchoring cytotrophoblast differentiation. Aspirin (acetylsalicylic acid, ASA) acts in a selective manner on cyclooxygenase-1 (COX-1) and this could have an indirect effect on PG production by induction of COX-2 (Castro-Rendón et al., 2006).

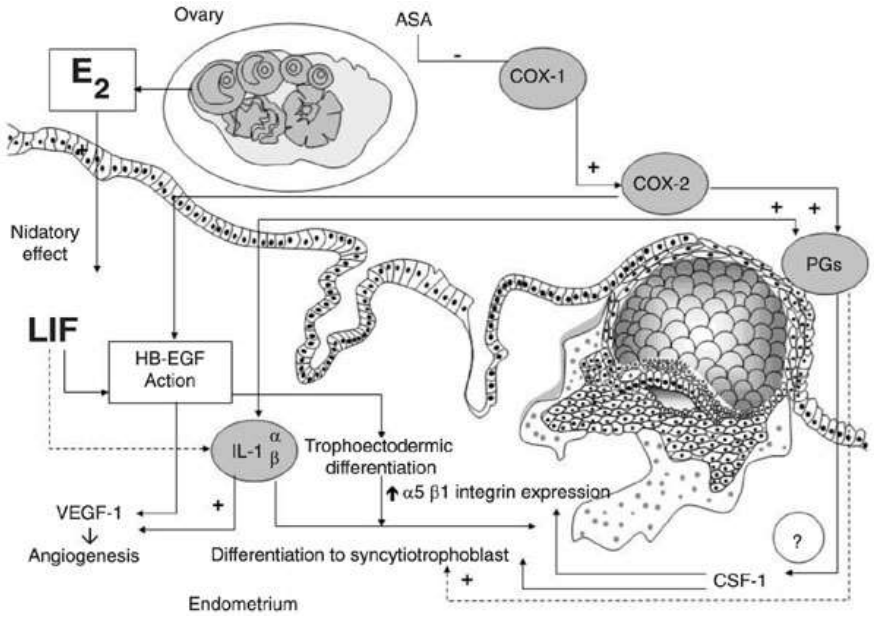


Figure 1.7 Model for the interaction between cytokines and mediators of inflammation associated with embryo implantation (Castro-Rendón et al., 2006).

The other theory proposed the role of two distinct cytokines, IL-1 system and IL-18 system families in addition to the role of the matrix metalloproteinase (MMP) and their inhibitors as well as VEGF system in early embryonic development. The period what called the "window of implantation" is characterized by morphological and biochemical changes to the endometrium including plasma membrane transformation as well as the presence of various specific adhesion molecules, cytokines, growth factors, proteinases and neoangiogenesis factors, all of which can have a wide range of paracrine, autocrine and endocrine activities. Successful embryo implantation requires an appropriate interaction between the blastocyst and a well-prepared uterine endometrium that has had adequate steroid hormonal stimulation during the luteal phase of the cycle (Huang, 2006).

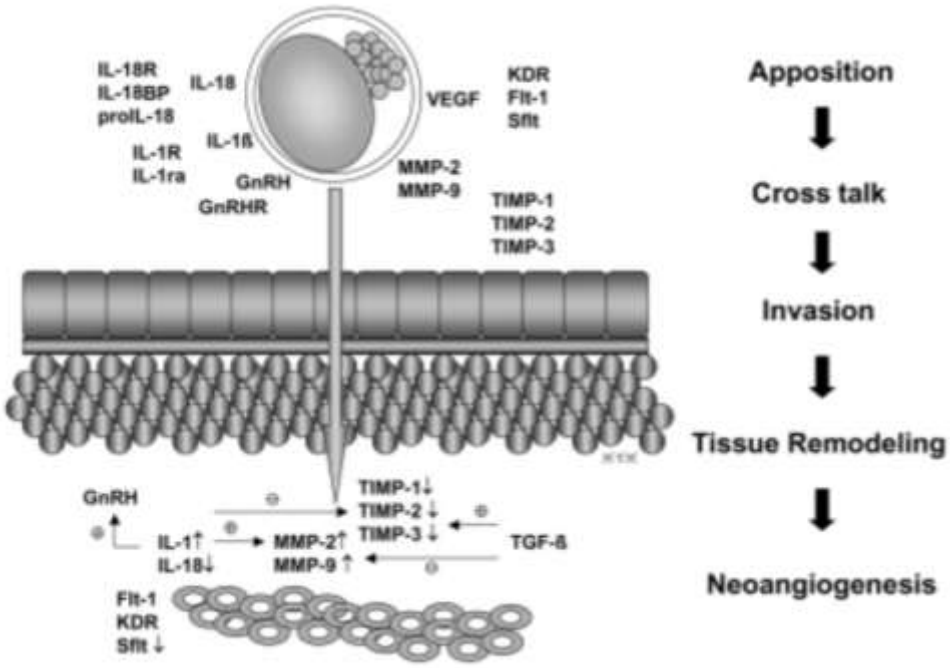


Figure 1.8 Schematic illustration of the cytokine network during embryo implantation (Huang, 2006).

1.7 Ovarian hyperstimulation syndrome (OHSS)

OHSS is an iatrogenic condition characterized by increased capillary permeability. Although the ultimate physiologic mechanism of OHSS is not yet known, there are well-known risk factors that must be considered during the administration of medications to treat infertility. Clinical features are consequences of third-spaced intravascular fluid. OHSS may become life-threatening secondary to thromboembolism or compromised pulmonary or cardiovascular function (Alper et al., 2009). OHSS can cause a rapid accumulation of volume (from 1.5 to 17 liters) in the peritoneal cavity that can lead to organ dysfunction, including respiratory impairment and oliguria (Grossman et al., 2010). The obstructive uropathy should be considered as a possible etiology in OHSS patients how develop oliguria or acute renal failure (Marak et al., 2013).

The main goal of conventional ovarian stimulation protocols for IVF is to obtain greater numbers of mature follicles and to achieve maximum oocyte yields by suppressing premature surge of LH. Patients with increasing LH and estrogen levels are at risk for premature ovulation and require additional monitoring in both protocol types. It is important to cease the cycle if there is added risk of OHSS. Some studies implicated the role of antagonistic protocol in prevention of

OHSS especially in PCOS patients (Shresta et al., 2015). Oocytes retrieved from patients following controlled ovarian hyperstimulation show varying stages of meiotic maturity (Ebner et al., 2003). Conventional protocols, however, are associated with patient discomfort, increased risk of OHSS and higher costs. Higher egg yields produce more than 15 oocytes resulted in more embryos available for cryopreservation without compromising embryo quality. The generation of more frozen embryos results in a higher cumulative chance of pregnancy per oocyte retrieval and lower overall risk and cumulative costs by reducing the need for additional stimulated cycles (Alper and Fauser, 2017).

An experimental evidence has identified a provocative link to pathologic vasoactive cytokine actions (Alper et al., 2009). The more important well-studied vasoactive cytokine was VEGF. It has two basic roles in endometrium. First, it regulates endometrial vascularization and vascular permeability and the second itestablish a receptive endometrium to support blastocyst implantation and trophoblast invasion (Cuzeloglu-Kayisli et al., 2009). FF of the patients with OHSS is in an inflammatory status and may affects oocyte quality and embryo development. The two- pronuclei (2PN) fertility rate, high-quality embryo rate and available embryo rate were all lower in OHSS patients. The levels of IL-10, IL-12, IL-18 and IL-23 were all significantly higher in OHSS group than in control group (Shi et al., 2015). The study of Amano et al. (2014) revealed an association between low follicular fluid tyrosine level and the onset of OHSS. As in the serum, tyrosine concentration in the follicular fluid is easily measurable in the clinical practice and may be a potential predictive marker of OHSS (Amano et al., 2014). In severe OHSS patients, peritoneal fluid IL-6, IL-8 and TNF-α and serum IL-6 and IL-1β were higher significantly. These findings suggest that these substances could be involved in mediating the capillary hyperpermeability characterizing this syndrome (Revel et al., 1996).

According to the results of previous studies mentioned herein, we intend to study the association of a number of follicular fluid cytokines with ICSI outcome.

2

MATERIALS AND METHODS

2.1 Study protocol

This study was done under the instructions of the Ethics Committee of Ferdowsi University of Mashhad and Mashhad University of Medical Sciences. The follicular fluid samples were collected from August 2016 to May 2017 in Milad Infertility Center, Mashhad University of Medical Sciences.

We collected 169 FF samples from infertility center. They were categorized according to the causes of infertility into the following groups:

1) Male factor infertility
2) Female factor infertility
3) Mixed factor infertility
4) Unexplained infertility
5) Oocyte donor healthy women

Then, only 80 patients were included in the study according to the inclusion criteria (having female infertility). These eighty patients aged between 20 and 43 years old. They were undergoing ICSI with female factor infertility (either alone or accompanied with male factor infertility) which were suffering from one or more of the following causes:

1) Uterine abnormalities (e.g. endometriosis, myoma, polyp, fibromas, and congenital defects)
2) Tubal obstructions, peritoneal factors (e.g. adhesions)
3) Ovulation disorders (e.g. polycystic ovary syndrome (PCOS))

Patients with other infertility causes including male factor infertility alone, unexplained infertility, oocyte donors and patients with special diseases such as diabetes and obesity (BMI $\geq$ 28 kg/m^2) were excluded from the study. Number of previous IVF/ICSI tries, infertility period and type, superovulation protocol, and ultrasonography measured endometrial thickness were recorded.

Diagnosis of severe OHSS was made by presence of one of the clinical signs (clinical ascites with or without pleural effusion, edema, and oliguria), and the number of follicles at the day of human chorionic gonadotropin (hCG) injection (Ocal et al., 2011).

2.2 Superovulation protocol

All patients received controlled ovarian hyperstimulation (COH) with gonadotropin releasing hormone (GnRH) protocols. Either agonist long or antagonist were selected by each physician and were individualized based on the results of patient's ovarian reserve. Thirty two patients were daily administered with **agonist long** recombinant FSH (Gonal-F; Merk Serono, Germany). This began at the mid-luteal phase (day 21) of the previous cycle and continued to the day of hCG injection. The size of follicles was monitored with transvaginal ultrasound every 2-3 days along with the agonist treatment. Other 48 patients were administered **antagonist** drugs daily (Cetrotide; Merk Serono, Germany) at the mid-follicular phase until the size of follicles reached 12 mm at least. Final oocyte maturation was triggered in both protocols by the injection of hCG (Pregnyl; IBSA, the Netherlands). The oocyte pickup (OPU) was achieved about 36-38 hours following hCG intramuscular injection.

2.3 Follicular fluid samples

Under general anesthesia, follicles were punctured through transvaginal guided sonography to pick up the oocytes with their surrounding FF using a special needle. About half to one hour, the FF of all follicles was collected as a pool respective to each patient. Then FF were centrifuged with 2000 rpm for 10 minutes to relieve the cellular debris. The blood contaminated samples were discarded and only the clear FF samples were stored at -80C until they were assayed.

2.4 Multiplex cytokines determination

The pooled FF samples were analyzed by magnetic beads multiplex to estimate the concentration of their cytokines. The ten measured cytokines/chemokines were included: IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL8/IL-8, IL-10, GM-CSF, IFN-γ and TNF-α. FF samples were assayed by Flow Cytometry with Luminex Platform Magnetic Luminex. Human premixed multi-analytic kit (R&D System Inc. Kit code: LXSAMH-10) was purchased from Bio-Rad laboratories, Italy. All FF samples were transferred to Italy with respective to maintain the frozen serial of the FF samples using dry ice (Dry ice, Dal Wand, Mashhad, Iran). All analyses were undertaken in Lapospace, Milano, Italy laboratories by Luminex instrument (Bio-Rad) (Luminex Map Technology, Milano, Italy). Samples were arrayed using Bio-Plex Manger software V 6.0. The procedure of determination of multiplex cytokines was performed according to the manufacturer's instructions.

The procedure of multiplex cytokines determination according to Labospace explanation, briefly FF samples were diluted with the reagent in 1:1 ratio. FF were added with a quantity of 50 µl per well in duplicate. Then incubation of the buffer and standard with FF sample for 2 hours, addition of the detection antibodies, then incubated for 1 hour and the sample were washed and magnetic polystyrene were added and incubated for 30 minutes. Finally, samples were re-suspended and acquired data was gathered using Luminex Detection system. The values of the standard curve were compared with the values of the manufacture and the prescriptions of the kit. The fluorescence intensity of each well is then converted into a concentration using a specific algorithm that is calculated automatically by the instrument. Measured values of cytokines were 97.1%**.**

2.5 Follicles and oocytes assessment

2.5.1 Follicles size and number

The size of follicles and their number were measured by ultrasound about 48-72 hrs proceeding the oocyte pickup. Follicles with a small size were smaller than 12 mm, medium sized follicles ranged between 12 and 15 mm, and the follicles larger or equal to 16 were grouped within the large sized ones.

2.5.2 Oocyte quality

Oocytes were directly assessed after 20-30 minutes of their collection. Morphologically, the ideal oocyte is generally receptive to fertilization, was characterized by the presence of the cumulus cells still around the oocyte, and well expanded producing the typical “sunburst-like effect”. The good quality oocytes had an extended sun-flare corona radiata. The oocytes evaluated as necrotic, germinal vesicle (GV) and metaphase II stage (MII) according to their stage of division apparent under microscope. Only the oocytes of a class MII were inseminated, others including GV and necrotic oocytes were rejected. The proportion of each of these three stages were calculated by dividing the number of each item by the total number of retrieved oocytes per patient multiplied by 100 according to the following equations:

$$\text{Necrotic oocyte \%} = \frac{\text{NO. of necrotic oocytes}}{\text{Total NO. of retrieved oocytes}} \times 100$$

$$\text{Germinal vesicle (GV) oocyte \%} = \frac{\text{NO. of GV oocytes}}{\text{Total NO. of retrieved oocytes}} \times 100$$

$$\text{Metaphase II (MII) oocyte \%} = \frac{\text{NO. of MII oocytes}}{\text{Total NO. of retrieved oocytes}} \times 100$$

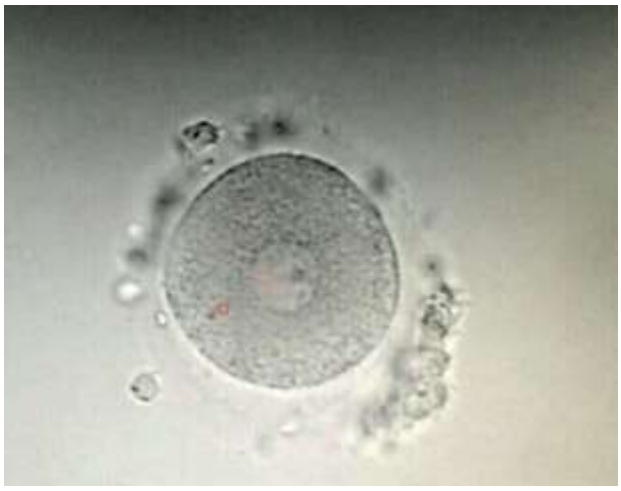


Figure 2.1 Germinal Vesicle (GV) oocyte. A large nucleus of the primary oocyte which its meiosis is not completed and polar bodies are not formed, corona radiata and cumulus oophorus tight together and cytoplasm is granulated. This oocyte is considered immature.

Figure 2.2 Metaphase (MII) oocyte. Fist polar body formation with disaperance of germinal vesicle. Expanded cumulus oophorus and corona radiata with a homogenous cytoplasm of mature oocyte.

2.6. ICSI Outcome

2.6.1 Fertilization rate (FR %)

Oocyte fertilization assessment was performed about 16-24 hrs after ICSI. Two pronuclei (2PN) were examined as an indication of fertilization and zygote formation. Fertilization rate (FR) was subsequently calculated by dividing 2PN to the number of MII oocytes and then multiplied by 100 according to the following equation:

$$\text{Fertilization rate (FR \%)} = \frac{\text{NO. of zygotes with 2PN}}{\text{NO. of MII oocyte}} \times 100$$

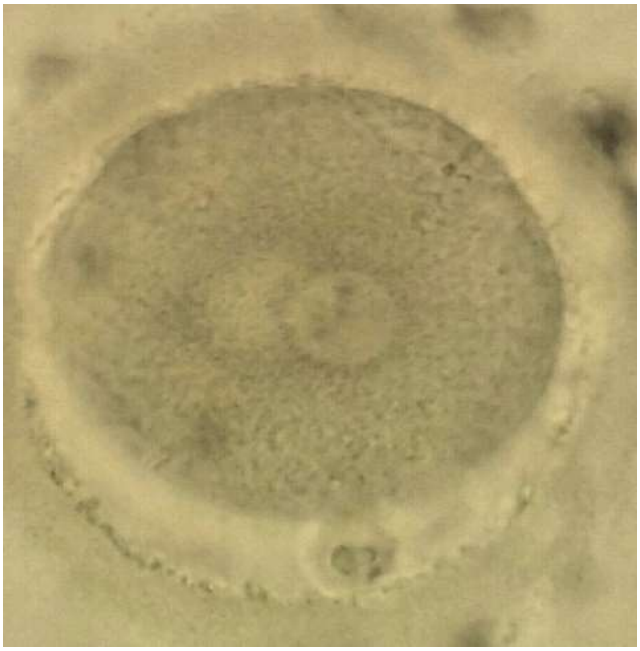


Figure 2.3 Normal zygote with two pronuclei (2PN) of equal size which are located at the center of ooplasm. 16 to 18 hours after ICSI.

2.6.2 Embryo grading

Embryo grading and assessment was achieved at day two/three of ICSI. The produced embryos were graded as follow:

Grade I characteristics: A transparent embryo, thin zona pellucida, the number of blastomeres were larger than 6 cells at day 3 with a regular blastomeres, fragmentation rate lesser or equal to 10%, equal size of blastomeres, and no multinucleated blastomeres.

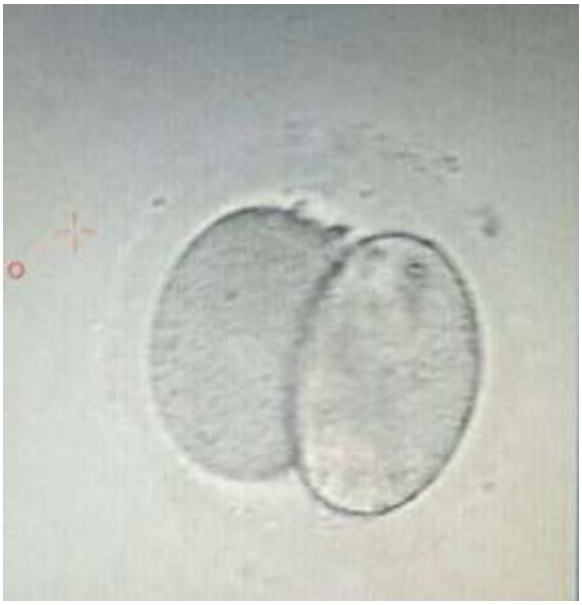


Figure 2.4 Two cell embryo grade I on day two. This embryo is grade I since there are no fragments and blastomeres size is equal.

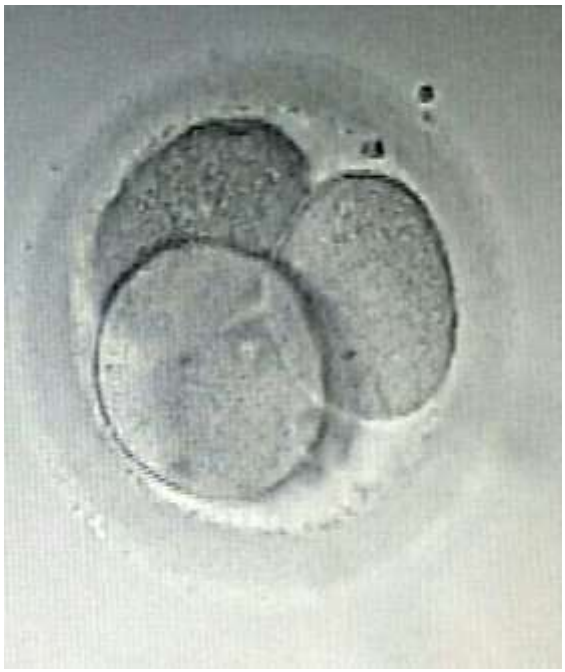


Figure 2.5 Four cell embryo grade I on day two. The embryo has no fragmentation and the size of the blastomeres is even and regular.

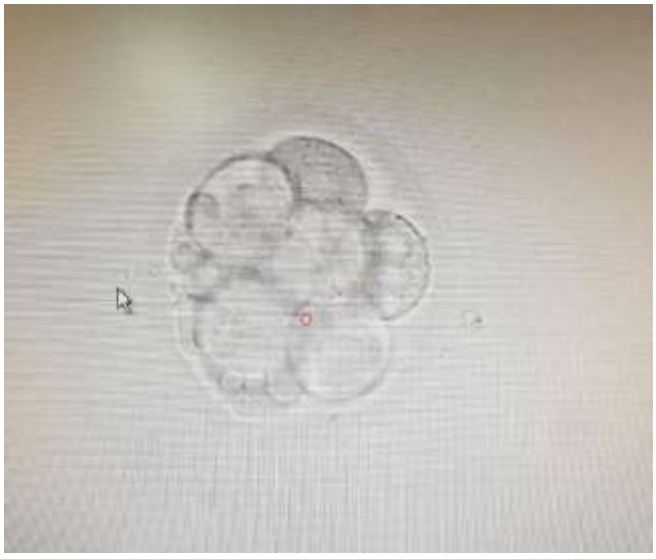


Figure 2.6 Eight cell embryo grade I on day two/three.

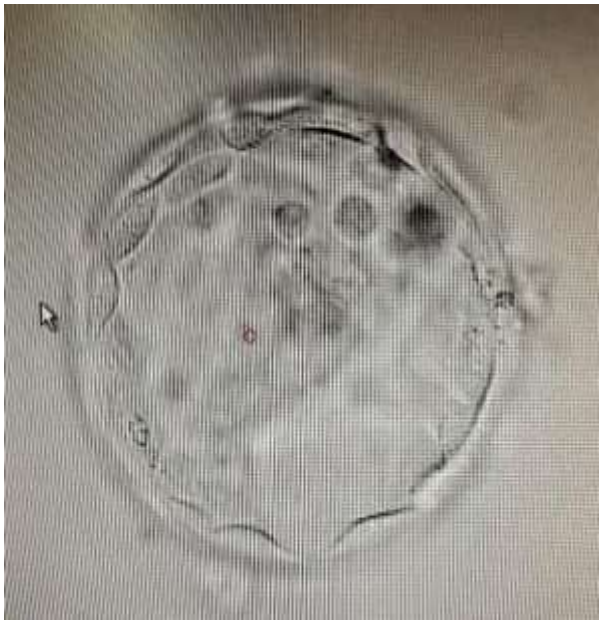


Figure 2.7 Blastocyst grade I on day 5 after ICSI. These blastocysts are fully expanded, with normal inner cell mass and good integrity of the trophoectoderm.

Grade II characteristics: Other embryos were included in this grade of embryos, fragmentation more than 20%, thick zona pellucida, unequal size of blastomeres, untransparent cytoplasm with few cell number lesser than 6 cells.

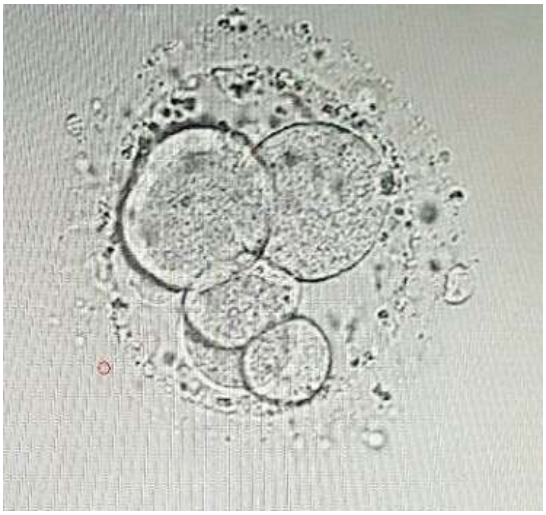


Figure 2.8 Four cell embryo grade II on day two. The embryo has fragmentations and the size of the blastomeres is uneven and irregular.

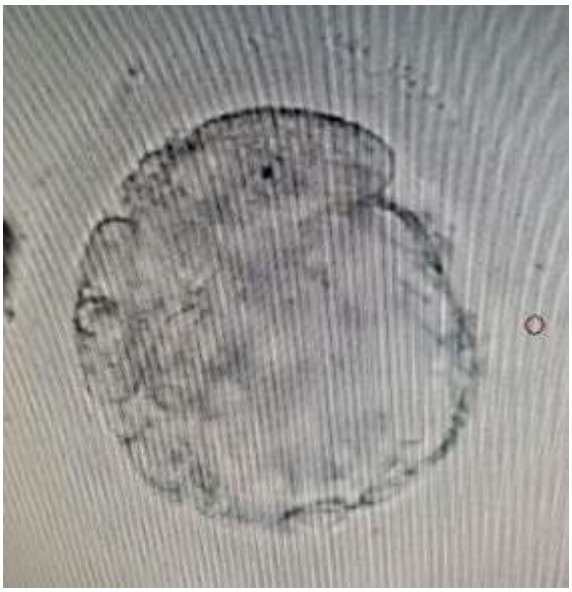


Figure 2.9 Blastocyst Grade II on day 5 after insemination.

2.6.3 Biochemical pregnancy

Biochemical pregnancy was diagnosed by the positive hCG in the blood after 16 days of intrauterine transfer of the embryo(s).

2.6.4 Implantation rate (IR %)

IR was calculated as the number of gestational sacs by sonography divided by the number of transferred embryos per patient (for pregnant women only).

$$IR\ \% = \frac{\text{No. of gestational sacs}}{\text{No. of transferred embryos}} \times 100$$

2.6.5 Clinical pregnancy

It was confirmed by the presence of one or more gestational sacs by sonography, positive heart sounds after 36 days of intrauterine transfer of the embryo(s), and amenorrhea for about 6-8 weeks.

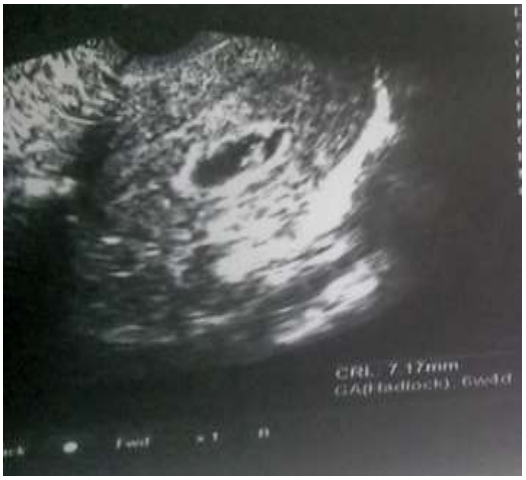


Figure 2.10 Picture of ultrasonography showing a clinical pregnancy (6 week ± 4 day)

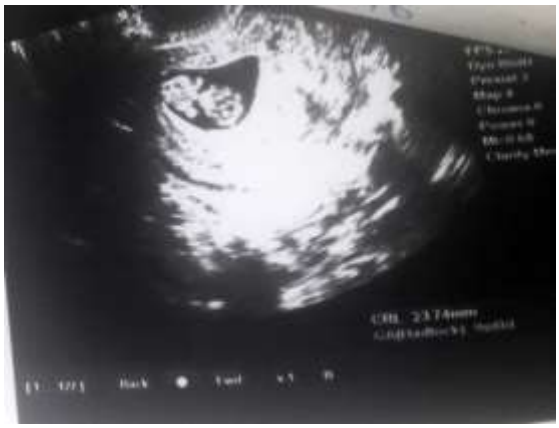


Figure 2.11 Picture of ultrasonography showing a clinical pregnancy (9 week ± 0day)

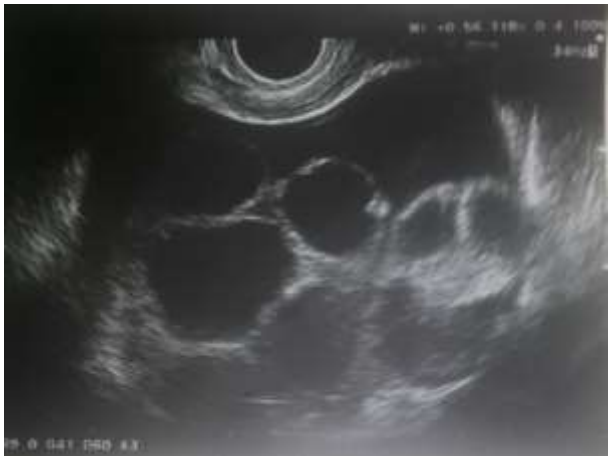


Figure 2.12 Picture of ultrasonography showing severe OHSS. Ovaries develop too many small to medium size follicles as they over-respond to the medication.

2.7 Statistical analysis

Mean ± SD of cytokines level in pooled follicular fluid of patients were reported. Comparison of the demographics and ICSI outcome between pregnant and non-pregnant women were done using Chi-square and independent samples *t*-test for the qualitative and quantitative variables respectively. To evaluate the effects of FF cytokines on ICSI outcome, at the first step, univariate correlation of cytokines level with quantitative and binary outcome were analyzed by correlation and logistic regression test, respectively. Then cytokines which were associated with ICSI outcomes at $p < 0.2$ were selected for inclusion in the multivariate analysis.

Controlling for the effects of age, type of superovulation protocol, history of previous IVF and infertility period, correlation of cytokines with quantitative outcomes including number of follicles, number of oocyte, proportion of oocyte quality, FR%, IR%, and embryo grading were evaluated by linear regression test. Also, controlling for the effects of age, type of superovulation protocol, history of previous IVF, infertility period and endometrial thickness, correlation of cytokines with binary outcomes such as results of biochemical, clinical pregnancy, and severe OHSS were evaluated by logistic regression test.

Receiver operating characteristic (ROC) curve was used to evaluate the performance of each cytokine on prediction of pregnancy and the chance of OHSS. The AUC guidline was presented in table (2.1). Statistical analysis was performed by SPSS version 21. A value of $p < 0.05$ was considered statistically significant.

Table 2.1 AUC Interpretation (Swets, 1988)

Rough AUC guidelines	
0.50 - 0.60	Not so good
0.60 - 0.75	Fair
0.75 - 0.90	Good
0.90 - 0.97	Very good
0.97 – 1.00	Excellent

3

RESULTS

3.1 Characterization of study population and ICSI cycles

3.2 Univariate comparison of demographics and ICSI outcome between pregnant (P) and non-pregnant (NP) women

3.3 Multivariate analysis of cytokines correlaton with follicles, oocyte quality and ICSI outcome

3.3.1 Number of follicles

3.3.2 Size of follicles

3.3.3 Oocytes number

3.3.4 Germinal vesicle (GV %)

3.3.5 Metaphase II (MII %)

3.3.6 Fertilization rate (FR %)

3.3.7 Embryo grading

3.3.8 Biochemical pregnancy

3.3.9 Implantation rate (IR %)

3.3.10 Clinical pregnancy

3.4 Multiplex measured cytokines

3.5 Receiver Operating Characteristics (ROC) curve and prediction of biochemical Pregnancy

3.6 Ovarian Hyperstimulation Syndrome (OHSS)

3.6.1 Description of severe OHSS in study population

3.6.2 Correlation of demographics and cytokines on severe OHSS

3.6.3 ROC curve and prediction of severe OHSS

3.1 Characterization of study population and ICSI cycles

Embryo transfer was achieved in 72.5% (58/80) and was canceled in 27.5% (22/80) of the patients (n=80). The transfer was canceled due to several causes such as ovarian hyperstimulation syndrome (OHSS), uterine polyp, inappropriate endometrium, no fertilization, increase progesterone concentration, severe pain and unknown causes (Table 3.1).

Fertilization rate% was 0.70 ± 0.28. Demographics of the study population (mean ± SD with their maximum and minimum values), number and size of follicle proportion, oocytes number, the proportion of each stage (MII, germinal vesicles, and necrotic oocytes), embryo grading, biochemical pregnancy according to the hCG blood test, and the clinical pregnancy% based on sonography are presented in Table 3.2. From the biochemical pregnancy stage, the pregnancy data for two patients were missing (n=78). The results of implantation rate % are not equal to 1.00, because four patients have no fertilization (both embryo grade I and grade II = 0).

Table 3.1 patients with canceled embryo transfere according to the cause of cancellation

Cause of cancellation	Number of patients
OHSS	13/22
No fertilization	4/22
Uterine polyp	1/22
Inappropriate endometrium	1/22
Increase progesterone concentration	1/22
Severe pain	1/22
Unknown	1/22

Table 3.2 Description of study population and characteristics of ICSI cycles. Quantitative and qualitative variables were presented as mean ± standard deviation and frequency (%), respectively.

	Mean ± SD	Minimum	Maximum	Frequency (%)
Age (year)	31.35 ± 5.23	20	43	-
Infertility type				
Female	-	-	-	29/80 (36.2%)
Mixed	-	-	-	51/80 (63.8%)
Infertility period (year)	5.98 ± 3.98	5	17	-
Superovulation protocol				
Agonist	-	-	-	32/80 (40%)
Antagonist	-	-	-	48/80 (60%)
Previous IVF/ICSI attempts				
Yes	-	-	-	23/80 (28.8%)
No	-	-	-	57/80 (71.2 %)
Endometrial thickness (mm)	9.22 ±2.14	3.5	15	-
Number of follicles	16.25 ± 11.56	1	50	-
Size of follicles				
Large (%)	0.53 ± 0.32	0.00	1.00	-
Medium (%)	0.38 ± 0.24	0.00	1.00	-
Small (%)	0.09 ± 0.17	0.00	0.80	-
Number of oocytes	10.34 ± 7.29	1	45	-
Oocyte quality				
MII (%)	0.91 ± 0.19	0.00	1.00	-
GV (%)	0.02 ± 0.07	0.00	0.50	-
Necrotic (%)	0.07 ± 0.15	0.00	0.70	-
FR %	0.70 ± 0.28	0.00	1.00	-
Embryo grading				
Grade I	0.61 ± 0.27	0.00	1.00	-
Grade II	0.34 ±0.24	0.00	1.00	-
IR %	0.25 ± 0.23	0.33	1.00	-
Biochemical pregnancy				
Positive	-	-	-	18/78 (23.1%)
Negative	-	-	-	60/78 (76.9%)
Clinical pregnancy				
Positive	-	-	-	17/78 (21.8 %)
Negative	-	-	-	61/78 (78.2%)

MII: metaphase II; GV: germinal vesicle; FR: fertilization rate; IR: implantation rate.

3.2 Univariate comparison of demographics and ICSI outcome between pregnant (P) and non-pregnant (NP) women

Result of biochemical pregnancy for 18 patients was positive, while it was negative for 60 patients. A number of quantitative variables did not show a significant difference between non-pregnant (NP) and pregnant (P) women using independent sample t-test including: age, number of follicles, large, medium sized follicles %, oocyte number, necrotic oocytes, grade I and II embryo, and IR %. Whereas other parameters showed significant differences ($p < 0.05$) between the two groups including: endometrial thickness, small oocytes%, MII%, and GV% (Table 3.3). The only cytokine which was significantly ($p = 0.033$) higher in the FF of NP women was IL-5. Although IL-6 was higher in the FF of the pregnant women and showed a trend to be linked with the biochemical pregnancy, it was not significant ($p = 0.195$) (Table 3.3). None of the qualitative variables showed a significant difference between the two study groups according to Chi-square test. These variables include infertility type, superovulation protocol, and the history of previous IVF/ICSI (Table 3.4).

Table 3.3 Demographics, cycle characteristics, and cytokines in non-pregnant (NP) and pregnant (P) women. Comparisons of these quantitative variables between P and NP were performed using univariate analysis independent sample t-test.

	Non pregnant	Pregnant	
	Mean ± SD (n=60)	**Mean ± SD (n=18)**	***P* Value**
Age (year)	31.67 ± 5.57	31.06 ± 3.64	0.663
Endometrial thickness (mm)	8.93 ±2.02	10.14 ± 2.40	0.036*
Number of follicles	17.17 ± 12.88	13.77 ± 4.93	0.272
Large follicles (%)	0.52 ± 0.34	0.55 ± 0.27	0.684
Medium follicles (%)	0.37 ±0.23	0.41 ± 0.25	0.625
Small follicles (%)	0.112 ± 0.18	0.04 ± 0.09	0.035*
Number of oocytes	10.57 ± 7.98	9.61 ± 4.49	0.520
MII (%)	0.89 ± 0.19	0.97 ± 0.89	0.020*
GV (%)	0.026 ± 0.084	0.003 ± 0.014	0.042*
Necrotic (%)	0.08 ± 0.016	0.24 ± 0.089	0.071
FR %	0.67 ± 0.29	0.79 ± 0.23	0.140
Grade I embryo (%)	0.60 ± 0.29	0.65 ± 0.25	0.102

	Non pregnant	Pregnant	
	Mean ± SD (n=60)	**Mean ± SD (n=18)**	***P* Value**
Grade II embryo (%)	0.33 ± 0.25	0.35 ± 0.20	0.422
IL-1β (pg/ml)	13.17 ± 0.75	13.06 ± 0.60	0.592
IL-2	30.14 ± 4.68	29.98 ± 3.55	0.894
IL-4	15.70 ± 1.19	15.88 ± 0.91	0.559
IL-5	10.37 ± 0.81	9.92 ± 0.51	0.033*
IL-6	51.09 ± 65.83	81.00 ± 131.64	0.195
CXCL8/IL8	789.50 ± 395.83	664.97 ± 365.78	0.238
IL-10	46.42 ± 9.74	44.43 ± 10.52	0.457
IFN-γ	17.80 ± 1.13	17.63 ± 0.63	0.574
GM-CSF	12.41 ± 6.32	11.63 ± 0.74	0.603
TNF-α	11.60 ± 0.90	11.47 ± 0.83	0.602

* Differences were statistically significant ($p < 0.05$).

MII: metaphase II; GV: germinal vesicle; FR: fertilization rate; IL: interleukin; INF-γ: interferon gamma; GM-CSF: Granulocyte macrophage-colony stimulating factor; TNF-α: tumor necrosis factor-alpha.

Table 3.4 Demographics and cycle characteristics in non-pregnant (NP) and pregnant (P) women. Comparisons of these qualitative variables between P and NP were performed using univariate analysis Chi-square test.

	Non pregnant	Pregnant	
	N (%) (n=60)	**N (%) (n=18)**	***P* Value**
Infertility type			
Female factor infertility n (%)	24/28 (86%)	4/28 (14%)	0.168
Mixed infertility n (%)	36/50 (72%)	14/50 (28%)	
Previous IVF/ICSI			
No n (%)	16/23(69.6%)	7/23(30.4%)	0.319
Yes n (%)	44/ 55 (80%)	11/55(20%)	
Superovulation protocol			
Agonist n (%)	20/28(71.4%)	8/28(28.6%)	0.389
Antagonist n (%)	40/50(80%)	10/50(20%)	

3.3 Multivariate analysis of cytokines correlation on follicles, oocyte quality and ICSI outcome

The effect of cytokines on the number and size of follicles, oocyte number, GV%, MII%, embryo grading, FR%, and IR% were analysed using multivariate linear regression test, whereas the effect of cytokines on binary ICSI outcomes including clinical and biochemical pregnancy were analysed by the logistic regression test.

3.3.1 Number of follicles

The results of multivariate analysis showed that the number of follicles was significantly affected by age and infertility period; as a whole, the number of follicles increased with the prolonged infertility period, and decreased with aged patients. Other predictors, including: FF cytokines, superovulation protocol, and the history of previous IVF/ICSI attempts, had no significant effect on the follicle number.

3.3.2 Size of follicles

In this study, the results showed that the patients with a history of previous IVF/ICSI attempts significantly correlated to the large follicles proportion (58.6%) in comparison to their proportion in the patients with first IVF/ICSI attempt (39.5%). Other predictors including cytokines, age, and infertility period have no effect on size proportion of large follicles.

3.3.3 Oocytes number

The age was significantly and inversely correlated with the number of oocytes. The ten measured cytokines and other predictors had no significant effect on the number of oocytes. IL-10 was linked to an increase in oocyte number, but did not reach to the significant level ($p = 0.083$).

3.3.4 Germinal vesicle (GV %)

In all patients, the infertility period was significantly and directly correlated to GV proportion. IL-5 had a significant correlation to the GV% ($p = 0.036$), and this cytokine was positively proportioned to GV%; each one unit increase in the FF IL-5 concentration, there was a 2.3% increase in GV% (Figure 3.1 and table 3.5). GM-CSF was correlated to increased GV%, but not reached to the

significancy level (p = 0.071). Other predictors including the other nine measured cytokines, in addition to the previously mentioned predictors had no any significant effect on the GV%.

Table 3.5 Correlation between follicular fluid IL-5 and germinal vesicle oocytes proportion (GV %).

	B	S.E.	p-value	95% CI for B	
				Lower Bound	Upper Bound
(Constant)	-.245	.122			
age	.002	.002	.310	-.002	.005
superovulation_protocol	-.011	.017	.531	-.044	.023
infertility_period	-.004	.002	.100	-.008	.001
history_of_previous_IVF	.028	.019	.146	-.010	.065
IL_5	.023	.011	.036	.002	.044

B: Regression coefficient; S.E.: Standard error; CI: Confidence interval

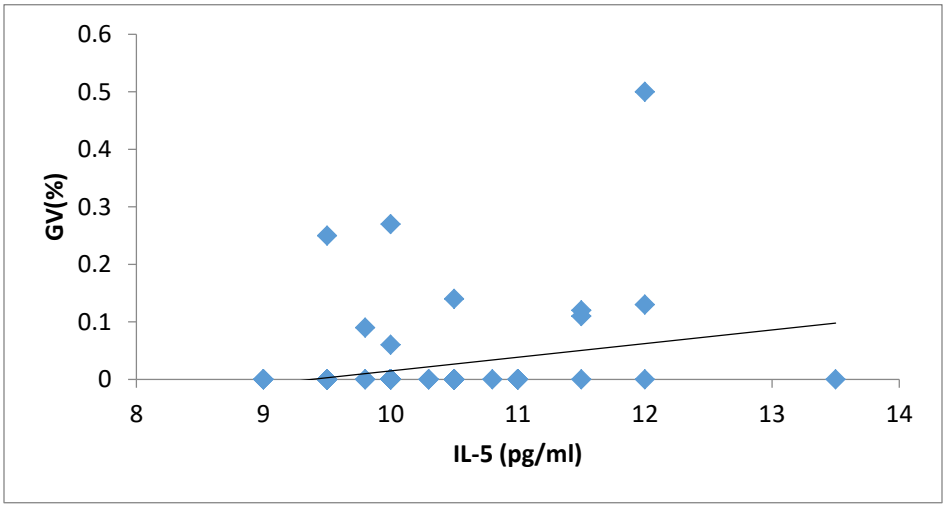


Figure 3.1 Correlation between follicular fluid IL-5 and germinal vesicle oocyte proportion

3.3.5 Metaphase II (MII %)

IL-5 had a significant correlation with the MII% (p = 0.046), with one unit increase in FF IL-5 concentration, the proportion of metaphase II oocyte was decreased 5.6% (Figure 3.2 and table 3.6). Also, GM-CSF had a significant correlation to the MII%, on average, with a unit increase in FF GM-CSF concentration, oocyte II proportion decreased by 1% (p = 0.012) (Table 3.6). Other eight measured cytokines and predictors had no significant effect on the MII% (Figure 3.3 and Table 3.7).

Table 3.6 Correlation between follicular fluid GM-CSF and metaphase II oocytes proportion (MII %).

	B	S. E	P-value	95.0% CI for B	
				Lower Bound	Upper Bound
(Constant)	1.309	.314	.000	.683	1.935
age	.002	.004	.549	-.006	.011
superovulation_protocol	.010	.044	.817	-.077	.097
infertility_period	.007	.005	.183	-.004	.018
history_of_previous_IVF	.046	.048	.347	-.051	.142
IL_5	-.056	.027	.046	-.111	-.001

B: Regression coefficient; S.E.: Standard error; CI: Confidence interval

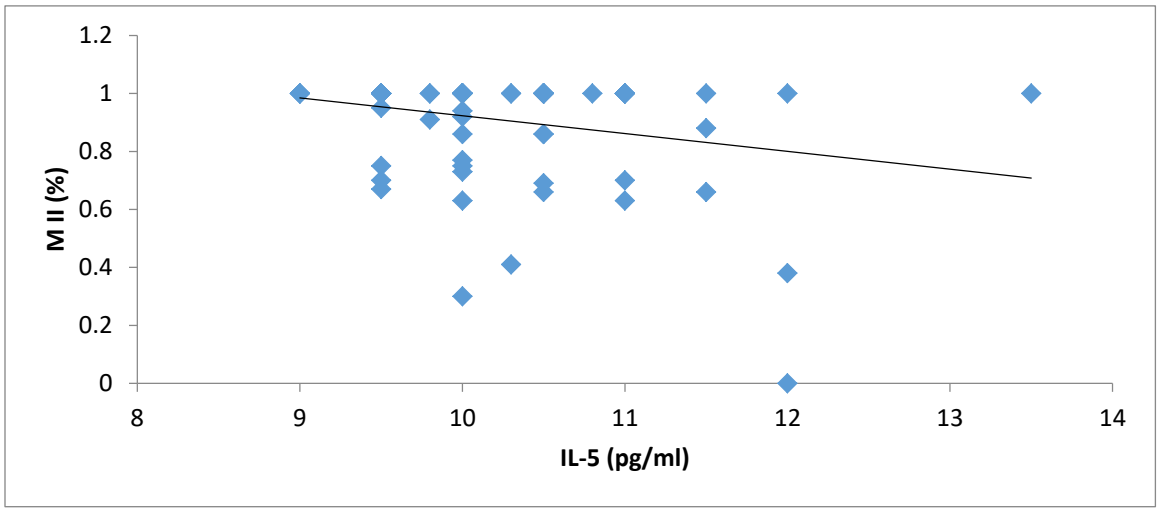


Figure 3.2 Correlation between follicular fluid IL-5 and metaphase II oocyte proportion

Table 3.7 Correlation between follicular fluid GM-CSF and metaphase II oocyte proportion

	B	S.E.	P-value	95% CI for B	
				Lower Bound	Upper Bound
(Constant)	.885	.158			
age	.002	.004	.651	-.006	.010
superovulation_protocol	.009	.043	.828	-.076	.095
infertility_period	.008	.005	.127	-.002	.019
history_of_previous_IVF	.028	.048	.561	-.068	.124
GM_CSF	-.010	.004	.012	-.017	-.002

B: Regression coefficient; S.E.: Standard error; CI: Confidence interval

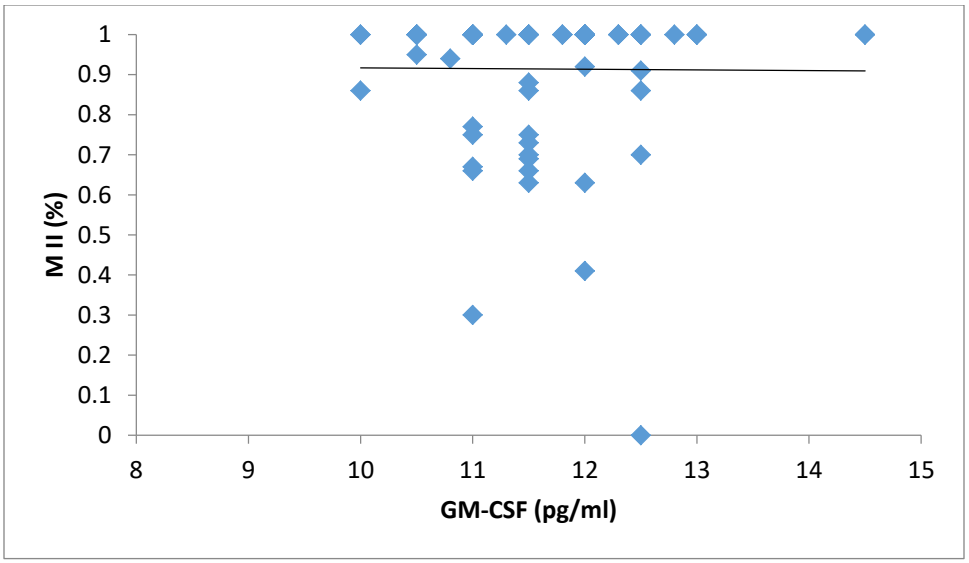


Figure 3.3 Correlation between follicular fluid GM-CSF and metaphase II oocyte proportion

3.3.6 Fertilization rate (FR %)

In general, patients with previous IVF/ICSI attempts had a fertilization rate of 74.4%), which was significantly greater than of those patients undergoing first ICSI attempt (FR% = 59.7%). The only significantly correlated measured cytokine was IL-4; an increase in each unit of FF IL-4 was correlated with 7.5% increase in FR% (p = 0.007) (Figure 3.4 and table 3.8).

Table 3.8 Correlaton between follicular fluid IL-4 and fertilization rate proportion (FR %).

	B	S.E	P-value.	95.0% CI for B	
				Lower Bound	Upper Bound
(Constant)	-.273	.456			
age	-.003	.006	.589	-.015	.009
superovulation_protocol	.052	.061	.394	-.069	.174
infertility_period	-.015	.008	.063	-.030	.001
history_of_previous_IVF	.166	.069	.018	.029	.303
infertility_type	-.086	.063	.175	-.212	.039
IL_4	.075	.027	.007	.021	.129

B: Regression coefficient; S.E.: Standard error; CI: Confidence interval

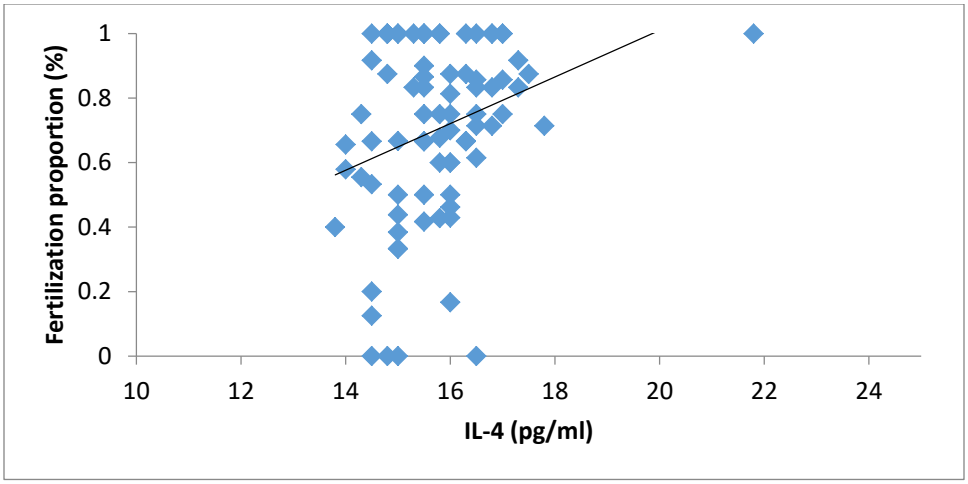


Figure 3.4 Correlation between follicular fluid IL-4 and fertilization rate proportion

3.3.7 Embryo grading

None of the ten estimated cytokines or other predictors had any significant correlation with the Embryo grading (Figures 2.4 – 2.9).

3.3.8 Biochemical pregnancy

IL-5 was inversely and significantly correlated to the chance of biochemical pregnancy (OR = 0.269; p = 0.029). Other nine measured cytokines and predictors had no significant effect on the chance of biochemical pregnancy (Table 3.9).

Table 3.9 Correlation between follicular fluid IL-5 and biochemical pregnancy.

	B	S.E.	P-value	OR	95% CI for OR	
					Lower	Upper
age	.003	.061	.962	1.003	.891	1.129
infertility_period	-.079	.084	.346	.924	.784	1.089
endometrial_tickness	.262	.155	.090	1.300	.960	1.762
infertility_type	.912	.730	.212	2.489	.595	10.406
superovulation_protocol	-.033	.628	.958	.968	.282	3.317
history_of_previous_IVF	-.174	.670	.795	.840	.226	3.123
IL_5	-1.314	.604	.029	.269	.082	.877
Constant	7.647	5.814	.188	2094.864		

B: Regression coefficient; S.E.: Standard error; CI: Confidence interval

3.3.9 Implantation rate (IR %)

In all patients, the endometrial thickness was significantly correlated to the IR%; in general, the thicker the endometrial thickness, the greater the implantation rate. None of the ten measured cytokines had a significant correlation with IR%. IR% was proportioned directly with (IFN-γ, IL-4, IL-6, TNF-α) and inversely with (IL-1β, IL-2, IL-5, IL-8, IL-10, GM-CSF), but it was insignificant.

3.3.10 Clinical pregnancy

In all patients, the endometrial thickness was significantly correlated to the chance of clinical pregnancy; as a general, the larger the endometrial thickness, the greater the chance of clinical pregnancy.

IL-5 was the only of the measured cytokines had a significant correlation to the chance of clinical pregnancy. The increased FF IL-5 concentration was correlated to the decrease in the chance of clinical pregnancy (OR = 0.223; p = 0.026) (Table 3.10).

Table 3.10 Correlation between follicular fluid IL-5 and clinical pregnancy.

	B	S.E.	P-value	OR	95% CI for OR	
					Lower	Upper
	.020	.062	.742	1.021	.903	1.153
infertility_period	-.136	.096	.154	.873	.723	1.053
infertility_type	.815	.757	.282	2.258	.512	9.951
superovulation_protocol	-.124	.655	.850	.884	.245	3.187
history_of_previous_IVF	-.209	.690	.762	.812	.210	3.141
endometrial_tickness	.432	.181	.017	1.541	1.081	2.196
IL_5	-1.499	.675	.026	.223	.059	.839
Constant	7.876	6.295	.211	2633.400		

B: Regression coefficient; S.E.: Standard error; OR: Odds ratio; CI: Confidence interval

3.4 Multiplex measured cytokines

Multiplex magnetic bead-based flow cytometry measured cytokines in the follicular fluid samples are shown in (Table 3.11). IL-5 is the only measured cytokine which was significantly correlated with the chance of biochemical pregnancy using multivariate logistic regression test.

Table 3.11 Mean± Standard deviation of multiplex bead-based measured cytokines concentrations in follicular fluid. Receiver operating characteristics (ROC) curve was done for estimation of performance of each cytokine on prediction of biochemical pregnancy status. Area under curve (AUC) is presented for each cytokine.

	Mean ± SD (pg/ml)	**AUC**
IL-1β	13.13 ± 0.72	0.534
IL-2	30.07 ± 4.45	0.531
IL-4	15.74 ± 1.13	0.583
IL-5	10.23 ± 0.77	0.654*
IL-6	57.19 ± 84.55	0.571
CXCL8/IL8	757.39 ± 385.97	0.592
IL-10	45.95 ± 9.95	0.576
IFN-γ	17.75 ± 1.03	0.519
GM-CSF	12.21 ± 5.48	0.518
TNF-α	11.57 ± 0.87	0.542

* Multivariate logistic regression test showed that only IL-5 significantly correlate with the chance of biochemical pregnancy. AUC for IL-5 shows that this cytokine has a medium ability in prediction of biochemical pregnancy.

IL: interleukin; INF-γ: interferon gamma; GM-CSF: Granulocyte macrophage-colony stimulating factor; TNF-α: tumor necrosis factor-alpha.

3.5 Receiver Operating Characteristics (ROC) curve and prediction of biochemical pregnancy

Using the ROC curve in the prediction of biochemical pregnancy, the area under curve (AUC) for IL-5 was 0.654, i.e. this cytokine has a medium ability in predicting the chance of biochemical pregnancy, whereas other nine measured cytokines had a poor ability to predict the biochemical pregnancy (Figure 3.5 and table 3.11).

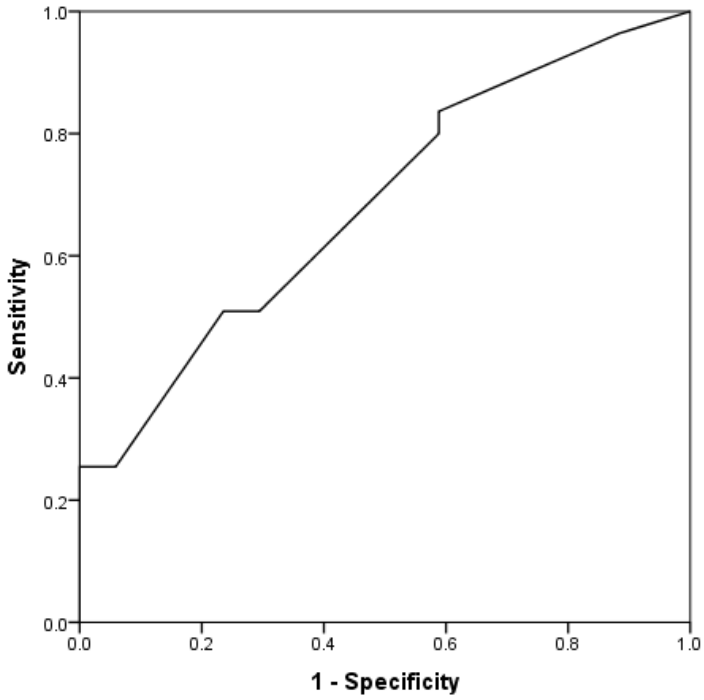


Figure 3.5 Receiver operating characteristics (ROC) curve showing the performance of IL-5 in prediction of biochemical pregnancy.

3.6 Ovarian Hyperstimulation Syndrome (OHSS)

3.6.1 Description of severe OHSS in study population

Thirteen patients were suffering from severe OHSS according to their clinical signs and/or the number of follicles at the day of HCG injection (Table 3.12).

Table 3.12 Description of severe OHSS in study population

OHSS		Frequency	Percent
	No	67	83.8
	Yes	13	16.3

3.6.2 Effects of demographics and cytokines on severe OHSS

According to the multivariate logistic regression test, none of the ten estimated cytokines had any significant correlation to the incidence of OHSS. Demographic predictors had no significant correlation to the incidence of OHSS except age of patients. The age was inversely and significantly correlated to the incidence of the OHSS in all the 80 patients included in the study.

3.6.3 ROC curve and prediction of severe OHSS

The area under curve (AUC) of IL-10 and TNF-α was 0.631 and 0.615 respectively. So these two cytokines have a medium ability in the prediction of OHSS. The other eight cytokines have poor ability in prediction of OHSS (Table 3.13) and (figures 3.6 and 3.7).

Table 3.13 Area under curve (AUC) of the estimated cytokines

Cytokine	AUC	Cytokine	AUC
IL-6	0.573	IFN-γ	0.513
IL-5	0.523	TNF-α	0.615
GM-CSF	0.541	IL-8	0.586
IL-1β	0.513	IL-10	0.631
IL-2	0.566	IL-4	0.544

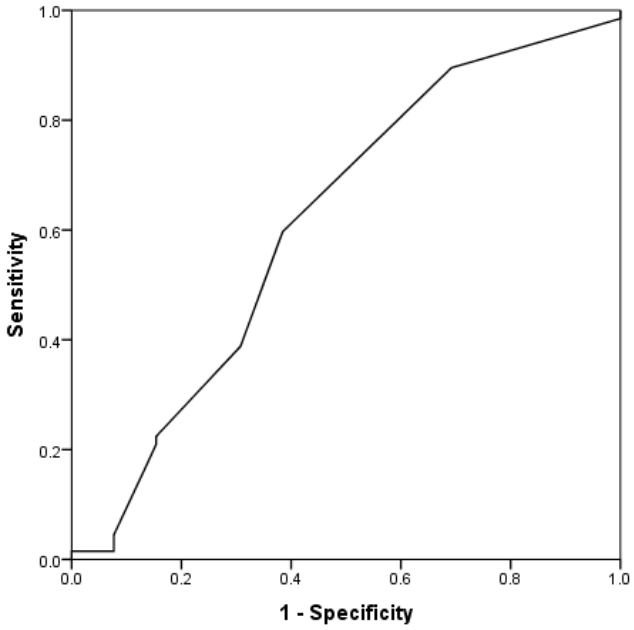


Figure 3.6 Receiver operating characteristics (ROC) curve showing the performance of TNF-α in prediction of severe OHSS. Area under curve (AUC) = 0.615.

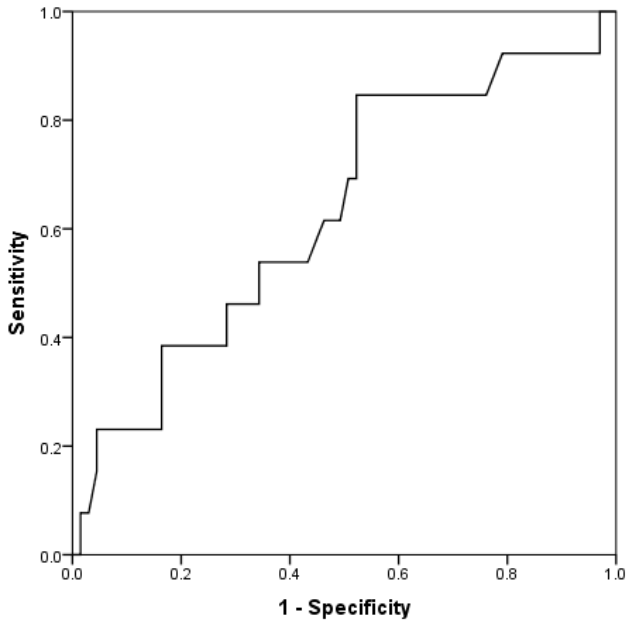


Figure 3.7 Receiver operating characteristics (ROC) curve showing the performance of IL-10 in prediction of severe OHSS. Area under curve AUC =0.6

4

DISCUSSION

4.1 Correlation of cytokines with follicle, oocyte quality and ICSI outcome

4.1.1 IL-5 and oocyte quality and pregnancy

4.1.2 IL-4 and fertilization rate

4.1.3 GM-CSF and oocyte quality

4.1.4 Embryo quality

4.1.5 Follicle and oocyte number

4.2 The correlation of cytokines with demographics

4.2.1. Infertility period

4.2.2 History of IVF/ICSI

4.2.3 Age

4.3 The correlation of cytokines and demographics with OHSS

4.3.1 Correlation of cytokines and demographics on OHSS

4.3.2 ROC curve in prediction of OHSS

4.4 Conclusions and Perspectives

4.4.1 Conclusions

4.4.2 Perspectives

In this study, the quantities of multiplex measured cytokines in FF were approximately near to the results of previous studies including Yildizfer et al. (2015) for TNF-α, Mendoza et al. (2002) and Rahiminejad et al. (2015) for IL-1β and TNF-α, Malhotra et al. (2013) for GM-CSF, Amato et al. (2003) for IL-6 and TNF-α, and the study of Lèdèe et al. (2008) for IL-2, IL-6, IL-8 and GM-CSF. Nonetheless, Lèdèe et al. (2011), Sarapik et al. (2012), Rehman et al. (2015), and Niu et al. (2017) reported different quantities.

4.1 Correlation of cytokines with follicle, oocyte quality and ICSI outcome

The main finding of this study is that some measured FF cytokines such as IL-5, IL-4, and GM-CSF from preovulatory follicles were significantly ($p < 0.05$) associated with ICSI outcome. Elevated FF IL-5 levels were associated with poor oocyte quality, which decreases the chance of pregnancy. Higher FF GM-CSF was associated with a decrease in mature oocytes, while higher FF IL-4 concentrations linked to good ICSI outcome through increased fertilization rate.

4.1.1 IL-5 and oocyte quality and pregnancy

We observed that the elevated FF IL-5 concentration was associated significantly and negatively with ICSI outcome and resulted in poor oocyte quality through increasing GV% and decreasing MII%. It has been reported that IL-5 is a well-known T helper cell type 1 (Th1) cytokine which is produced by both hematopoietic and non-hematopoietic cells with a pleiotropic activities (Takusta et al., 2011). It affects the differentiation of myeloid cells, increment of chemotactic activity and adhesion capacity of eosinophils (Akdis et al., 2011; Robertson et al., 2000). It also stimulates B-cells and act as a differentiation factor for eosinophils (Dor et al., 1996; Altun et al., 2011). In reproductive organs, IL-5 contributes to tissue remodeling in mice ovaries (Boden et al., 2010) and endometrial tissue remodeling associated with estrus cycle (Robertson et al., 2000). Recently, Terenina et.al. (2017) suggested that IL-5 acts as one of the upstream key regulators of porcine ovarian follicular atresia.

Previous studies which investigated the possible role of IL-5 in IVF/ICSI outcome, could not detect IL-5 in the FF of women undergoing ICSI using a bead-based multiplex sandwich immunoassay (Lèdèe et al., 2008) and Bio-Rad multiplex microbead assay system (Malhotra et al., 2013). Another study detected IL-5 in 25% of FF samples using the R&D systems. In the

latter study, the implantation rate, embryo morphology, fragmentation or early cleavage were not significantly different between oocytes which lead to a successful birth and those that do not (Lèdèe et al., 2010). In a recent study, Niu et al. (2017) did not find a significant correlation between FF IL-5 from the largest follicle with top-quality embryo and the embryo developmental potential in a cohort of patients with or without metabolic syndrome undergoing IVF.

It was suggested that Th1 cytokines promote allograft rejection and may compromise pregnancy, whereas the Th2 cytokines promote allograft tolerance and therefore may improve fetal survival (Raghupathy et al., 2000; Piccinni, 2007). Recent animal model study documented that Th-2 baised response in peripheral blood mononuclear cells (PMNC) during early pregnancy is essential for successful pregnancy in cattle (Yang et al., 2016). Interestingly, IL-5, is known with its Th2 anti-inflammatory properties, decreased significantly in FF of pregnant women and was inversely correlated with both biochemical and clinical pregnancy. In addition, it has a medium ability in prediction of biochemical pregnancy based on ROC curve in our study. Pregnancy is not only as a simplistic Th1/Th2 paradigm, where the 'bad' Th1-type cytokines induce abortion and 'good' Th2-type cytokines are responsible for successful pregnancy (Piccinni, 2007). Many cytokines, not only Th1 and Th2 cytokines are produced by other immune and non-immune cells present at the fetomaternal interface. Those cells are pluripotent in that their functions could depend on their relative concentrations. Their activity could be mitigated by receptors and antagonists, and they could have stage-dependent functions (Dealtry et al., 2000; Piccinni, 2007). Furthermore, our findings identify detrimental implications of increased infertility period on the outcome of IVF. Infertility period was significantly and directly correlated to GV proportion in all patients and resulted in poor oocyte quality. We hypothesize that one of the detreminal effects of increased infertility period was through increased GV, a less competent oocyte. This assumption merits further exploration in future studies.

4.1.2 IL-4 and fertilization rate

The other cytokine in our study with significant difference between successful and unsuccessful ICSI cycles, was IL-4. This interleukin was increased significantly in FF of pregnant women and resulted in increased fertilization rates. IL-4 is extensively expressed from the cumulus oophorus that surrounds the oocyte during ovulation and the embryo during the first 72 h before the implantation, and its effect is favored by progesterone produced by cumulus luteal cells (Piccinni

et al., 2001). The resolution of inflammation plays a critical role throughout pregnancy and is largely mediated by immune cells that produce IL-4 and IL-10 (Chatterjee et al., 2014). The study of Marzi et al. (1996) demonstrated that the normal pregnancy was correlated with an increase in serum IL-4 and IL-10. However, our study does not reveal any significant correlations between FF concentrations of IL-10 and the implantation rate.

4.1.3 GM-CSF and oocyte quality

Also, GM-CSF was significantly correlated with poor oocyte quality, as an increase in FF concentrations of this cytokine was associated with decrease in MII oocytes. GM-CSF is expressed from granulosa cell (GC) and theca cell (TC) and participates in ovulation and lutenization by enhancing recruitment of macrophages (Field et al., 2014). It is a pro-inflammatory cytokine which regulates myeloid cell proliferation and development. It is also critical for the functions of monocytes, macrophages and dendritic cells, and is produced in large amounts under inflammatory conditions by activated cells of immune system (Ushach and Zlotnik, 2016). The study of Lèdèe et al. (2011) suggested that follicular GM-CSF concentrations are not related to corresponding ongoing pregnancy rates.

The use of COH to stimulate a multi-follicular response using exogenous gonadotropins for ART treatment can be detrimental to oogenesis, embryo quality, and endometrial receptivity, and consequent perturbation to the intrafollicular cytokines networks, in terms of both cytokine levels and their interrelationships. This may impact oocyte maturation/fertilization and embryo developmental competence by comparing to the intrafollicular milieu (Baskind et al., 2014). This forces on the production of oocytes from follicles that do not reach optimal maturation possibly yield oocytes that are not fully competent. The similar sized pre-ovulatory follicles of unstimulated cycles have a different hormonal milieu when compared with the growing follicles as COH cycles which affect not only immune processes but also meiosis and ovulation pathways. Nevertheless, these differences do not seem to be related to early stage embryonic morphology (de los Santos et al., 2012). GM-CSF was higher in the FF from the antagonist superovulation protocol (Malhotra et al., 2013), while in our study we did not find any significant differences in FF concentrations of GM-CSF between the two protocols.

4.1.4 Embryo quality

Little evidence is available that non-invasive selection at the oocyte stage may be of prognostic value. Certain patterns of pronuclei such as the number and distribution of pronuclei at the zygote stage was found to correlate to treatment outcome in IVF and ICSI cycles (Ebner et al., 2003). While our results revealed neither significant correlation between embryo grading at the day three after ICSI and its outcome, nor with FF fluid concentration of cytokines.

4.1.5 Follicle and oocyte number

At ovulation, the ovulatory follicle undergoes maturational changes that are associated with increasing follicular size and alterations accompanied with increasing its ability to produce estradiol. The use of COH in ART treatments affects the maturation of follicles and oocytes at the induced ovulation including the interval from induced luteolysis to induced ovulation, ovulatory follicles growth rate, and ovulatory follicles size in animal models (Geary et al., 2013). Günther et al. (2016) demonstrated a significant correlation between increased FF IL-18 concentration and successful pregnancy after IVF/ICSI. The author attributed this success to increased number of oocyte retrieved due to the increase in response to ovarian stimulation. While none of the measured cytokines significantly affected the number of oocytes retrieved among our ICSI patients. Previous comparative study between GnRH agonist long and antagonist protocol and their effect on implantation rate and serum cytokines concentrations reported that FF IL-1β, IL-8, IL-12, and TNF-α had no significant differences between the two protocols (Ficicioglu et al., 2010). We confirmed these findings regarding most of these cytokines, in addition to other cytokines including IFN-γ, GM-CSF, IL-2, IL-5, and IL-10.

4.2 Correlation of cytokines with demographics

4.2.1. Infertility period

An intriguing result in this study was regarding the infertility period. We found that longer infertility period associated with a higher number of follicles; however, none of the ten measured cytokines was associated. The overall likelihood of successful treatment for infertility is nearly 50%, but this varies by reason, age of female partner, history of previous fertility, and duration of infertility. A shorter duration of infertility and previous fertility increases the chances of achieving pregnancy (Pandian et al., 2015). Kalu et al. (2011) concluded that young women who

had a live birth and those who experienced an early miscarriage after IVF had a greater chance of achieving pregnancy and a live birth in a second cycle. The outcome of the first IVF cycle, however, does not predict subsequent IVF success in older women. Rehman et al. (2015) demonstrated that higher FF IL-1β concentrations was associated with an increase in the number of retrieved, mature and fertilized oocytes in ICSI patients.

4.2.2 History of IVF/ICSI

In this study, we found that the patients with previous IVF/ICSI attempts have a high number of large follicles proportion compared with patients undergoing their first ICSI trial. Likewise, FR% was higher (74.4%) in patients with previous IVF/ICSI attempts compared with patients at first attempt (59.7%). This may be attributed to the previous ovary stimulation with a controlled ovarian hyperstimulation cycles in the previous IVF/ICSI trials that may override the ovarian response in these patients. Our results are consistent with those of Hendriks et.al. (2008) in which patients within a certain age produce a poor ovarian response to gonadotropin stimulation in their first IVF cycle.

4.2.3 Age

Our results revealed that the age was inversely proportioned with the number of follicles and the number of oocytes. Age is the most prognostic factor for IVF/ICSI success. Age associated infertility appears to be primarily related to ovarian aging and the diminishing ovarian follicle count (Liu and Case, 2011). During the reproductive life, once ovary has exhausted its reserve of follicles throughout many of functions are lost (Orsi et al., 2014). Previous studies reported that the deleterious effects of advanced patient age associated with IL-6 and its negative effects on the uterine receptivity to the embryo, hence, poor IVF/ICSI outcome (Altun et al., 2011). In our female factor infertility patients, results showed no significant correlation between age and FF concentrations of IL-6 from successful ICSI cycles and unsuccessful ones. We also revealed that the endometrial thickness was linked to increase implantation rate and the chance of clinical pregnancy, but none of the ten measured cytokines had been significantly associated to increase endometrial thickness. While the study of Rehman et al. (2015) demonstrate that the IL-1β is correlated with increase of endometrial thickness and reproductive outcome rates in ICSI patients.

4.3 The correlation of cytokines and demographics with OHSS

4.3.1 Correlation of cytokines and demographics with OHSS

In our study, embryo transfer of severe OHSS patients were canceled with cryopreservation of all embryos according to the "Freeze-all-policy". OHSS continues to be a serious complication of assisted reproductive therapy (ART), with no universally agreed upon best method of prevention. Coasting and cryopreservation of all embryos are the most commonly used approaches in the literature, but cycle cancellation is the only method that can completely prevent the development of OHSS (Alper et al., 2009).

Age was the only demographic had significantly and inversely correlated with the incidence of severe OHSS in our ICSI patients. It has been reported in most studies that women suffering from OHSS are significantly younger than those who do not (Shibahara et al., 2005; Delvigne, 2009; Busso et al., 2010; Pandian et al., 2015; Griesinger et al., 2016). Busso et al. (2010) considered young age as one of OHSS risk factors before and during gonadotropin administration. Patients suffering from OHSS are generally young because OHSS depends on the patient's ovarian reserve (Shibahara et al., 2005).

Many previous studies revealed a significant differences (increase) in some cytokines of FF (Chen et al., 2000; Shi et al., 2015), of peritoneal fluid (Revel et al., 1996), and of ascites (Chung et al., 2004), and PBMCs (Orvieto et al., 2014) from patients with severe OHSS. A suggested model for involvement of the immune system in the pathogenesis of OHSS. hCG, LH, and other factors stimulate ovarian macrophages and granulosa cells to produce TNF-α and other cytokines such as IL-2. The secreted TNF-α stimulates lymphocytes and natural killer cells which resulted in activation of endometrium to increase vascular permeability and micro thrombi, thereby appearance of the clinical manifestations of OHSS (Mathur et al., 1997). Inflammatory response to hCG leading to dysregulation of IL-2 expression and suppressor of cytokine signaling (SOCS)-1 activation in the PBMCs, might be the culprit of OHSS. May therefore hypothesize that patients at risk to develop OHSS possess an inherited paradoxical immune response to hCG, with IL-2 instead of SOCS-1 dominance, leading to systemic inflammatory response with the consequent development of OHSS (Orvieto et al., 2014).

FF IL-6 at the time of oocyte retrieval and serum IL-8 concentration at the day of embryo transfer may serve as early predictor for OHSS (Chen et al., 2000). In severe OHSS patients,

peritoneal fluid IL-6, IL-8 and TNF-α and serum IL-6 and IL-1β were higher significantly. These findings suggest that these substances could be involved in mediating the capillary hyperpermeability characterizing this syndrome (Revel et al., 1996). However, our study did not show any significant differences of the ten estimated cytokines in the FF of severe OHSS patients and they did not have.

4.3.2 ROC curve in prediction of OHSS

Our study revealed that IL-10 and TNF-α had a medium ability in the prediction of OHSS incidence using ROC curve. IL-10 is one of the cytokines had been detected in the peritoneal fluid of women with OHSS and implicated in the pathogenesis of severe OHSS (Soares et al., 2008). Study of Shi et al. (2015) revealed that the concentration of IL-10 were higher significantly in FF of OHSS patients than in control group. TNF-α, an important cytokine in the antral follicle growth and selection (Field et al., 2014). TNF-α is considered the main cytokine involved in luteal function (Dor et al., 1996). In addition, TNF-α is an important mediator of ovulation in terms of decreasing the number of released oocytes and inducing granulosa cell death of un-ruptured follicles could induce follicle atresia via apoptosis and autophagy for remodeling ovarian tissues. TNF-α considered to be one of the important factors in the preovulatory stage; however, the actual role of TNF-α and its effects in the ovary are not completely understood. (Yamamato et al., 2015).

4.4 Conclusions and Perspectives

4.4.1 Conclusions

In summary, our data suggests several detrimental effects of elevated FF IL-5 on ICSI outcome. One is associated with poor oocyte quality through a significant increase in oocytes with a germinal vesicle stage along with a decrease in MII oocytes proportion. The second one was associated with decreased chance of both biochemical and clinical pregnancy. Moreover, IL-5 had medium prediction ability predicting the chance of biochemical pregnancy. So, we can conclude that the elevated intrafollicular concentrations of IL-5 seem to be a negative predictor to the pregnancy outcome in ICSI cycles. In addition, elevated FF GM-CSF was associated with poor oocyte quality, while higher FF IL-4 concentrations were linked to good ICSI outcome. Using ROC curve, IL-10 and TNF-α had a medium ability in the prediction of severe OHSS incidence.

4.4.2 Perspectives

Taken together, our results and previously published data suggest that the alterations in cytokine balance and changes in their expression may function as an important diagnostic tool in IVF/ICSI cycles. Determination of their concentration could be used routinely in addition to the hormonal and morphological criteria. Collectively, cytokines appear to be promising markers for ICSI outcome.

Additional prospective studies with higher patient numbers are required to understand the regulatory mechanisms in ICSI treatments, which might show the importance of some cytokines as biomarkers for the success of ICSI.

As the most of female infertility causes in our study and other similar investigations were mixed (e.g. endometriosis, PCOS, etc.), it is difficult to determine which cytokine is correlated to a corresponding cause of infertility. So, future studies should monitor the levels of parameters in the patients with a single cause of female infertility in order to reveal more definite relationship. Moreover, other cytokines/chemokines can be considered in future studies to find their probable prognostic performance for ICSI outcome.

Finally, further directions should focus on intrafollicular cytokine concentrations and extracellular vesicles of healthy and infertile women to confirm the value of these prognostic markers for the pregnancy outcome in ICSI cycles.

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بسم الله الرحمن الرحيم

دانشکده دامپزشکی

گروه علوم پایه

پایان نامه برای اخذ درجه دکتری (Ph. D) در رشته دامپزشکی گرایش فیزیولوژی

عنوان

بررسی ارتباط برخی سایتوکین‌های مایع فولیکولی با میزان باروری در روش تزریق داخل سیتوپلاسمی اسپرم

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نگارنده

میعاد جبار صاحب الهلالی

تابستان ۱۳۹۷

تعهدنامه

اینجانب میعاد جبار صاحب الهلالی دانشجوی دکتری رشته دامپزشکی **دانشکده** دامپزشکی **پردیس بین الملل دانشگاه فردوسی مشهد نویسنده** پایان نامه جهت اخذ درجه دکتری (PH. D) در رشته دامپزشکی گرایش فیزیولوژی بررسی ارتباط برخی سایتوکین‌های مایع فولیکولی با میزان باروری در روش تزریق داخل سیتوپلاسمی اسپرم **تحت راهنمایی دکتر** عباس پرهام **و دکتر** آرمین عطاران زاده، دکتر ملیحه امیریان و دکتر محمد عزیز زاده **متعهد می‌شوم:**

- تحقیقات در این رساله/پایان نامه توسط اینجانب انجام شده است و از صحت و اصالت برخوردار است.
- در استفاده از نتایج پژوهش‌های محققان دیگر به مرجع مورد استفاده استناد شده است.
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امضاء دانشجو:

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جلسه دفاع از رساله خانم **میعاد جبار المعالی** به شماره دانشجویی **۹۳۲۶۶۰۸۰۱۰** رشته **دکتری تخصصی فیزیولوژی** گرایش - در ساعت ۱۱ روز دوشنبه مورخ **۱۳۹۷/۵/۱** در محل دانشکده دامپزشکی و با عنوان **بررسی ارتباط برخی سایتوکاین های مایع فولیکولی با میزان باروری در روش تزریق داخل سیتوپلاسمی اسپرم** با حضور امضاکنندگان ذیل برگزار گردید و بر اساس کیفیت رساله و دستاوردهای آن، ارائه دفاعیه و نحوه پاسخ به سئوالات، رای نهایی هیات داوران به شرح ذیل اعلام گردید:

درجه رساله: **بسیار خوب** نمره رساله به عدد: **۱۸/۸۰** به حروف: **هجده و هشتاد**

استاد(ان) راهنما و مشاور:

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۱- داور: **دکتر نسیره خادم** استاد دانشگاه علوم پزشکی مشهد

۲- داور: **دکتر حسام دهقانی** استاد گروه علوم پایه دانشکده دامپزشکی دانشگاه فردوسی مشهد

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نماینده تحصیلات تکمیلی: دکتر **فرشید حمیدی** مدیر گروه: **دکتر عباس پرهام**

معاون پژوهش و فناوری دانشکده: **دکتر محمد محسن زاده** رئیس دانشکده: **دکتر محسن ملکی**

گواهی اعضای کمیته‌ی پایان نامه

بررسی ارتباط برخی سایتوکین‌های مایع فولیکولی با میزان باروری در روش تزریق داخل سیتوپلاسمی اسپرم

به کوشش :

میعاد جبار صاحب الهلالی

پایان‌نامه

ارائه شده به تحصیلات تکمیلی دانشگاه فردوسی مشهد به عنوان بخشی از فعالیات‌های تحصیلی لازم جهت اخذ درجه دکتری تخصصی

در رشته‌ی: فیزیولوژی

از دانشگاه فردوسی مشهد

جمهوری اسلامی ایران

ارزیابی کمیته‌ی پایان نامه با درجه: بسیار خوب **و نمره** : ۱۸ /۸۰

دکتر عباس پرهام دانشیار گروه علوم پایه دانشکده دامپزشکی دانشگاه فردوسی مشهد

دکتر آرمین عطاران زاده دانشکاه علوم پزشکی مشهد

دکتر ملیحه امیریان دانشیار دانشکاه علوم پزشکی مشهد

دکتر محمد عزیززاده دانشیار گروه علوم درمانگاهی بهداشت وپیشگیری بیمارهای دامی دانشکده دامپزشکی دانشگاه فردوسی مشهد

دکتر نیره خادم استاد دانشکاه علوم پزشکی مشهد

دکتر حسام دهقانی استاد گروه علوم پایه دانشکده دامپزشکی دانشگاه فردوسی مشهد

دکتر پژمان میرشکرایی دانشیار گروه علوم درمانگاهی بهداشت وپیشگیری بیمارهای دامی دانشکده دامپزشکی دانشگاه فردوسی مشهد

مرداد ماه ۱۳۹۷

تشکر و قدردانی

با تقدیر و سپاس فراوان از استادان گرامی

دکتر عباس پرهام، دکتر آرمین عطاران زاده، و دکتر محمد عزیز زاده

به پاس حمایت ها و راهنمایی های دلسوزانه شان در به ثمر رساندن این پایان نامه

این پایان نامه را ضمن تشکر و سپاس بیکران و در کمال افتخار و امتنان تقدیم می نمایم به:

-محضر ارزشمند پدر و مادر عزیزم به خاطر همه ی تلاشهای محبت آمیزی که در دوران مختلف زندگی ام انجام داده اند و با مهربانی چگونه زیستن را به من آموخته اند.

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و

به استادانم که در راه کسب علم و معرفت مرا یاری نمودند و بدون کمک های آن ها انجام این پایان نامه برایم مقدور نبود

خلاصه فارسی مبسوط

۱- مقدمه

سایتوکین‌ها تنظیم کننده‌های کلیدی فیزیولوژی تخمدان هستند و به ویژه به فرآیند رشد و تکوین فولیکول و تخمک گذاری، که در آن به ایجاد محیطی مطلوب برای حمایت از انتخاب و رشد فولیکول‌ها نیاز است، کمک می‌کنند. کاربردهای چندگانه آنها عبارتند از: تنظیم تکثیر / تمایز سلولی، بقای فولیکول / آترزی فولیکول و بلوغ اووسیت [۱-۴]. برخی از سایتوکین‌ها از طریق مسیرهای سیگنالینگ داخل سلولی خاص، اثرات پیش التهابی و ضد التهابی اعمال می‌کنند [۴]. چندین سایتوکین، مانند اعضای فوق خانواده فاکتور رشد تغییردهنده بتا (TGF-β) در تمام مراحل فولیکولوژنز دخیل هستند، در حالی که برخی دیگر از قبیل اعضای خانواده سایتوکین گلیکوپروتئین ۱۳۰ (IL-11 و LIF)، فاکتورهای تحریک کننده کلونی (CSFs)، IL-1، IL-15 و IL-33 وابسته به مرحله هستند و در مراحل خاصی دخالت دارند [۳]. برخی از سایتوکین‌ها مانند IL-1β بر لقاح اووسیت و کیفیت جنین تأثیر می‌گذارند و برخی دیگر مانند IL-8، IL-18 و MIP-1β با بارداری موفق همبستگی دارند [۵].

انتقال پیام دوطرفه‌ی سلول سوماتیک- اووسیت برای ایجاد یک ریزمحیط داخل فولیکولی برای کنترل رشد فولیکول‌های اولیه، که در نهایت منجر به انتخاب یک فولیکول آنترال برای تخمک گذاری می‌شود، ضروری است [۶]. کیفیت و ماندگاری اووسیت‌ها به طور مستقیم با ترکیب سایتوکینی داخل تخمدانی/اطراف فولیکولی ارتباط نزدیکی دارد [۳]. مایع فولیکولی (FF)، به عنوان ریز محیط اووسیت، مایع بیولوژیکی منحصر به فردی است که غنی از عوامل رشد و سایتوکین‌ها است و با اثرات پاراکرینی و اتوکرینی خود بر روی رشد اووسیت، کیفیت آن و حتی لانه گزینی اعمال اثر می‌کند. وقایعی از قبیل تخمک گذاری، بلوغ فولیکولی و ارتباط سلول‌های سوماتیک - سلول‌های جنسی در مایع فولیکولی رخ می‌دهد. با توجه به نزدیکی و مجاورت مایع فولیکولی با اووسیت در حال رشد، این مایع یک دریچه منحصر به فرد برای بررسی فرآیندهایی است که در زمان بلوغ فولیکول رخ می‌دهد [۶، ۷، ۸]. علاوه بر این، مایع فولیکولی به راحتی در زمان اخذ اووسیت در دسترس است و ارائه دهنده منبعی مطلوب از فاکتورهای بیوشیمیایی غیر تهاجمی پیش بینی کننده‌ی کیفیت اووسیت است [۹].

فاکتور محرک کلونی گرانولوسیت (G-CSF)، تنها سایتوکین شناخته شده در مایع فولیکولی است که به طور کامل با موفقیت تکنیک‌های کمکی تولید مثل (ART) در ارتباط است و در سه آزمایش مستقلی که توسط شبکه اروپایی برای تحقیق و کنترل لانه گزینی جنین (EMBIC) انجام شد، این قضیه تایید شده است [۱۰]. G-CSF بیومارکری غیر تهاجمی برای لانه گزینی است، زیرا با پتانسیل رویانی که در روش لقاح آزمایشگاهی منجر به تولد نوزاد زنده می‌شود، مرتبط است (11]. همچنین غلظت این فاکتور، همراه با اینترلوکین ۵ در مایع

فولیکولی، پیش بینی کننده‌ی شایستگی و قابلیت اووسیت و تولد نوزاد است [۱۲]. علاوه بر این، در مقایسه با روشی که انتخاب فقط بر اساس مورفولوژی صورت می‌گیرد، میزان G-CSF سبب انتخاب مناسب تر رویان‌ها و کاهش حاملگی‌های چندگانه می‌شود [۱۳]. سایر سایتوکین‌ها مانند IL-1β[۱۴]، IL-6 [۱۶] و TNF-α [۱۷و ۱۸و ۱۹] نتایج متناقضی را در همبستگی با نتایج IVF/ICSI دارند. علاوه بر این، برخی از سایتوکین‌های دیگر به ندرت مورد مطالعه قرار گرفته اند و یا مانند IL-3 و IL-5 در درمان‌های ART تاثیری نداشته اند.

در مطالعات قبلی که رابطه بین بیومارکرها و پتانسیل آنها در پیش بینی نتایج IVF/ICSI را مورد بررسی قرار داده اند، از نمونه‌های انفرادی مایع فولیکولی یعنی فقط از یک فولیکول [۱۰، ۱۱، ۱۲، ۱۳، ۲۰، ۲۱، ۲۲] یا نمونه‌های تجمیع شده مایع فولیکولی هر فرد [۲۷-۲۳] استفاده شده است. مایع فولیکولی تجمیع شده می‌تواند به طور جامعی محیط زیست میکرودینامیکی که در آن اووسیت رشد کرده و بالغ می‌شود را منعکس نماید [۲۱]. علاوه بر این، آسپیراسیون فولیکول انفرادی برای بیمار و پزشک بسیار سخت و آزاردهنده است زیرا در آن چندین سوراخ واژینال ضروری است که طبعا با افزایش خطر خونریزی واژینال همراه است [۹]. ناباروری ناشی از اختلالات تخمدان، اختلالات رحمی، انسداد لوله و علل شایع صفاقی یا گردن رحمی است [۲۸]. لذا با توجه به توضیحات فوق، در این مطالعه ما مایع فولیکولی فولیکول‌های قبل از تخمک گذاری بیماران تحت درمان برای ICSI را که به علت ناباروری عامل زنانه مراجعه کرده بودند جمع آوری کردیم تا غلظت ده سایتوکین را اندازه گیری کنیم و در نهایت نقش احتمالی هر سایتوکین را به عنوان یک بیومارکر غیر تهاجمی برای ارزیابی قابلیت اووسیت و پیش بینی نتیجه ICSI ارزیابی کنیم.

۲- مواد و روش‌ها

۲-۱- پروتکل مطالعه

این مطالعه بر اساس دستورالعمل های کمیته اخلاقی دانشگاه فردوسی مشهد و دانشگاه علوم پزشکی مشهد انجام شد. نمونه‌های مایع فولیکولی (FF) از مردادماه سال ۱۳۹۵ تا اردیبهشت ماه سال ۱۳۹۶ در مرکز ناباروری میلاد دانشگاه علوم پزشکی مشهد جمع آوری شد. بر اساس معیارهای ورود به مطالعه، هشتاد بیمار در دامنه سنی ۲۰ تا ۴۳ سال در این مطالعه وارد شدند. تعداد تلاش IVF/ICSI قبلی، دوره ناباروری و نوع آن، پروتکل سوپراوولاسیون و ضخامت اندومتر ثبت شد. تمام بیماران تحت درمان ICSI با علت ناباروری فاکتور زنانه (به تنهایی یا همراه با ناباروری عامل مردان) به علت موارد ذیل تحت درمان بودند: اختلالات رحمی (مثلا اندومتریوز، مایوما، پولیپ، فیبروم، و نقص مادرزادی)، انسداد لوله، عوامل صفاقی (مانند چسبندگی) و اختلالات تخمک گذاری (مانند سندرم تخمدان پلی کیستیک (PCOS)). بیماران با سایر علل ناباروری از قبیل فقط ناباروری فاکتور مردانه، ناباروری غیرقابل توصیف، اهداکنندگان اووسیت و بیماران مبتلا به بیماری‌های خاص

مانند دیابت و چاقی ($BMI \geq 28\ kg/m^2$) از مطالعه حذف شدند. تشخیص OHSS شدید بر اساس وجود یکی از علایم بالینی مربوطه و تعداد فولیکول‌ها در روز تزریق hCG انجام شد (اوکال و همکاران، ۲۰۱۱).

۲-۲- پروتکل سوپراوولاسیون

همه بیماران، تحت درمان سوپراوولاسیون کنترل شده با پروتکل‌های آگونیست یا آنتاگونیست قرار گرفتند. روش آگونیست طولانی و یا آنتاگونیست به وسیله پزشک متخصص زنان انتخاب شد. در روش آگونیست، به ۳۲ بیمار روزانه FSH نوترکیب (Gonal-F، Merk Serono، آلمان) تجویز شد. تزریق در فاز میانی لوتئال (روز ۲۱) سیکل قبلی شروع شد و تا روز تزریق hCG ادامه داشت. اندازه فولیکول‌ها با سونوگرافی ترانس واژینال هر ۲-۳ روز مورد بررسی قرار می‌گرفت. ۴۸ بیمار دیگر به طور روزانه (Cetrotide، Merk Serono، آلمان) داروهای آنتاگونیست را در فاز میانی فولیکولی تا زمانی که اندازه فولیکول‌ها حداقل به ۱۲ میلی متر می‌رسید، دریافت کردند. بلوغ نهایی اووسیت در هر دو پروتکل به وسیله تزریق گنادوتروپین کوریونی انسانی (Pregnyl) (hCG؛ IBSA، هلند) القا می‌شد. اخذ اووسیت‌ها (OPU) حدود ۳۶-۳۸ ساعت پس از تزریق داخل عضلانی hCG انجام می‌شد.

۲-۳- اخذ نمونه‌های مایع فولیکولی و تعیین غلظت سایتوکین‌ها

تحت بیهوشی عمومی کوتاه مدت، فولیکول‌ها همراه با سونوگرافی هدایت شده ترانس واژینال سوراخ شدند تا با استفاده از یک سوزن مخصوص، اووسیت‌ها همراه با مایع فولیکولی اطرافشان برداشته شوند. حدود نیم تا یک ساعت، مایع فولیکولی تمام فولیکول‌های هر فرد جمع آوری شد و سپس با ۱۰ دقیقه سانتریفیوژ با دور ۲۰۰۰، بقایای سلولی حذف شد. نمونه‌های آلوده به خون کنار گذاشته می‌شدند و فقط نمونه‌های مایع فولیکولی روشن مورد استفاده قرار می‌گرفتند و تا زمان اندازه گیری غلظت سایتوکاین ها، در دمای ۸۰- درجه سانتی گراد نگهداری شدند. ۱۰ سایتوکین مورد اندازه گیری شامل: IL-1β، IL-2، IL-4، IL-5، IL-6، IL-8 / CXCL8، IL-10، GM-CSF، IFN-γ و TNF-α بودند. نمونه‌های مایع فولیکولی با رعایت زنجیره انجماد (در کنار یخ خشک) به آزمایشگاه مقصد منتقل شدند و غلظت سایتوکاین‌ها توسط روش فلوسیتومتری (Luminex Platform Magnetic Luminex) اندازه گیری شد. کیت Multi-Anaylte Premixed ((R & D System Inc کد کیت: LXSAMH-10) از آزمایشگاه Bio-Rad ایتالیا خریداری شد. اندازه گیری و تحلیل مقادیر سایتوکاین‌ها در آزمایشگاه Labospace میلان ایتالیا توسط ابزار Luminex Map Technology (Luminex (Bio-Rad) انجام شد. نمونه‌ها با نرم افزار Bio-Plex Manger V 6.0 مورد تجزیه و تحلیل قرار گرفتند. روش تعیین غلظت سایتوکین‌ها بر اساس دستورالعمل شرکت سازنده انجام شد. بر اساس توضیح آزمایشگاه، نمونه‌های مایع

فولیکولی با واکنش دهنده به نسبت ۱: ۱ رقیق شدند و مقدار ۵۰ میکرولیتر در هر چاهک ریخته شد (به صورت دوتایی). مقادیر منحنی استاندارد با مقادیر به دست آمده مقایسه شد. سپس شدت فلورسانس هر چاهک با استفاده از یک الگوریتم خاص که به وسیله دستگاه به صورت خودکار محاسبه می‌شود، به یک غلظت تبدیل شد.

۲-۴- ارزیابی فولیکول‌ها و اووسیت‌ها

اندازه فولیکول‌ها و تعداد آنها با استفاده از سونوگرافی در حدود ۴۸ تا ۷۲ ساعت قبل از اخذ اووسیت اندازه گیری شد. فولیکول‌های با ابعاد کوچکتر از ۱۲ میلی متر به عنوان فولیکول کوچک، فولیکول‌های بین ۱۲ تا ۱۵ میلی متر به عنوان فولیکول متوسط و فولیکول‌های بزرگتر یا برابر با ۱۶ میلی متر به عنوان فولیکول بزرگ دسته بندی شدند. پس از ۲۰ تا ۳۰ دقیقه از جمع آوری اووسیت ها، کیفیت اووسیت‌ها به طور مستقیم بررسی شد. اووسیت‌های با کیفیت خوب دارای سیتوپلاسمی یکنواخت و تاج شعاعی سالمی بودند. با ارزیابی میکروسکوپی، اووسیت‌ها در گروه‌های نکروتیک، وزیکول زایا (GV)، متافاز یک و متافاز دو (MII) دسته بندی شدند. فقط اووسیت‌های گروه MII تحت آزمایش قرار گرفتند و سایر موارد از جمله اووسیت‌های GV و نکروتیک حذف شدند. نسبت (درصد) هر یک از این سه دسته با تقسیم تعداد هر گروه بر تعداد کل اووسیت‌های اخذ شده از هر بیمار ضربدر ۱۰۰ محاسبه شد.

۲-۵- نتیجه تزریق داخل سیتوپلاسمی اسپرم (ICSI)

برای بررسی نتیجه ICSI موارد مختلفی شامل: ارزیابی لقاح، میزان باروری، ارزیابی کیفیت رویان، میزان لانه‌گزینی و میزان بارداری بیوشیمیایی و بالینی مورد ارزیابی قرار گرفت.

ارزیابی لقاح اووسیت حدود ۲۴ ساعت پس از ICSI انجام شد. وجود دو پیش هسته (2PN) به عنوان نشانه‌ای از لقاح و تشکیل زیگوت در نظر گرفته شد. پس از آن، میزان باروری (FR%) با تقسیم تعداد 2PN به تعداد اووسیت‌های MII محاسبه و پس از آن در ۱۰۰ ضرب شد. درجه بندی و ارزیابی رویان در روز ۳ پس از ICSI انجام شد. رویان‌های تولید شده به صورت رویان‌های درجه یک (I) و دو (II)تقسیم شدند: رویان‌های درجه I شفاف، دارای لایه شفاف نازک، تعداد بلاستومر ۶ یا بیشتر و با اندازه برابر و میزان فراگمنتاسیون کمتر از ۱۰ درصد بودند و هیچ بلاستومر چند هسته‌ای نداشتند. سایر رویان‌ها به عنوان درجه دوم درجه بندی شدند. میزان لانه گزینی (IR) به صورت زیر محاسبه شد: تعداد کیسه‌های بارداری تقسیم بر تعداد رویان‌های منتقل شده در هر بیمار ضربدر ۱۰۰. بارداری بیوشیمیایی عبارت بود از: تایید حضور hCG در خون ۱۶ روز پس از انتقال داخل رحمی رویان. حاملگی بالینی بدین صورت تعریف می‌شد: حضور یک یا چند کیسه بارداری با تایید سونوگرافی همراه با صداهای قلب ۳۶ روز پس از انتقال داخل رحمی رویان و آمنوره به مدت حدود ۸-۶ هفته.

۲-۶- تحلیل آماری

مقدار سایتوکین‌ها در مایع فولیکولی بیماران به صورت میانگین ± انحراف استاندارد گزارش شد. مقایسه نتایج جمعیت شناختی (دموگرافیک) و نتایج ICSI بین زنان باردار و غیر باردار به ترتیب با استفاده از آزمون کای مربع و آزمون t مستقل برای مولفه‌های کیفی و کمی انجام شد. برای بررسی اثرات سایتوکین‌های مایع فولیکولی بر نتیجه ICSI، در مرحله اول، همبستگی یک طرفه سطح سایتوکین‌ها با نتایج کمی و باینری (کیفی) به ترتیب با استفاده از آزمون همبستگی و رگرسیون لجستیک مورد تجزیه و تحلیل قرار گرفت. سپس، سایتوکین‌هایی که با $p < 0.2$ با نتایج ICSI مرتبط بودند برای تجزیه و تحلیل چند متغیری انتخاب شدند. کنترل اثرات سن، نوع پروتکل تحریک سوپراوولاسیون، سابقه IVF قبلی و دوره نازایی، همبستگی سایتوکین‌ها با نتایج کمی از قبیل تعداد فولیکول ها، تعداد اووسیت و درصد کیفیت اووسیت توسط آزمون رگرسیون خطی مورد ارزیابی قرار گرفت. همچنین کنترل اثرات سن، نوع پروتکل سوپراوولاسیون، سابقه IVF قبلی، دوره نازایی و ضخامت اندومتر، همبستگی سایتوکین‌ها با نتایج باینری مانند نتایج بارداری بیوشیمیایی و بالینی با آزمون رگرسیون لجستیک مورد ارزیابی قرار گرفت. منحنی ROC (Receiver operating characteristic) برای ارزیابی عملکرد هر سایتوکین در پیش بینی بارداری و OHSS شدید مورد استفاده قرار گرفت. تجزیه و تحلیل آماری با استفاده از نرم افزار SPSS نسخه ۲۱ انجام شد. مقدار $p < 0.05$ به لحاظ آماری معنی‌دار درنظر گرفته شد.

۳- نتایج

انتقال رویان در ٪ ۷۲.۵ موارد (۵۸ نفر از ۸۰ نفر کل) انجام شد و در٪ ۲۷.۲۵ موارد (۲۲ نفر از ۸۰ نفر) لغو شد. انتقال به علل متعددی از قبیل سندرم تحریک بیش از حد تخمدان (OHSS)، پولیپ رحمی، مایوما، اندومتر نامناسب و عدم لقاح لغو شد. میزان باروری٪ ۰.۲۸ ± ۰.۷۰ بود. داده‌های دموگرافیک جمعیت مورد مطالعه (میانگین ± انحراف استاندارد با حداکثر و حداقل مقادیر آنها)، میانگین ± انحراف استاندارد برای تعداد و نسبت فولیکول، تعداد اووسیت، نسبت هر گروه از اووسیت‌ها (MII، وزیکول زایا و اووسیت‌های نکروتیک)، درجه بندی رویان‌ها و همچنین نتایج درصد لانه گزینی ، بارداری بیوشیمیایی و بارداری بالینی در جدول ۱ ارائه شده است. از مرحله بارداری بیوشیمیایی، داده‌های حاملگی دو نفر در دسترس نبود ($n = 78$).

جدول ۱- اطلاعات توصیفی جمعیت مورد مطالعه و خصوصیات چرخه‌های ICSI. داده‌های متغیرهای کمی به صورت میانگین ± انحراف استاندارد و متغیرهای کیفی به صورت فراوانی (٪) ارائه شده اند.

	میانگین ± SD	کمترین	بیشترین	فراوانی (٪)
سن (سال)	31.35 ± 5.23	20	43	-
نوع ناباروری				
زن	-	-	-	29/80 (36.2%)
ترکیبی	-	-	-	51/80 (63.8%)
دوره ناباروری (سال)	5.98 ± 3.98	5	17	-
پروتکل سوپراوولاسیون				
آگونیست	-	-	-	32/80 (40%)
آنتاگونیست	-	-	-	48/80 (60%)
تلاش‌های قبلی IVF / ICSI				
بله	-	-	-	23/80 (28.8%)
نه	-	-	-	57/80 (71.2%)
ضخامت آندومتر (میلی متر)	9.22 ±2.14	3.5	15	-
تعداد فولیکول ها	16.25 ± 11.56	1	50	-
اندازه فولیکول ها				
بزرگ (٪)	0.53 ± 0.32	0.00	1.00	-
متوسط (٪)	0.38 ± 0.24	0.00	1.00	-
کوچک (٪)	0.09 ± 0.17	0.00	0.80	-
تعداد اووسیت ها	10 34 ± 7.29	1	45	-
کیفیت اووسیت				
MII (٪)	0.91 ± 0.19	0.00	1.00	-
GV (٪)	0.02 ± 0.07	0.00	0.50	-
ناباروری (٪)	0.07 ± 0.15	0.00	0.70	-
FR٪	0.70 ± 0.28	0.00	1.00	-
ارزیابی رویان				
درجه اول	0.61 ± 0.27	0.00	1.00	-
درجه دوم	0.34 ±0.24	0.00	1.00	-
IR٪	0.25 ± 0.23	0.33	1.00	-
بارداری بیوشیمیایی				
مثبت	-	-	-	18/78 (23.1%)
منفی	-	-	-	60/78 (76.9%)
بارداری بالینی				
مثبت	-	-	-	17/78 (21.8%)
منفی	-	-	-	61/78 (78.2%)

نتیجه حاملگی بیوشیمیایی ۱۸ بیمار مثبت بود و در ۶۰ بیمار منفی بود. با استفاده از آزمون t مستقل مشخص شد که در تعدادی از متغیرهای کمی، اختلاف معنی داری بین زنان غیر باردار (NP) و باردار (P) وجود ندارد. این موارد عبارت بودند از: سن، تعداد فولیکول ها، درصد فولیکول‌های بزرگ و متوسط، تعداد اووسیت، درصد اووسیت‌های نکروتیک، رویان‌های درجه I و II و میزان لانه گزینی. در عین حال، پارامترهای دیگر اختلاف معنی داری بین دو گروه نشان دادند ($p<$ ۰/ ۰۵) که شامل ضخامت اندومتر، درصد فولیکول‌های کوچک، درصد MII، درصد GV و درصد لقاح بود (جدول ۲). تنها سایتوکینی که به طور معنی داری ($p= 0.033$) در مایع فولیکولی زنان غیرباردار بالاتر بود، IL-5 بود. اگر چه IL-6 در مایع فولیکولی زنان باردار بالاتر بود و روندی مرتبط با بارداری بیوشیمیایی داشت، اما این تفاوت معنی دار نبود ($p = 0.195$) (جدول ۲). بر اساس آزمون مربع کای، بین هیچ کدام از متغیرهای کیفی تفاوت معنی داری بین دو گروه مطالعه وجود نداشت. این متغیرها عبارت بودند از نوع ناباروری، پروتکل سوپراوولاسیون و سابقه IVF/ICSI قبلی (جدول ۳).

نتایج تجزیه و تحلیل چند متغیره نشان داد که تعداد فولیکول‌ها به طور معنی داری تحت تاثیر سن و ناباروری قرار می‌گیرد. به طور کلی، تعداد فولیکول‌ها با افزایش دوره ناباروری افزایش می‌یابد و با افزایش سن بیماران کاهش می‌یابد. در عین حال، پیش آگهی دهنده‌های دیگر از قبیل سایتوکین‌های مایع فولیکولی، پروتکل سوپراوولاسیون و سابقه دفعات IVF/CSI قبلی تاثیر قابل توجهی بر تعداد فولیکول نداشتند. در این مطالعه، نتایج نشان داد که بیماران با سابقه قبلی تلاش برای IVF/ICSI، همبستگی معنی داری با درصد فولیکول‌های بزرگ (۵۸/۶٪) نسبت به بیمارانی که اولین تلاش IVF / ICSI (۳۹.۵٪) را داشتند، دارند. این در حالی است که پیش آگهی دهنده‌های دیگر از جمله سایتوکین ها، سن و ناباروری، هیچ تاثیری بر نسبت فولیکول‌های بزرگ نداشتند. سن به طور معنی دار و معکوس با تعداد اووسیت‌ها ارتباط داشت. ده مورد سایتوکین اندازه گیری شده و پیش آگهی دهنده‌های دیگر، اثر معنی داری بر تعداد اووسیت نداشتند. IL-10 با افزایش تعداد اووسیت ارتباط داشت، اما به میزان قابل توجهی ($p =$ ۰/۰۸۳) نبود.

جدول ۲- خصوصیات جمعیتی (دموگرافیک)، ویژگی‌های چرخه‌ها و سایتوکین‌های اندازه گیری شده در زنان غیر باردار (NP) و باردار (P). مقایسه این متغیرهای کمی بین دو گروه P و NP با استفاده از آزمون t تحلیل تک متغیره مستقل تجزیه و تحلیل شد.

	غیر باردار	باردار	
	Mean ± SD (تعداد=۶۰)	Mean ± SD (تعداد=۱۸)	*P* Value
سن (سال)	31.67 ± 5.57	31.06 ± 3.64	0.663
ضخامت آندومتر (میلی متر)	8.93 ±2.02	10.14 ± 2.40	0.036*
(٪) تعداد فولیکول‌ها	17.17 ± 12.88	13.77 ± 4.93	0.272
(٪) فولیکولهای بزرگ	0.52 ± 0.34	0.55 ± 0.27	0.684
(٪) فولیکول‌های متوسط	0.37 ±0.23	0.41 ± 0.25	0.625
(٪) فولیکول‌های کوچک	0.112 ± 0.18	0.04 ± 0.09	0.035*
تعداد اووسیت ها	10.57 ± 7.98	9.61 ± 4.49	0.520
MII (%)	0.89 ± 0.19	0.97 ± 0.89	0.020*
GV (%)	0.026 ± 0.084	0.03 ± 0.014	0.042*
Necrotic (%)	0.08 ± 0.016	0.24 ± 0.089	0.071
FR %	0.67 ± 0.29	0.79 ± 0.23	0.140
(%) رویان درجه یک	0.60 ± 0.29	0.65 ± 0.25	0.102
(%) رویان درجه دو	0.33 ± 0.25	0.35 ± 0.20	0.422
IL-1β	13.17 ± 0.75	13.06 ± 0.60	0.592
IL-2	30.14 ± 4.68	29.98 ± 3.55	0.894
IL-4	15.70 ± 1.19	15.88 ± 0.91	0.559
IL-5	10.37 ± 0.81	9.92 ± 0.51	0.033*
IL-6	51.09 ± 65.83	81.00 ± 131.64	0.195
CXCL8/IL8	789.50 ± 395.83	664.97 ± 365.78	0.238
IL-10	46.42 ± 9.74	44.43 ± 10.52	0.457
IFN-γ	17.80 ± 1.13	17.63 ± 0.63	0.574
GM-CSF	12.41 ± 6.32	11.63 ± 0.74	0.603
TNF-α	11.60 ± 0.90	11.47 ± 0.83	0.602

* تفاوت از لحاظ آماری معنی‌دار بود ($p < ۰/۰۵$).

MII : متافازII ؛GV : وزیکول زایا؛ FR٪ :میزان لقاح؛ :IL اینترلوکین؛ :INF-γ اینترفرون گاما؛ GM-CSF: فاکتور محرک کلونی ماکروفاژ-گرانولوسیت؛ TNF-α: فاکتور نکروز دهنده توموری آلفا.

جدول ۳. خصوصیات جمعیتی و چرخه‌های زنان غیر باردار (NP) و باردار (P). مقایسه این متغیرهای کیفی بین دو گروه P و NP با استفاده از آزمون تحلیل تک متغیره مربع کای انجام شد.

	غیر باردار	باردار	
	N (%) (n=60)	N (%) (n=18)	*P* Value
نوع نازایی			
n (%) ناباروری بافاکتور زنانه	24/28 (86%)	4/28 (14%)	0.168
n (%) ناباروری ترکیبی	36/50 (72%)	14/50 (28%)	
IVF/ICSI قبلی			
n (%) خیر	16/23(69.6%)	7/23(30.4%)	0.319
n (%) بلی	44/ 55 (80%)	11/55(20%)	
پروتکل سوپراوولاسیون			
n (%) آگونیست	20/28(71.4%)	8/28(28.6%)	0.389
n (%) آنتاگونیست	40/50(80%)	10/50(20%)	

در همه بیماران، دوره ناباروری به طور معنی دار و مستقیم با نسبت اووسیت‌های در مرحله وزیکول زایا (GV) ارتباط داشت. IL-5 همبستگی معنی دار و مثبتی با GV (٪0.036= p) داشت و برای هر واحد افزایش غلظت IL-5 در مایع فولیکولی، ۲.۳ درصد افزایش در GV مشاهده شد. GM-CSF با افزایش درصد GV ارتباط داشت اما به سطح معنی داری نرسیده بود (۰/۰۷۱ = p). پیش آگهی دهنده‌های دیگر از جمله ۹ سایتوکین اندازه گیری شده دیگر، تاثیر معنی داری بر درصد GV نداشتند. GM-CSF همبستگی معنی داری با درصد MII داشت. به طور متوسط با افزایش هر واحد در غلظت GM-CSF در مایع فولیکولی، نسبت MII به میزان % ۱ کاهش یافت (۰.۰۱۲ = p). همچنین، IL-5 همبستگی معنی داری با MII (0.046 = p) داشت و با افزایش یک واحد غلظت IL-5 در مایع فولیکولی، نسبت اووسیت متافاز II به میزان ٪ ۵.۶ کاهش می‌یافت. ۸ سایتوکین اندازه گیری شده دیگر و سایر پیش آگهی دهنده ها، تاثیر معنی داری بر درصد MII نداشتند. هیچ کدام از ۱۰ سایتوکین یا سایر پیش آگهی دهنده‌های دیگر هیچ ارتباطی با درجه بندی رویان درجه یک و دو نداشتند. به طور کلی، میزان لقاح در بیماران با IVF/ICSI قبلی (٪۷۴/۴) به طور معنی داری بیشتر از کسانی بود که تحت درمان اولین ICSI بودند (% ۵۹.۷). IL-4 تنها سایتوکینی بود که همبستگی قابل توجهی داشت. با افزایش هر واحد IL-4در مایع فولیکولی، میزان درصد FR ٪۷.۵ افزایش می‌یافت (0.007 = p). IL-5 به طور معکوس و به طور قابل توجهی با احتمال بارداری بیوشیمیایی ارتباط داشت (۰/۰۲۶ = p، 0.223 = OR). ۹ مورد سایتوکین دیگر و سایر پیش آگهی دهنده ها، تاثیر معنی داری بر احتمال بارداری بیوشیمیایی نداشتند. در همه بیماران، ضخامت اندومتر به طور معنی داری با درصد لانه گزینی (٪ IR) ارتباط داشت. به طور کلی، هر چه ضخامت اندومتر بیشتر بود، نرخ لانه گزینی بالاتر بود. هیچ یک از ۱۰ سایتوکین اندازه گیری شده با شاخص IR٪

همبستگی معنی داری نداشتند. درصد IR به طور مستقیم با IFN-γ، IL-4، IL-6، TNF-α و به طور معکوس با IL-1β، IL-2، IL-5، IL-8، IL-10، GM-CSF متناسب بود، اما مقدار آن ناچیز بود.

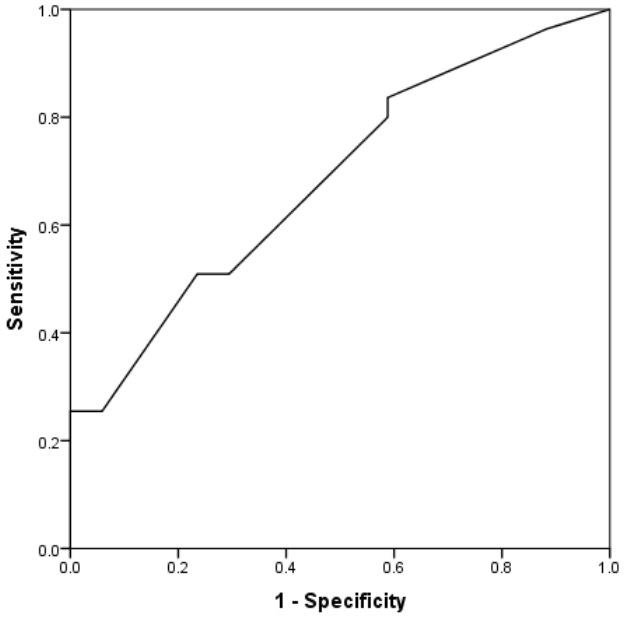
غلظت سایتوکین‌های اندازه گیری شده در مایع فولیکولی با روش فلوسیتومتری (multiplex magnetix bead-based Cytometry) در جدول ۴ نشان داده شده است. IL-5 تنها سایتوکین اندازه گیری شده‌ای بود که در آزمون رگرسیون چند متغیره، به طور قابل توجهی بر احتمال بارداری بیوشیمیایی تأثیر داشت. با استفاده از منحنی ROC در پیش بینی بارداری بیوشیمیایی، سطح زیر منحنی (AUC) برای IL-5 ۰.۶۵۴ بود. این بدین معناست که این سایتوکین دارای توانایی متوسط در پیش بینی شانس بارداری بیوشیمیایی است. در حالی که ۹ مورد سایتوکین اندازه گیری شده دیگر توانایی پیش بینی بارداری بیوشیمیایی ضعیفی داشتند (شکل ۱ و جدول ۴).

سیزده بیمار مبتلا به OHSS شدید بودند. با توجه به آزمون رگرسیون لجستیک چند متغیره، هیچ کدام از ده سایتوکین اندازه‌گیری شده ارتباط معنی‌داری با میزان بروز OHSS نداشتند. به جز سن بیماران، سایر پیش آگهی دهنده هایخصوصیات جمعیتی ارتباط معنی‌داری با میزان بروزOHSS نداشتند. سن به طور معکوس و به طور قابل توجهی با میزان بروز OHSS در ۸۰ بیمار مورد مطالعه همبستگی داشت. سطح زیر منحنی (AUC) IL-10و TNF-α به ترتیب ۰.۶۳۱ و ۰.۶۱۵ بود، بنابراین می‌توان گفت که این دو سایتوکین توانایی متوسطی در پیش‌بینی OHSS داشتند.

جدول ۴- میانگین ± انحراف معیار غلظت سایتوکین‌های اندازه گیری شده در مایع فولیکولی به روش فلوسیتومتری مغناطیسی. منحنی ROC برای ارزیابی عملکرد هر سایتوکین در پیش بینی وضعیت بارداری بیوشیمیایی انجام شد. سطح زیر منحنی (AUC) برای هر سایتوکین ارائه شده است.

	Mean ± SD (pg/ml)	AUC
IL-1β	13.13 ± 0.72	0.534
IL-2	30.07 ± 4.45	0.531
IL-4	15.74 ± 1.13	0.583
IL-5	10.23 ± 0.77	0.654*
IL-6	57.19 ± 84.55	0.571
CXCL8/IL8	757.39 ± 385.97	0.592
IL-10	45.95 ± 9.95	0.576
IFN-γ	17.75 ± 1.03	0.519
GM-CSF	12.21 ± 5.48	0.518
TNF-α	11.57 ± 0.87	0.542

*آزمون رگرسیون لجستیک چند متغیره نشان داد که تنها IL-5 به طور قابل توجهی بر احتمال حاملگی بیوشیمیایی تأثیر می گذارد. AUC برای IL-5 نشان می‌دهد که این سایتوکین دارای توانایی متوسط در پیش بینی بارداری بیوشیمیایی است. IL: اینترلوکین؛ INF-γ: اینترفرون گاما؛ GM-CSF: فاکتور تحریک کننده کلونی گرانولوسیت- ماکروفاژ؛ TNF-α: فاکتور نکروز دهنده توموری آلفا.



شکل ۱- منحنی ROC اینترلوکین ۵ که نشان دهنده کارآیی IL-5 در پیش بینی بارداری بیوشیمیایی است.

۴- بحث

یافته اصلی این مطالعه این است که برخی از سایتوکین‌های مایع فولیکولی مانند IL-5، IL-4 و GM-CSF در فولیکول‌های پیش تخمک گذاری به طور قابل توجهی با نتیجه ICSI ارتباط دارند. سطح بالای IL-5 با کیفیت اووسیت ضعیف مرتبط بود که احتمال حاملگی را کاهش می‌دهد. غلظت بالای GM-CSF با کاهش میزان اووسیت‌های بالغ همراه بود. غلظت بالای IL-4 در مایع فولیکولی با افزایش میزان لقاح منجر به بهبود نتیجه ICSI شد.

در این مطالعه مشخص شد که افزایش غلظت IL-5در مایع فولیکولی به طور معنی دار و معکوس با نتیجه ICSI همراه است که ناشی از افزایش درصد اووسیت‌های GV و کاهش درصد اووسیت‌های مرحله MIIبود. گزارش شده است که IL-5 یک سایتوکین شناخته شده سلول‌های Th1 است که هم توسط سلول‌های خونی و هم سلول‌های غیر خونی تولید می‌شود [۲۹] و بر تمایز سلول‌های میلوئیدی، افزایش فعالیت شیمیایی و ظرفیت چسبندگی ائوزینوفیل‌ها تأثیر می‌گذارد [۳۰، ۳۱]. همچنین سلول‌های B را تحریک می‌کند و به عنوان فاکتور تمایز برای ائوزینوفیل‌ها عمل می‌کند [۲۵، ۳۲]. در اندام‌های تولیدمثلی، IL-5 به بازسازی بافت تخمدان موش [۳۳] و بازسازی بافت اندومتر در چرخه فحلی کمک می‌کند [۳۱]. اخیرا، ترنینا و همکاران [۳۴] پیشنهاد کردند که IL-5 به عنوان یکی از تنظیم کننده‌های اصلی بالادست در آترزی فولیکول تخمدان خوک عمل می‌کند.

مطالعات قبلی، که نقش احتمالی IL-5 را در نتایج IVF/ICSI بررسی کردند، نتوانستند IL-5 را در FF زنان مبتلا به ICSI با استفاده از روش ایمونواسی ساندویچی چندگانه (bead-based multiplex sandwich immunoassay) ردیابی کنند [۱۱]. مطالعه دیگری، IL-5 را در ٪۲۵ نمونه‌های FF با استفاده از سیستم‌های R & D تشخیص داد و در این مطالعه، نرخ لانه گزینی، مورفولوژی رویان، قطعه قطعه شدن و یا تسهیم زودهنگام تفاوت معنی داری در بین اووسیت هایی که به تولد موفقیت آمیز منجر شدند و آنهایی که نشدند، نداشت [۱۰]. در تحقیق نیو و همکاران [35] همبستگی معنی داری بین IL-5 مایع فولیکولی مربوط به بزرگترین فولیکول با رویان با کیفیت بالا و پتانسیل رشد جنین در یک گروه از بیماران مبتلا به سندرم متابولیک که تحت درمان IVF قرار داشتند و یا نداشتند، پیدا نکردند.

پیشنهاد شده است که سایتوکین‌های Th1 پس زدن آلوگرافت را تسریع می‌کنند و ممکن است بارداری را به خطر بیندازند، در حالی که سایتوکین‌های Th2 تحمل آلوگرافت را افزایش می‌دهند و بنابراین ممکن است بقای جنین را بهبود بخشند. مطالعه اخیر مدل حیوانی تایید کرده است که عملکرد Th-2 در دوران اولیه بارداری برای بارداری موفق در گاوها ضروری است [۳۷]. به طور جالبی، IL-5 با ویژگی‌های ضد التهابی شناخته شده Th2، در مایع فولیکولی زنان باردار به طور قابل توجهی کاهش یافته و هم با بارداری بیوشیمیایی و هم بارداری بالینی همبستگی معکوس دارد. علاوه بر این، بر اساس منحنی ROC دارای توانایی متوسطی در پیش آگهی بارداری بیوشیمیایی در مطالعه ما است. بارداری تنها یک پارادایم Th1/Th2 ساده نیست که در آن سایتوکین‌های "بد" Th1 باعث سقط جنین شوند و سایتوکین‌های "خوب" Th2 مسئول بارداری موفق باشند [۱]. نه تنها سایتوکین‌های Th1 و Th2، بلکه بسیاری از سایتوکین‌های دیگر در ناحیه جنینی-مادری توسط سلول‌های ایمنی و غیر ایمنی تولید می‌شوند. این سلول‌ها توانایی بالایی دارند و بسته به تعدادشان اثرات خود را اعمال می‌کنند. فعالیت آنها می‌تواند توسط گیرنده‌ها و آنتاگونیست‌ها کاهش یابد و آنها می‌توانند کاربردهایی در هر مرحله داشته باشند [۱، ۳۸].

IL-4 سایتوکین دیگری در مطالعه ما بود که تفاوت معنی داری بین چرخه ICSI موفق و ناموفق داشت. این اینترلوکین به طور قابل توجهی در مایع فولیکولی زنان باردار افزایش یافته و منجر به افزایش میزان لقاح شد. IL-4 به طور گسترده‌ای از سلول‌های کومولوس اووفوروس ترشح می‌شود که اووسیت را در طی تخمک گذاری و رویان را در طی ۷۲ ساعت اول قبل از لانه گزینی احاطه می‌کنند [۳۹]. قطع التهاب نقشی حیاتی در طول بارداری ایفا می‌کند و عمدتا از طریق سلول‌های ایمنی تولید کننده IL-4 و IL-10 میانجیگری می‌شود [۴۰].

مطالعه مارزی و همکاران [41] نشان داد که حاملگی طبیعی با افزایش IL-4 و IL-10 در سرم ارتباط دارد. با این حال، مطالعه ما هیچ ارتباط معنی داری بین غلظت IL-10 در مایع فولیکولی و نرخ لانه گزینی نشان نداد.

همچنین GM-CSF به طور معنی داری با کیفیت اووسیت ضعیف ارتباط داشت، زیرا افزایش غلظت این سایتوکین در مایع فولیکولی با کاهش اووسیت MII همراه بود. GM-CSF از سلول گرانولوزا و سلول تکا ترشح می‌شود و با افزایش فراخوانی ماکروفاژها، در تخمک گذاری و لوتئینی شدن شرکت می‌کند [۳]. GM-CSF سایتوکینی پیش التهابی است که تکثیر سلول‌های میلوئیدی را تنظیم می‌کند. همچنین برای عملکرد مونوسیت ها، ماکروفاژها و سلول‌های دندریتیکی حیاتی است و در شرایط التهاب توسط سلول‌های فعال سیستم ایمنی در مقادیر زیاد تولید می‌شود [۴۲]. مطالعه لدی و همکاران [۱۳] نشان می‌دهد که غلظت GM-CSF در مایع فولیکولی با نرخ حاملگی پایدار متناظر مرتبط نیست. استفاده از تحریک تخمدانی کنترل شده (COH) برای القای رشد چندین فولیکول با استفاده از گنادوتروپین‌های خارجی در درمان ART می‌تواند برای لقاح، کیفیت رویان، پذیرش آندومتر و همچنین سطح سایتوکین‌های داخل فولیکولی و ارتباطات آنها، مضر باشد. لذا این مسئله ممکن است بر بلوغ اووسیت/بارور شدن اووسیت و شایستگی رشد رویان به دلیل تاثیرپذیری آنها از محیط داخل فولیکول تاثیر بگذارد (۲۲). این موضوع باعث تولید اووسیت از فولیکول هایی می‌شود که به بلوغ مطلوب نمی رسند و احتمالا اووسیت هایی را تولید می‌کنند که کاملا شایسته نیستند. فولیکول‌های هم اندازه‌ی چرخه‌های تحریک نشده، دارای محیط هورمونی متفاوتی از فولیکول‌های چرخه‌های تحریک شده می‌باشند، و لذا چرخه‌های تحریک شده نه تنها پروتئین‌های ایمنی را تحت تاثیر قرار می‌دهند، بلکه مسیرهای میوز و تخمک گذاری را نیز متاثر می‌کنند. با این وجود، به نظر نمی رسد که این تفاوت‌ها با مورفولوژی رویان اولیه مرتبط باشد [۲۰]. GM-CSF در مایع فولیکولی فولیکول‌های رشد کرده در پروتکل سوپراوولاسیون آنتاگونیستی بالاتر بوده است [۴۳]، در حالی که در مطالعه ما هیچ تفاوت معنی داری در غلظت GM-CSF مایع فولیکولی فولیکول‌های بین دو پروتکل مشاهده نشد.

شواهد کمی در دسترس است که تایید کند انتخاب غیر تهاجمی در مرحله اووسیت بتواند توانایی پیش آگهی داشته باشد. ارتباطی میان برخی از الگوهای پیش هسته ها، مانند تعداد و توزیع پیش هسته‌ها در مرحله زیگوت، با نتیجه درمان در چرخه IVF و ICSI یافت شده است [۴۴]، در حالی که نتایج ما نشان داد همبستگی قابل توجهی بین درجه بندی رویان در روز سوم بعد از ICSI و نتیجه اش، و یا با غلظت سایتوکین‌های مایع فولیکولی وجود ندارد.

در تخمک گذاری، فولیکول تخمک گذاری متحمل تغییرات بلوغ می‌شود که با افزایش اندازه فولیکول و افزایش توانایی آن در تولید استرادیول همراه است. استفاده از تحریک کنترل شده تخمدان (COH) در درمان‌های ART بر بلوغ فولیکول‌ها و اووسیت‌ها در مدل‌های حیوانی تاثیر می‌گذارد [۴۵]. گونتر و همکاران [۴۶] ارتباط معنی داری بین افزایش غلظت IL-18 در مایع فولیکولی و بارداری موفق پس از IVF/ICSI نشان دادند. نویسندگان این موفقیت را به افزایش تعداد اووسیت‌های اخذ شده به خاطر افزایش پاسخ به تحریک تخمدان نسبت دادند. این در حالی است که هیچ کدام از سایتوکین‌های اندازه گیری شده در مطالعه ما تاثیر قابل توجهی بر تعداد اووسیست‌های اخذ شده در بیماران ICSI نداشتند. یک بررسی مقایسه‌ای قبلی گزارش داد که در پروتکل آگونیست و آنتاگونیست و تاثیر آن بر نرخ لانه گزینی و غلظت سایتوکین‌های سرم شامل FF IL-1β، IL-8، IL-12 و TNF-α هیچ تفاوت معنی داری بین دو پروتکل وجود ندارد [۴۷]. ما نیز در این مطالعه، این یافته‌ها را در مورد بسیاری از این سایتوکین‌ها و همچنین سایر سایتوکین‌ها شامل IFN-γ، GM-CSF، IL-2، IL-5 و IL-10 تایید کردیم.

یک نتیجه جالب در این مطالعه مربوط به دوره ناباروری بود. ما دریافتیم که دوره ناباروری طولانی تر با تعداد بیشتر فولیکول‌ها ارتباط دارد، اما هیچ کدام از ۱۰ سایتوکین مورد بررسی با آن مرتبط نبودند. احتمال کلی درمان موفقیت آمیز ناباروری تقریبا ٪۵۰ است، اما این میزان به دلیل عامل ناباروری، سن زن، پیشینه باروری قبلی و طول مدت ناباروری متفاوت است. مدت ناباروری کوتاهتر و باروری قبلی شانس رسیدن به حاملگی را افزایش می‌دهد [۴۸]. کالو و همکاران [49] نتیجه گرفتند که زنان جوانی که تولد زنده داشته اند در مقایسه با کسانی که پس از IVF سقط جنین داشته اند، شانس بیشتری برای رسیدن به حاملگی و تولد زنده در دوره دوم دارند. با این وجود، نتیجه اولین چرخه IVF، موفقیت IVF بعدی در زنان مسن را پیش بینی نمی کند. رهمان و همکاران [۱۴] نشان دادند که افزایش غلظت IL-1β با افزایش تعداد اووسیت‌های اخذ شده بالغ در بیماران ICSI همراه است.

در این مطالعه، ما دریافتیم که بیماران با سابق قبلی IVF/ICSI دارای تعداد بیشتری از فولیکول‌های بزرگ نسبت به بیماران تحت درمان اولین ICSI هستند. به همین ترتیب، درصد لقاح (FR) در بیماران با سابقه قبلی IVF/ICSI و بیماران مرتبه اول به ترتیب ٪ ۷۴.۴ و ٪ ۵۹.۷ بود. این ممکن است به دلیل تحریک قبلی تخمدان در یک چرخه کنترل شده تحریک تخمدان در درمان‌های IVF/ICSI قبلی باشد. نتایج ما با نتایج هندریکس و همکاران [50] مطابقت داشت، یعنی بیماران در اولین چرخه IVF پاسخ تخمدانی ضعیفی به تحریک گنادوتروپین می‌دهند.

نتایج ما نشان داد که سن به طور معکوس با تعداد فولیکول‌ها و تعداد اووسیت ها مرتبط است. سن بهترین پیش آگهی برای موفقیت برای IVF/ICSI است. به نظر می‌رسد ناباروری مربوط به سن، بیشتر مربوط به پیری تخمدان و کاهش تعداد فولیکول‌های تخمدان باشد [۵۱]. در طول عمر باروری، به محض آنکه تخمدان ذخایر فولیکولی را به اتمام برساند، بسیاری از عملکردها از دست می‌روند [۵۲]. مطالعات قبلی گزارش داده اند که اثرات زیان آور سن بالای بیمار با IL-6 و اثرات منفی آن بر روی پذیرش رحم برای رویان مرتبط است و لذا نتیجه IVF/ICSI ضعیف است [۲۵]. در بیماران نابارور با عامل زنانه در مطالعه ما، نتایج نشان داد که بین سن و غلظت IL-6 مایع فولیکولی چرخه ICSI موفق و ناموفق رابطه‌ای وجود ندارد. ما همچنین نشان دادیم که ضخامت اندومتر با افزایش نرخ لانه گزینی و احتمال حاملگی بالینی مرتبط است، اما هیچکدام از ۱۰ سایتوکین اندازه گیری شده به طور معنی داری با افزایش ضخامت اندومتر مرتبط نبودند. این در حالی است که مطالعه رهمان و همکاران [۱۴] نشان داد که IL-1β با افزایش ضخامت اندومتر و نرخ تولید مثل در بیماران ICSI مرتبط است.

سن بیماران تنها خصوصیت جمعیتی بود که در بیماران ICSI این مطالعه به طور معنی دار و معکوس با میزان بروز OHSS شدید ارتباط داشت. در اغلب مطالعات گزارش شده است که زنان مبتلا بهOHSS به طور قابل توجه جوانتر از افرادی هستند که مبتلا به این سندرم نیستند [۵۳- ۵۷]. بوسو و همکاران (۲۰۱۰) یکی از عوامل خطر ابتلا به OHSS قبل و بعد از مصرف گنادوتروپین را جوانی عنوانن کردند. بیماران مبتلا به OHSS عموما جوان هستند، زیرا OHSS به ذخیره تخمدان بیمار وابسته است [۵۳].

به طور خلاصه، اطلاعات ما نشان دهنده چندین اثر مضر میزان بالای IL-5 در مایع فولیکولی بر روی نتیجه ICSI است. یکی از دلایل اصلی آن کاهش کیفیت اووسیت است که با افزایش قابل ملاحظه اووسیت ها در مرحله وزیکول زایا و کاهش اووسیت های مرحله MII بروز می‌نماید. کاهش شانس بارداری بیوشیمیایی و بالینی اثر مضر دیگر این اینترلوکین بود. علاوه بر این، IL-5 توانایی متوسطی در پیش بینی شانس حاملگی بیوشیمیایی داشت. بنابراین، می‌توان نتیجه گرفت که افزایش غلظت داخل فولیکولی IL-5 به عنوان یکی از پیش آگهی دهنده‌های منفی برای نتیجه حاملگی در چرخه‌های ICSI است. علاوه بر این، افزایش GM-CSF مایع فولیکولی با کیفیت ضعیف اووسیت همراه بود، در حالی که غلظت بالای IL-4 در مایع فولیکولی با نتیجه ICSI خوب ارتباط داشت. با توجه به منحنی‌های ROC، TNF-α و IL-10 نیزتوانایی پیش آگهی متوسطی برای پیش بینی احتمال ابتلا بهOHSS شدید داشتند.

عنوان:

بررسی ارتباط برخی سایتوکین‌های مایع فولیکولی با میزان باروری در روش تزریق داخل سیتوپلاسمی اسپرم

چکیده

هدف از این مطالعه، تعیین توانایی پیش آگهی غلظت برخی از سایتوکین‌های مایع فولیکولی بر روی نتیجه تزریق داخل سیتوپلاسمی اسپرم / انتقال جنین (ICSI/ET) در زنانی بود که تحت درمان تحریک تخمدانی قرار گرفته بودند. بدین منظور، در مجموع ۸۰ بیمار به دنبال تحریک تخمدان و انجام ICSI مورد مطالعه قرار گرفتند. مایع فولیکولی (FF) در روز اخذ اووسیت جمع آوری شد و ده سایتوکین شامل: فاکتور نکروز دهنده توموری آلفا(TNF-α) ، IL-1β، IL-2، IL-4، IL-5، IL-6، IL-8 /CXCL، IL-10، فاکتور تحریک کننده کلونی گرانولوسیت- ماکروفاژ (GM-CSF) و اینترفرون گاما (IFN-γ) با استفاده از روش فلوسیتومتری (multiplex magnetix bead-based Cytometry) اندازه گیری شد. نتایج حاصله حاکی از این بود که فقط غلظتIL-5 ، IL-4 و GM-CSF در مایع فولیکولی آن دوره‌هایی از ICSIکه منجر به حاملگی شده بودند و آنهایی که منجر به حاملگی نشدند تفاوت معنی‌داری ($p < 0.05$) دارد. سطوح بالای IL-5 مایع فولیکولی با کیفیت پایین اووسیت مرتبط بود که احتمال حاملگی بیوشیمیایی و بالینی را کاهش می‌داد. غلظت بالاتر M-CSF در مایع فولیکولی با کاهش اووسیت های بالغ ارتباط داشت، در حالی که غلظت بالای IL-4 در مایع فولیکولی با افزایش میزان لقاح و به دنبال آن نتیجه بهتر ICSIهمراه بود. در مجموع، افزایش غلظت IL-5 مایع فولیکولی به عنوان یک پیش گوی منفی برای پیامد حاملگی در دوره های ICSI به نظر می‌رسد. همچنین، TNF-α وIL-10 دارای توانایی پیش آگهی متوسطی در احتمال ابتلا به سندرم تحریک بیش از حد تخمدان شدید (OHSS) بودند.

واژه‌های کلیدی: سایتوکین ها، اینترلوکین -۵، تزریق داخل سیتوپلاسمی اسپرم (ICSI)، ناباروری زنان.

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عنوان

بررسی ارتباط برخی سایتوکین‌های مایع فولیکولی با میزان باروری در روش تزریق داخل سیتوپلاسمی اسپرم

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تابستان ۱۳۹۷